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# Hydrocarbon circulation and colonial signature in *Pachycondyla villosa*

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**Abstract**

In ants, both cuticular and postpharyngeal gland (PPG) hydrocarbons (HCs) have been involved in nestmate recognition. However, no detailed comparison is available. A comparative study including also high density lipophorin (HDLp), an internal HC carrier, was therefore undertaken on *Pachycondyla villosa*. Purified HDLp is an 820 kDa lipoprotein with a density of 1.114 g/ml and two 245 and 80 kDa apo-proteins. Its hydrocarbon profile is very similar with the cuticular one, in agreement with its hydrocarbon carrier function. Conversely, *n*-alkanes and externally branched monomethylalkanes are markedly decreased in the PPG. According to their physical properties, this suggests that they are involved in waterproofing on the cuticle. The PPG actually contains only internally branched mono-, dimethylalkanes or monomethylalkenes; their greater fluidity is more adequate for chemical communication. The percentages of some of them are statistically not different between the cuticle and PPG. Their mixtures vary with colonies and they may thus be involved in colonial signature. A scheme for hydrocarbon circulation is discussed, involving lipophorin, cuticle, PPG and self-grooming in one individual, a pathway complementary or alternative to the selective delivery by lipophorin in some other insects. HCs are then distributed between nestmates' cuticles through allo-grooming and physical contacts.


*Keywords:* Cuticle; Lipophorin; Postpharyngeal gland; Hydrocarbons; Ants' nestmate recognition

## 1. Introduction

Insect hydrocarbons (HCs) play important roles in waterproofing and chemical communication. They make up an effective barrier against desiccation due to their hydrophobicity and organization on the cuticle (Noble-Nesbitt, 1991; Gibbs, 1998; Rouault et al., 2000; Young et al., 2000). Interestingly, their involvement in chemical communication induces various behaviours in solitary as well as social insects: male–female mating (Antony et al., 1985; Singer, 1998), reproductive isolation (Blomquist et al., 1987; Cobb and Jallon, 1990; Chase et al., 1992), chemical mimicry (Dettner and Liepert, 1994), species-/colony-/caste-recognition (Vander Meer and Morel, 1998; Lenoir et al., 1999) and the signalling of fertility status (Monnin et al., 1998; Cuvillier-Hot et al., 2002; Heinze et al., 2002).

The site of biosynthesis of insect hydrocarbons has been shown to be abdominal, internal but close to the integument (Dillwith and Blomquist, 1982; Gu et al., 1995; Ferveur et al., 1997). Since the studies of Diehl (1975) on *Schistocerca gregaria* and Romer (1980) on *Tenebrio molitor* larvae, oenocytes are thought to synthesize hydrocarbons in adult insects also and this has been recently demonstrated by Fan et al. (2003) with an 'oenocyte-enriched fraction' of the abdominal integument of *Blatella germanica*.

Transfer of internally synthesized hydrocarbons to the cuticle of several insect species is performed by lipophorin (Schal et al., 2001 and references therein). This ubiquitous lipoprotein shuttles different classes of

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lipids between their sites of synthesis or absorption and storage or utilization in various target organs or tissues through the aqueous environment of hemolymph. Hydrocarbons are transported in the hydrophobic core of high density lipophorin (HDLp), as has been shown in different structural studies (Van der Horst, 1990), and delivered to the cuticle.

In social insects, cuticular long chain HCs have been often involved in nestmate recognition. Typically, ants from other colonies that do not bear the colony-specific blend of these long chain hydrocarbons ('gestalt') are rejected and even killed. Similar long chain HCs are also found in abundance in the post-pharyngeal gland (PPG), an exocrine gland that is unique to ants (Jackson and Morgan, 1993) and occupies a situation very propitious to secretion/exchanges through oral contact and grooming. These hydrocarbons originate behaviours similar to those elicited by cuticular HCs. Thus, in a colony, an individual coated with alien PPG HCs undergoes aggressive behaviour from its nestmates, whereas an alien ant treated with PPG HCs from this colony is accepted as a nestmate (Soroker et al., 1994; Lahav et al., 1999).

Despite this interesting observation, detailed chemical comparisons of HCs from cuticle and PPG have been rarely done. A question of central interest in the present study is what the origin of these HCs may be. Are they synthesized by the PPG, brought in internally by lipophorin through the hemolymph or transferred from the cuticle by self-grooming (Hefetz et al., 2001)? The PPG of an individual, however, has also been shown to contain HCs of other nestmates brought through physical contacts, allo-grooming and trophallaxis (Soroker et al., 1994, 1995, 2003; Dahbi et al., 1998; Hefetz et al., 2001). Together, these considerations led to the hypothesis that HDLp, PPG and cuticle are involved in the HC transport pathway within and between individuals.

The complete description of the composition of cuticular hydrocarbons has already been performed on three *Pachycondyla* close species (Lucas et al., 2002). *Pachycondyla villosa* exhibits the broadest pattern of hydrocarbons. We have purified the HDLp of this primitive ant. This enabled us to compare HCs associated to HDLp to those of the cuticle and the PPG and investigate their roles in these social insects. Our data shed new light on the potential roles of different types of HCs found on the cuticle and in the PPG.

## 2. Material and methods

### 2.1. Ants

Queen-right colonies of the species *P. villosa* (Lucas et al., 2002) were collected in the experimental fields of the Cocoa Research Center at Ilhéus, Bahia, Brazil. All the colonies were reared in the laboratory in artificial nests at least 6 months before being analysed. The nests were maintained at $27 \pm 1\ ^{\circ}C$, with about 60–80% relative humidity, and a 12L:12D photoperiod. All the colonies were provided with an identical diet (honey/apple mixture, *Calliphora* sp. larvae) twice weekly.

### 2.2. Extraction procedure and characterization of hydrocarbons

Three colonies were used (6–9 foragers per colony) for chemical analysis. Each individual was picked using clean forceps and placed into chilled vials for 10 min before analysis. Secretion of the Dufour gland compounds—the only contaminants otherwise observed—was thus inhibited on subsequent solvent addition. Cuticular hydrocarbons (HCs) were extracted from each entire individual by 5 min immersion in 200 μl of heptane with agitation.

Workers were then dissected and the PPGs were individually collected. The PPG is glove-shaped, with two symmetrical halves terminating in a varying number of finger-shaped projections (Fig. 1). PPG's hydrocarbons were extracted under the same conditions.

Samples were dried under nitrogen and dissolved in 50 μl of heptane. Each sample was analysed using gas chromatography–mass spectrometry (GC–MS) carried out with a Fisons mass spectrometer MD 800 (electron

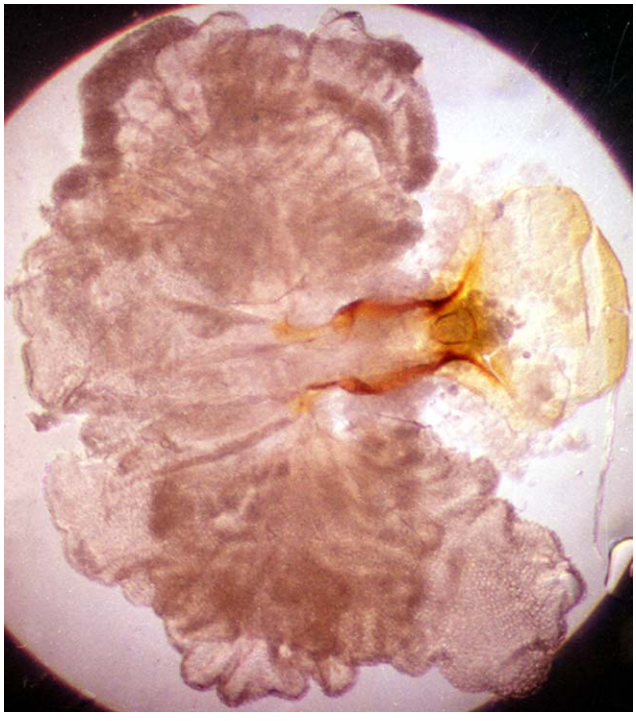

Fig. 1. *Pachycondyla villosa* postpharyngeal gland.

impact at 70 eV) coupled directly with a Carlo Erba gas chromatograph GC 8065MS. The GC–MS was fitted with a 25QC2 BP1 methylsilicone capillary column (25 m × 0.22 mm ID × 0.1 μm). Injections of 5 μl of solution were done at 60 °C. The oven temperature was increased 1 min after injection from 60 to 225 °C at 20 °C/min and from 225 to 320 °C at 3 °C/min (isotherm 10 min). Data were integrated using an IBM-PC with MassLab 1.27 data acquisition, plotting and analysis software. Masses were scanned between 40 and 700 amu at 0.45 scan/s. The mass spectra of HCs were interpreted according to published criteria (McCarthy et al., 1968; Nelson et al., 1972; Nelson, 1978; Pomonis et al., 1978, 1980).

### 2.3. Collection of hemolymph proteins

Forty workers (only foragers) were picked from one colony using clean forceps and put into chilled vials. Cuticular HCs were extracted from this pool by immersion for 5 min in 40 ml of heptane with three-dimensional agitation. The ants were put at 5 °C into an extraction buffer containing protease inhibitors (1 ml/worker). This buffer consisted of 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, 2 mM EDTA, 5 mM benzamidin, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin and aprotinin at 20 μg/ml, glutathione at 2 mg/ml, soybean trypsin inhibitor at 2 mg/ml and 0.01% sodium azide. Heads and legs were removed to facilitate the collection of hemolymph. Individual heads were stored separately for later dissection of PPGs and extraction of the HCs as described above. Hemolymph was obtained by centrifuging the remaining parts of the ants with 10 μm membrane tubes (VectaSpin 20, Whatman, Kent, UK) at 2000 g/4 °C for 2 min (Sigma centrifuge). The collected hemolymph and extraction buffer were centrifuged at 8 °C in a Kontron T 124 centrifuge (Kontron Instruments, Milano, Italy) with an A824 rotor at 39,120 $g^{max}$ for 35 min. The protein content of the supernatant was precipitated by adding 0.533 g/ml ammonium sulphate at 4 °C, and the precipitate was collected by centrifugation under the conditions described above (39,120 $g^{max}$ for 35 min). The pellet was redissolved in extraction buffer and recentrifuged to get a clear supernatant containing the dissolved proteins.

### 2.4. Lipophorin purification

HDLp was isolated by density gradient ultracentrifugation (Shapiro et al., 1984). The last supernatant was stained with 400 μl of Sudan Black (5 mg/ml in ethylene glycol), mixed with 2.3 g of solid sodium bromide and adjusted to a final volume of 5 ml. The solution was transferred to a 11.5 ml ultracentrifuge tube and overlaid with a solution of 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, 2 mM EDTA and 0.01% sodium azide. Tubes were sealed and centrifuged with a slow acceleration mode at 235,340 $g^{max}$ for 60 min at 4 °C in a Kontron TVF 65.13 vertical rotor. The stained Lp band was withdrawn and its concentration and salt removal were performed by ultrafiltration through Biomax-100 membrane (100 kDa cut-off, Ultrafree-4 centrifugal filter unit, Millipore, Bedford, MA, USA). For further purification, this stained Lp material was diluted in a final volume of 5 ml extraction buffer without protease inhibitors and containing 2.3 g solid sodium bromide, and subjected to ultracentrifugation again. The purity of HDLp was checked by native and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoreses (PAGE) (Laëmmli, 1970) using a concave exponential gradient of acrylamide (5–25%) for the resolving gel in both types of electrophoresis. Silver staining was performed according to Blum et al. (1987). The molecular mass of Lp and its subunits were determined with high and low molecular standard kits from Pharmacia (Saint-Quentin-en-Yvelines, France). Platelet myosin was added to the latter kit.

The density of isolated HDLp was determined by comparative centrifugation with a density gradient reference tube. Fractions (400 μl) were collected from the top of the tubes. Each fraction was measured for absorbance at 280 nm (Lp tube) and for refractive index (blank tube) to calculate the density.

### 2.5. Lipid extraction from lipophorin

Isolated HDLp was submitted to lipid extraction (Folch et al., 1957). The chloroform extract was dried under nitrogen and redissolved in heptane. The HCs were separated from other lipids by thin layer chromatography (TLC) on Silica Gel 60 F-254 in heptane/diethyl ether/acetic acid solvent (80/20/2, v/v). The HC band was located by reference to control lipid standards subjected to the same run and stained with $I_2$, scraped from the plates and extracted with heptane, before analysis by GC–MS.

### 2.6. Statistical analysis

Mann–Whitney *U*-tests were performed using Statistica 5.5 software (Statsoft Inc., Tulsa, OK, USA) with the sequential Bonferroni correction of the *P*-values. Principal component analysis (PCA), on normalized variables, used SPAD 3.0 software (Decisia, Paris, France).

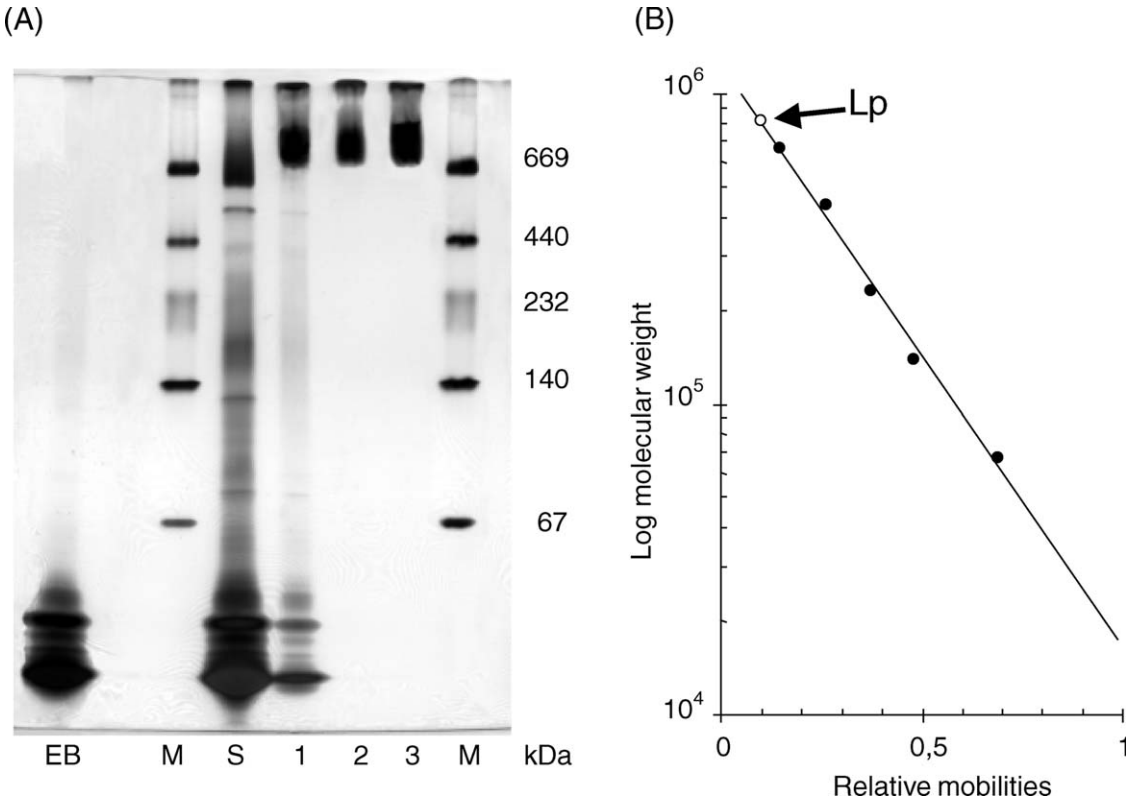


Fig. 2. (A) Electrophoretic analysis of NaBr gradient fractions by native slab gel PAGE. EB: extraction buffer with antiproteases; M: molecular weight markers (in kDa) were thyroglobulin (669), ferritin (440), catalase (232), lactate dehydrogenase (140) and bovine serum albumin (67); S: starting $(NH_4)_2SO_4$ precipitated material redissolved in EB; 1: lipophorin after ultracentrifugation with EB; 2–3: lipophorin after ultracentrifugations without antiproteases; (B) Molecular mass determination. Open point: native lipophorin (Lp), 820 kDa.

## 3. Results

Comparison of the HC profiles of the cuticle, HDLp and PPG implies two prerequisites: (1) HDLp be pure and (2) the cuticle, HDLp and PPG originate from the same individuals because in social insects, like ants, differences do exist between HC profiles of workers from different colonies and even within colonies (Tentschert et al., 2002; Greene and Gordon, 2003).

### 3.1. Lipophorin purification

The HDLp isolated using density gradient ultracentrifugation was pure as shown by slab gel native (Fig. 2, lanes 2–3) and SDS PAGE (Fig. 3, lanes 2–3) with a concave exponential gradient of polyacrylamide (5–25%). On the top of each slab gel shown (Figs. 2 and Fig. 3), which corresponds to the interface between the concentrating and resolving gels, a stained band is visible in all lanes. This is due to the formation of aggregates, which cannot enter the resolving gel. Otherwise, the lane with the starting precipitated material (Figs. 2 and 3, lane S) shows a weak band of HDLp and a multitude of proteins whereas lanes 2 and 3, show a single Sudan Black-stainable native protein (Fig. 2) or two polypeptides characteristic of HDLp (Fig. 3) when ultracentrifugations were performed without antiproteases. The native HDLp has a molecular weight of approximately 820 kDa (Fig. 2B). Its band is broad, due to the high concentration which was used in order to reveal any kind of contaminant. As shown in Fig. 4, there is only one peak of absorption ($\lambda = 280$ nm) for purified HDLp. A density gradient reference tube permits to locate it at a density of 1.114 g/ml (Fig. 4). The molecular mass of the two subunits, apoLp-I and apoLp-II, are approximately 245 and 80 kDa, respectively (Fig. 3B).

Before this purification procedure was set up, direct puncture collection of hemolymph had been tried. Electrophoresis of this hemolymph showed the same Sudan black-stained native protein and the same predominant subunits, with less protein or polypeptide contaminants (not shown). However, as clotting rapidly occurred, insufficient volumes of hemolymph were available for purification. Nevertheless, these data show that the different steps of our present procedure did not alter the purified HDLp.

### 3.2. Lipophorin and cuticle hydrocarbons

In several insect species, HDLp has been shown to carry HCs from the site of biosynthesis, probably

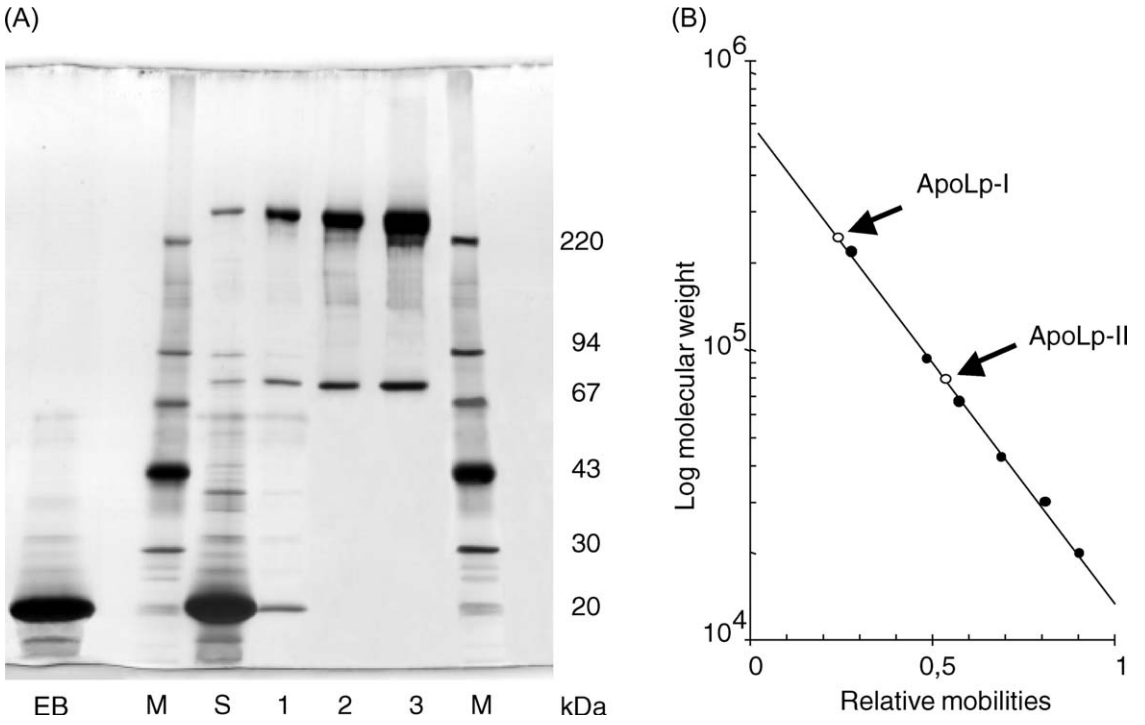


Fig. 3. (A) Electrophoretic analysis of NaBr gradient fractions by SDS slab gel PAGE. M: molecular weight markers (in kDa) were platelet myosin heavy chain (220), phosphorylase B (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), and soybean trypsin inhibitor (20). EB, S, 1 and 2–3: as in Fig. 2; (B) Molecular mass determination. Open points: apoLp-I: 245 kDa, apoLp-II: 80 kDa.

oenocytes as in *B. germanica* (Fan et al., 2003), to the cuticle. This has never been studied in Hymenoptera where it is known that HCs are especially important for chemical communication. Therefore, lipid extraction was performed on the purified HDLp and its HCs isolated by TLC and analysed by GC–MS. The HCs associated with *P. villosa* HDLp are qualitatively and quantitatively similar to those of the cuticle (Table 1 and Fig. 5, cuticle pool and lipophorin). As for the cuticle (Lucas et al., 2002), HDLp HCs cover a large range of chain lengths (C26–47). They have even- or odd-numbered chain lengths, are saturated or unsaturated, linear or branched with branching occurring on even- or odd-numbered carbon atoms. Internally branched monomethylalkanes (on carbons inner than $C_6$) appear as mixtures of unresolved isomers among which the 11- to 13-isomers are dominant. Dimethylalkanes are also observed as mixtures, but there is more diversity in branchings. Trimethylalkanes are present in very small quantities whereas *n*-alkadienes—which are more prevalent HCs in another species, *Pachycondyla apicalis* (Hefetz et al., 2001), are completely absent.

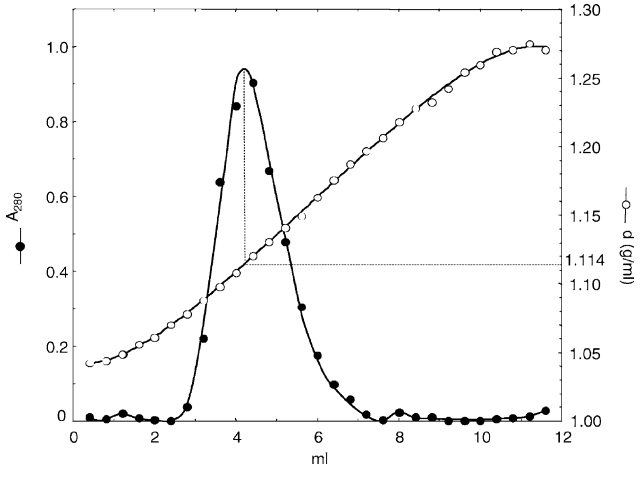


Fig. 4. Density determination of isolated HDLp.

### 3.3. Lipophorin and postpharyngeal gland hydrocarbons

Detailed comparison of hydrocarbons associated with HDLp or extracted from PPGs of the same set of individuals showed clear quantitative differences, even if we do not take into account the unresolved isomers (Fig. 5, PPG pool and lipophorin). Both HDLp and cuticle HC profiles are divided into two subgroups (Fig. 5). The first eluting subgroup (peaks 1–27, Table 1) represents, in HDLp for instance, 20.6% of all HCs, *n*-alkanes and externally branched monomethylalkanes (ext-MeA) being its main components (0.7% and 16.2%, respectively). The later eluting subgroup (peaks 28–63) represents 79.4% and is essentially

Table 1
Percentages of HCs from cuticle, PPG (mean values ± s.d., $n = 18$) and HDLp in order of increasing retention times

| Peak | Compound | Cuticle | | PPG | | Cuticle pool | PPG pool | HDLp | Diagnostic ions |
|---|---|---|---|---|---|---|---|---|---|
| 1 | nC26 | tr | | tr | | tr | tr | tr | 366 |
| 2 | nC27 | tr | | tr | | 0.11 | tr | tr | 380 |
| 3 | 13-, 11-MeC27 | tr | | tr | | tr | tr | tr | 196/7, 224/5; 168/9, 252/3; 379 (M − 15) |
| 4 | 9-MeC27 | tr | | tr | | tr | tr | tr | 140/1, 280/1, 379 (M − 15) |
| 5 | 7-MeC27 | tr | | tr | | tr | tr | tr | 112/3, 308/9, 379 (M − 15) |
| 6 | 5-MeC27 | tr | | tr | | tr | tr | tr | 84/5, 336/7, 379 (M − 15) |
| 7 | nC28 | tr | | tr | | 0.17 | tr | tr | 394 |
| **8** | **2-MeC28** | **2.60** | (**0.37**) | tr | | **2.91** | tr | tr | 365 (M − 43), 393 (M − 15), 408 |
| 9 | X-nC29:1 | tr | | tr | | tr | tr | tr | 406 |
| 10 | X′-nC29:1 | tr | | tr | | tr | tr | tr | 406 |
| 11 | 2,16-; 2,14-diMeC28 | tr | | tr | | tr | tr | tr | 196/7, 252/3; 224/5; 407 (M − 15) |
| **12** | **nC29** | 1.90 | (0.35) | tr | | **2.09** | tr | 0.44 | 408 |
| 13 | 15-, 13-, 11-, 7-MeC29 | 0.35 | (0.08) | 0.14 | (0.03) | 0.35 | tr | 0.97 | 224/5; 196/7, 252/3; 168/9, 280/1; 407 (M − 15) |
| 14 | 13,17-diMeC29 | tr | | tr | | 0.13 | tr | 0.34 | 196/7, 267, 421 (M − 15) |
| 15 | 11,19-diMeC29 | tr | | tr | | tr | tr | tr | 168/9, 295, 421 (M − 15) |
| 16 | 2-MeC29 | tr | | tr | | tr | tr | tr | 379 (M − 43), 407 (M − 15), 422 |
| 17 | 9,17-diMeC29 | tr | | tr | | tr | tr | tr | 140/1, 196/7, 267, 323, 421 (M − 15) |
| **18** | **3-MeC29** | **8.43** | (**0.85**) | 0.15 | (0.02) | **6.70** | tr | **9.25** | 364/5 (M − 57), 393 (M − 29), 422 |
| 19 | nC30 | 0.82 | (0.14) | tr | | 0.58 | tr | 0.25 | 422 |
| 20 | 14-, 13-, 11-MeC30 | tr | | tr | | tr | tr | 0.47 | 210/1, 252/3; 196/7, 266/7; 168/9, 294/5, 421 (M − 15) |
| 21 | 8-, 9-MeC30 | tr | | tr | | tr | tr | 0.43 | 126/7, 336/7; 140/1, 322/3; 421 (M − 15) |
| **22** | **2-MeC30** | **4.53** | (**0.50**) | 0.16 | (0.02) | **5.45** | 0.10 | **5.09** | 393 (M − 43), 421 (M − 15), 436 |
| 23 | 2,20-; 2,18-; 2,16-diMeC30 | tr | | tr | | 0.40 | tr | 1.02 | 168/9, 308/9; 196/7, 280/1; 224/5, 252/3, 435 (M − 15) |
| 24 | nC31 | 0.41 | (0.08) | tr | | tr | tr | tr | 436 |
| 25 | 15-, 13-, 11-MeC31 | 0.20 | (0.05) | 0.17 | (0.03) | 0.23 | tr | 0.52 | 224/5, 252/3; 196/7, 280/1; 168/9, 308/9; 435 (M − 15) |
| 26 | 13,19-; 11,19-diMeC31 | tr | | 0.13 | (0.02) | tr | tr | tr | 196/7, 295; 168/9, 196/7, 295, 323, 449 (M − 15) |
| 27 | 3-MeC31 | 1.56 | (0.21) | tr | | 1.91 | tr | 1.84 | 392/3 (M − 57), 421 (M − 29), 450 |
| 28 | Xi-MeC33:1 | tr | | tr | | tr | tr | tr | 476 |
| 29 | 17-, 15-, 13-, 11-MeC33 | tr | | 0.18 | (0.02) | tr | tr | tr | 252/253; 224/5, 280/1; 196/7, 308/9; 168/169, 336/7, 463 (M − 15) |
| 30 | 13,17-diMeC33 | tr | | 0.37 | (0.04) | tr | tr | tr | 196/7, 252/3, 267, 323, 477 (M − 15) |
| 31 | 17-, 15-, 14-, 13-, 12-MeC34 | tr | | tr | | tr | tr | tr | 252/3, 266/7; 224/5, 294/5; 210/1, 308/9; 196/7, 322/3; 182/3, 336/7, 477 (M − 15) |
| 32 | 15,19-; 13,17-diMeC34 | tr | | 0.22 | (0.02) | tr | 0.11 | tr | 224/5, 238/9, 295, 309; 196/7, 266/7, 337, 491 (M − 15) |
| 33 | Xi-MeC35:1 | 0.20 | (0.05) | 0.97 | (0.13) | 0.42 | 0.31 | 0.60 | 504 |
| **34** | **17-, 15-, 13-MeC35** | 0.59 | (0.12) | **2.09** | (**0.15**) | 0.81 | 0.95 | 1.24 | 252/3, 280/1; 224/5, 308/9; 196/7, 336/7, 491 (M − 15) |
| 35 | Xi-diMeC35:1 | tr | | tr | | tr | tr | tr | 518 |
| **36** | **13,19-; 13,17-diMeC35** | **6.65** | (**0.69**) | **15.44** | (**0.94**) | **3.99** | **6.41** | **4.09** | 196/7, 252/3, 295, 351; 196/7, 267, 280/1, 351, 505 (M − 15) |
| 37 | Xi-MeC36:1 | tr | | 0.16 | (0.02) | tr | tr | 0.14 | 518 |
| 38 | 14-MeC36 | tr | | 0.40 | (0.03) | 0.25 | 0.26 | 0.29 | 210/1, 336/7, 505 (M − 15) |
| **39** | **14,18-; 12,16-diMeC36** | 0.66 | (0.14) | **2.65** | (**0.11**) | 1.12 | 1.66 | 1.63 | 210/1, 280/1, 351; 182/3, 253, 308/9, 379, 519 (M − 15) |
| **40** | **Xi-MeC37:1** | 1.39 | (0.39) | **2.83** | (**0.24**) | **3.30** | 1.72 | **2.96** | 532 |
| **41** | **19-, 17-, 15-, 13-MeC37** | **5.73** | (**0.31**) | **4.50** | (**0.43**) | **7.69** | **6.45** | **7.69** | 280/1; 252/3, 308/9; 224/5, 336/7; 196/7, 364/5, 519 (M − 15), |
| **42** | **15,19-; 13,17-diMeC37** | **50.23** | (**3.26**) | **50.10** | (**1.99**) | **35.95** | **61.78** | **42.44** | 224/5, 280/1, 295, 351; 196/7, 267, 308/9, 379, 533 (M − 15) |
| 43 | 13,17,21-triMeC37 | tr | | 0.69 | (0.09) | 0.75 | 0.53 | 0.64 | 196/7, 252/3, 267, 323, 337, 393, 547 (M − 15) |
| 44 | Xi-MeC38:1 | tr | | 0.24 | (0.03) | 0.52 | 0.22 | 0.37 | 546 |

Table 1 (*continued*)

| Peak | Compound | Cuticle | | PPG | | Cuticle pool | PPG pool | HDLp | Diagnostic ions |
|---|---|---|---|---|---|---|---|---|---|
| 45 | 15-, 14-, 13-, 12-, 11-, 7-MeC38 | tr | | 0.34 | (0.03) | 0.60 | 0.37 | 0.49 | 224/5, 350/1; 210/1, 364/5; 196/7, 378/9; 182/3, 392/3; 168/9, 406/7; 112/3, 462/3, 533 (M – 15) |
| 46 | 14,18-; 13,17-diMeC38 | 0.50 | (0.14) | 1.16 | (0.08) | 1.19 | 1.14 | 1.11 | 210/1, 281, 308/9, 379; 196/7, 267, 322/3, 393, 547 (M – 15) |
| 47 | 12,16,20-triMeC38 | tr | | 0.10 | (0.01) | tr | tr | tr | 182/3, 253, 280/1, 323, 351, 421, 561 (M – 15) |
| **48** | **Xi-MeC39:1** | 1.08 | (0.36) | 1.33 | (0.13) | **3.24** | 1.40 | 1.84 | 560 |
| **49** | **19-, 17-, 15-, 14-, 13-MeC39** | **3.33** | **(0.48)** | **2.74** | **(0.20)** | **5.53** | **2.95** | **3.59** | 280/1, 308/9; 252/3, 336/7; 224/5, 364/5; 210/1, 378/9; 196/7, 392/3, 547 (M – 15) |
| **50** | **13,19-diMeC39** | **8.11** | **(0.84)** | **8.08** | **(0.28)** | **8.70** | **8.87** | **7.00** | 196/7, 295, 308/9, 407, 561 (M – 15) |
| 51 | 13,17,21-triMeC39 | tr | | 0.53 | (0.05) | 0.69 | 0.57 | 0.63 | 196/7, 267, 280/1, 337, 351, 421, 575 (M – 15) |
| 52 | 20-, 14-MeC40 | tr | | 0.11 | (0.01) | 0.17 | tr | 0.13 | 294/5, 308/9; 210/1, 392/3, 561 (M – 15) |
| 53 | 14,20-diMeC40 | tr | | 0.23 | (0.02) | 0.23 | 0.22 | 0.18 | 210/1, 308/9, 407, 575 (M – 15) |
| 54 | Xi-MeC41:1 | tr | | 0.18 | (0.03) | 0.28 | 0.13 | 0.16 | 588 |
| 55 | X′i-MeC41:1 | tr | | tr | | 0.30 | 0.11 | 0.17 | 588 |
| 56 | 21-, 19-, 17-, 15-, 13-MeC41 | 0.16 | (0.07) | 0.48 | (0.05) | 0.81 | 0.46 | 0.55 | 308/9; 280/1, 336/7; 252/3, 364/5; 224/5, 392/3; 196/7, 420/1, 575 (M – 15) |
| 57 | 15,21-; 13,21-; 13,19-diMeC41 | 0.25 | (0.11) | 0.99 | (0.09) | 0.98 | 0.98 | 0.76 | 224/5, 308/9, 323, 407; 196/7, 308/9, 323, 435; 196/7, 295, 336/7, 435, 589 (M – 15) |
| 58 | 13,17,21-triMeC41 | tr | | 0.17 | (0.02) | 0.14 | 0.17 | tr | 196/7, 267, 308/9, 337, 379, 449, 603 (M – 15) |
| 59 | 14,20-diMeC42 | tr | | tr | | tr | tr | tr | 210/1, 309, 336/7, 435, 603 (M – 15) |
| 60 | 13-MeC43 | tr | | 0.15 | (0.02) | 0.20 | 0.25 | tr | 196/7, 448/9, 603 (M – 15) |
| 61 | 13,19-; 13,21-diMeC43 | 0.12 | (0.06) | 0.65 | (0.11) | 0.47 | 0.56 | 0.30 | 196/7, 295, 364/5, 463; 196/7, 323, 336/7, 463, 617 (M – 15) |
| 62 | 13,17,21-triMeC43 | tr | | tr | | tr | tr | tr | 196/7, 267, 336/7, 407, 477, 631 (M – 15) |
| 63 | 13,21-diMeC45 | tr | | 0.52 | (0.07) | 0.26 | 0.41 | 0.18 | 196/7, 323, 364/5, 491, 645 (M – 15) |

The 'cuticle pool', 'PPG pool' and the HDLp extract came from the same 40 workers. Compounds in bold type: >2%; trace (tr): <0.1%; X: unknown position of double bond; Xi: unknown position of internal methyl branch.

composed of successive associations of small amounts of Xi-methylalkenes (methylalkenes with undetermined double bond and internal methyl branching positions, peaks 33, 40 and 48), monomethylalkanes (mainly the 13-isomer) (34, 41 and 49) and 13,17- and/or 13,19-dimethylalkanes (36, 42 and 50), in order of increasing abundance (5.4%, 12.5% and 53.5%). In the PPG, the first subgroup of *n*-alkanes and ext-MeA is almost completely absent and represents only 1.1% of the total. The second subgroup represents 98.9% and conserves the same organization (same peak number) with similar increase order (5.1%, 9.3% and 73.6%, respectively). Representative mass spectra are shown for externally branched monomethylalkanes (Fig. 6A), internally branched monomethylalkanes (Fig. 6B) Xi-monomethylalkenes (Fig. 6C), and internally branched dimethylalkanes (Fig. 6D).

The results of PCA of the percentages of the prevalent HCs (mean percentage >2%) from PPG and cuticle are shown in Fig. 7. The first two factors, F1 and F2, represent 44% and 26% of the total variance, respectively. The 'cuticle pool' and 'PPG pool' data arose from the total HC extracts of cuticles and PPGs from the same 40 workers used to isolate the HDLp. The PPG and the cuticle points cluster at opposite sides along the F1 axis. The HDLp point is also found within the cuticle cluster whereas the 'PPG pool' point is close to the PPG cluster.

Plotting the percentages of HCs by structural classes confirms these observations (Fig. 8). The *n*-alkanes and externally branched monomethylalkanes are mostly absent from the PPG compared to HDLp or the cuticle ($P < 0.001$). The internally branched monomethylalkanes are not statistically significantly different ($P > 0.05$) between PPG and cuticle. Conversely, the dimethylalkanes (and also trimethylalkanes which are in small quantities) are statistically significantly ($P < 0.001$) lower in cuticle than in PPG. The percentages of this class of HCs from HDLp and 'cuticle pool' are also lower than that of the 'PPG pool'. Concerning alkenes, the cuticle presents lower quantities than the PPG ($P < 0.01$). Unfortunately, the

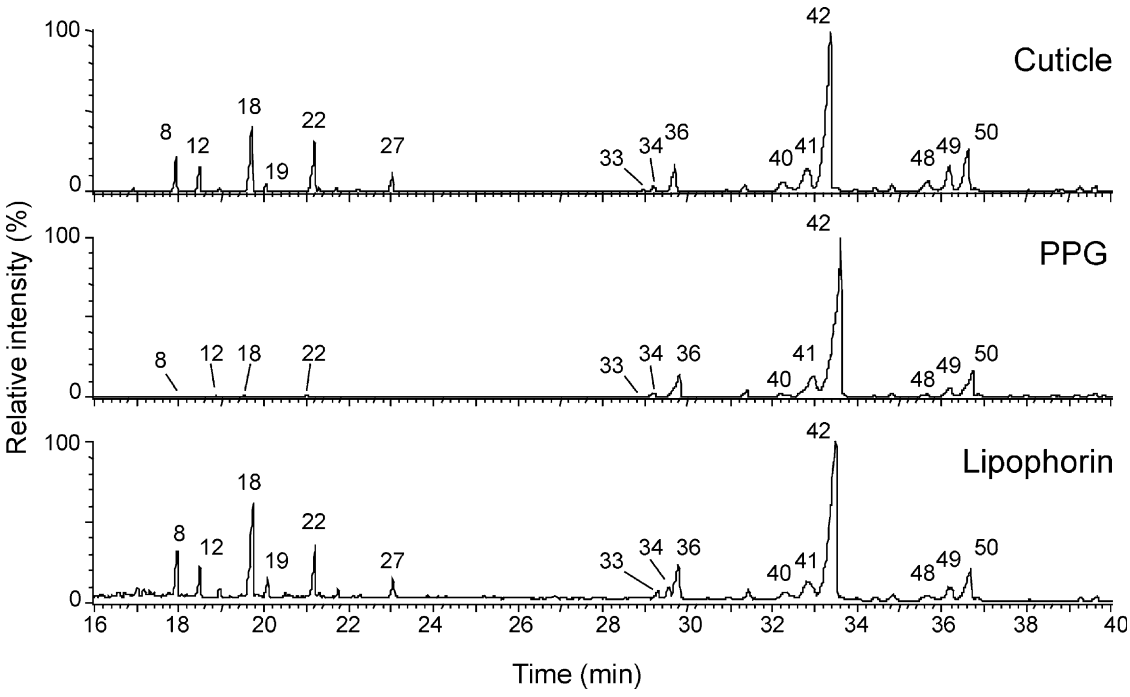


Fig. 5. Total ion GC–MS chromatograms of the HCs from cuticle, PPG and HDLp of the same 40 workers. Numbers refer to the peaks listed in Table 1.

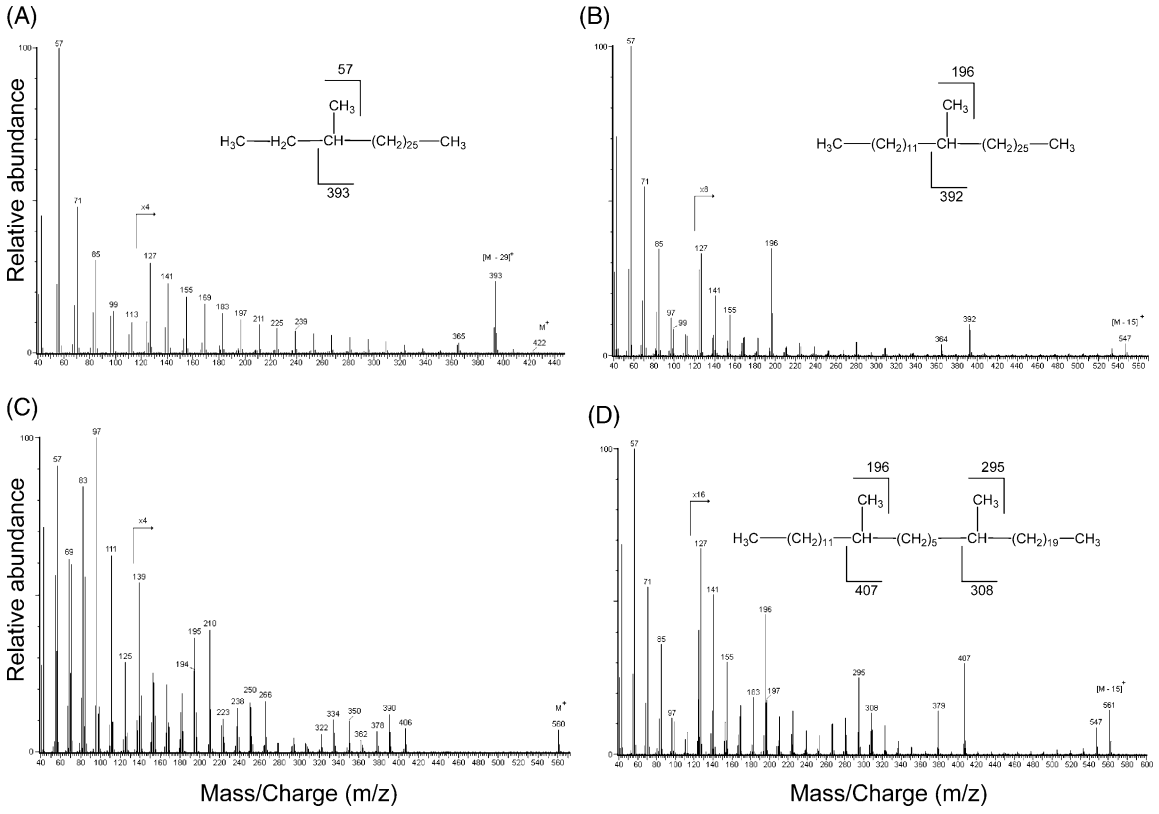


Fig. 6. (A) Mass spectrum of 3-MeC29 (peak 18); (B) mass spectrum of 13-Me C39 (peak 49); (C) mass spectrum of Xi-MeC39:1 (peak 48), probably a mixture of isomers of monounsaturated homologs of 13-MeC39 (peak 49): the $m/z$ values of M+ and fragment ions are those of monounsaturated species, and the diagnostic fragments, arising from cleavages α and β of the branching points, are in clusters 1–3 amu lower than the corresponding doublets of the related methylalkanes ($m/z$ range 139–560). Furthermore, the peak is at about −0.3 equivalent chain length from peak 49. Nevertheless, precise structural determinations (methyl branching and double bond position) would need isolation in sufficient amounts and chemical modifications for further spectral analyses; (D) mass spectrum of 13,19-diMeC39 (peak50).

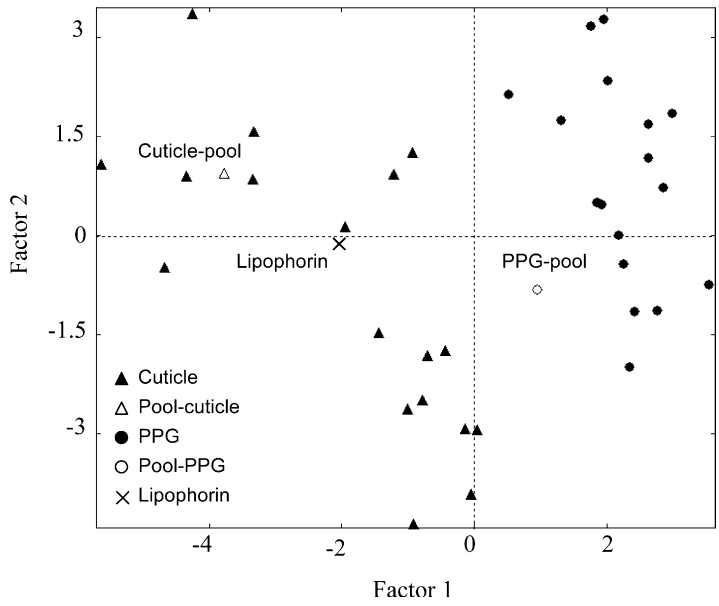


Fig. 7. PCA of HC percentages of cuticle, PPG and lipophorin. The first two factors F1 and F2 represent respectively 44% and 26% of the total variance. The variables were normalized during the analysis in order to obtain a better comparison. The 'cuticle pool' and 'PPG pool' points on the PCA represent the total HC extracts from the same 40 workers that have been used to isolate the lipophorin.

quantities of alkenes are too small to be sorted by class like alkanes, but they are mainly internally branched methylalkenes. *n*-Alkanes and ext-MeA being discarded, the mean percentages of the other HCs have been recalculated: they clearly illustrate the structural similarities between the HCs common to both HDLp and PPG.

Comparison of the HC profiles of the cuticle and the PPG have also been performed, on the two close species *Pachycondyla* cf. *inversa* and *Pachycondyla subversa* (Lucas, 2002), and led to the same conclusions: qualitative similarity but marked decrease of *n*-alkanes and MeA-ext and opposite clustering of cuticle and PPG points in PCA analyses.

### 3.4. Variations between colonies

Statistical comparison of the recalculated data between cuticular and PPG HCs of *P. villosa* shows that several HCs are not significantly different ($P > 0.05$) and are therefore considered as 'stable'. Such is the case, for instance, in Pv3 colony, for 11 HCs listed underlined in Table 2. All are internally branched (on carbons inner than $C_6$) mono-, dimethylakanes or monomethylalkenes. Four of them are prevalent (>2%) and none of them are mixed externally/internally branched dimethylalkanes.

Furthermore, this table also shows that such stable HCs vary between colonies (Table 2), in comparisons

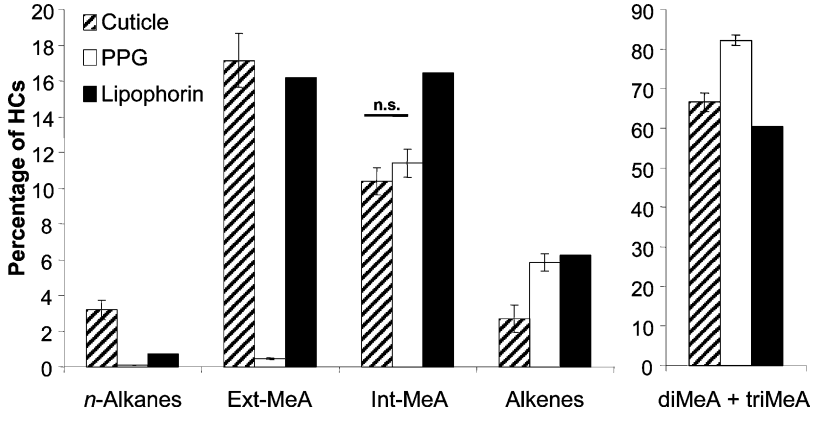


Fig. 8. HC percentages of cuticle, PPG and lipophorin (mean ± s.d.). There are significant differences ($P < 0.05$) between cuticle and PPG values ($n = 18$) except for the group marked n.s. The 'cuticle pool' and 'PPG pool': as in Fig. 7. Ext-MeA: externally branched monomethylalkanes; Int-MeA: internally branched monomethylalkanes; diMeA: dimethylalkanes; triMeA: trimethylalkanes.

Table 2
Recalculated percentages of HCs from cuticle, PPG of three *P. villosa* colonies (mean values ± s.d., $n = 6$, 5 and 7) in order of increasing retention times

| Peak | Compound | Cuticle Pv3 | | PPG Pv3 | | PPG Pv6 | | PPG Pv21 | |
|---|---|---|---|---|---|---|---|---|---|
| 13 | 15-, 13-, 11-, 7-MeC29 | 0.77 | (0.19) | 0.19 | (0.08) | 0.20 | (0.02) | tr | |
| 25 | 15-, 13-, 11-MeC31 | 0.48 | (0.13) | 0.24 | (0.07) | 0.19 | (0.03) | *0.10* | (*0.01*) |
| 26 | 13,19-; 11,19-diMeC31 | tr | | 0.19 | (0.04) | 0.10 | (0.01) | tr | |
| 29 | 17-, 15-, 13-, 11-MeC33 | 0.18 | (0.03) | 0.22 | (0.03) | 0.22 | (0.02) | *0.11* | (*0.01*) |
| 30 | 13, 17-diMeC33 | 0.23 | (0.03) | 0.41 | (0.10) | 0.45 | (0.03) | 0.27 | (0.05) |
| 32 | 15,19-; 13,17-diMeC34 | tr | | 0.20 | (0.04) | 0.31 | (0.02) | 0.17 | (0.03) |
| 33 | Xi-MeC35 : 1 | 0.50 | (0.13) | 0.83 | (0.21) | *1.64* | (*0.09*) | 0.64 | (0.09) |
| **34** | **17-, 15-, 13-MeC35** | 1.31 | (0.20) | **2.05** | (**0.18**) | ***2.88*** | (***0.11***) | 1.58 | (0.16) |
| 35 | Xi-diMeC35:1 | 0.14 | (0.04) | tr | | tr | | tr | |
| **36** | **13, 19-; 13, 17-diMeC35** | **10.16** | (**1.65**) | **15.90** | (**2.49**) | **17.74** | (**0.72**) | **13.62** | (**0.79**) |
| 37 | Xi-MeC36:1 | tr | | 0.11 | (0.02) | *0.29* | (*0.02*) | 0.11 | (0.01) |
| 38 | 14-MeC36 | tr | | 0.33 | (0.02) | *0.57* | (*0.02*) | 0.35 | (0.02) |
| **39** | **14,18-; 12,16-diMeC36** | 1.55 | (0.12) | **2.28** | (**0.12**) | ***3.24*** | (***0.06***) | **2.58** | (**0.1**) |
| **40** | **Xi-MeC37:1** | **4.43** | (**0.29**) | **2.54** | (**0.46**) | ***3.82*** | (***0.22***) | **2.42** | (**0.31**) |
| **41** | **19-, 17-, 15-, 13-MeC37** | **7.40** | (**0.30**) | **4.09** | (**0.61**) | **6.20** | (**0.82**) | **3.71** | (**0.54**) |
| **42** | **15, 19-; 13, 17-diMeC37** | **47.89** | (**4.74**) | **50.33** | (**2.98**) | ***42.31*** | (***1.61***) | **56.12** | (**2.82**) |
| 43 | 13,17,21-triMeC37 | tr | | 0.44 | (0.14) | *1.08* | (*0.11*) | 0.64 | (0.13) |
| 44 | Xi-MeC38:1 | tr | | 0.18 | (0.04) | *0.35* | (*0.04*) | 0.21 | (0.05) |
| 45 | 15-, 14-, 13-, 12-, 11-, 7-MeC38 | tr | | 0.27 | (0.05) | *0.47* | (*0.05*) | 0.32 | (0.05) |
| 46 | 14, 18-; 13, 17-diMeC38 | 1.29 | (0.38) | 1.06 | (0.15) | 1.40 | (0.11) | 1.09 | (0.14) |
| **48** | **Xi-MeC39:1** | **3.42** | (**0.90**) | 1.18 | (0.26) | 1.76 | (0.16) | 1.18 | (0.17) |
| **49** | **19-, 17-, 15-, 14-, 13-MeC39** | **5.78** | (**1.15**) | **2.84** | (**0.48**) | **3.17** | (**0.16**) | **2.39** | (**0.28**) |
| **50** | **13, 19-diMeC39** | **12.45** | (**1.96**) | **8.21** | (**0.73**) | **7.53** | (**0.25**) | **8.46** | (**0.30**) |
| 51 | 13,17,21-triMeC39 | tr | | 0.42 | (0.09) | *0.76* | (*0.06*) | 0.47 | (0.08) |
| 52 | 20-, 14-MeC40 | tr | | 0.11 | (0.02) | 0.12 | (0.01) | 0.10 | (0.02) |
| 53 | 14,20-diMeC40 | tr | | 0.22 | (0.03) | 0.21 | (0.02) | 0.25 | (0.03) |
| 54 | Xi-MeC41:1 | tr | | 0.16 | (0.04) | 0.10 | (0.01) | 0.26 | (0.05) |
| 55 | X′i-MeC41:1 | tr | | 0.12 | (0.03) | tr | | tr | |
| 56 | 21-, 19-, 17-, 15-, 13-MeC41 | 0.62 | (0.18) | 0.61 | (0.10) | *0.38* | (*0.04*) | 0.45 | (0.07) |
| 57 | 15, 21-; 13, 21-; 13, 19-diMeC41 | 0.96 | (0.32) | 1.32 | (0.13) | *0.65* | (*0.07*) | *0.97* | (*0.13*) |
| 58 | 13,17,21-triMeC41 | tr | | 0.22 | (0.05) | 0.16 | (0.02) | 0.14 | (0.03) |
| 60 | 13-MeC43 | tr | | 0.22 | (0.04) | *0.11* | (*0.02*) | *0.11* | (*0.03*) |
| 61 | 13,19-; 13,21-diMeC43 | 0.46 | (0.21) | 1.11 | (0.21) | *0.45* | (*0.08*) | *0.41* | (*0.09*) |
| 62 | 13,17,21-triMeC43 | tr | | 0.14 | (0.02) | 0.11 | (0.02) | tr | |
| 63 | 13,21-diMeC45 | tr | | 0.88 | (0.09) | *0.41* | (*0.07*) | *0.28* | (*0.05*) |

Statistically not different values between cuticle and PPG of Pv3 are underlined.
Significantly different values between Pv3 PPG with either Pv6 or Pv21 PPG are in italics.
Bold type, tr and Xi: as in Table 1.

by pairs (Pv3–Pv6, Pv3–Pv21). Similarly, the same kind of comparison of Pv6 with either Pv3 or Pv21, or Pv21 with either Pv3 or Pv6 leads to similar conclusions (data not shown).

## 4. Discussion

Over the past decades, numerous studies have emphasized the importance of hydrocarbons in insects. Due to their large surface/volume ratio, protection against desiccation is vital, and their hydrophobicity as well as their alignment on the cuticle provide a passive but effective barrier to water loss. Moreover, many insect species utilize hydrocarbons in chemical communication. In social insects particularly, long chain hydrocarbons are involved in species and nestmate recognition. The variety of their structures (chain lengths, number and positions of methyl branching or double bonds) as well as their combinations allow multiple information processing. All these hydrocarbons are deposited on—and not synthesized in—the cuticle. They come from internally synthesized hydrocarbons and are carried by HDLp. In ants, hydrocarbons are also found in the PPG and induce behavioural effects as do the cuticular hydrocarbons. To further investigate the circulation and eventually differentiate the roles of the components of the mixtures of hydrocarbons in each case, we have undertaken a comparative study of cuticular, lipophorin and PPG hydrocarbons.

This comparison implied prior purification of the lipoprotein. We report here the first purification and

characterization of an ant HDLp. The native HDLp has a molecular weight of approximately 820 kDa. This molecular mass is higher than published data on lipophorins of other species which ranged from 580 (*Locusta migratoria*, Van der Horst, 1990) to 730 kDa (*Bombyx mori*, Kim and Kim, 1994). The unique peak of absorbance at 280 nm obtained on recentrifugation, the density of 1.114, the composition in subunits and their molecular masses showed that HDLp had been purified. This purification has allowed a detailed analysis of the HCs associated with this lipoprotein.

Few quantitative differences between HDLp and cuticular extracts were observed as confirmed by the bivariate plot of all the extracts. This supports a possible role of HDLp as an HC carrier from the internal sites of biosynthesis, probably oenocytes (Fan et al., 2003), to the cuticle as already shown by studies on a number of insect species. However, the mechanism of HC transfer from this hemolymph lipoprotein to the epicuticle is not yet clear, in any insect species. The existence of potential specialized structures has been suggested (Noirot and Quennedey, 1991; Quennedey, 1998), but clear-cut evidence remains to be found.

Interestingly, clear differences were observed between PPG and HDLp hydrocarbons: whereas long chain internally branched monomethyl-, dimethylalkanes or alkenes were present in comparable percentages, shorter chain normal and externally branched methylalkanes were almost completely absent from PPG. Do these differences come from the ability of the gland to synthesize hydrocarbons on its own? In the closely related species, *Pachycondyla inversa* (Lucas, 2002) as well as in the more advanced ant *Cataglyphis niger* (Soroker and Hefetz, 2000), when PPGs were incubated with radioactive acetate as a precursor, no labelled HCs were found whereas other radioactive lipids were produced. PPGs thus appear unable to synthesize HCs. Nevertheless, after abdominal injection of the same precursor to ants of the related species *Pachycondyla apicalis*, an appreciable amount of labelled HCs was found in the PPGs (Soroker et al., 1998, 2003).

These HCs may be transferred from hemolymph HDLp but the differences observed in HC compositions between HDLp and PPG would then require some selectivity in this transfer to exclude normal and externally branched methylalkanes. Such selectivity has been observed in the tiger moth *Holomelina aurantiaca* (Schal et al., 1998a, b), and the house fly *Musca domestica* (Schal et al., 2001).

In *Pachycondyla apicalis*, an external mechanism for the transfer of cuticular HCs to the PPG has been suggested to involve self-grooming (Soroker et al., 1998; Hefetz et al., 2001). During this behaviour, ants pass the tarsal brushes of their front legs through their mouths into which the PPG opens. And, indeed, after injection of radioactive acetate precursor, the amounts of labelled HCs in PPG markedly decreased when ants' mouthparts were blocked or front legs immobilized. Such a mechanism may be efficient as these brushes represent a large surface of the cuticle which does bear a large proportion of cuticular HCs (more than one fourth). Such brushes are also present on front legs in *P. villosa* and involved in self-grooming. They may thus be favoured unloading sites for HCs towards the PPG.

This transfer does not occur solely within an individual; labelled HC exchanges were observed between nestmates in several ant species (Soroker et al., 1994, 1995, 2003; Meskali et al., 1995). In those studies, an HC probe first present on only one worker was rapidly transmitted to the cuticles of all other nestmates encountered as well as to their PPGs. In *Pachycondyla apicalis*, this interindividual HC transfer occurred mainly through physical contact and less so through allo-grooming (Soroker et al., 2003).

Taken together, published data and those presented here agree with and complement the general hydrocarbons circulation scheme, for *Pachycondyla* ants, hypothesized by Lenoir et al. (1999). HCs internally synthesized in oenocytes are transported by HDLp through the hemolymph to the cuticle, especially to the basitarsal brushes of the front legs. These are licked during self-grooming and individual cuticular HCs transferred to the PPG. A complementary direct and selective transfer of internally branched methylakanes and alkenes from lipophorin to PPG is possible, but its mechanism has to be clarified (Schal et al., 2003). PPG hydrocarbons are dispatched again on the other parts of the same individual's cuticle or on its nesmates' cuticles during physical contacts and allo-grooming. This recurrent and mutual redistribution of HCs on the cuticle of every member of a colony might allow the formation of a colonial signature.

The chemical properties of the different HC classes present or not in the PPG may be related to different roles. Cuticular *n*-alkanes and externally branched monomethylalkanes have high melting temperatures (Tms) and are more efficiently organized to protect insects from water loss (Gibbs and Pomonis, 1995; Gibbs, 1998 and references in these reviews; Wagner et al., 1998, 2001; Kaib et al., 2000; Young et al., 2000). For the same reason, they are also less easily collected into PPG during grooming. On the contrary, it is well known that internal methyl branching as well as unsaturation markedly lower the Tms of HCs (Gibbs, 1998) and hence increase their fluidity. This might explain why internally branched monomethyl- and dimethylalkanes and alkenes are easily collected into PPG and redistributed during self- or allo-grooming and physical contacts, which supports their possible role in chemical communication. Indeed, despite the

absence of normal and externally branched methylalkanes, PPG HCs keep a behavioural role in nestmate recognition (Soroker et al., 1994; Lahav et al., 1999). Which of the remaining HCs common to cuticle and PPG could have a role in nestmate recognition? Those which are in the same proportion in both of them may be good candidates. *n*-Alkanes and ext-MeA being discarded, we have recalculated the mean percentages of the other HCs. Statistical comparison between cuticle and PPG HCs then showed that several compounds were not significantly different. These 'stable' HCs might be important in chemical communication. Moreover, comparison between different colonies shows that these HCs vary from colony to colony. They might thus be involved in colonial signature. It remains to compare these statistical data with behavioural studies using different hydrocarbon structural classes or components isolated from the extracts.

On the cuticle, the two groups of HCs (waterproofing and insect communication) are precariously balanced. Depending on the environment or life cycle, the use of HCs for waterproofing or chemical communication is favoured. PPG could be considered as an evolutionary answer to the problem raised by the coexistence of the two different types of cuticular HCs. While resistance against high temperatures may be a relatively slow process, colonial signature must be rapidly restored for nestmate recognition to avoid aggressive and even fatal reactions. From this point of view, the PPG constitutes a reservoir for a great quantity of rapidly accessible recognition cues.

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