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Chemical heterogeneity in inbred European population of the invasive hornet *Vespa velutina*
nigrithorax

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Key words

Invasive species, Inbreeding, Cuticular hydrocarbons, Queen pheromone, Yellow-legged
hornet.

Abstract

Invasive social insect populations that have been introduced to a new environment through a
limited number of introduction events generally exhibit reduced variability in their chemical
signatures (cuticular hydrocarbons) compared to native populations of the same species. The
reduced variability in these major recognition cues could be caused by a reduction of genetic
diversity due to a genetic bottleneck. This hypothesis was tested in an inbred European
population of the invasive hornet *Vespa velutina nigrithorax*. Our results show that, in spite of
the limited amount of genetic diversity present in the European population, the chemical
signatures of individuals were highly heterogeneous according to their caste, sex, and colony
origin. In queens, some specific saturated and unsaturated hydrocarbons were identified.
These results suggest that epigenetic and/or environmental factors could play a role in
modifying cuticular hydrocarbon profiles in this introduced hornet population despite the
observed reduction of genetic diversity.

Introduction

The insect cuticle is covered by a thin lipid layer, which largely consists of cuticular
hydrocarbons (CHCs). Because of their chemical properties, CHCs primarily act to limit
insect water loss through the creation of a surface barrier. In addition, they also serve as
contact pheromones, i.e. recognition cues, for inter- and intraspecific recognition among
insects. The CHC profile of an individual serves as a personalized chemical signature
(Blomquist and Bagnères 2010; Ginzl and Blomquist 2016). Several studies have
demonstrated a strong link between the social behavior of insects and their cuticular
signatures (Bagnères et al. 1991; Howard and Blomquist 2005; Leconte and Hefetz 2008).

CHCs are mainly involved in the recognition of nestmates (Howard and Blomquist 2005; van Zweden and D'Ettorre 2010), castes (Bagnères et al. 1998; Kaib et al. 2000; Smith and Taylor 1990), and worker's tasks (Rahman et al 2016). Therefore, chemical signatures can vary, and the degree of variation depends on a wide range of endogenous factors, such as the endocrine system or enzymes (Darrouzet et al. 2014b; Fan et al. 2004; Lengyel et al. 2007), and exogenous factors, such as climate and season (Bagnères et al. 1990), diet (Liang and Silverman 2000), parasitism (Bagnères et al. 1996; Lebreton et al. 2010; LeConte et al. 2015), or time (Bagnères et al. 2011; Lebreton et al. 2009). The relative importance of these factors varies among species (VanderMeer and Morel 1998). However, CHC profiles are modified not only by the social or biotic environment but are also under genetic control in both social and solitary insects (Bonavita-Cougourdan et al. 1996; Dronnet et al. 2006; Etges et al. 2009; Thomas and Simmons 2008; Vauchot et al. 1996). Consequently, variation in CHC profiles has been used to identify species (Bagnères and Wicker-Thomas 2010) and to differentiate populations (Bonelli et al. 2015; Nowbahari et al. 1990). This type of chemotaxonomy, i.e. the use of chemical compounds, has been productively used in numerous insect species. However, less attention has been paid to the extent or function of chemical variation within invasive species, which in their introduced ranges often experience habitats and species interactions that are strikingly different from those of their native environments (Perdereau et al. 2010).

Native to China, the invasive yellow-legged hornet, *Vespa velutina nigrithorax*, was accidentally introduced to southwestern France around 2004, probably in imported ceramic pottery (Haxaire et al. 2006). The species has successfully expanded its range to cover more than 75% of France, and is currently colonizing neighboring countries (Spain, Portugal, Italy, Belgium, Germany, and England) (Darrouzet et al. 2014a; Goldarazena et al. 2015; Monceau et al. 2013; Robinet et al. 2017). Thus far, *V. velutina* invasion in France has two main negative impacts. First, the species preys on several insect and arthropod taxa, thus potentially affecting local biodiversity. In particular, *V. velutina* is a predator of the domestic honeybee, *Apis mellifera*, and could induce economic losses via the destruction of bee colonies (Darrouzet et al. 2014a; Monceau et al. 2013). Second, the species presents a risk to human health. Accidents have occurred, some fatal, when people have inadvertently approached the hornet's nests (de Haro et al. 2010).

New colonies of *V. velutina* are established in the spring by mated queens, after the overwintering period. Colonies first pass through a period in which an increasingly large number of workers are produced in order to ensure colony growth, and then produce sexual

individuals (males and gynes) at the end of the summer (Monceau et al. 2013; Rome et al. 2015). These reproductive individuals emerge between late August and November. After reproductive flights, the gynes disperse and the males die. However, Darrouzet et al. (2015) showed that several colonies produced diploid males all throughout the colony life. As authors explained, this result strongly suggests the occurrence of inbreeding in this species in its invaded range (Europe). This hypothesis was supported by genetic analyses based on a combination of mitochondrial and nuclear markers (Arca et al. 2015; Takeuchi et al. 2017). The authors suggested that the yellow-legged hornet population in France was derived from a single introduction event, and they showed evidence of a strong founder effect. Thus the French populations sampled in that study suffered a notable loss of genetic diversity compared to native populations in China.

In the present study, we analyzed the chemical signature of the invasive hornet *V. velutina nigrithorax* in France, i.e. in an inbreeding context, to test whether this reduced genetic diversity translates into homogeneity in the insects' chemical signatures, as has been reported for other invasive social insect species (*Linepithema humile*: Brandt et al. 2009; *Wasmannia auropunctata*: Errard et al. 2005; *Reticulitermes flavipes*: Perdereau et al. 2010).

Materials and Methods

Insect collection

Hornet colonies were collected in 16 different locations in the Centre-Val-de-Loire region in France (Table 1). In each colony, individuals from each caste and sex present (workers, gynes, males, and queens when possible) were randomly selected, with a maximum of 10 per caste. Insects were weighed and stored at -20°C before being further analyzed.

Cuticular hydrocarbon (CHC) analyses

CHCs were collected by plunging each hornet in 1 ml pentane for 2 x 1 min (vortex). A 500- μ L aliquot of pentane extract was transferred to a new tube. Immediately prior to analyses, 10 μ L of *n*-C20 (*n*-eicosane; concentration 10^{-3} g/ml) was added to each tube as an internal standard. Then, 2- μ L samples were analyzed using gas chromatography (GC) in a CPG Agilent Technologies 7820A GC System equipped with a flame-ionization detector (FID) and a capillary column (HP-5 Agilent Technology, Santa Clara, USA; 30 m x 0.32 mm x 0.25 μ m) using helium as a carrier gas (1.7 ml/min). The following temperature program was used: a first temperature ramp from 50 to 200°C at 8°C/min, a second temperature ramp from 200 to 315°C at 5°C/min, and a 5-min final hold at 315°C.

To identify the compounds (CHCs) in the chemical signatures according to caste and sex (workers, gynes, and males), CHC extracts were pooled from nine random hornets, each from a different randomly selected colony (Table 1S). Workers and gynes were discriminated according to their size and weight (Rome et al. 2015). CHCs from five random queens were also extracted and pooled. The samples were dried, then stored at -20°C. Before being analyzed, 300 µl of pentane was added to each, with the internal standard as in the previous analysis. To determine which compounds were present, we used an Agilent Technologies 7000C GC/MS Triple Quad with a 7890B GC System equipped with the same column as above; we also used the same temperature program as above. Compounds were identified following Blomquist (2010) and using standard *n*-alkanes.

To characterize the CHC profiles, the areas of the main peaks were determined using ChemStation software (Agilent Technologies), and the relative proportions of each peak were calculated as described in Bagnères et al. (1990).

Calculations and statistical analyses

To determine whether we could use chemical (CHC) profiles to discriminate among colonies and/or among individuals within a colony on the basis of caste and sex, we performed multivariate principal component analyses (PCAs) using the R 2.14.1 software.

Differences in CHC composition among and within colonies were quantified using the Nei distance (Dronnet et al., 2006; Nowbahari et al 1990). The dissimilarity matrix, which included all possible types of pairs (intra-caste, inter-caste within a colony, and inter-colony within a caste), was constructed from the previously calculated mean relative areas of cuticular hydrocarbon peaks and were analyzed with a Kruskal-Wallis test using R 2.14.1.

For each individual, the quantity of each CHC was estimated with the following formula: $Q_i = (Q_{C20} \times x_i) / (x_{C20i})$. The quantity of each CHC was then divided by the mass of the individual to obtain the per-gram quantity of each compound.

Results

Analyses of CHCs

Out of the peaks detected by GC, only the 75 most stable peaks (of which four were queen-specific) in individuals in each group were retained and used in our statistical analyses. Among the identified CHCs, which ranged in chain length from 21 to 35 carbon atoms, we found *n*-alkanes (13.48%, 12 compounds), mono- and dimethyl-alkanes (57.30%, 51 compounds), alkenes (17.98%, 16 compounds), and unknown compounds (11.23%, 10

compounds). Queens possessed four specific alkenes (C27:1, C31:1, C33:1, and C35:1) which were never detected in other castes (Table 2, Figure 1).

Caste- and colony-related CHC differences

In all colonies analyzed, PCAs showed that workers, males, gynes, and queens displayed different CHC profiles, with queens always clearly differentiated on axis 2 (Figure 2, Table 3). A comparison of chemical signature among colonies also showed significant differences in PCAs, with queens more or less following their offspring but always well differentiated from other castes (Figure 3).

Chemical distances in CHC profiles

With regard to the chemical distances associated with CHC profiles (Figure 4), we found more difference between gynes and other castes within a given colony than among gynes from different colonies (Kruskal-Wallis, $P < 0.001$). Instead, the chemical signatures of males were not significantly different from those of other castes in the same colony or among different colonies ($P > 0.05$; Figure 4). For workers, among-colony differences were larger than the chemical distances within colonies and among castes ($P < 0.001$; Figure 4). For gynes, males, and workers, intra-caste chemical distances (i.e. among individuals in the same caste, whatever the colony) were always shorter than those among castes in the same colony or between colonies (all $P < 0.001$). Moreover, a comparison of intra-caste chemical distances revealed that these distances were largest among workers (workers vs males $P < 0.001$, workers vs gynes $P < 0.01$), and greater in gynes than in males ($P < 0.05$). Among colonies, the chemical distances among workers were larger than those among gynes and males ($P < 0.001$ in both cases), and were similar between gynes and males ($P > 0.05$).

Figure 5 shows chemical distances between castes. The shortest distances were between comparisons of males and gynes and males and workers. Comparisons with queens always produced the highest distances.

Quantitative analysis of CHC profiles

Quantitatively, queens possessed more CHCs per gram of body weight than did the other individuals within a colony (Figure 6) (Kruskal-Wallis, $P < 0.001$). Gynes and males were similar to each other in terms of quantities of CHCs ($P > 0.05$), but each had less than workers ($P < 0.001$). The chromatograms of queens were characterized by the presence of two main alkanes, *n*-C25 and *n*-C27, in greater quantities than in other castes (Figure 1) [*n*-C25: 7.7x

more than in workers (Kruskal-Wallis $P < 0.001$), 11.5x more than in gynes ($P < 0.001$) and 10.9x more than in males ($P < 0.001$); *n*-C27: 3.9x more than in workers ($P < 0.001$), 10.1x more than in gynes ($P < 0.001$), and 7.7x more than in males ($P < 0.001$)], plus the queen-specific alkenes described above in the paragraph “Analyses of CHCs”.

Figure 7 depicts differences between castes and genders in the chemical makeup of CHCs. With respect to the amounts present in gynes, males, and workers, queens possessed higher quantities of *n*-alkanes ($P < 0.001$ for each comparison), monomethyl-alkanes ($P < 0.001$), and alkenes ($P < 0.001$), but the same quantities of dimethyl-alkanes ($P > 0.05$). Workers possessed more *n*-alkanes than gynes and males ($P < 0.05$ with males; $P < 0.001$ with gynes), more monomethyl-alkanes than gynes ($P < 0.05$) but a similar quantity as males ($P > 0.05$), and more dimethyl-alkanes than males and gynes ($P < 0.001$ for each comparison). Gynes possessed less *n*-alkanes than males ($P < 0.001$), but the same quantities of monomethyl-alkanes, dimethyl-alkanes, and alkenes ($P > 0.05$ respectively).

Discussion

Our results demonstrated that, in spite of inbreeding (Darrouzet et al. 2015) linked to a genetic bottleneck (Arca et al. 2015), the chemical signature of the invasive hornet *Vespa velutina* in France is clearly heterogeneous. Each colony, caste, and gender possesses a specific CHC profile. This study thus suggests that CHCs could serve as a useful marker for discriminating yellow-legged hornet populations in France, the main invaded area of this species in Europe.

The chemical heterogeneity that we observed within the introduced hornet population in France was unexpected. Arca et al. (2015) demonstrated that native *V. velutina* populations near Shanghai (China) contained at least three different haplotypes. Little is currently known about the chemical signatures of yellow-legged hornets in their native area, but it is possible, indeed probable, that these populations have strongly heterogeneous CHC profiles. Instead, the introduced population in France has just one haplotype (Arca et al. 2015), but has notable chemical heterogeneity in CHC profiles. This result is surprising, because to our knowledge this type of hydrocarbon heterogeneity has not previously been detected in other introduced social insects. On the contrary, recent research has revealed homogeneity in CHC profiles in introduced ants (*Linepithema humile*: Brandt et al. 2009; *Wasmannia auropunctata*: Errard et al. 2005) and termites (*Reticulitermes flavipes*: Perdereau et al. 2010). These previous studies suggested that the reduced chemical variability of introduced populations may arise from the limited number of introduction events into a new environment. Specifically, the reduced variability of recognition cues, such as CHCs, in these introduced populations of ants and

termite has been attributed to a reduction of genetic diversity through a genetic bottleneck (Tsutsui et al. 2000) or to ‘genetic cleansing’, i.e. selection against the less-common recognition alleles (Giraud et al. 2002).

In the case of the invasive hornet *V. velutina* in France, which is an inbred population with a genetic bottleneck, the heterogeneity in CHC profiles may be affected by environmental and/or epigenetic parameters. For example, diet or laboratory rearing conditions are known to alter the chemical signature in termites (Shelton and Grace 1997; Polizzi and Forschler 1998; Florane et al. 2004). A change in ants’ chemical signature could occur over a short period of time, which is due to the ants walking over their prey (Liang and Silverman 2000). Because *V. velutina* is a generalist predator and can consume several different types of prey, it is possible that a particular diet specific to the area around a given colony could explain the CHC heterogeneity we observed. However, as hornets have a general diet, it is unlikely that any great overall differences could appear in such conditions. But this idea needs further studies to be verified. Seasonal variations could also alter *V. velutina*’s chemical signatures, as has been observed in termite species (Bagnères et al. 1990); however, this cannot explain the differences reported here since we collected all hornet colonies in the same period of time. Another possible contributing factor could be the composition of the endosymbiont community in the digestive tract, as this has been reported to affect host chemical communication in termites (Matsuura 2001, 2003). Since these symbionts are probably obtained by new individuals thanks to trophallaxis with adult workers, it is possible that each *V. velutina* colony could possess a unique endosymbiont profile with specific effects on the CHC profiles of their host. Further studies are clearly necessary to analyze all these factors and their relative contributions to the hornet CHC profile.

These specific chemical signatures probably enable each individual to identify the colony and caste origins of conspecifics. In social insect colonies, labor is divided between a reproductive queen, reproductive individuals (gynes and males), and sterile workers (Wilson 1971). The distribution of tasks among individuals requires interactions among colony members which involve the recognition of caste membership and perhaps specific tasks inside the caste (more particularly in the worker caste) (Green and Gordon 2003). This process is generally based on chemical cues such as CHCs. For example, in bees and termites, castes can be distinguished by differences in CHC profiles (honeybees: Kather et al. 2011; termites: Bagnères et al. 1998, 2011; Darrouzet et al. 2014b; Haverty et al. 1996; Kaib et al. 2002; LeConte and Hefetz 2008; Nunes et al. 2009). Such differences were also observed across different age groups in *Apis cerana* workers and could serve as the basis for task allocation in

that species (Rahman et al. 2015). Wagner et al. (1998) supposed that these caste-related differences in CHCs may be representative of the tasks performed by individuals, that is, the type and quantity of CHCs present could be linked to the tasks and specific environment of a given individual. For example, bee foragers are exposed more often than other workers to unfavorable environmental conditions, such as hot temperatures, high humidity, and harmful chemicals. Consequently, Rahman et al. (2015) suggested that the increased levels of *n*-alkanes observed in *A. cerana* foragers, compared with those of workers remaining inside the hive, could play an important role in regulating water impermeability, as had been previously proposed (Gibbs and Pomonis 1995). In the present study, we observed similar differences between *V. velutina* workers and reproductive individuals collected in nests (gynes and males). Such differences could be explained by the environment of individuals: most workers must contend with variable environmental conditions during their foraging activities while the reproductive individuals we analyzed (i.e. collected in their nests) stayed in the nest until their nuptial flight. However, eventually these gynes and males have to leave the nests and, like workers, will be confronted with variable environmental conditions. It is possible that their chemical signature adapts prior to leaving the nest by increasing the amount of *n*-alkanes present, though further studies are necessary to verify this hypothesis.

Interestingly, the CHC profiles of *V. velutina* queens possess two alkanes in great quantities (*n*-C25 and *n*-C27) which seem to act as queen pheromones in other hymenopteran species, such as *Lasius* ants (Holman et al 2010, 2013), the bumble bee *Bombus terrestris* (Holman 2014), and the wasp *Vespula vulgaris* (Van Oystaeyen et al. 2014). These *n*-alkanes, which are present on queen cuticle, could induce sterility in workers. This reproductive inhibition of the sterile caste seems to occur via two distinct processes: first, by inhibiting the functioning of workers' ovaries, and second, by causing oocyte resorption (Van Oystaeyen et al. 2014). These saturated linear alkanes seem to be the most conserved class of queen pheromones in several hymenopterans (Van Oystaeyen et al. 2014), and could be used as a fertility signal in a common solitary ancestor of social hymenopteran species (Wilson et al. 2013). Our result seems consistent with this idea and adds a second *Vespa* species to the group of species, spanning major clades of social hymenoptera, which has yielded clues about the evolutionary history of queen and fertility signals. However, Amsalem et al (2015) tested three alkanes (*n*-C23, *n*-C25 and *n*-C27) in *Bombus impatiens* and found no evidence to support this theory that a conserved class of hydrocarbons serve as queen pheromones or queen signals. Moreover, we observed four unsaturated hydrocarbons (C27:1, C31:1, C33:1, and C35:1) specific to the chemical profile of *V. velutina* queens. As observed by Van

Oystaeyen et al. (2014), some other compounds, including unsaturated hydrocarbons, can also be used as queen pheromones. In the *Polistes*, *Apis*, *Bombus*, and *Lasius* species in which they have been studied, these alkenes derive from saturated hydrocarbons, and can be obtained after biochemical modification during pheromone biosynthesis, from the up-regulation of specific desaturases (Blomquist & Vogt 2003; Blomquist and Bagnères 2010; Qiu et al. 2012). Some alkene compounds are used as male attractants (sex pheromones), particularly in solitary species (Blomquist and Vogt 2003; Blomquist and Bagnères 2010). As hypothesized by Van Oystaeyen et al. (2014), different queen pheromone compounds have arisen as a result of the multiple independent origins of eusociality, particularly by evolving from a fertility signal directed to attract males in solitary ancestors. Further studies are necessary to test the function of these alkenes in *V. velutina nigrithorax*.

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Legends

Figure 1: Typical chromatograms of queens, gynes, males, and workers.

Figure 2: Plot of the first two axes of the Principal Components Analysis (PCA) of the proportion of CHCs in the different castes within *V. velutina* colonies.

Figure 3: Plot of the first two axes of the Principal Components Analysis (PCA) of the proportion of CHCs in the different castes (queens, males, workers, and gynes) among different colonies.

Figure 4: Calculation of chemical distances based on comparisons of CHC profiles of workers, gynes, and males. “Intra-caste” represents the distance between individuals in the same caste and in the same colony, “inter-caste” is between the analyzed caste and the others castes in the same colony, and “inter-colony” is for the same caste but among colonies.

Figure 5: Comparisons of inter-caste chemical distances. The different letters for each distance were obtained with $P < 0.01$ (Kruskal-Wallis test). G=gynes, m=males, q=queens, w=workers.

Figure 6: Quantitative analysis of total CHC profiles (gram of CHCs per gram of insect) in workers, queens, gynes, and males.

Figure 7: Quantitative analysis of alkanes, alkenes, monomethyl-alkanes, and dimethyl-alkanes in CHC profiles (gram of CHCs per gram of insect) from workers, queens, gynes, and males.

498 **Table 1** Origins and dates of sample collection and sample sizes.

Colonies' origines	Lambert 93 coordinates	Dates of sample collections	Number of			
			Males	Workers	Gynes	Queens
Chinon	X : 491725 Y : 6677696	07/10/2011	10	0	0	0
Sepmes	X : 522947 Y : 6665711	17/10/2011	10	10	10	0
Barrou	X : 530076 Y : 6643026	24/10/2011	8	4	10	0
Ligueil (A)	X : 534307 Y : 6662641	25/10/2011	5	10	10	1
Azay sur Indre	X : 544621 Y : 6680754	21/11/2011	10	0	0	0
Ligueil (B)	X : 534307 Y : 6662641	26/11/2011	10	10	4	0
Civray de touraine	X : 552648 Y : 6694251	01/12/2011	10	10	10	0
Reignac sur Indre	X : 542394 Y : 6683401	06/12/2011	0	10	0	0
Lussault sur Loire	X : 542876 Y : 6701668	16/10/2012	10	10	0	0
Montlouis sur Loire	X : 536567 Y : 6700904	22/10/2012	10	10	10	1
Lerné	X : 482765 Y : 6674503	23/10/2012	10	10	10	0
Artannes	X : 518507 Y : 6688647	08/11/2012	10	10	10	0
Athée sur Cher	X : 542527 Y : 6693243	09/11/2012	10	10	0	0
St Avertin	X : 528458 Y : 6698865	27/08/2013	10	10	0	1
Tours	X : 525709 Y : 6701535	16/09/2013	10	10	0	1
Ligueil (C)	X : 534307 Y : 6662641	11/10/2013	10	10	0	1

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Table 1S Origins and dates of sample collection for insect pools to identify CHC chemical signatures (one individual was isolated from each colony).

pool of	Colonies from	Dates of sample collections
Males	Bourgueil	17/10/2011
	Sepmes	17/10/2011
	Azay sur Indre	21/11/2011
	Civray de touraine	01/12/2011
	Lussault	16/10/2012
	Montlouis sur Loire	22/10/2012
	Lerné	23/10/2012
	Athée	09/11/2012
	Tours	16/09/2013
Workers	Sepmes	17/10/2011
	Chinon	28/10/2011
	Azay sur Indre	21/11/2011
	Ligueil	26/11/2011
	Civray de touraine	01/12/2011
	Reignac sur Indre	06/12/2011
	Montlouis sur Loire	22/10/2012
	Lerné	23/10/2012
	Artannes	08/11/2012
Gynes	Sepmes	17/10/2011
	Barrou	24/10/2011
	Ligueil (<i>b</i>)	25/10/2011
	Ligueil(<i>a</i>)	26/11/2011
	Civray de touraine	01/12/2011
	Montlouis sur Loire	22/10/2012
	Lerné	23/10/2012
	Artannes	08/11/2012
	Orléans	12/12/2012
Queens	Ligueil (<i>b</i>)	25/10/2011
	Montlouis sur Loire	22/10/2012
	St Avertin	27/08/2013
	Tours	16/09/2013
	Ligui (<i>c</i>)	11/10/2013

Table 2 Identification and relative proportion of the cuticular hydrocarbon compounds (CHCs) in the chemical signature of each caste of *Vespa velutina*. Compounds are the same between males and females (workers and gynes). Peaks pQ1 to pQ4 are queen-specifics.

Peaks	RT (min)	ECL	Identification	Workers		Males		Gynes		Queens	
				%	± SE	%	± SE	%	± SE	%	± SE
C20	23,12	20,00	n-eicosane								
p1	24,66	21,00	n-C21	0,23	0,31	0,48	0,63	0,56	1,22	0,20	0,06
p2	25,27	21,38	11-MeC21	0,59	1,17	0,50	0,46	0,26	0,35	0,19	0,12
p3	26,26	22,00	n-C22	0,03	0,05	0,10	0,17	0,08	0,13	0,04	0,01
p4	27,47	22,75	C23:1	0,09	0,13	0,14	0,16	0,24	0,24	0,03	0,02
p5	27,58	22,81	C23:1	0,03	0,09	0,03	0,05	0,03	0,05	0,01	0,00
p6	27,89	23,00	n-C23	2,03	2,41	4,14	3,83	5,01	6,65	2,99	0,58
p7	28,45	23,36	11-MeC23	0,11	0,14	0,21	0,20	0,29	0,20	0,05	0,02
p8	28,55	23,42	7-MeC23	0,01	0,02	0,02	0,05	0,03	0,04	0,01	0,00
p9	28,68	23,51	5-MeC23	0,00	0,00	0,00	0,01	0,01	0,02	0,01	0,00
p10	29,03	23,73	3-MeC23	0,11	0,07	0,16	0,11	0,24	0,11	0,09	0,03
p11	29,46	24,00	n-C24	0,36	0,18	0,59	0,51	0,48	0,21	0,83	0,13
p12	30,02	24,34	12-MeC24	0,08	0,06	0,10	0,06	0,13	0,03	0,02	0,01
p13	30,68	24,75	C25:1	1,21	0,63	1,27	0,74	2,10	1,46	1,53	0,73
p14	30,78	24,81	C25:1	0,17	0,10	0,23	0,10	0,31	0,09	0,09	0,03
p15	31,08	25,00	n-C25	14,93	4,56	17,94	5,38	14,96	4,98	39,84	4,60
p16	31,59	25,34	13-+11-MeC25	2,09	1,45	2,77	1,54	5,79	1,06	0,65	0,21
p17	31,70	25,41	7-MeC25	0,18	0,13	0,25	0,12	0,49	0,11	0,05	0,02
p18	31,83	25,50	5-MeC25	0,40	0,14	0,39	0,16	0,54	0,40	0,39	0,09
p19	32,05	25,65	C26:1	0,09	0,06	0,06	0,07	0,17	0,05	0,01	0,01
p20	32,19	25,73	3-MeC25	4,34	1,28	4,67	1,42	4,15	1,11	4,12	0,85
p21	32,33	25,83	5,11-+5,9-diMeC25	0,21	0,09	0,14	0,05	0,25	0,06	0,08	0,02
p22	32,58	26,00	n-C26	1,32	0,54	1,38	0,95	1,00	0,41	2,25	0,34
p23	32,68	26,07	diMe or triMeC25	0,15	0,07	0,08	0,04	0,19	0,11	0,14	0,05
p24	33,09	26,33	12-+10-MeC26	0,56	0,21	0,56	0,16	0,97	0,22	0,12	0,03
p25	33,21	26,41	?	0,00	0,00	0,01	0,02	0,03	0,04	0,02	0,00
p26	33,27	26,45	?	0,05	0,03	0,03	0,03	0,03	0,02	0,02	0,00
p27	33,35	26,50	?	0,02	0,04	0,01	0,02	0,03	0,03	0,03	0,01
p28	33,47	26,58	4-MeC26	0,37	0,16	0,25	0,09	0,17	0,05	0,29	0,04
pQ1	33,64	26,69	C27:1	0,00	0,00	0,00	0,00	0,00	0,00	1,59	0,86
p29	33,78	26,78	C27:1	7,98	5,83	5,88	1,66	6,58	2,23	3,39	1,31
p30	33,87	26,84	C27:1	3,57	5,99	0,78	0,22	0,86	0,15	0,19	0,13
p31	34,12	27,00	n-C27	10,73	6,18	12,23	3,76	8,21	2,06	19,95	2,62
p32	34,61	27,34	13-+11-+9-MeC27	6,99	2,58	8,79	2,67	13,37	3,34	1,19	0,60
p33	34,72	27,42	7-MeC27	0,96	0,38	1,04	0,38	1,79	0,59	0,39	0,13
p34	34,85	27,51	5-MeC27	0,80	0,43	0,63	0,39	0,65	0,21	0,69	0,12
p35	35,00	27,62	11,15-diMeC27	3,40	1,26	2,42	0,75	3,06	1,07	0,33	0,23
p36	35,06	27,66	9,15-diMeC27	0,33	0,60	0,04	0,18	0,37	0,53	0,00	0,00

p37	35,20	27,75	3-MeC27	7,47	2,56	7,22	3,24	4,68	1,46	5,66	0,83
p38	35,31	27,83	5,15-diMeC27	1,40	2,43	0,54	0,28	0,72	0,29	0,19	0,05
p39	35,38	27,88	?	0,39	0,64	0,41	0,39	0,10	0,11	0,07	0,04
p40	35,45	27,92	?	0,06	0,35	0,00	0,01	0,05	0,04	0,00	0,00
p41	35,56	28,00	<i>n</i> -C28	0,89	0,69	0,74	1,05	0,40	0,33	0,33	0,03
p42	35,64	28,06	diMe or triMeC27	0,89	0,76	0,72	0,49	0,55	0,28	0,50	0,12
p43	36,02	28,32	12-MeC28	0,50	0,47	0,44	0,23	0,67	0,21	0,15	0,06
p44	36,28	28,50	8-MeC28	0,54	1,32	0,18	0,76	0,02	0,02	0,05	0,02
p45	36,42	28,60	4-MeC28	0,67	1,01	0,83	1,89	0,28	0,14	0,15	0,04
p46	36,55	28,70	C29:2	0,58	1,00	0,60	1,47	0,10	0,05	1,42	0,62
p47	36,70	28,80	C29:1	3,94	2,68	2,81	1,17	2,29	0,81	0,62	0,20
p48	36,82	28,88	C29:1	2,45	1,72	0,98	0,30	0,71	0,17	0,28	0,15
p49	36,99	29,00	<i>n</i> -C29	1,49	0,76	1,61	1,13	0,84	0,34	1,32	0,20
p50	37,29	29,27	?	0,57	0,63	0,67	0,40	0,26	0,13	0,21	0,08
p51	37,44	29,40	13-+11-+9-MeC29	1,52	0,63	1,97	0,64	3,00	0,87	0,44	0,17
p52	37,57	29,51	7-MeC29	0,07	0,13	0,09	0,06	0,20	0,11	0,05	0,01
p53	37,82	29,73	11,15-+11,17-diMeC29	2,34	0,62	1,85	0,66	2,12	0,79	0,26	0,15
p54	37,90	29,81	3-MeC29	0,37	0,30	0,39	0,27	0,67	0,48	0,14	0,05
p55	38,02	29,92	5,17-diMeC29	0,56	0,31	0,44	0,20	0,24	0,15	0,48	0,07
p56	38,12	30,00	<i>n</i> -C30	0,46	0,22	0,26	0,15	0,27	0,11	0,18	0,05
p57	38,81	30,43	4-MeC30	1,30	1,30	0,94	0,90	0,78	0,59	0,20	0,09
pQ2	39,31	30,74	C31:1	0,00	0,00	0,00	0,00	0,00	0,00	0,94	0,28
p58	39,47	30,84	C31:1	0,49	0,28	0,40	0,15	0,28	0,10	0,12	0,03
p59	39,61	30,93	?	0,20	0,08	0,15	0,08	0,09	0,04	0,05	0,01
p60	39,73	31,00	<i>n</i> -C31	0,34	0,29	0,57	0,64	0,32	0,27	0,29	0,05
p61	40,14	31,31	15-+13-+11-MeC31	0,69	0,39	1,06	0,35	1,39	0,53	0,31	0,07
p62	40,36	31,48	?	0,06	0,18	0,21	0,41	0,04	0,09	0,03	0,01
p63	40,49	31,58	11,15-diMeC31	1,51	0,62	1,53	0,65	1,86	0,80	0,24	0,13
pQ3	41,94	32,69	C33:1	0,00	0,00	0,00	0,00	0,00	0,00	1,65	0,80
p64	42,07	32,79	C33:1	0,17	0,17	0,15	0,11	0,12	0,13	0,07	0,02
p65	42,34	33,00	<i>n</i> -C33	0,16	0,26	0,30	0,42	0,15	0,13	0,11	0,02
p66	42,75	33,26	15-+13-+11-MeC33	0,40	0,23	0,66	0,27	0,78	0,40	0,18	0,05
p67	43,15	33,53	11,19-+11,17-diMeC33	1,04	0,47	0,99	0,49	1,01	0,49	0,23	0,09
p68	44,46	34,38	?	0,36	0,52	0,41	0,44	0,22	0,30	0,42	0,23
pQ4	44,73	34,56	C35:1	0,00	0,00	0,00	0,00	0,00	0,00	0,41	0,27
p69	45,80	35,26	11-MeC35	0,24	0,14	0,38	0,21	0,37	0,23	0,12	0,04
p70	46,27	35,57	13,19-+11,17-diMeC35	1,19	0,63	1,10	0,65	0,95	0,54	0,25	0,09
p71	46,49	35,71	?	0,16	0,12	0,09	0,08	0,06	0,05	0,06	0,03

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512 **Table 3** Caste comparisons of each cuticular hydrocarbon compound (CHCs) in the chemical
513 signature (Kruskal-Wallis chi-squared test).

Peaks	global comparison	Caste comparisons					
		Male-Workers	Male-Queen	Males-Gynes	Workers-Queens	Workers-Gynes	Gynes-Queens
p1	$\chi^2 = 23.7183$, df = 3, p = 2.86e-05	p=0.001	N.S	N.S	N.S	p=0.01	N.S
p2	$\chi^2 = 12.9491$, df = 3, p = 0.004748	N.S	N.S	p=0.01	N.S	N.S	N.S
p3	$\chi^2 = 21.2598$, df = 3, p = 9.297e-05	p=0.01	N.S	N.S	N.S	p=0.001	N.S
p4	$\chi^2 = 30.3086$, df = 3, p = 1.188e-06	N.S	N.S	p=0.01	N.S	p=0.001	p=0.001
p5	$\chi^2 = 14.4091$, df = 3, p = 0.002398	N.S	N.S	N.S	N.S	p=0.05	N.S
p6	$\chi^2 = 41.2662$, df = 3, p = 5.742e-09	p=0.001	N.S	N.S	p=0.01	p=0.001	N.S
p7	$\chi^2 = 68.7282$, df = 3, p = 7.99e-15	p=0.001	p=0.01	p=0.01	N.S	p=0.001	p=0.001
p8	$\chi^2 = 47.6831$, df = 3, p = 2.487e-10	p=0.05	N.S	p=0.05	p=0.01	p=0.001	N.S
p9	$\chi^2 = 99.9361$, df = 3, p < 2.2e-16	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
p10	$\chi^2 = 61.3123$, df = 3, p = 3.082e-13	p=0.01	N.S	p=0.001	N.S	p=0.001	p=0.001
p11	$\chi^2 = 55.6694$, df = 3, p = 4.942e-12	p=0.001	p=0.001	N.S	p=0.001	p=0.01	p=0.001
p12	$\chi^2 = 67.5535$, df = 3, p = 1.426e-14	N.S	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001
p13	$\chi^2 = 14.5332$, df = 3, p = 0.002262	N.S	N.S	p=0.01	N.S	p=0.01	N.S
p14	$\chi^2 = 92.0405$, df = 3, p < 2.2e-16	p=0.01	p=0.001	p=0.001	p=0.05	p=0.001	p=0.001
p15	$\chi^2 = 55.2756$, df = 3, p = 5.997e-12	p=0.05	p=0.001	p=0.01	p=0.001	N.S	p=0.001
p16	$\chi^2 = 146.472$, df = 3, p < 2.2e-16	N.S	p=0.001	p=0.001	p=0.01	p=0.001	p=0.001
p17	$\chi^2 = 140.5225$, df = 3, p < 2.2e-16	N.S	p=0.001	p=0.001	p=0.01	p=0.001	p=0.001
p18	$\chi^2 = 21.9075$, df = 3, p = 6.819e-05	N.S	N.S	p=0.001	N.S	p=0.01	N.S
p19	$\chi^2 = 98.4494$, df = 3, p < 2.2e-16	p=0.05	N.S	p=0.001	p=0.05	p=0.001	p=0.001
p20	$\chi^2 = 7.2278$, df = 3, p = 0.06498	N.S	N.S	N.S	N.S	N.S	N.S
p21	$\chi^2 = 114.3282$, df = 3, p < 2.2e-16	p=0.001	N.S	p=0.001	p=0.001	N.S	p=0.001
p22	$\chi^2 = 63.2816$, df = 3, p = 1.169e-13	N.S	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001
p23	$\chi^2 = 79.0834$, df = 3, p < 2.2e-16	p=0.001	p=0.01	p=0.001	N.S	N.S	N.S
p24	$\chi^2 = 134.2695$, df = 3, p < 2.2e-16	N.S	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001
p25	$\chi^2 = 56.1558$, df = 3, p = 3.891e-12	N.S	p=0.01	N.S	p=0.001	p=0.01	p=0.05
p26	$\chi^2 = 35.8875$, df = 3, p = 7.91e-08	p=0.001	N.S	N.S	p=0.01	p=0.001	N.S
p27	$\chi^2 = 32.1362$, df = 3, p = 4.899e-07	N.S	p=0.01	p=0.001	p=0.05	p=0.05	N.S
p28	$\chi^2 = 86.5888$, df = 3, p < 2.2e-16	p=0.001	N.S	p=0.001	N.S	p=0.001	p=0.001
p29	$\chi^2 = 25.3108$, df = 3, p = 1.329e-05	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
p30	$\chi^2 = 94.0832$, df = 3, p < 2.2e-16	p=0.001	p=0.001	N.S	p=0.001	p=0.001	p=0.001
p31	$\chi^2 = 82.2795$, df = 3, p < 2.2e-16	N.S	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001
p32	$\chi^2 = 132.0132$, df = 3, p < 2.2e-16	p=0.05	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001
p33	$\chi^2 = 107.1492$, df = 3, p < 2.2e-16	N.S	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001
p34	$\chi^2 = 12.6864$, df = 3, p = 0.005366	p=0.01	N.S	N.S	N.S	N.S	N.S
p35	$\chi^2 = 70.7854$, df = 3, p = 2.898e-15	p=0.001	p=0.001	p=0.01	p=0.001	N.S	p=0.001
p36	$\chi^2 = 27.8999$, df = 3, p = 3.812e-06	N.S	N.S	p=0.01	N.S	N.S	N.S
p37	$\chi^2 = 50.9824$, df = 3, p = 4.935e-11	N.S	N.S	p=0.001	N.S	p=0.001	N.S
p38	$\chi^2 = 88.9472$, df = 3, p < 2.2e-16	p=0.001	p=0.01	p=0.01	p=0.001	p=0.01	p=0.001
p39	$\chi^2 = 47.5966$, df = 3, p = 2.595e-10	p=0.001	p=0.01	p=0.001	N.S	N.S	N.S
p40	$\chi^2 = 94.4655$, df = 3, p < 2.2e-16	N.S	N.S	p=0.001	N.S	p=0.001	p=0.001
p41	$\chi^2 = 79.079$, df = 3, p < 2.2e-16	p=0.001	N.S	p=0.01	p=0.001	p=0.001	N.S

p42	$X^2 = 23.715$, df = 3, p = 2.865e-05	p=0.01	N.S	N.S	p=0.01	p=0.001	N.S
p43	$X^2 = 86.0122$, df = 3, p < 2.2e-16	N.S	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001
p44	$X^2 = 27.807$, df = 3, p = 3.987e-06	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
p45	$X^2 = 53.2645$, df = 3, p = 1.61e-11	N.S	p=0.001	N.S	p=0.001	p=0.001	p=0.01
p46	$X^2 = 31.2824$, df = 3, p = 7.413e-07	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
p47	$X^2 = 72.9925$, df = 3, p = 9.757e-16	N.S	p=0.001	N.S	p=0.001	p=0.001	p=0.001
p48	$X^2 = 152.917$, df = 3, p < 2.2e-16	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001	p=0.01
p49	$X^2 = 63.6709$, df = 3, p = 9.652e-14	N.S	N.S	p=0.001	N.S	p=0.001	p=0.001
p50	$X^2 = 72.4929$, df = 3, p = 1.248e-15	p=0.001	p=0.001	p=0.001	p=0.01	p=0.01	N.S
p51	$X^2 = 121.4764$, df = 3, p < 2.2e-16	p=0.01	p=0.001	p=0.001	p=0.01	p=0.001	p=0.001
p52	$X^2 = 73.8372$, df = 3, p = 6.431e-16	p=0.01	p=0.05	p=0.001	N.S	p=0.001	p=0.001
p53	$X^2 = 58.9537$, df = 3, p = 9.834e-13	p=0.001	p=0.001	N.S	p=0.001	N.S	p=0.001
p54	$X^2 = 28.7926$, df = 3, p = 2.476e-06	N.S	p=0.05	p=0.01	N.S	p=0.001	p=0.001
p55	$X^2 = 75.3837$, df = 3, p = 2.998e-16	N.S	N.S	p=0.001	N.S	p=0.001	p=0.001
p56	$X^2 = 61.4547$, df = 3, p = 2.873e-13	p=0.001	N.S	N.S	p=0.001	p=0.001	N.S
p57	$X^2 = 40.9767$, df = 3, p = 6.614e-09	N.S	p=0.001	N.S	p=0.001	p=0.01	p=0.001
p58	$X^2 = 72.5886$, df = 3, p = 1.191e-15	N.S	p=0.001	p=0.001	p=0.001	p=0.001	p=0.001
p59	$X^2 = 96.7315$, df = 3, p < 2.2e-16	p=0.01	p=0.001	p=0.001	p=0.001	p=0.001	N.S
p60	$X^2 = 13.5855$, df = 3, p = 0.003527	p=0.01	N.S	p=0.001	N.S	N.S	N.S
p61	$X^2 = 96.2835$, df = 3, p < 2.2e-16	p=0.001	p=0.001	p=0.05	p=0.05	p=0.001	p=0.001
p62	$X^2 = 31.5711$, df = 3, p = 6.444e-07	N.S	p=0.05	N.S	p=0.001	N.S	p=0.05
p63	$X^2 = 49.9463$, df = 3, p = 8.202e-11	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
p64	$X^2 = 21.9726$, df = 3, p = 6.609e-05	N.S	p=0.001	p=0.05	p=0.01	N.S	N.S
p65	$X^2 = 17.8008$, df = 3, p = 0.0004835	p=0.001	N.S	N.S	N.S	N.S	N.S
p66	$X^2 = 78.504$, df = 3, p < 2.2e-16	p=0.001	p=0.001	N.S	p=0.01	p=0.001	p=0.001
p67	$X^2 = 40.1883$, df = 3, p = 9.719e-09	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
p68	$X^2 = 15.8611$, df = 3, p = 0.001211	N.S	N.S	p=0.01	N.S	N.S	p=0.01
p69	$X^2 = 45.4726$, df = 3, p = 7.342e-10	p=0.001	p=0.001	N.S	p=0.01	p=0.01	p=0.001
p70	$X^2 = 36.0571$, df = 3, p = 7.283e-08	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
p71	$X^2 = 34.6872$, df = 3, p = 1.418e-07	p=0.001	N.S	N.S	p=0.01	p=0.001	N.S
pQ1	$X^2 = 243.6771$, df = 3, p < 2.2e-16	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
pQ2	$X^2 = 243.6771$, df = 3, p < 2.2e-16	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
pQ3	$X^2 = 243.6771$, df = 3, p < 2.2e-16	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001
pQ4	$X^2 = 243.6771$, df = 3, p < 2.2e-16	N.S	p=0.001	N.S	p=0.001	N.S	p=0.001

515 **Figure 1**

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519 **Figure 2**

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539 **Figure 5**

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542 **Figure 6**

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