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

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7

8 **Key words**

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

11

12 **Abstract**

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

25

26 **Introduction**

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

35 CHCs are mainly involved in the recognition of nestmates (Howard and Blomquist 2005; van
36 Zweden and D’Ettorre 2010), castes (Bagnères et al. 1998; Kaib et al. 2000; Smith and Taylor
37 1990), and worker’s tasks (Rahman et al 2016). Therefore, chemical signatures can vary, and
38 the degree of variation depends on a wide range of endogenous factors, such as the endocrine
39 system or enzymes (Darrouzet et al. 2014b; Fan et al. 2004; Lengyel et al. 2007), and
40 exogenous factors, such as climate and season (Bagnères et al. 1990), diet (Liang and
41 Silverman 2000), parasitism (Bagnères et al. 1996; Lebreton et al. 2010; LeConte et al. 2015),
42 or time (Bagnères et al. 2011; Lebreton et al. 2009). The relative importance of these factors
43 varies among species (VanderMeer and Morel 1998). However, CHC profiles are modified
44 not only by the social or biotic environment but are also under genetic control in both social
45 and solitary insects (Bonavita-Cougourdan et al. 1996; Dronnet et al. 2006; Etges et al. 2009;
46 Thomas and Simmons 2008; Vauchot et al. 1996). Consequently, variation in CHC profiles
47 has been used to identify species (Bagnères and Wicker-Thomas 2010) and to differentiate
48 populations (Bonelli et al. 2015; Nowbahari et al. 1990). This type of chemotaxonomy, i.e.
49 the use of chemical compounds, has been productively used in numerous insect species.
50 However, less attention has been paid to the extent or function of chemical variation within
51 invasive species, which in their introduced ranges often experience habitats and species
52 interactions that are strikingly different from those of their native environments (Perdereau et
53 al. 2010).

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

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

69 individuals (males and gynes) at the end of the summer (Monceau et al. 2013; Rome et al.
70 2015). These reproductive individuals emerge between late August and November. After
71 reproductive flights, the gynes disperse and the males die. However, Darrouzet et al. (2015)
72 showed that several colonies produced diploid males all throughout the colony life. As
73 authors explained, this result strongly suggests the occurrence of inbreeding in this species in
74 its invaded range (Europe). This hypothesis was supported by genetic analyses based on a
75 combination of mitochondrial and nuclear markers (Arca et al. 2015; Takeuchi et al. 2017).
76 The authors suggested that the yellow-legged hornet population in France was derived from a
77 single introduction event, and they showed evidence of a strong founder effect. Thus the
78 French populations sampled in that study suffered a notable loss of genetic diversity
79 compared to native populations in China.

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

85

86 **Materials and Methods**

87 *Insect collection*

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

92

93 *Cuticular hydrocarbon (CHC) analyses*

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

103 To identify the compounds (CHCs) in the chemical signatures according to caste and
104 sex (workers, gynes, and males), CHC extracts were pooled from nine random hornets, each
105 from a different randomly selected colony (Table 1S). Workers and gynes were discriminated
106 according to their size and weight (Rome et al. 2015). CHCs from five random queens were
107 also extracted and pooled. The samples were dried, then stored at -20°C. Before being
108 analyzed, 300 µl of pentane was added to each, with the internal standard as in the previous
109 analysis. To determine which compounds were present, we used an Agilent Technologies
110 7000C GC/MS Triple Quad with a 7890B GC System equipped with the same column as
111 above; we also used the same temperature program as above. Compounds were identified
112 following Blomquist (2010) and using standard *n*-alkanes.

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

116

117 *Calculations and statistical analyses*

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

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

126 For each individual, the quantity of each CHC was estimated with the following
127 formula: $Q_i = (Q_{C_{20}} \times x_i) / (x_{C_{20}i})$. The quantity of each CHC was then divided by the mass of
128 the individual to obtain the per-gram quantity of each compound.

129

130 **Results**

131 *Analyses of CHCs*

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

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

139

140 *Caste- and colony-related CHC differences*

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

146

147 *Chemical distances in CHC profiles*

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

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

164

165 *Quantitative analysis of CHC profiles*

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

171 more than in workers (Kruskal-Wallis $P < 0.001$), 11.5x more than in gynes ($P < 0.001$) and
172 10.9x more than in males ($P < 0.001$); *n*-C27: 3.9x more than in workers ($P < 0.001$), 10.1x
173 more than in gynes ($P < 0.001$), and 7.7x more than in males ($P < 0.001$), plus the queen-
174 specific alkenes described above in the paragraph “Analyses of CHCs”.

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

184

185 Discussion

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

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

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

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

228 These specific chemical signatures probably enable each individual to identify the
229 colony and caste origins of conspecifics. In social insect colonies, labor is divided between a
230 reproductive queen, reproductive individuals (gynes and males), and sterile workers (Wilson
231 1971). The distribution of tasks among individuals requires interactions among colony
232 members which involve the recognition of caste membership and perhaps specific tasks inside
233 the caste (more particularly in the worker caste) (Green and Gordon 2003). This process is
234 generally based on chemical cues such as CHCs. For example, in bees and termites, castes can
235 be distinguished by differences in CHC profiles (honeybees: Kather et al. 2011; termites:
236 Bagnères et al. 1998, 2011; Darrouzet et al. 2014b; Haverty et al. 1996; Kaib et al. 2002;
237 LeConte and Hefetz 2008; Nunes et al. 2009). Such differences were also observed across
238 different age groups in *Apis cerana* workers and could serve as the basis for task allocation in

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

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

273 Oystaeyen et al. (2014), some other compounds, including unsaturated hydrocarbons, can also
274 be used as queen pheromones. In the *Polistes*, *Apis*, *Bombus*, and *Lasius* species in which they
275 have been studied, these alkenes derive from saturated hydrocarbons, and can be obtained
276 after biochemical modification during pheromone biosynthesis, from the up-regulation of
277 specific desaturases (Blomquist & Vogt 2003; Blomquist and Bagnères 2010; Qiu et al.
278 2012). Some alkene compounds are used as male attractants (sex pheromones), particularly in
279 solitary species (Blomquist and Vogt 2003; Blomquist and Bagnères 2010). As hypothesized
280 by Van Oystaeyen et al. (2014), different queen pheromone compounds have arisen as a result
281 of the multiple independent origins of eusociality, particularly by evolving from a fertility
282 signal directed to attract males in solitary ancestors. Further studies are necessary to test the
283 function of these alkenes in *V. velutina nigrithorax*.

284

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289

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470

471 **Legends**

472

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

474

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

477

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

481

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

486

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

490

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

493

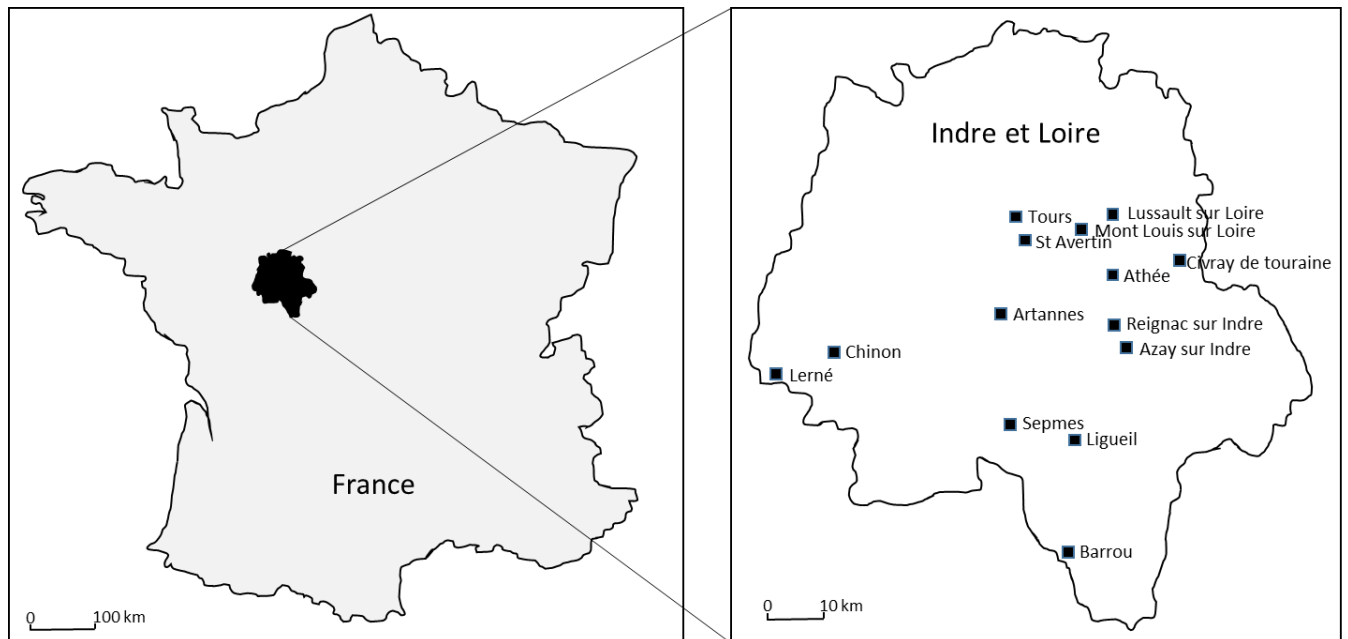
494 Figure 7: Quantitative analysis of alkanes, alkenes, monomethyl-alkanes, and dimethyl-
495 alkanes in CHC profiles (gram of CHCs per gram of insect) from workers, queens, gynes, and
496 males.

497

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

Colonies' origins	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

499



500
501

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

504

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

505

506 **Table 2** Identification and relative proportion of the cuticular hydrocarbon compounds
 507 (CHCs) in the chemical signature of each caste of *Vespa velutina*. Compounds are the same
 508 between males and females (workers and gynes). Peaks pQ1 to pQ4 are queen-specifics.
 509

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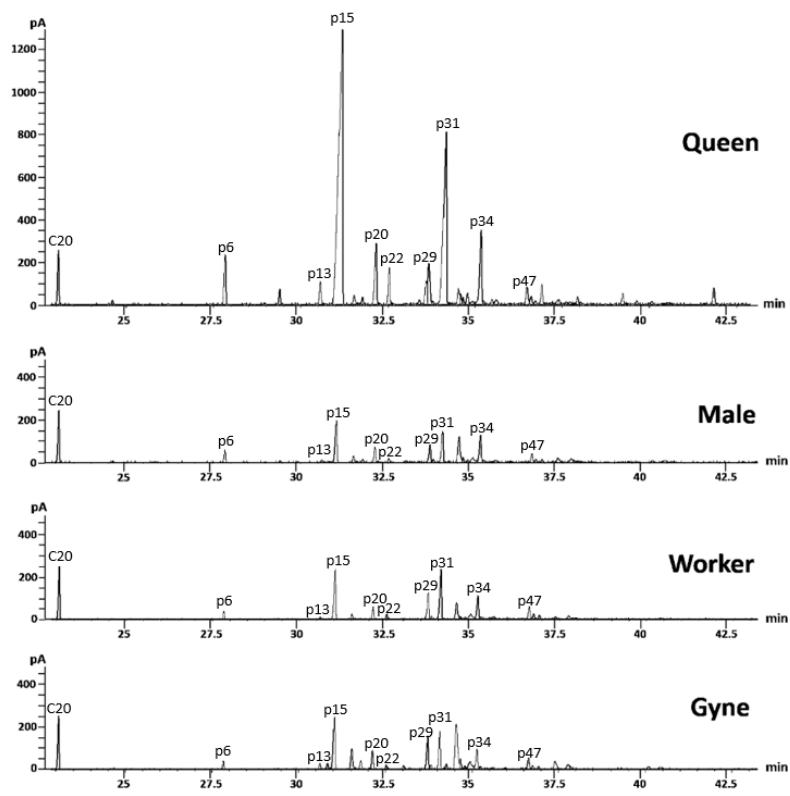
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512 **Table 3** Caste comparisons of each cuticular hydrocarbon compound (CHCs) in the chemical
 513 signature (Kruskal-Wallis chi-squared test).

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

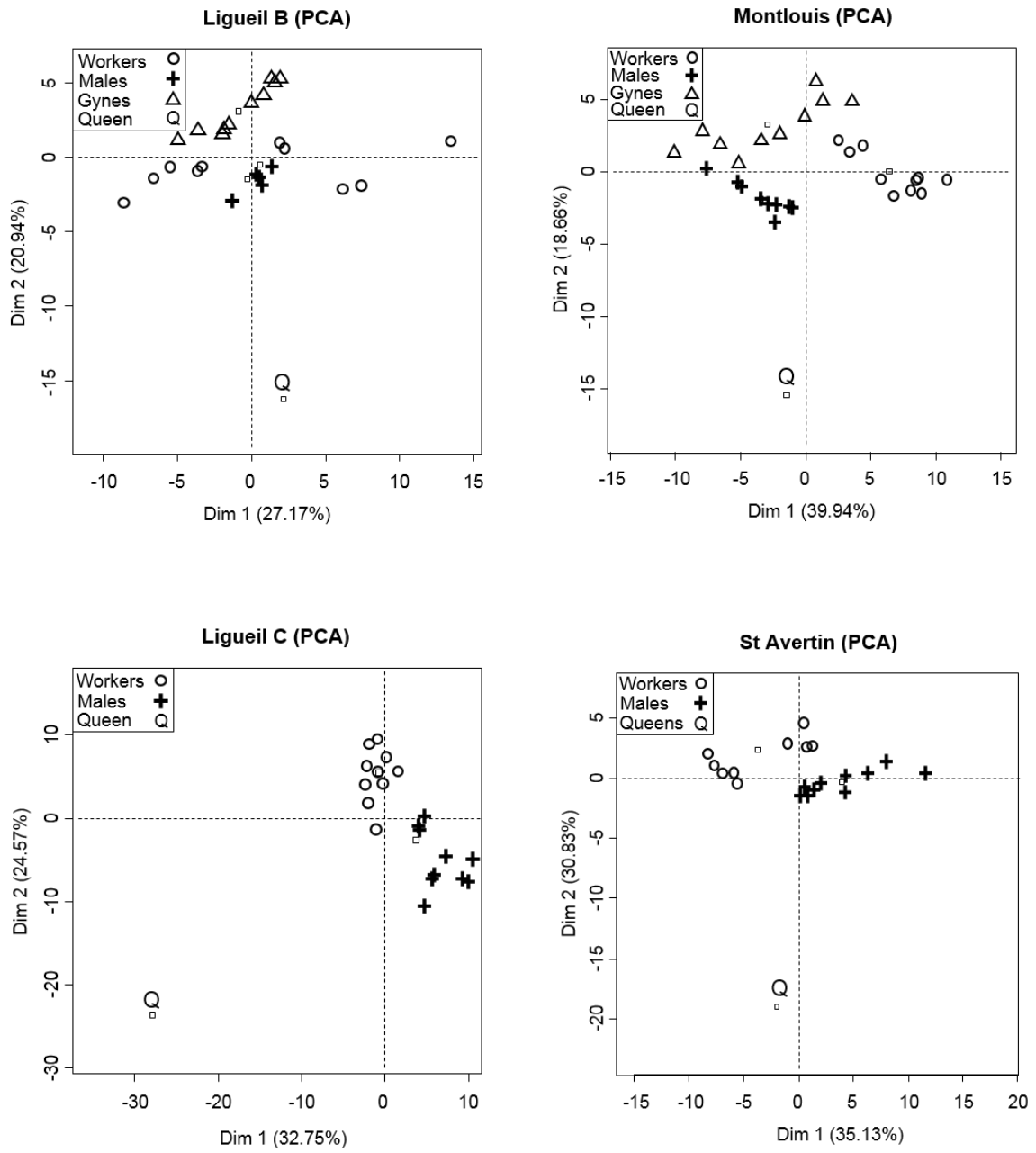


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519 **Figure 2**



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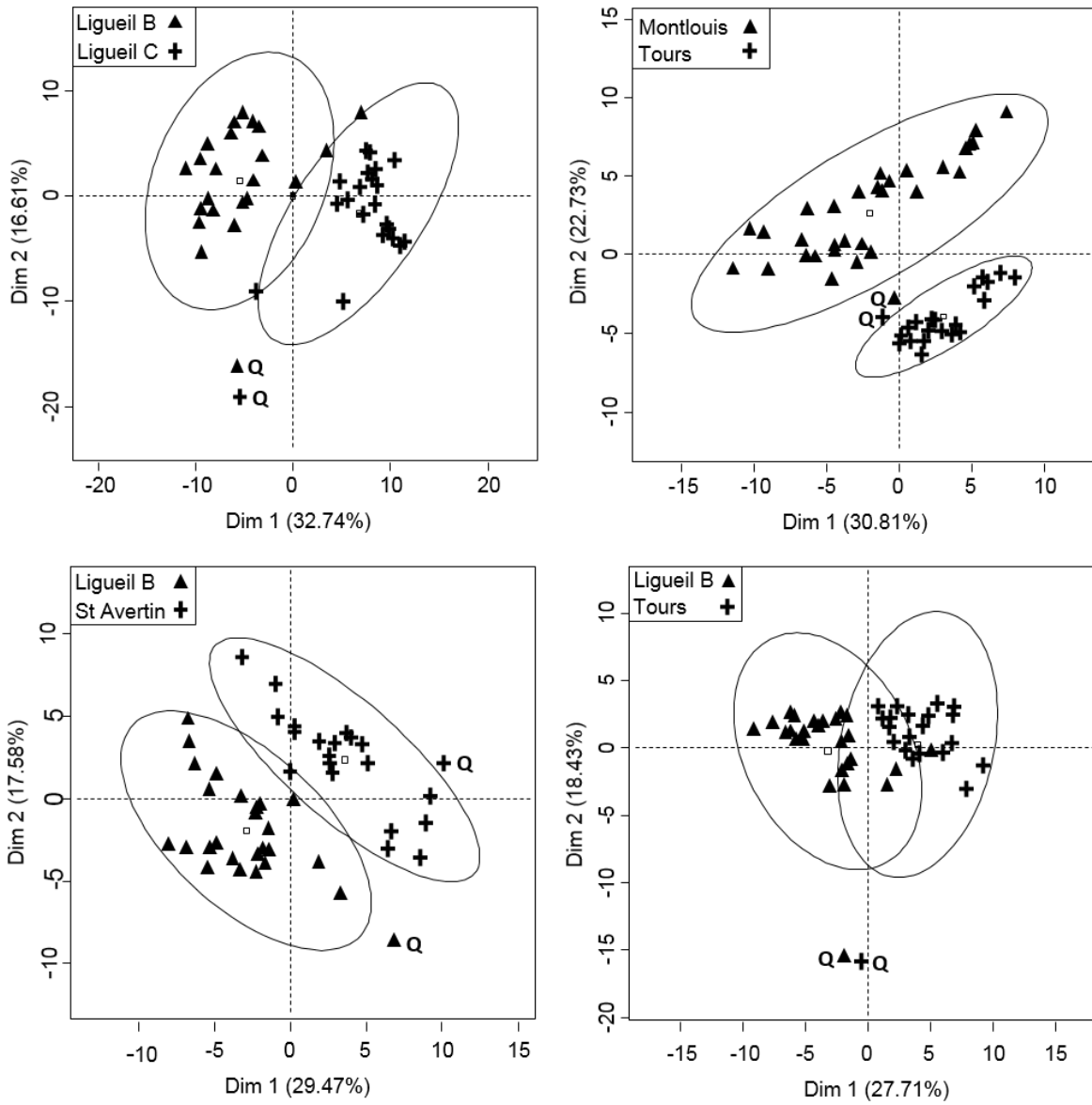
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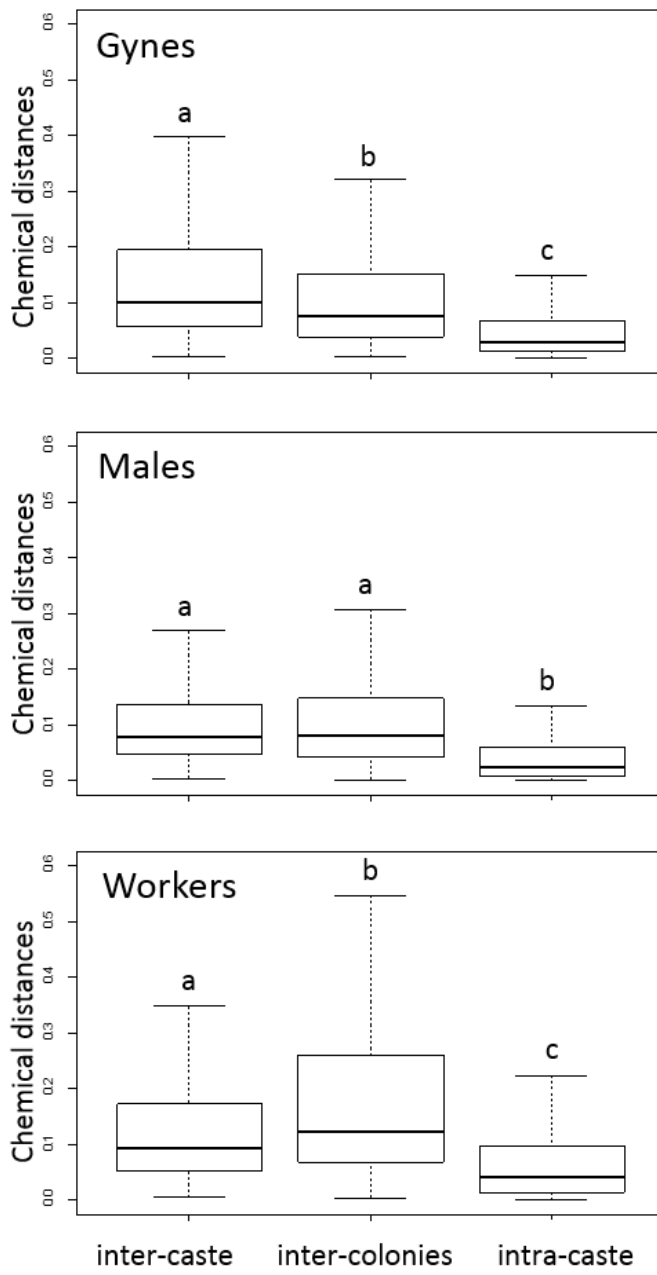
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525 **Figure 3**



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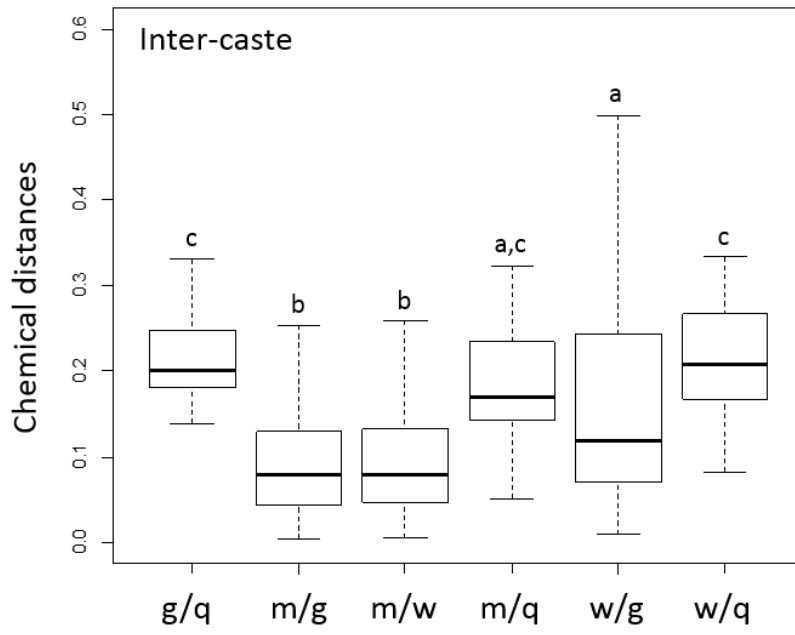
536 **Figure 4**



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539 **Figure 5**



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542 **Figure 6**



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