

Callows have their own odour but not for long: Fast acquisition of the chemical signature in the yellow-legged hornet, Vespa velutina nigrithorax

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1	Callows have their own odour but not for long: Fast acquisition of the chemical
2	signature in the yellow-legged hornet, Vespa velutina nigrithorax
3	
4	Short title: Chemical signature dynamics in callows' hornets
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23 Abstract

24 In eusocial insects, the discrimination between conspecifics and intruders is essential for the functioning of most insect societies. This capacity mediated by pheromones, and in particular 25 by Cuticular Hydrocarbon Compounds (CHCs) is the main mechanism of social nestmate 26 recognition which can include heritable cues, as well as acquired cues from nestmates and/or 27 their environment. In this study, we analysed the acquisition of the chemical signature of 28 callows over time, compared to nestmate workers and their nest materials in the invasive 29 hornet Vespa velutina nigrithorax. We show that callows present their own chemical 30 signature, different from both the oldest individuals and nest materials. Interestingly, this 31 32 chemical signature evolves rapidly, the first two days after emergence, and is not influenced by the presence of a chemical template (workers and/or nests) in laboratory conditions. 33 Moreover, quantities of branched alkanes increased with age. All these results suggest that the 34 chemical signature in *V. velutina nigrithorax* is probably more influenced by some parameters 35 like physiological processes or genetic factors. 36

37

38 Keywords: Vespidae, cuticular hydrocarbons, nestmate, nest, age, Asian hornet.

39 Introduction

In social insects, the increasing complexity of social organization involves coordinating
activities among nestmates in a variety of contexts, which therefore requires a wide diversity
of signals (Leonhardt et al. 2016). Chemical signals are the main communication system
employed, using pheromones (Abd El-Ghany 2020).

On insect's cuticle, Cuticular Hydrocarbon Compounds (CHCs) are involved in 44 protection against desiccation (Gibbs 2002), but also in many behaviours which include 45 recognition processes. In such processes, CHC profiles are considered to be specific and called 46 47 chemical signature of insects (Ginzel and Blomquist 2016). This allows insects to convey their identity to their congeners (Blomquist and Bagnères 2010). Many studies demonstrated that the 48 chemical signature of insects is complex and dynamic, with CHCs varying quantitatively and 49 qualitatively (Blomquist and Ginzel 2021). Depending on the species, CHCs are generally a 50 mixture of saturated (alkanes) and/or unsaturated compounds, which may be methylated or not. 51 52 These CHC mixtures allow complex profiles with functional significance (Ginzel and Blomquist 2016). CHCs are produced in oenocytes from the fatty acid biosynthesis pathway 53 (Krupp et al. 2008). The Acetyl-coA, a fatty acid precursor, is converted to alcohol and then 54 55 oxidized to aldehyde. It then undergoes decarboxylation which converts it into hydrocarbons (Kent et al. 2008). Three different pathways can be used in order to obtain linear alkanes, 56 branched alkanes or alkenes, of different lengths (*i.e.*, with different number of carbon atoms). 57 58 Then, CHCs will circulate into the hemolymph to the cuticle of insects thanks to lipophorins (Lucas et al. 2004; Blomquist and Ginzel 2021). In Vespidae (like several other species), these 59 CHCs can vary according to species and colonies (Soares et al. 2017), but also according to 60 castes and sexes (Gévar et al. 2017). These compounds can thus be employed as contact 61 pheromones with the use of antennae for inter and intra-specific recognition processes 62 63 (Blomquist and Bagnères 2010).

New-emerging insects, *i.e.* callows, may have a period of weak chemical signature 64 called "cuticular chemical insignificance", followed by a period of integration where they 65 synthesize and obtain their own CHCs from nestmates and/or nest materials like it has been 66 shown in ants (Lenoir et al. 1999). The Gestalt model suggests that all nestmates share their 67 own recognition cues with colony members to create a colonial identity. This model explain 68 how insects recognize and discriminate nestmates from intruders (Hefetz 2007). In 69 Hymenoptera, the proportion of CHCs can change rapidly after emergence to acquire the 70 colonial profile (Lorenzi et al. 2004), through trophallaxis and social grooming, as previously 71 demonstrated in ants (Lahav et al. 2001). In the ant Rhytidoponera confusea, individuals could 72 73 acquire colonial odour from nest materials (Crosland 1989). The acquisition of the chemical 74 signature in callows is therefore of crucial importance for their social integration and the maintenance of a stable social organization at the colony level. 75

The invasive yellow-legged hornet, Vespa velutina nigrithorax, was accidentally 76 introduced to southwest France from China in 2004 (Arca et al. 2015). Since, then it has rapidly 77 78 invaded several countries throughout Europe (Robinet et al. 2018). In the European population, despite the presence of an inbred population (Darrouzet et al. 2015), the chemical signature of 79 individuals is heterogeneous (Gévar et al. 2017). Studying the dynamics of the chemical 80 81 signature, and environmental factors that may influence this heterogeneity is crucial to understand the social life and the invasive success of V. velutina nigrithorax. Here, we aimed 82 to investigate the chemical signatures of hornet callows, and more particularly the cuticular 83 profiles dynamics according to ages and local environments. Indeed, the local environment, 84 with the presence of conspecifics and/or nest materials, could serve as chemical sources. To 85 86 test this hypothesis, the chemical signature of callows was analysed over time, then in relation of their local environment, *i.e.*, in the presence of congeners and/or nest materials. 87

89 Materials and Methods

90 *Hornets sampling*

91 Three different *V. velutina nigrithorax* colonies were collected in Indre et Loire region 92 (France), in the cities of Bléré, Saint-Épain and Truyes. For each colony, after their collection, 93 workers (W) were isolated in plastic breeding boxes with aeration (L95 x W65 x H65 mm) at 94 ambient temperature, then fed with honey and water *ad libitum*. Combs were also collected, 95 checked daily, awaiting the emergence of callows at ambient temperature. Each callow (C) was 96 isolated on the day of its emergence and reared in the same manner as adult workers.

97

98 *Experimental setups*

99 Chemical analyses were performed to study (i) a potential "cuticular insignificance" of 100 callows, (ii) the evolution of their chemical signatures over time, and (iii) the chemical signature 101 acquisition according to their local environment.

102

103 *Cuticular chemical insignificance of callows*

To highlight a "cuticular chemical insignificance" of callows, chemical analyses were 104 105 performed to compare callows on their day of emergence (less than 24 hours of age: C₀), adult old workers (W, unknown age since these workers were isolated when the colony was collected 106 in the field) and nest material (N). For the nest material, envelopes were chosen for their ease 107 of sampling and because they possess a colonial odour (Haouzi et al. 2021). In this first 108 experiment, 6 technical replicates were performed per colony for each modality (C₀, W, N). 109 Each hornet (n=36) was frozen killed, placed in a vial with 1ml of heptane, and vortexed 1 min 110 head up and 1 min head down. Then, 500 µl of the extract was placed in a new vial (Gévar et 111

al. 2017). Immediately prior to the analyses, 10 μ l of n-eicosane at 10⁻³ mg/ μ l was added, and 112 vortexed for 10 s. For each nest, 150 mg of nest material (N) corresponding to envelopes were 113 ground into small particles, and was placed in a vial with 5 ml of heptane and vortexed for 1 114 min. The extracts were left to stand for 24 h, allowing the particles to settle. Subsequently, 1.5 115 116 ml of the supernatant was transferred into a new vial and completely evaporated under nitrogen flux. Then, 150 µl of heptane was added, and the mixture was vortexed for 30 s. Afterward, a 117 100 µl aliquot was transferred to a vial (Haouzi et al. 2021). Immediately before analyses, 3 µl 118 of n-eicosane (n-C20) internal standard at 10^{-3} mg/µl were added and vortexed for 10 s. 119

120

121 *Evolution of the chemical signatures over time*

Just after emergence, callows were isolated in plastic boxes, as described above. To study the evolution of the chemical signature over time, callows were frozen killed at different ages: on the day of emergence (between 0 and 24 h: C_0), two days later (between 48 and 72 h: C_2), four days later (between 96 and 120 h: C_4), six days later (between 144 and 168 h: C_6), or eight days later (between 192 and 216 h: C_8). CHCs from frozen individuals (n=90) were immediately extracted following the same procedure as in the first experiment. For each age modality (C_0 , C_2 , C_4 , C_6 , C_8), 6 technical replicates were performed per colony.

129

130 *Chemical signature acquisition depending on local environment*

Environmental conditions of callows were manipulated from the day of emergence (C_0) according to four modalities: a callow was placed alone (C), with an old nestmate worker (CW, condition Callow-Worker), with a piece of 0.5 g of nest material (CN, condition Callow-Nest), or with both a worker nestmate and a piece of 0.5g of nest material (CWN, condition Callow-Worker-Nest). Each callow was frozen on the sixth day of the experiments (6-days-old callows) and CHCs (n=57) were extracted in the same way as previously described. For each modality
(C, CW, CN, CWN), five technical replicates were performed per colony, except for SaintÉpain (5 samples C, 3 CW, 4 CN and 5 CWN). For each replicate, workers, callows and nest
material were provided from the same nest.

140

141 *Chemical analyses*

In each experiment, 2 μ l of each chemical extract were injected in a gas chromatograph (Agilent Technologies 7820A GC) equipped with a Flame Ionization Detector (FID), and a capillary column HP-5 (Agilent Technology 19091J-413, 30 m × 320 μ m × 0.25 μ m). Helium was the carrier gas at 1.7 ml/min. The temperature program started from 50°C to 200°C at 8°C/min, then 200°C to 315°C at 5°C/min, and a 5-min hold at 315°C.

To identify CHCs, a second series of injections were performed on a gas chromatograph 147 (Agilent Technologies 7890B GC) coupled to a mass spectrometer (Agilent Technologies 148 149 7000C GC/MS Triple Quad) using an HP-5 Ultra Inert capillary column (Agilent Technology, USA; 30 m x 250 µm x 0.25 µm) and a Gerstel MPS autosampler. The same temperature 150 program described above was used. Injectors were in splitless mode with a splitless time of 2 151 152 min at a constant temperature of 250 °C. Helium was the carrier gas set up at 1.2 ml/min. Electron impacts at 70 eV were recorded in scanning mode every 0.3 s with a scan lapse of 0.1 153 s and a mass scan range of 40-600 amu. Data were analysed using MassHunter B.07.00 154 software (Agilent). Compounds were identified according to fragmentation analyses and 155 compared to published spectra (Gévar et al. 2017), and spectrum library AMDIS v2.0g (NIST, 156 157 2017). Chromatograms were integrated using the ChemStation B.04.03[16] software (Agilent Technologies). 158

161 Statistical analyses were performed using R software (v4.1.0). Technical replicates were 162 pooled and averaged (*i.e.* sample replicates by modalities and by nest). Relative abundances of 163 CHCs were calculated by dividing the area of each compound by the sum of all the CHCs, and 164 the total was multiplied by 100.

Permutational Analysis of Variance (PERMANOVA, n = 999 permutations) were 165 performed to test the effects of the different modalities on the chemical profiles, for the three 166 experiments called (i) a potential "cuticular insignificance" of callows (Co vs W vs N), (ii) the 167 evolution of their chemical signatures over time (C₀ vs C₂ vs C₄ vs C₆ vs C₈), and (iii) the 168 169 chemical signature acquisition according to their local environment (C vs CW vs CN vs CWN). 170 PERMANOVA were executed on the Bray-Curtis dissimilarity matrices calculated with the chemical profile between each pair of samples, using the adonis2 function from the vegan 171 package (v2.6-2). To illustrate results, Principal Coordinate Analysis (PCoA) were performed 172 using the ape package (v5.6-2). 173

To further cluster the age of callows ($C_0 vs C_2 vs C_4 vs C_6 vs C_8$) according to their 174 chemical similarities, Partial Least Squares - Discriminant Analysis (PLS-DA) were performed 175 using mixOmics (v6.16.3) and RVAideMemoire (v.0.9-81-2) packages. Four PLS-DA models 176 were carried out: all age groups (i, C₀, C₂, C₄, C₆, and C₈), four age groups where C₆, and C₈ 177 were clustered (ii, C₀, C₂, C₄, and C₆-C₈), three age groups were C₄, C₆ and C₈ were clustered 178 (iii, C₀, C₂, and C₄-C₆-C₈) and two age groups where C₂, C₄, C₆, and C₈ were clustered (iv, C₀, 179 and C₂-C₄-C₆-C₈). The performance of each model was assessed by a Classification Error Rate 180 (CER) computed using a double cross-validation scheme. Permutation tests (999 permutations) 181 based on the averaged CER were used to conclude the significance of the differences between 182 clusters. Then, pairwise correlation tests based on cross-model validation of models were 183 performed, and *p*-values were corrected using the False Discovery Rate (FDR) method. For 184

185 each model, we measured a Variable Importance in the Projection (VIP) scores to acknowledge186 the importance of each variable.

For each sample, the quantity of each CHC was estimated with the following formula: 187 $Q_i = \frac{(Q_{C20} \times X_i)}{(X_{C20})}$ where Q corresponds to the quantity, X to the peak area and C20 to the internal 188 standard. Quantities were divided by the mass of hornets or nest materials to obtain the per-189 190 gram quantity of each compound. Quantities were log-transformed to normalize data. To analyse which CHC quantities differ between modalities, in the three above experiments, we 191 performed Linear Mixed Effect Models in R (LMER) with colony factors as random effect 192 using the lmer function (package lme4, v1.1-30). For all these analyses, chromatogram peaks 193 containing a mix of compounds were excluded. Prerequisites of each LMER models were 194 confirmed by the DHARMa package (v0.4.6). Afterwards, ANOVAs using models were carry 195 out using the car package (v3.1-1), and estimated marginal means were calculated using 196 pairwise comparisons. 197

198

199 **Results**

The 21 main chromatogram peaks, including compounds ranging from 21 to 31 carbon atoms in chain length (**Table S1, Fig. S1**) with a relative area higher than 1% of the entire profiles, were analysed. It includes chemical compounds with mixtures of chemical families in the same peaks or not, *i.e.* 6 alkanes, 8 alkenes and 10 branched alkanes.

204

205 Cuticular chemical insignificance of callows

On the two first axis of the PCoA (**Fig. 1**), 87% of the chemical variability is explained by modalities (C₀ vs W vs N) with a significant effect (PERMANOVA test, $R^2 = 0.87$; p = 0.003).

The quantitative analyses between modalities showed significative differences for 209 alkanes (ANOVA, p < 0.001), alkenes (p < 0.001) and branched alkanes (p = < 0.001). For 210 branched alkanes, callows (C_0) are significantly different from workers (W) (p < 0.05, Table 211 S2). Callows (C_0) and nest material (N) are significantly different in their alkanes and alkenes 212 quantities (p < 0.05, Table S2). For both alkenes and branched alkanes quantities, workers (W) 213 are significantly different from nest materials (N) (p < 0.005, Table S2). Callows (C₀) have 214 fewer branched alkanes than workers (W), but they have more alkanes and alkenes than nest 215 materials (N) (Table S3, Fig. 2). Workers (W) have more CHCs compared to nest materials 216 (N) for both alkenes and branched alkanes quantities (Table S3, Fig. 2). 217

218

219 *Evolution of the chemical signatures over time*

As callows (C_0) are chemically different from older workers, a new analysis was 220 performed on their age ($C_0 vs C_2 vs C_4 vs C_6 vs C_8$), which showed its influence on the chemical 221 profiles (PERMANOVA, $R^2 = 0.77$, p = 0.001, Fig. S2). For PLS-DA models, the cross-model 222 validations (2CV) gave 54% ± 2.9 SE (i, Fig. S3 A), 36% ± 1.5 SE (ii, Fig. S3 B), 12.7% ± 1.6 223 SE (iii, Fig. 3A) and $0\% \pm 0$ SE (iv, Fig. 3B) of the CER. Differences between sample groups 224 have been highlighted for models with (ii) four age classes (CER = 0.39, p = 0.005), (iii) three 225 226 age classes (CER = 0.13, p = 0.002) and (iv) two age classes (CER = 0, p = 0.003) contrary to the model (i) with all age classes (CER = 0.63, p = 0.063). In the first model (i) and the second 227 model (ii), no differences have been observed between callows (pairwise.MVA.test, p > 0.05, 228 229 for all comparisons, **Table S4**). However, in the third model (iii) with three age classes, C₀ and C_2 are significantly separated from C_4 - C_6 - C_8 cluster (p = 0.021 and 0.044 respectively) but also 230 in the fourth model (iv) where C_0 is significantly separated from C_2 - C_4 - C_6 - C_8 cluster (p = 0.005). 231 VIP scores were measured and indicated in Table S1. 232

The quantitative analysis showed significant differences for branched alkanes 233 (ANOVA, p = 0.002). Compared in pairs, C₀ is significantly different from C₆ and C₈ (Pairwise 234 tests, p < 0.05, Table S5). Branched alkanes quantities increase with time (Fig. 4, Table S5, 235 **S6**). No significant differences have been demonstrated for alkanes and alkenes (p > 0.05). We 236 have a large dispersion of data among callows aged from C₀ to C₄. On the Fig. 4, alkanes 237 quantities seem to decrease with the age of callows whereas branched alkanes quantities 238 increase, as demonstrated in the Table S6. In the model, which included old workers and C8 239 callows for branched alkanes quantities, no significative effect has been observed (ANOVA, p 240 = 0.156). 241

242

243 Chemical signature acquisition depending on local environment

Callows were placed in different contexts of local environments (C vs CW vs CN vs CWN). As the second experiment showed no differences in cuticular profiles of C₄, C₆, and C₈ callows, the experiment was done up to 6^{th} day.

247 The local environment conditions were not significantly determinant on callows CHCs 248 (PERMANOVA, $R^2 = 0.21$; p = 0.083, Fig. S4).

Finally, the quantitative analysis for callows in different local environments showed no significant differences for all chemical families (ANOVA, p > 0.05). However, there are nonsignificant trends where the amounts of all CHCs increase with the presence of a chemical template (CN, CW, CWN) compared to callows alone (C) (**Fig. 5, Table S7**).

253

254 **Discussion**

Upon emergence, *V. velutina nigrithorax* callows have their own chemical signature, clearly different from those of their nest and adult nestmates. For all chemical compound families, differences have been highlighted between callows, adult workers, and nest materials.

For example, quantities of branched alkanes are significantly lower in callows compared to 258 workers. These compounds employed in communication can be used as signals for the colonial 259 chemical identity (Martin et al. 2008a) and nestmate recognition (Lucas et al. 2004; Blomquist 260 and Ginzel 2021). We assume that the colonial signature shared between nestmates requires 261 more time to be obtained. In fact, in Pachycondyla apicalis ants, cuticular lipids distribution 262 was homogenized between 5 and 10 days (Soroker et al. 2003). It has been demonstrated in 263 Polistes dominulus wasps, that young individuals can absorb hydrocarbons contrary to adults 264 (Lorenzi et al. 2004). This phenomenon could be the same in V. velutina nigrithorax. However, 265 for the most part of CHC quantities, individuals (callows and/or workers) have more 266 267 compounds than nest materials.

Callows cuticular profiles could be chemically undetectable and considered as "chemical 268 insignificant" (Martin et al. 2008b). Consequently, our results support previous publications in 269 other Hymenoptera (Lenoir et al. 1999; Uboni et al. 2012). Callows possess a weak chemical 270 signature, probably to avoid being rejected by nestmates and to more easily acquire the colonial 271 272 odour. Indeed, in the ant Cataglyphis cursor, newly emerged workers are adopted by an unrelated colony during their first four days and rejected after this date (Nowbahari and Lenoir 273 1989). In Myrmica sp. during the first days of adult life, chemical mimicry was observed by 274 grooming hosts (Lenoir et al. 1997). Moreover, callows of V. velutina possess more linear 275 alkanes than branched alkanes, contrary to adult workers which possess more branched alkanes 276 than linear alkanes. The weak chemical signature for callows is probably linked to a weak 277 quantity of compounds and in the same time with this difference in linear alkane and branched 278 alkane composition. 279

In our study, we observed that the chemical profiles of callows evolve during the first 48 hours after emergence. The chemical signature is dynamic and can depend on the age of the insects, as demonstrated in honey bees (Winston 1987) and the ants *Formica truncorum* (Nielsen et al. 1999). In Vespidae, the cuticular profile changes between 24 and 72 hours after
emergence (Panek et al. 2001). In Diptera, the same phenomenon has been observed, where
CHC quantities increase during the first four days (Jackson and Bartelt 1986).

286 The quantities of branched alkanes increase in callows with time (0.003 mg for C₀ compared to 0.007 mg in C₈), as it was observed in *Polistes dominulus* (Lorenzi et al. 2004). No difference 287 has been shown between C₈ callows and old workers, suggesting that their branched alkanes 288 quantities are stable at this age. An important number of branched alkanes may be present in 289 individuals which can leave the nest. In ants, it has been demonstrated that individuals which 290 stay in the nest had a lower ratio of branched alkanes (Wagner et al. 1998). Therefore, in these 291 species, branched alkanes could be considered as an indicator of the adult stage. It has been 292 suggested that CHC profiles are correlated with both the capability of insects to work outside 293 and their age (Bonavita-Cougourdan et al. 1993). 294

In different local environments (i.e., in the presence of conspecifics and/or a piece of nest), 295 callows show the same cuticular profiles. It suggests that these epigenetic factors are not 296 responsible for the acquisition of CHCs after emergence. This is true for the 6 days of our 297 experiments, but more time might be necessary to observe further CHC modifications. 298 299 Moreover, performing a local environment more representative of their social and natural conditions, for example with more hornets or a real nest environment, could be important, and 300 might be tested. However, some other epigenetic factors can also modify the cuticular profiles 301 302 like gut microbiota (García-Roa et al. 2022) or hormones (Wicker-thomas and Jallon 1995; Holze et al. 2021). 303

304

305 To conclude, our results showed that the chemical signature in *V. velutina nigrithorax* 306 is dynamic. Callows have a weak chemical signature which could be probably characterized as

a "cuticular insignificance chemical signature" in early life. This chemical signature evolves
rapidly during the first days and is not influenced by the environment (nestmate and/or nest
material). We also show that quantities of branched alkanes increase in callows, to reach those
corresponding to older workers. Further studies are needed to determine the factors able to
modulate CHC profiles at the beginning of adult life, like genetic and/or physiological factors.

312

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439 Figure legends

Fig. 1. Plot on the first two axis of the Principal Coordinate Analysis (PCoA) with Bray-Curtis
distances. C₀ corresponds to callows on their first day of emergence, N: nest materials and W:
workers.

443

Fig. 2. Boxplots representing quantities of the different chemical compound families according to modalities. C₀: callows on their first day [between 0 and 48 hours], N: nest materials and W: workers. Modalities with common letters are not significantly different (p > 0.05).

447

Fig. 3. Score and correlation circle plots on the first two axis from the PLS-DAs based on the chemical composition on different models. **Fig** (**A**) corresponds to the third model with three age classes where C_4 , C_6 and C_8 were clustered, and **Fig** (**B**) corresponds to the fourth model with two age classes where C_2 , C_4 , C_6 and C_8 were clustered. Correlation circle plots allow visualizing the discriminant compounds (Table S1), and how they contribute to each component. Ellipses represent confidence levels set up at 95%.

454

Fig. 4. Boxplots representing chemical compounds families' quantities according to modalities. Callows at different age: C_0 , C_2 , C_4 , C_6 , and C_8 . Modalities with common letter are not significantly different (p > 0.05).

458

Fig. 5. Boxplots representing chemical compound families' quantities according to modalities.
Callows were placed in different conditions: Callow only (C), Callow-Nest (CN), Callow-

- 461 Worker (CW), and Callow-Worker-Nest (CWN). Modalities with common letter are not
- 462 significantly different (p > 0.05).

464 Figures

Fig.1





468 Fig.2



Fig. 3



Fig. 4







478 **Fig. S1.** Chromatogram of a callow C_0 (less than 24 hours) with peaks identification.

479

Fig. S2. Plot on the first two axis of the Principal Coordinate Analysis (PCoA) on the chemical diversity between age modalities calculated with Bray-Curtis distances. Callows were collected at different days of emergence: C_0 , C_2 , C_4 , C_6 , and C_8 .

483

Fig. S3. Score plots and correlation circle plots on the first two axis from the chemical composition of PLS-DA models. (**A**) corresponds to the first model with five age classes, (**B**) to the second model with four age classes where C_6 and C_8 were clustered. Correlation circle plots allow visualizing the discriminant compounds (Table S1), and how they contribute to each component. Ellipses represent confidence levels set up at 95%.

489

490 Fig. S4. Plot on the first two axis of the Principal Coordinate Analysis (PCoA) on the

491 chemical diversity between modalities calculated with Bray-Curtis distances. Callows were

492 placed in different conditions: Callow only (C), Callow-Nest (CN), Callow-Worker (CW),

493 and Callow-Worker-Nest (CWN).



Fig. S2



Fig. S3



Fig. S4



504	Table S1. Identification of the main cuticular hydrocarbons (CHCs) of callows, workers and
505	nest materials of Vespa velutina nigrithorax. Variable Importance in the Projection (VIP) and
506	Equivalent Chain Lenght (ECL) were calculated. PLS-DA analyses based on age resulted in
507	VIP scores represented with * first model, ¤ second model, ♦ third model and # fourth model.
508	The percentage and standard error were calculated for emergent individual at different ages:
509	C ₀ , C ₂ , C ₄ , C ₆ and C ₈ .

Peaks	Compound identity	Retention Time (min)	VIP	ECL	C0 %	C2 %	C4 %	C6 %	C8 %
P1	<i>n</i> -C21	27.13	*¤ ♦ #	21.03	1.64 ±	$0.37 \pm$	$0.47 \pm$	$0.24 \pm$	0.13 ±
					0.45 SE	0.18 SE	0.30 SE	0.13 SE	0.06 SE
P2	<i>n</i> -C23	30.54	♦#	23.00	20.16 ±	$8.72 \pm$	6.23 ±	$3.45 \pm$	$2.57 \pm$
					2.60 SE	2.94 SE	3.02 SE	1.23 SE	1.39 SE
P3	X-C25:1	33.43	*¤♦#	24.76	$2.22 \pm$	$1.49\pm$	1.75 ±	$1.79\pm$	$1.30 \pm$
					3.18 SE	0.27 SE	0.28 SE	0.43 SE	0.34 SE
P4	<i>n</i> -C25 + Unknown	33.84	*¤♦	25.00	23.02 ±	$24.85 \pm$	$23.02\pm$	$19.69\pm$	$19.50 \pm$
					1.55 SE	3.06 SE	2.54 SE	3.25 SE	4.40 SE
P5	11-; 13-MeC25	34.36		25.33	$3.044 \pm$	$2.48 \pm$	2.13 ±	$1.69\pm$	$1.66 \pm$
					0.59 SE	0.26 SE	0.39 SE	0.58 SE	0.55 SE
P6	3-MeC25 + X-C26:1	34.99	*¤	25.74	3.37 ±	$3.29 \pm$	$3.78 \pm$	$3.73 \pm$	$3.80 \pm$
					0.43 SE	0.31 SE	0.39 SE	0.71 SE	0.87 SE
P7	<i>n</i> -C26	35.40	¤♦#	26.00	$0.80 \pm$	$1.13 \pm$	$1.25 \pm$	$1.23 \pm$	$1.32 \pm$
					0.61 SE	0.09 SE	0.16 SE	0.16 SE	0.19 SE
P8	X-C27:1 + X'-C27:1	36.64		26.78	8.78±	11.25 ±	12.16±	14.75 ±	13.12 ±
					1.17 SE	2.96 SE	2.90 SE	3.42 SE	3.36 SE
Р9	X''-C27:1	36.74		26.84	0.62 ±	0.61 ±	0.56 ±	0.60 ±	0.61 ±
					0.19 SE	0.38 SE	0.19 SE	0.15 SE	0.27 SE

P10	<i>n</i> -C27+X-MeC27:1	36.99	*¤♦	27.00	13.88 ±	$18.46 \pm$	$17.90 \pm$	$18.59 \pm$	20.12 ±
					1.59 SE	1.38 SE	0.92 SE	1.64 SE	2.93 SE
P11	X'-MeC27:1 + 13-; 11-;	37.48	*¤♦#	27.34	$9.57 \pm$	$9.56 \pm$	8.73 ±	$7.97\pm$	8.69 ±
	9-MeC27				1.00 SE	0.72 SE	0.85 SE	0.92 SE	1.36 SE
P12	11-; 15-; 9-; X-	37.87	♦#	27.6	0.99	$1.49 \pm$	$2.20 \pm$	$2.33 \pm$	$2.52 \pm$
	diMeC27				± 0.21 SE	0.23 SE	0.50 SE	0.62 SE	0.53 SE
P13	3-MeC27	38.09	#	27.75	$2.47 \pm$	5.02	$6.95\pm$	$7.88\pm$	$8.90\pm$
					0.39 SE	± 0.40 SE	1.15 SE	1.69 SE	0.58 SE
P14	5-,15-diMeC27 + 2-	38.17		27.81	$0.26 \pm$	$0.38\pm$	$0.60 \pm$	$1.08 \pm$	$0.86 \pm$
	,13-diMeC27 + 2-,15-				0.05 SE	0.08 SE	0.15 SE	1.58 SE	0.25 SE
	diMeC27								
P15	X,X'-diMeC27	38.56	•	28.07	$0.19\pm$	$0.42 \pm$	$0.85 \pm$	$1.24 \pm$	1.55 ±
	+ X-MeC28				0.04 SE	0.11 SE	0.28 SE	0.31 SE	0.46 SE
P16	X-C29:1	39.66		28.8	$3.08 \pm$	$4.36\pm$	$4.68\pm$	$6.41 \pm$	$5.32 \pm$
					0.66 SE	1.16 SE	1.08 SE	2.49 SE	1.88 SE
P17	X'-C29:1 + Unknown	39.78	*	28.88	$0.26\pm$	$0.29\pm$	$0.29\pm$	$0.50\pm$	$0.67 \pm$
					0.07 SE	0.18 SE	0.12 SE	0.19 SE	0.57 SE
P18	n-C29	39.96	*¤#	29.00	$2.25 \pm$	$1.86 \pm$	$1.72 \pm$	$1.65 \pm$	$1.69 \pm$
					0.29 SE	0.22 SE	0.22 SE	0.20 SE	0.57 SE
P19	13-; 11-; 9-; 7-MeC29	40.41	*¤	29.39	$2.25 \pm$	$2.26 \pm$	$1.99\pm$	$1.85 \pm$	$2.02 \pm$
					0.18 SE	0.22 SE	0.19 SE	0.19 SE	0.43 SE
P20	5-MeC29 + 11-,15-	40.49	¤♦#	29.71	$0.64 \pm$	$1.03 \pm$	$1.62 \pm$	$1.91 \pm$	$2.04 \pm$
	diMeC29				0.12 SE	0.11 SE	0,45 SE	0.39 SE	0.51 SE
P21	X,X'-diMeC31	43.69		31.63	$0.47 \pm$	$0.67 \pm$	1.12 ±	1.43 ±	1.60 ±
					0.08 SE	0.08 SE	0.24 SE	0.31 SE	0.54 SE

510 **Table S2.** P-values obtained by pairwise comparisons for CHCs quantities between modalities.

511 C₀: callows on their first day [between 0 and 48 hours], N: nest materials and W: workers. P-

values were adjusted by the Tukey method. **Bold** values are statistically significant (p < 0.05).

		C_0	Ν
Alkanes	N	0.043	-
	W	0.130	0.860
Alkenes	N	0.005	-
	W	0.840	0.001
Branched alkanes	N	0.950	-
	W	< 0.001	< 0.001

Table S3. Mean quantities $(\pm SE)$ calculated for callows on their first day [between 0 and 48]

515 hours], nest materials and workers.

	C0	Ν	W
Alkanes	0.016 ± 0.007	0.004 ± 0.001	$0.004 \pm < 0.001$
Alkenes	$< 0.001 \pm < 0.001$	$0.003 \pm < 0.001$	0.013 ± 0.004
Branched alkanes	$0.003 \pm < 0.001$	0.004 ± 0.001	0.011 ± 0.003

518	among the age of callows for each PLS-DA model. P-values are obtained by pairwise							
519	comparisons using factor fitting to an ordination. First PLS-DA model (i) with five age classes							
520	$(C_0, C_2, C_4, C_6, C_8)$, the second PLSDA model (ii) with four age classes (C_0, C_2, C_4, C_6-C_8) , the							
521	third PLSDA model (iii) with three age classes (C_0 , C_2 , C_4 - C_6 - C_8) and the fourth PLSDA model							
522	(iv) with two age classes (C_0 , C_2 - C_4 - C_6 - C_8). Bold values are statistically significant (adjusted							
523	by the False Discovery Rate, $p < 0.05$).							

Table S4. Age cross-tabulations, showing all pairwise differences of sum relative percentages

Models	Age	C_{0}	C_2	C4	C6
	C ₂	0.150	-	-	-
	C4	0.150	0.150	-	-
(i)	C ₆	0.150	0.150	0.740	-
	C ₈	0.150	0.150	0.190	0.740
	C ₂	0.130	-	-	-
	C4	0.140	0.080	-	-
(ii)	C ₆ - C ₈	0.060	0.060	0.190	-
<i>(</i>)	C ₂	0.110	-	-	-
(111)	C4- C6- C8	0.020	0.044	-	-
(iv)	C ₂ - C ₄ - C ₆ - C ₈	0.005	-	-	-

Table S5. P-values obtained by pairwise comparisons of the quantities of branched alkanes according to the age of the callows. Emergent individuals were collected at different ages: C_0 , C_2 , C_4 , C_6 , and C_8 . P-values obtained were adjusted by the Tukey method. **Bold** values are statistically significant (p < 0.05).

	Age	C_0	C ₂	C ₄	C ₆
	C ₂	0.630	-	-	-
Branched	C ₄	0.060	0.710	-	-
alkanes	C ₆	0.037	0.570	0.990	-
	C ₈	0.003	0.160	0.850	0.930

	C_{0}	C ₂	C4	C_6	C_8
Alkanes	0.016	0.007	0.007	0.004	0.003
	± 0.007	± 0.002	± 0.002	± 0.001	$\pm < 0.001$
Alkenes	< 0.001	0.012	0.013	0.014	0.014
	$\pm < 0.001$	± 0.004	± 0.004	± 0.004	± 0.004
Branched alkanes	0.003	0.004	0.006	0.006	0.007
	$\pm < 0.001$	$\pm < 0.001$	± 0.001	± 0.001	± 0.001

Table S6. Mean quantities $(\pm SE)$ calculated for callows at different ages: C₀, C₂, C₄, C₆, and

531 C₈.

Table S7. Mean quantities (± SE) calculated for callows. Callows were placed in different
conditions: Callow only (C), Callow-Nest (CN), Callow-Worker (CW), and Callow-WorkerNest (CWN).

	С	CN	CW	CWN
Alkanes	0.004	0.008	0.011	0.006
	± 0.001	± 0.002	± 0.005	± 0.002
Alkenes	0.014	0.041	0.031	0.025
	± 0.004	± 0.010	± 0.010	± 0.009
Branches alkanes	0.006	0.009	0.009	0.010
	± 0.001	± 0.002	± 0.002	± 0.002