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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

5
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22

23 **Abstract**

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

37

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

39 **Introduction**

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

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

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

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

88

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*

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

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

120

121 *Evolution of the chemical signatures over time*

122 Just after emergence, callows were isolated in plastic boxes, as described above. To
123 study the evolution of the chemical signature over time, callows were frozen killed at different
124 ages: on the day of emergence (between 0 and 24 h: C₀), two days later (between 48 and 72 h:
125 C₂), four days later (between 96 and 120 h: C₄), six days later (between 144 and 168 h: C₆), or
126 eight days later (between 192 and 216 h: C₈). CHCs from frozen individuals (n=90) were
127 immediately extracted following the same procedure as in the first experiment. For each age
128 modality (C₀, C₂, C₄, C₆, C₈), 6 technical replicates were performed per colony.

129

130 *Chemical signature acquisition depending on local environment*

131 Environmental conditions of callows were manipulated from the day of emergence (C₀)
132 according to four modalities: a callow was placed alone (C), with an old nestmate worker (CW,
133 condition Callow-Worker), with a piece of 0.5 g of nest material (CN, condition Callow-Nest),
134 or with both a worker nestmate and a piece of 0.5g of nest material (CWN, condition Callow-
135 Worker-Nest). Each callow was frozen on the sixth day of the experiments (6-days-old callows)

136 and CHCs (n=57) were extracted in the same way as previously described. For each modality
137 (C, CW, CN, CWN), five technical replicates were performed per colony, except for Saint-
138 Épain (5 samples C, 3 CW, 4 CN and 5 CWN). For each replicate, workers, callows and nest
139 material were provided from the same nest.

140

141 *Chemical analyses*

142 In each experiment, 2 μ l of each chemical extract were injected in a gas chromatograph
143 (Agilent Technologies 7820A GC) equipped with a Flame Ionization Detector (FID), and a
144 capillary column HP-5 (Agilent Technology 19091J-413, 30 m \times 320 μ m \times 0.25 μ m). Helium
145 was the carrier gas at 1.7 ml/min. The temperature program started from 50°C to 200°C at
146 8°C/min, then 200°C to 315°C at 5°C/min, and a 5-min hold at 315°C.

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

159

160 *Statistical analyses*

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.

165 Permutational Analysis of Variance (PERMANOVA, $n = 999$ permutations) were
166 performed to test the effects of the different modalities on the chemical profiles, for the three
167 experiments called (i) a potential “cuticular insignificance” of callows (C_0 vs W vs N), (ii) the
168 evolution of their chemical signatures over time (C_0 vs C_2 vs C_4 vs C_6 vs C_8), and (iii) the
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
171 chemical profile between each pair of samples, using the *adonis2* function from the *vegan*
172 package (v2.6-2). To illustrate results, Principal Coordinate Analysis (PCoA) were performed
173 using the *ape* package (v5.6-2).

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

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

187 For each sample, the quantity of each CHC was estimated with the following formula:

188 $Q_i = \frac{(Q_{C20} \times X_i)}{(X_{C20})}$ where Q corresponds to the quantity, X to the peak area and C20 to the internal

189 standard. Quantities were divided by the mass of hornets or nest materials to obtain the per-
190 gram quantity of each compound. Quantities were log-transformed to normalize data. To
191 analyse which CHC quantities differ between modalities, in the three above experiments, we
192 performed Linear Mixed Effect Models in R (LMER) with colony factors as random effect
193 using the lmer function (package lme4, v1.1-30). For all these analyses, chromatogram peaks
194 containing a mix of compounds were excluded. Prerequisites of each LMER models were
195 confirmed by the DHARMA package (v0.4.6). Afterwards, ANOVAs using models were carry
196 out using the car package (v3.1-1), and estimated marginal means were calculated using
197 pairwise comparisons.

198

199 **Results**

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

204

205 *Cuticular chemical insignificance of callows*

206 On the two first axis of the PCoA (**Fig. 1**), 87% of the chemical variability is explained
207 by modalities (C₀ vs W vs N) with a significant effect (PERMANOVA test, R² = 0.87; p =
208 0.003).

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

218

219 *Evolution of the chemical signatures over time*

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

233 The quantitative analysis showed significant differences for branched alkanes
234 (ANOVA, $p = 0.002$). Compared in pairs, C_0 is significantly different from C_6 and C_8 (Pairwise
235 tests, $p < 0.05$, **Table S5**). Branched alkanes quantities increase with time (**Fig. 4, Table S5,**
236 **S6**). No significant differences have been demonstrated for alkanes and alkenes ($p > 0.05$). We
237 have a large dispersion of data among callows aged from C_0 to C_4 . On the Fig. 4, alkanes
238 quantities seem to decrease with the age of callows whereas branched alkanes quantities
239 increase, as demonstrated in the **Table S6**. In the model, which included old workers and C_8
240 callows for branched alkanes quantities, no significative effect has been observed (ANOVA, p
241 $= 0.156$).

242

243 *Chemical signature acquisition depending on local environment*

244 Callows were placed in different contexts of local environments (C vs CW vs CN vs
245 CWN). As the second experiment showed no differences in cuticular profiles of C_4 , C_6 , and C_8
246 callows, the experiment was done up to 6th day.

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

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

253

254 **Discussion**

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

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

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

280 In our study, we observed that the chemical profiles of callows evolve during the first
281 48 hours after emergence. The chemical signature is dynamic and can depend on the age of the
282 insects, as demonstrated in honey bees (Winston 1987) and the ants *Formica truncorum*

283 (Nielsen et al. 1999). In Vespidae, the cuticular profile changes between 24 and 72 hours after
284 emergence (Panek et al. 2001). In Diptera, the same phenomenon has been observed, where
285 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
287 0.007 mg in C₈), as it was observed in *Polistes dominulus* (Lorenzi et al. 2004). No difference
288 has been shown between C₈ callows and old workers, suggesting that their branched alkanes
289 quantities are stable at this age. An important number of branched alkanes may be present in
290 individuals which can leave the nest. In ants, it has been demonstrated that individuals which
291 stay in the nest had a lower ratio of branched alkanes (Wagner et al. 1998). Therefore, in these
292 species, branched alkanes could be considered as an indicator of the adult stage. It has been
293 suggested that CHC profiles are correlated with both the capability of insects to work outside
294 and their age (Bonavita-Cougourdan et al. 1993).

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

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

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

312

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437

438

439 **Figure legends**

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

443

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

447

448 **Fig. 3.** Score and correlation circle plots on the first two axis from the PLS-DAs based on the
449 chemical composition on different models. **Fig (A)** corresponds to the third model with three
450 age classes where C₄, C₆ and C₈ were clustered, and **Fig (B)** corresponds to the fourth model
451 with two age classes where C₂, C₄, C₆ and C₈ were clustered. Correlation circle plots allow
452 visualizing the discriminant compounds (Table S1), and how they contribute to each
453 component. Ellipses represent confidence levels set up at 95%.

454

455 **Fig. 4.** Boxplots representing chemical compounds families' quantities according to modalities.
456 Callows at different age: C₀, C₂, C₄, C₆, and C₈. Modalities with common letter are not
457 significantly different ($p > 0.05$).

458

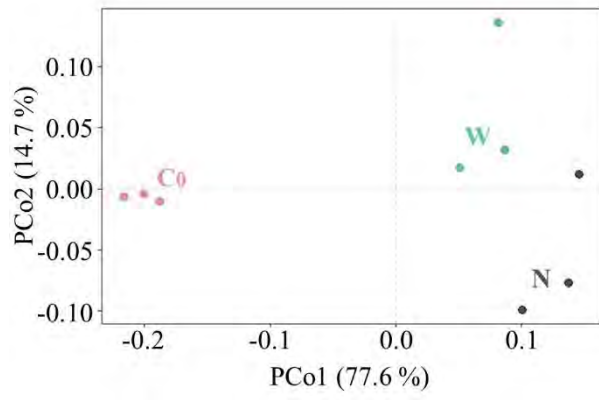
459 **Fig. 5.** Boxplots representing chemical compound families' quantities according to modalities.
460 Callows were placed in different conditions: Callow only (C), Callow-Nest (CN), Callow-

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

463

464 **Figures**

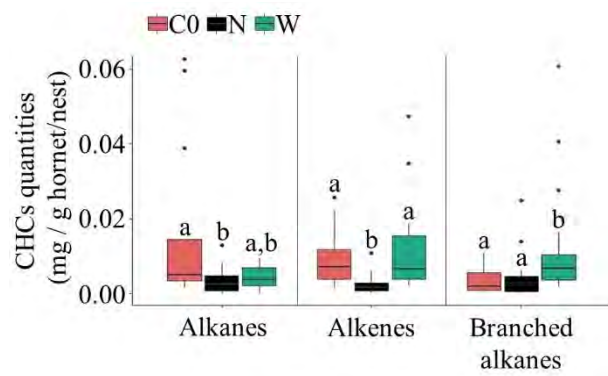
465 **Fig.1**



466

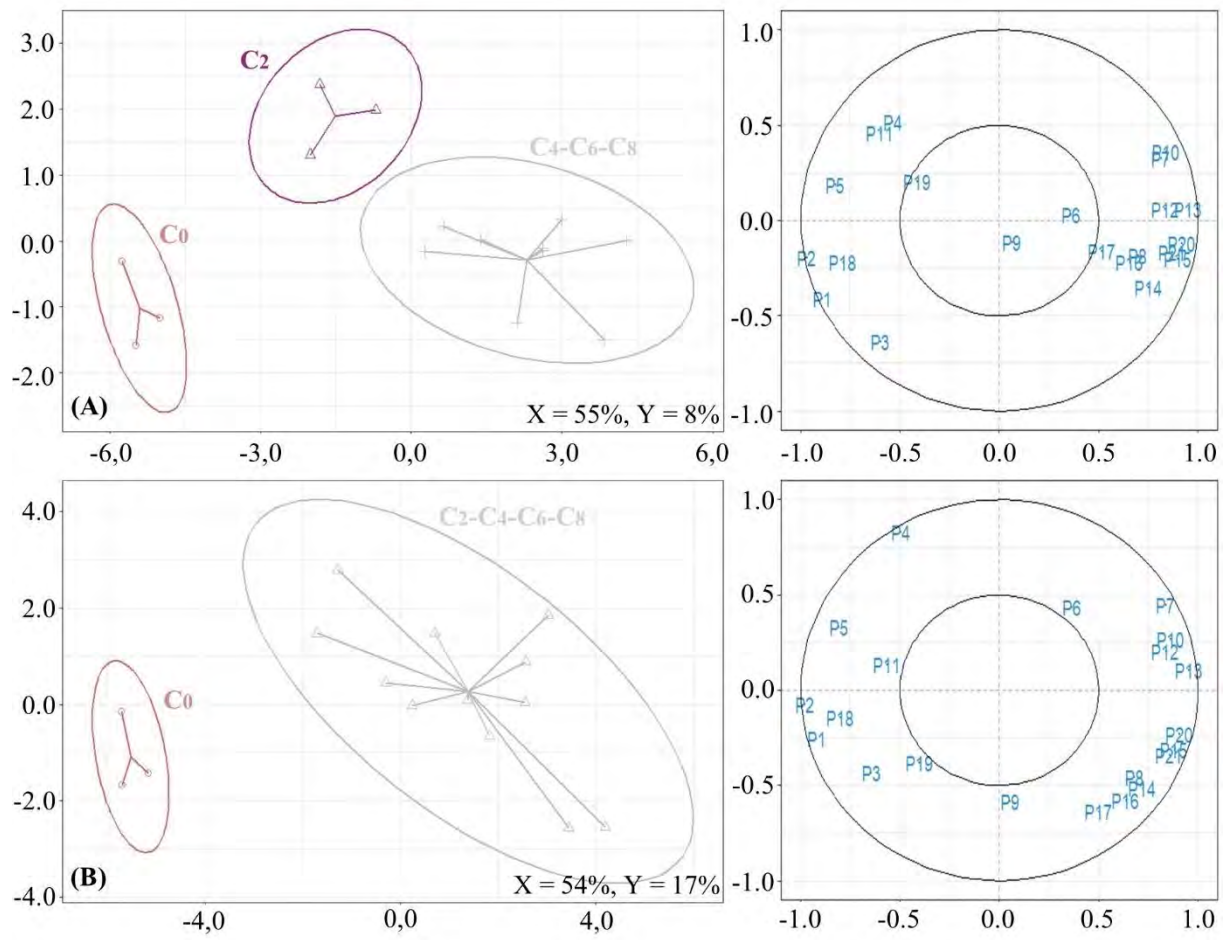
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468 **Fig.2**



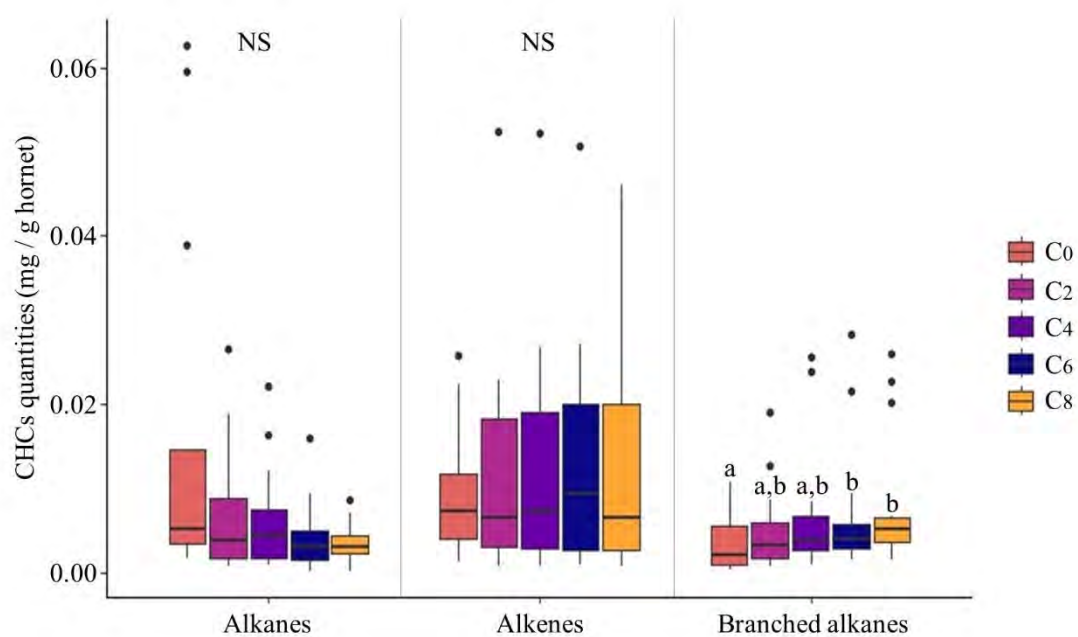
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470 **Fig. 3**



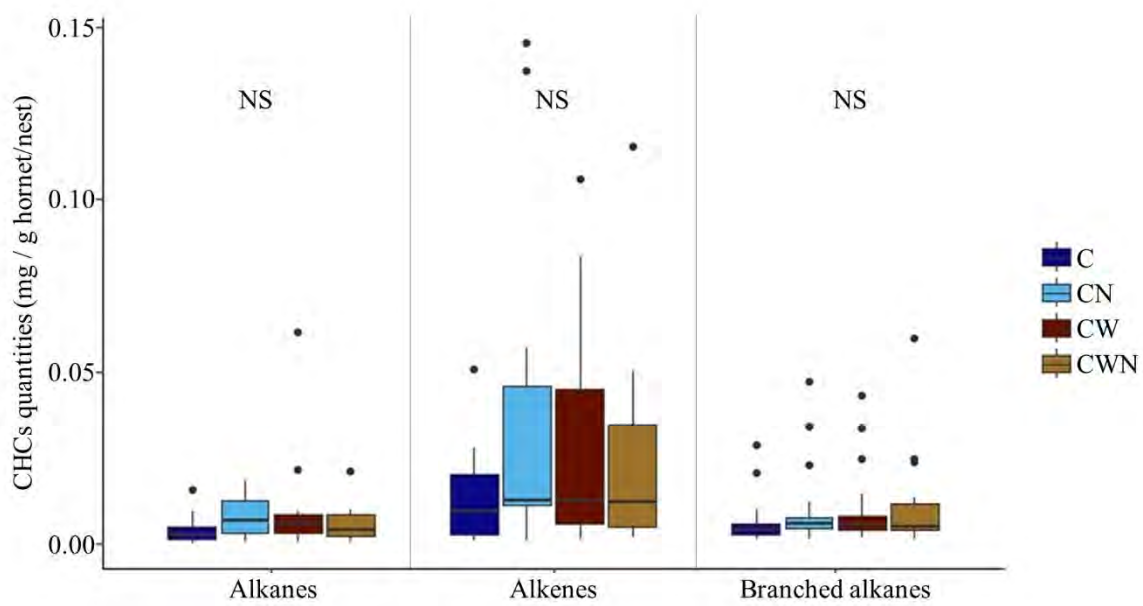
471

472 **Fig. 4**



473
474

475 **Fig. 5**



476

477 **Supplementary files**

478 **Fig. S1.** Chromatogram of a callow C₀ (less than 24 hours) with peaks identification.

479

480 **Fig. S2.** Plot on the first two axis of the Principal Coordinate Analysis (PCoA) on the chemical
481 diversity between age modalities calculated with Bray-Curtis distances. Callows were collected
482 at different days of emergence: C₀, C₂, C₄, C₆, and C₈.

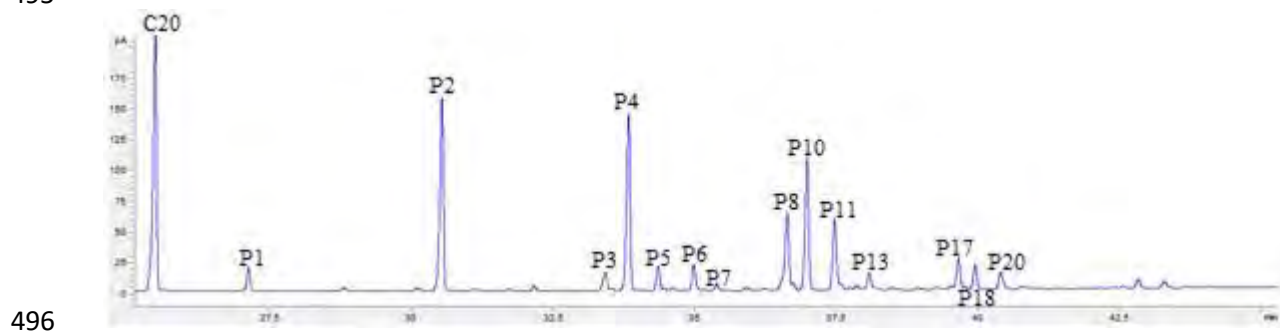
483

484 **Fig. S3.** Score plots and correlation circle plots on the first two axis from the chemical
485 composition of PLS-DA models. **(A)** corresponds to the first model with five age classes, **(B)**
486 to the second model with four age classes where C₆ and C₈ were clustered. Correlation circle
487 plots allow visualizing the discriminant compounds (Table S1), and how they contribute to each
488 component. Ellipses represent confidence levels set up at 95%.

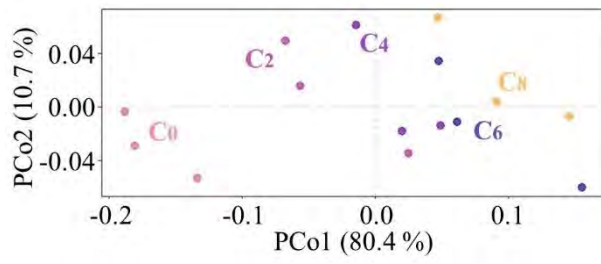
489

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).

494 **Fig. S1**
495

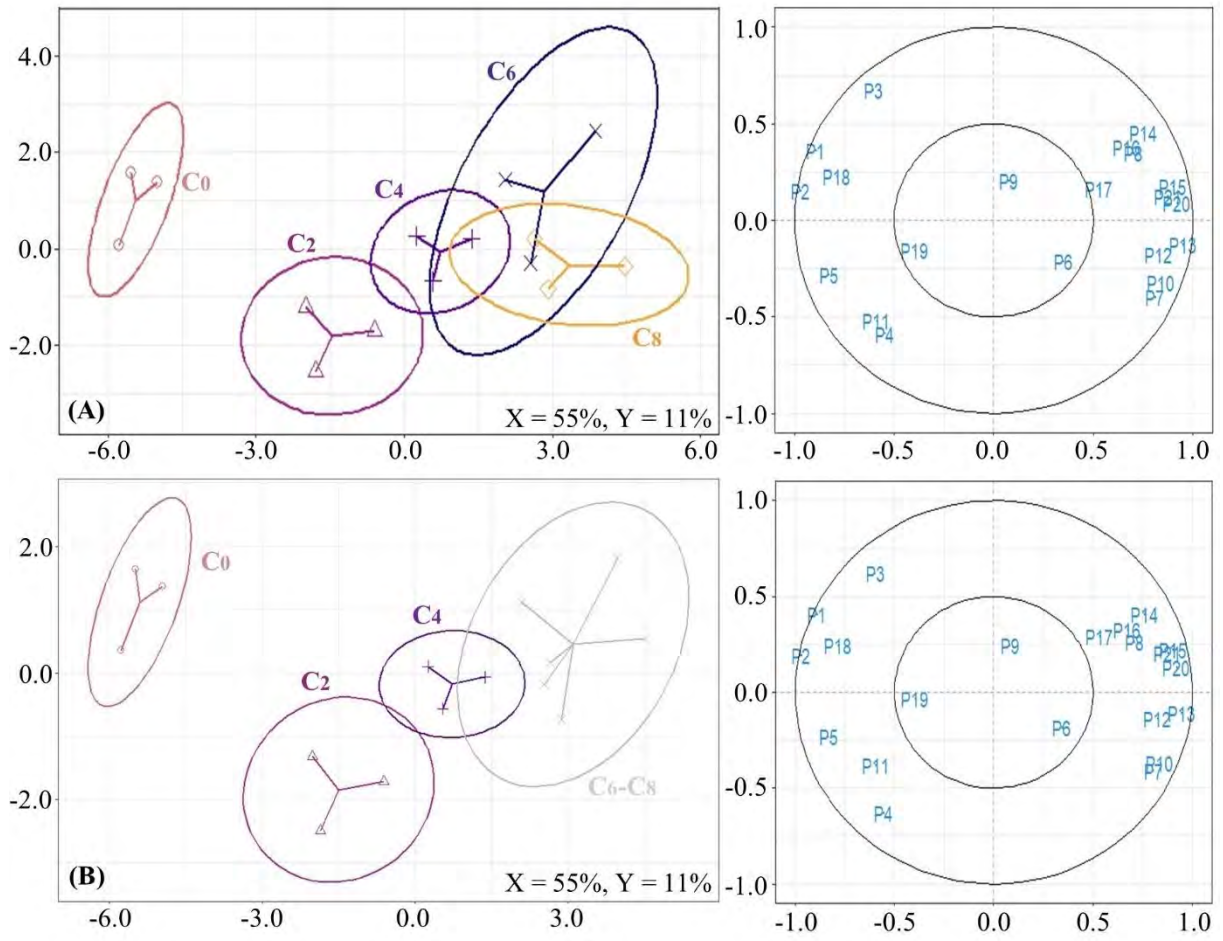


497 **Fig. S2**



498

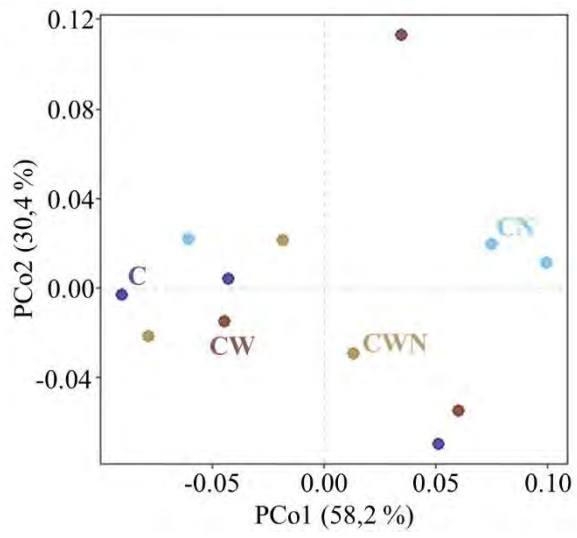
499 **Fig. S3**



500

501

502 **Fig. S4**



503

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 Length (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 C₀, C₂, C₄, C₆ and C₈.

Peaks	Compound identity	Retention Time (min)	VIP	ECL	C ₀ %	C ₂ %	C ₄ %	C ₆ %	C ₈ %
P1	<i>n</i> -C21	27.13	*□◆#	21.03	1.64 ± 0.45 SE	0.37 ± 0.18 SE	0.47 ± 0.30 SE	0.24 ± 0.13 SE	0.13 ± 0.06 SE
P2	<i>n</i> -C23	30.54	◆#	23.00	20.16 ± 2.60 SE	8.72 ± 2.94 SE	6.23 ± 3.02 SE	3.45 ± 1.23 SE	2.57 ± 1.39 SE
P3	X-C25:1	33.43	*□◆#	24.76	2.22 ± 3.18 SE	1.49 ± 0.27 SE	1.75 ± 0.28 SE	1.79 ± 0.43 SE	1.30 ± 0.34 SE
P4	<i>n</i> -C25 + Unknown	33.84	*□◆	25.00	23.02 ± 1.55 SE	24.85 ± 3.06 SE	23.02 ± 2.54 SE	19.69 ± 3.25 SE	19.50 ± 4.40 SE
P5	11-; 13-MeC25	34.36		25.33	3.044 ± 0.59 SE	2.48 ± 0.26 SE	2.13 ± 0.39 SE	1.69 ± 0.58 SE	1.66 ± 0.55 SE
P6	3-MeC25 + X-C26:1	34.99	*□	25.74	3.37 ± 0.43 SE	3.29 ± 0.31 SE	3.78 ± 0.39 SE	3.73 ± 0.71 SE	3.80 ± 0.87 SE
P7	<i>n</i> -C26	35.40	□◆#	26.00	0.80 ± 0.61 SE	1.13 ± 0.09 SE	1.25 ± 0.16 SE	1.23 ± 0.16 SE	1.32 ± 0.19 SE
P8	X-C27:1 + X'-C27:1	36.64		26.78	8.78 ± 1.17 SE	11.25 ± 2.96 SE	12.16 ± 2.90 SE	14.75 ± 3.42 SE	13.12 ± 3.36 SE
P9	X''-C27:1	36.74		26.84	0.62 ± 0.19 SE	0.61 ± 0.38 SE	0.56 ± 0.19 SE	0.60 ± 0.15 SE	0.61 ± 0.27 SE

P10	<i>n</i> -C27+ X-MeC27:1	36.99	*□◆	27.00	13.88 ± 1.59 SE	18.46 ± 1.38 SE	17.90 ± 0.92 SE	18.59 ± 1.64 SE	20.12 ± 2.93 SE
P11	X'-MeC27:1 + 13-; 11-; 9-MeC27	37.48	*□◆#	27.34	9.57 ± 1.00 SE	9.56 ± 0.72 SE	8.73 ± 0.85 SE	7.97 ± 0.92 SE	8.69 ± 1.36 SE
P12	11-; 15-; 9-; X- diMeC27	37.87	◆#	27.6	0.99 ± 0.21 SE	1.49 ± 0.23 SE	2.20 ± 0.50 SE	2.33 ± 0.62 SE	2.52 ± 0.53 SE
P13	3-MeC27	38.09	#	27.75	2.47 ± 0.39 SE	5.02 ± 0.40 SE	6.95 ± 1.15 SE	7.88 ± 1.69 SE	8.90 ± 0.58 SE
P14	5-,15-diMeC27 + 2- ,13-diMeC27 + 2-,15- diMeC27	38.17		27.81	0.26 ± 0.05 SE	0.38 ± 0.08 SE	0.60 ± 0.15 SE	1.08 ± 1.58 SE	0.86 ± 0.25 SE
P15	X,X'-diMeC27 + X-MeC28	38.56	◆	28.07	0.19 ± 0.04 SE	0.42 ± 0.11 SE	0.85 ± 0.28 SE	1.24 ± 0.31 SE	1.55 ± 0.46 SE
P16	X-C29:1	39.66		28.8	3.08 ± 0.66 SE	4.36 ± 1.16 SE	4.68 ± 1.08 SE	6.41 ± 2.49 SE	5.32 ± 1.88 SE
P17	X'-C29:1 + Unknown	39.78	*	28.88	0.26 ± 0.07 SE	0.29 ± 0.18 SE	0.29 ± 0.12 SE	0.50 ± 0.19 SE	0.67 ± 0.57 SE
P18	<i>n</i> -C29	39.96	*□#	29.00	2.25 ± 0.29 SE	1.86 ± 0.22 SE	1.72 ± 0.22 SE	1.65 ± 0.20 SE	1.69 ± 0.57 SE
P19	13-; 11-; 9-; 7-MeC29	40.41	*□	29.39	2.25 ± 0.18 SE	2.26 ± 0.22 SE	1.99 ± 0.19 SE	1.85 ± 0.19 SE	2.02 ± 0.43 SE
P20	5-MeC29 + 11-,15- diMeC29	40.49	□◆#	29.71	0.64 ± 0.12 SE	1.03 ± 0.11 SE	1.62 ± 0.45 SE	1.91 ± 0.39 SE	2.04 ± 0.51 SE
P21	X,X'-diMeC31	43.69		31.63	0.47 ± 0.08 SE	0.67 ± 0.08 SE	1.12 ± 0.24 SE	1.43 ± 0.31 SE	1.60 ± 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-
 512 values were adjusted by the Tukey method. **Bold** values are statistically significant ($p < 0.05$).

		C ₀	N
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

513

514 **Table S3.** Mean quantities (\pm SE) calculated for callows on their first day [between 0 and 48
515 hours], nest materials and workers.

	C0	N	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

516

517 **Table S4.** Age cross-tabulations, showing all pairwise differences of sum relative percentages
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 (C₀, C₂, C₄, C₆, C₈), the second PLSDA model (ii) with four age classes (C₀, C₂, C₄, C₆-C₈), the
521 third PLSDA model (iii) with three age classes (C₀, C₂, C₄-C₆-C₈) and the fourth PLSDA model
522 (iv) with two age classes (C₀, C₂-C₄-C₆-C₈). **Bold** values are statistically significant (adjusted
523 by the False Discovery Rate, p < 0.05).

Models	Age	C ₀	C ₂	C ₄	C ₆
(i)	C ₂	0.150	-	-	-
	C ₄	0.150	0.150	-	-
	C ₆	0.150	0.150	0.740	-
	C ₈	0.150	0.150	0.190	0.740
(ii)	C ₂	0.130	-	-	-
	C ₄	0.140	0.080	-	-
	C ₆ - C ₈	0.060	0.060	0.190	-
(iii)	C ₂	0.110	-	-	-
	C ₄ - C ₆ - C ₈	0.020	0.044	-	-
(iv)	C ₂ - C ₄ - C ₆ - C ₈	0.005	-	-	-

524

525 **Table S5.** P-values obtained by pairwise comparisons of the quantities of branched alkanes
 526 according to the age of the callows. Emergent individuals were collected at different ages: C₀,
 527 C₂, C₄, C₆, and C₈. P-values obtained were adjusted by the Tukey method. **Bold** values are
 528 statistically significant ($p < 0.05$).

	Age	C ₀	C ₂	C ₄	C ₆
Branched alkanes	C ₂	0.630	-	-	-
	C ₄	0.060	0.710	-	-
	C ₆	0.037	0.570	0.990	-
	C ₈	0.003	0.160	0.850	0.930

529

530 **Table S6.** Mean quantities (\pm SE) calculated for callows at different ages: C₀, C₂, C₄, C₆, and
 531 C₈.

	C ₀	C ₂	C ₄	C ₆	C ₈
Alkanes	0.016 \pm 0.007	0.007 \pm 0.002	0.007 \pm 0.002	0.004 \pm 0.001	0.003 \pm < 0.001
Alkenes	< 0.001 \pm < 0.001	0.012 \pm 0.004	0.013 \pm 0.004	0.014 \pm 0.004	0.014 \pm 0.004
Branched alkanes	0.003 \pm < 0.001	0.004 \pm < 0.001	0.006 \pm 0.001	0.006 \pm 0.001	0.007 \pm 0.001

532

533 **Table S7.** Mean quantities (\pm SE) calculated for callows. Callows were placed in different
534 conditions: Callow only (C), Callow-Nest (CN), Callow-Worker (CW), and Callow-Worker-
535 Nest (CWN).

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

536