

1 **Nest structures display specific hydrocarbon profiles: insights into the chemical ecology**
2 **of the invasive yellow-legged hornet *Vespa velutina nigrithorax***

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12

13 **Abstract**

14 In insects, chemical communication is the most common form of communication, and cuticular
15 hydrocarbons (CHCs) are employed in recognition processes. In social insects, CHCs also help
16 define colony identity and thus contribute to social cohesion among nestmates. Individuals can
17 deposit their chemical signatures on nest surfaces. This information serves as a reference for
18 newly emerged individuals and allows them to obtain the odor specific to their colony. This
19 study examined nest chemical profiles in an inbred invasive species: the yellow-legged hornet,
20 *Vespa velutina nigrithorax*. We demonstrated that nest structures (i.e., envelopes, combs, and
21 pillars) had specific hydrocarbon profiles, which were colony specific. There were similarities
22 between the chemical profiles of the nests and the CHC signatures of hornets. The loss of
23 genetic diversity previously documented in the yellow-legged hornet population in France does
24 not appear to have constrained nest chemical diversity.

25

26 **Keywords:** cuticular hydrocarbons, chemical marking, Hymenoptera, invasive species

27

28 **Introduction**

29 The members of social insect colonies operate as a unit and are thus considered to form
30 a higher-level organism (Wheeler 1911). The stability of social groups relies on group members
31 being able to differentiate between those who belong to the group and those who do not
32 (Hamilton 1987). This recognition system is based on chemical cues (Hölldobler 1995).

33 In insects, chemical communication can be carried out by pheromones, and substrates
34 may be marked with volatile or non-volatile compounds. In social insects, nestmates can

35 communicate using cuticular hydrocarbons (CHCs). CHCs are non-volatile compounds and
36 form a complex layer on the insect cuticle, providing essential protection against desiccation
37 (Hadley 1984; Gibbs 1998). They also play a key role in kin recognition (Espelie and Hermann
38 1990), nestmate recognition (Hefetz 2007; D’Ettorre and Lenoir 2010), caste recognition
39 (Howard et al. 1982; Smith and Taylor Jr 1990; Bagnères et al. 1998; Kaib et al. 2000), and the
40 division of labor (Bonavita-Cougourdan et al. 1993; Scholl and Naug 2011; Rahman et al.
41 2016). The CHC signatures of insects are often complex and dynamic, with compound levels
42 that vary qualitatively and quantitatively (Bonavita-Cougourdan et al. 1993; Lenoir et al. 1999,
43 2001). Indeed, insects have personalized chemical signatures that are shaped by endogenous
44 factors (Fan et al. 2004; Lengyel et al. 2007; Darrouzet et al. 2014), exogenous factors
45 (Bagnères et al. 1990), and parasitism (Bagnères et al. 1996; Darrouzet et al. 2010; Lebreton et
46 al. 2010). An individual’s chemical signature serves as a type of highly specific “visa,”
47 conveying the individual’s identity to conspecifics (Fresneau and Errard 1994).

48 Some invasive social insects are tolerant of small differences in CHC signatures, a trait
49 that enhances their invasive success (Suarez et al. 1999; Tsutsui et al. 2001; Pedersen et al.
50 2006; Brandt et al. 2009). In such species, colonies differ less in their chemical profiles and
51 thus compete less for resources. One example of this phenomenon is the Argentine ant,
52 *Linepithema humile*, which has successfully colonized several continents (Holway and Case
53 1999; Suarez et al. 2000). In this context, attention has turned to a species that recently invaded
54 Europe: the yellow-legged hornet, *Vespa velutina nigrithorax*. This hornet is native to China
55 and, more specifically, comes from two provinces near Shanghai (Arca 2012; Arca et al. 2015).
56 It was accidentally introduced into southwestern France around 2004 (Haxaire et al. 2006) and
57 then spread rapidly throughout Europe (Robinet et al. 2018). It is now found in Spain, Portugal,
58 Italy, Germany, and, more recently, in Belgium, the Netherlands, Switzerland, and the United
59 Kingdom (Bruneau 2011; López et al. 2011; Grosso-silva and Maia 2012; Demichelis et al.
60 2014; Monceau and Thiéry 2017; Robinet et al. 2017, 2018). To feed its larvae, it consumes
61 large quantities of insects (Villemant et al. 2011) and, more particularly, of honey bees
62 (Darrouzet and Gévar 2014; Darrouzet 2019). Bee colonies suffer as a result, and their foraging
63 activity decreases, thus impacting beekeeping (Requier et al. 2018, 2019; Leza et al. 2019). Not
64 only is the yellow-legged hornet a threat to biodiversity, but it is also responsible for drastic
65 economic losses as well as public health concerns (Beggs et al. 2011; Darrouzet 2019; Laborde-
66 Castérot et al. 2020).

67 Despite the lack of genetic diversity in the European yellow-legged hornet population
68 (Arca et al. 2015; Darrouzet et al. 2015), hornet chemical signatures, composed of CHCs, were

69 found to vary tremendously based on caste, sex, and colony (Gévar et al. 2017). This result was
70 surprising because such heterogeneity has not been observed in other invasive social insects,
71 such as ants [*Linepithema humile* (Tsutsui et al. 2000; Brandt et al. 2009); *Wasmannia*
72 *auropunctata* (Errard et al. 2005)] or termites [*Reticulitermes flavipes* (Perdereau et al. 2010)].
73 Previous studies have suggested that this lower level of chemical variability may arise from the
74 reduced genetic diversity that can result from genetic bottlenecks (Tsutsui et al. 2001) or
75 selection against less-common recognition alleles (Giraud et al. 2002). Gévar et al. (2017)
76 suggested that the heterogeneity of CHC profiles in *Vespa velutina nigrithorax* may be affected
77 by environmental and/or epigenetic factors. For example, in termites, changes in temperature
78 or diet can erase or modify colony odor in *Coptotermes formosanus* (Shelton and Grace 1997;
79 Florane et al. 2004). In *Reticulitermes flavipes*, seasonal variation can modify chemical
80 signatures (Bagnères et al. 1990); in *Cryptotermes brevis*, profiles shift depending on specific
81 combinations of temperature and relative humidity (Woodrow et al. 2000). The composition of
82 the intestinal bacteria community can also affect nestmate recognition in *Reticulitermes*
83 *speratus* (Matsuura 2001, 2003).

84 In eusocial insects, nest odors are present and attractive to colony members (Kukuk et
85 al. 1977; Gamboa et al. 1986; Ferguson et al. 1987). Indeed, colony odor helps maintain colony
86 unity in ants and termites (Wilson 1971). Insects may use visual cues for nest recognition, but
87 olfactory cues have also been observed in *Apis mellifera* (Butler et al. 1969, 1970; Free 1987)
88 and *Lasioglossum zephyrum* (Kukuk et al. 1977). Cuticular hydrocarbons may be deposited on
89 nest structures, like the walls and soil surfaces of ant nests (Bos et al. 2011), the waxy combs
90 of beehives (Couvillon et al. 2007), and the paper nests of social wasps (Espelie and Hermann
91 1990; Elia et al. 2017). For example, the ant *Lasius niger* passively deposits CHCs on the inner
92 walls of its nest via its tarsi (Devigne and Detrain 2006; Lenoir et al. 2009). *Camponotus*
93 *aethiops* indirectly transfers CHCs to its nest, creating a uniform colony odor (Bos et al. 2011).
94 In the social wasp *Polistes metricus*, the CHCs of adult wasps may be similar to those of the
95 nest (Espelie et al. 1990). The chemical compounds laid down within the nest allow newly
96 emerged individuals to recognize and obtain their colony's signature (Singer and Espelie 1992).
97 In the social wasp *Polistes biglumis*, there is temporal variation in nest chemical profiles, which
98 can also shift when colonies are invaded by the social parasite *Polistes atrimandibularis* (Elia
99 et al. 2017).

100 This study aimed to investigate (1) whether nests built by *Vespa velutina nigrithorax*
101 displayed hydrocarbon-based chemical profiles, (2) whether the three main nest structures
102 (envelopes, combs, and pillars) had similar or different chemical profiles, and (3) whether nests

103 differed in their chemical profiles. We hypothesized that, since hornet workers display colony-
104 specific CHC signatures (Gévar et al. 2017), the latter should be reflected in the chemical
105 profiles of nests, since hydrocarbons should be deposited onto nest materials. This process
106 could influence colony signatures across generations (Singer and Espelie 1992), overcoming
107 the effects of inbreeding within the invasive population (Darrouzet et al. 2015).

108

109 **Materials and Methods**

110 Vespidae nests are built with plant fibers mixed with water and saliva (Edwards 1980). Yellow-
111 legged hornet nests are located in different habitats but frequently occur in tree tops; they can
112 generally be distinguished from the nests of other social Vespidae in France by the presence of
113 a lateral entrance (Rome et al. 2009; Darrouzet 2013, 2019). Nests are composed of a stack of
114 several combs that are connected by pillars and covered by a protective envelope (Darrouzet
115 2019). The combs are composed of alveolar cells that open downward; these cells harbor larvae
116 and nymphs at different developmental stages. The top of the nest does not contain cells;
117 instead, it consists of a sponge-like structure that likely protects the lower part of the nest (where
118 the hornets are found) against harsh environmental conditions (e.g., rain). Nest construction
119 follows a cyclical pattern and varies depending on the colony's degree of development. After
120 overwintering, the foundress will begin nest building in early spring (March–April): she will
121 first affix the pedicel and then build an initial comb of a dozen alveolar cells that are surrounded
122 by a protective envelope. Around May, workers will emerge. They will enlarge and strengthen
123 the nest along the vertical axis by adding new combs and along the horizontal axis by adding
124 new cells to already established combs (Rome et al. 2015; Darrouzet 2019).

125

126 *Chemical analysis of different nest structures*

127 *Nest collection*

128 A total of 10 *Vespa velutina nigrithorax* nests (Table S1) were collected in France's Centre-
129 Val-de-Loire region between 2015 and 2017. Each nest was processed immediately and stored
130 at -20°C. Chemical extractions were carried out in 2018.

131

132 *Hydrocarbon extractions*

133 Hydrocarbons were extracted from three distinct nest structures: the envelopes, the combs, and
134 the pillars. For each structure, three replicates were performed per nest (n = 10 nests). To extract
135 the hydrocarbons, 150 mg of structural material was placed in a 20-ml glass vial and crushed

136 down to small particles. Then, 5 ml of heptane was added, and the whole mixture was vortexed
137 for 1 minute at 300 rpm. It was left to rest for 24 hours, allowing the particles to settle.
138 Subsequently, 1.5 ml of the supernatant was transferred into 2-ml glass vials and fully
139 evaporated under nitrogen flux. Then, 150 μ l of heptane was added to each vial, and the mixture
140 was vortexed for 30 seconds at 300 rpm. Afterward, 100- μ l aliquots of these extracts were
141 transferred into vials. Before the chemical analyses were performed, 3 μ l of C20 (n-eicosane
142 10^{-3} g/ml) was added as an internal standard.

143

144 *Detailed comb analyses*

145 *Nest collection and sampling*

146 To carry out a more detailed chemical analysis of the nests, three end-of-season nests (i.e., large
147 nests; Table S2) were collected in the Centre-Val-de-Loire, Brittany, and Pays-de-la-Loire
148 regions from February to March 2018. The combs' hydrocarbon profiles were analyzed by
149 taking samples from different parts of the nest: from the top comb to the bottom comb and from
150 the center, middle, and periphery of the individual combs. In each case, three replicates were
151 performed (n = 3 nests).

152

153 *Hydrocarbon extraction*

154 To extract the hydrocarbons, 150 mg of structural material was collected. Then, 5 ml of heptane
155 was added. The whole mixture was vortexed for 1 minute at 300 rpm and left to rest for 24
156 hours. Subsequently, 100 μ l of the supernatant was transferred to 2-ml glass vials containing
157 an insert. Before the chemical analyses were performed, 3 μ l of the internal standard (C20 n-
158 eicosane at 10^{-3} g/ml) was added to samples.

159

160 *Chemical analyses*

161 Samples were analyzed using gas chromatography (GC), employing an Agilent Technologies
162 7000C GC/MS Triple Quad with a 7890 BGC System equipped with a capillary column (HP-5
163 Agilent Technology, Santa Clara, USA; 30 m \times 0.32 mm \times 0.25 μ m); helium was the carrier
164 gas (1.7 ml/min). The following temperature program was used: an initial temperature ramp
165 from 50 $^{\circ}$ C to 200 $^{\circ}$ C at 8 $^{\circ}$ C/min, a second temperature ramp from 200 $^{\circ}$ C to 315 $^{\circ}$ C at 5
166 $^{\circ}$ C/min, and a 5-min final hold at 315 $^{\circ}$ C. The surface areas of the 27 major peaks were
167 determined using ChemStation software (Agilent Technologies). The hydrocarbon profiles of
168 the nests were compared to the species' chemical signature. The identities of the chemical
169 compounds present on the nest structures were determined via comparison with the *Vespa*

170 *velutina nigrithorax* CHCs analyzed using the same GC system (Gévar et al. 2017). Compound
171 identities were confirmed using GC-MS analyses performed with an Agilent Technologies
172 7000C GC/MS Triple Quad with a 7890B GC System equipped with the same column as above;
173 we also used the same temperature program as above.

174

175 *Calculations and statistical analyses*

176 Principal component analysis (PCA) was carried out using R software (v. 3.4.3) and the
177 packages FactoMineR (Lê et al. 2008) and RcmdrPluginFactoMineR (Fox 2005), which are
178 included in the software. Differences in the hydrocarbon profiles of the three nest structures
179 (envelopes, combs, and pillars) among and within nests were quantified using the Nei distance
180 (Nowbahari et al. 1990; Dronnet et al. 2006). A dissimilarity matrix, which included all possible
181 pairwise comparisons (within structures, among structures within nests, and among nests for a
182 given structure), was constructed using the mean relative areas of the hydrocarbon peaks. The
183 matrix was then analyzed using a Kruskal-Wallis test implemented in RStudio (v. 1.0.143;
184 ©2009–2016 RStudio, Inc). For each structure, hydrocarbon quantity was estimated using the
185 following formula: $Q_i = (QC_{20} \times x_i) / (x_{C_{20i}})$ (Gévar et al. 2017). The quantity of each
186 hydrocarbon was then divided by the mass of the nest sample to obtain the mass-specific
187 quantity of each compound. Then, Kruskal-Wallis nonparametric tests were performed using
188 RStudio software.

189

190 **Results**

191 *Hydrocarbon analyses*

192 The nest structures bore the same hydrocarbon compounds as the cuticle of *Vespa velutina*
193 *nigrithorax* (Gévar et al. 2017) (Table 1). There were 8 alkanes, 10 methyl alkanes, 3 dimethyl
194 alkanes, 1 di/trimethyl alkane, and 5 alkenes among the 27 major peaks analyzed.

195

196 *Differences in nest structure hydrocarbons*

197 The three nest structures (envelopes, combs, and pillars) had distinct hydrocarbon profiles (Fig.
198 1) in which certain compounds predominated. In particular, there were two alkanes and three
199 methyl alkanes specifically found in the envelopes and combs; one alkane and one methyl
200 alkane specifically found in the combs and pillars; and one alkane, five methyl alkanes, two
201 dimethyl alkanes, and two alkenes specifically found in the envelopes and pillars (Table 2).

202

203 *Chemical distances for nest structures*

204 Chemical distances were compared within and among nest structures (Fig. S1). Chemical
205 distances within structures (i.e., among samples from a given structure, regardless of nest
206 identity) were significantly smaller than chemical distances among structures (i.e., among nest
207 structures within the same nest, regardless of nest identity) (Kruskal-Wallis; p -value < 0.001).
208 Similarly, for each structure, chemical distances among nests were significantly greater than
209 chemical distances within structures (Kruskal-Wallis; p -value < 0.001).

210

211 *Quantitative analyses of the nest structures*

212 The envelopes had significantly lower quantities of total hydrocarbons than did the other two
213 structures (Kruskal-Wallis; p -value < 0.001). The combs had significantly higher quantities of
214 total hydrocarbons than did the pillars (Kruskal-Wallis; p -value < 0.05) (Fig. 2).

215 The structures all differed from each other in specific hydrocarbon quantities (Kruskal-Wallis;
216 p -value < 0.001 for most comparisons, with p -value < 0.05 for the comparisons of alkanes and
217 branched alkanes between the pillars and combs). For each compound, the envelopes displayed
218 the lowest quantities, and the combs displayed the highest quantities (Fig. 3).

219

220 *Qualitative analyses of the combs*

221 Within combs, we found no significant differences among center samples or between center
222 and middle samples (regardless of comb or nest) (Kruskal-Wallis; p -value > 0.05 ; Fig. S2). The
223 chemical distances between center and periphery samples were significantly higher than the
224 chemical distances among center samples (Kruskal-Wallis; p -value < 0.001 ; Fig. S2). Finally,
225 the chemical distances among middle samples and among periphery samples were significantly
226 different as were the chemical distances between middle and periphery samples (Kruskal-
227 Wallis; p -value < 0.001 ; Fig. S2).

228 The results for the chemical distances among combs (Fig. S3) revealed that only comb 1 (the
229 oldest comb, found at the top of the nest) was significantly different from all the others
230 (Kruskal-Wallis; p -value < 0.05), with the exception of comb 7 (Kruskal-Wallis; p -value $>$
231 0.05). The other combs were not significantly different from each other (Kruskal-Wallis; p -
232 value > 0.05).

233

234 *Quantitative analyses of the combs*

235 Within combs, hydrocarbon quantities decreased significantly from the center to the periphery
236 (Kruskal-Wallis; p-value < 0.001 for the center/periphery and middle/periphery comparisons
237 and p-value < 0.05 for center/middle comparison) (Fig. 4).

238 Across combs, hydrocarbon quantities differed significantly between combs 1, 2, 3, and 4 and
239 combs 6 and 7 (Kruskal-Wallis; p-value < 0.05); comb 5 was only significantly different from
240 comb 7 (Kruskal-Wallis; p-value < 0.05) (Fig. 5).

241

242 **Discussion**

243 Many studies have shown that nest recognition in social insects is based on chemical cues. Such
244 is the case in bumble bees (Saleh et al. 2007), in honey bees and wasps (Butler et al. 1969), and
245 in ants (Depickère et al. 2004). In this study, hydrocarbons previously found on the hornet's
246 cuticle (Gévar et al. 2017) were discovered to occur on different parts of the hornet's nest. This
247 finding suggests *Vespa velutina nigrithorax* deposits CHCs on nest structures. However, we do
248 not know if deposition is active or passive. Indeed, no data on *Vespa velutina* behavior within
249 the nest are currently available. Consequently, we do not know if the hornet interacts in a
250 particular way with the nest substrate. Some species are known to directly interact with nest
251 materials and/or with nestmates to obtain/deposit chemical markings (Akino 2008). For
252 example, some European *Polistes* species apply chemical markings via rubbing (Turillazzi and
253 Ugolini 1979). Conversely, in *Lasius niger*, ants passively mark the nest with their tarsi as they
254 walk across the substrate (Depickère et al. 2004; Devigne and Detrain 2006; Lenoir et al. 2009).
255 Further behavioral studies are thus needed to clarify marking mechanisms in *V. velutina*.

256 Chemical signatures are colony and caste specific in *V. velutina* (Gévar et al. 2017). In
257 our study, we observed pronounced qualitative and quantitative chemical differences among
258 nest structures. This finding may indicate that individuals differing in caste or reproductive
259 status (fertile vs. non-fertile females) occur in separate parts of the nest. For example, chemical
260 signatures can vary among castes: *V. velutina* queens bear four specific alkenes and have higher
261 levels of CHCs than do other castes (Gévar et al. 2017). However, the queen-specific
262 compounds were not found on the nest structures. The chemical signatures of nest structures
263 are likely shaped by the chemical deposits of colony members, whose CHC signatures vary in
264 their absolute and relative composition. This variation is functional and is likely influenced by
265 a variety of recognition-oriented behaviors and discrimination among individuals (Hölldobler
266 and Carlin 1987). For example, monomethyl alkanes often serve as signals (Nelson 1993).
267 Hydrocarbons could therefore function in recognition and communication within nests. Indeed,

268 the nest's interior is opaque, creating dark conditions. Individuals thus need a mechanism other
269 than vision to locate each other within the nest. Labeling nest structures so that they have
270 different chemical profiles could allow insects to orient themselves and communicate with each
271 other. The fact that combs and envelopes had the highest and lowest quantities of hydrocarbons,
272 respectively, might support this hypothesis. However, these differences could also arise from
273 the higher contact frequencies among individuals and between individuals and comb surfaces
274 inside the nest. Furthermore, there could be smaller quantities of hydrocarbons on the envelopes
275 because they are exposed to exterior conditions (e.g., rainfall could wash them away). More
276 experiments are necessary to explore these issues. Moreover, nest structures were also
277 qualitatively different from each other. This finding is interesting because branched alkanes and
278 alkenes help define the chemical identity of colonies and are involved in communication among
279 individuals (Howard and Blomquist 2005). Linear alkanes may play more of a role in physically
280 protecting individuals against desiccation (Hadley 1984; Singer 1998). Consequently, their high
281 levels on the nest's surface could provide protection against wet and humid conditions. Indeed,
282 because the hornet's nest is composed of "paper mache", it is likely that chemical protection
283 would be required. When n-alkanes and monomethyl alkanes are combined, they produce an
284 impermeable layer whose solid-liquid phase transition extends over a wide temperature range
285 (Gibbs 1995), allowing the nest to benefit from seasonal waterproofing. Finally, the chemical
286 heterogeneity of nest structures could be explained by temporal shifts. In *Bombus terrestris*,
287 cuticular lipids convey colony identity and change over time (Rottler-Hoermann et al. 2016).
288 Further research is needed to clarify the chemical dynamics of nests.

289 We also found that there was spatial heterogeneity in the combs' chemical profiles.
290 Hydrocarbon quantity decreased from the center to the periphery of the combs and from the top
291 combs to the bottom combs. It appeared that the oldest parts of the nest (i.e., the center of the
292 comb and the top comb) had greater quantities of hydrocarbons. This observation could be
293 explained by structure age, these structures had spent more time in contact with adult hornets.
294 There were qualitative differences along the horizontal axis of the individual combs, signaling
295 differences associated with time of construction. In contrast, there were no differences along
296 the vertical axis of the combs. These results suggest that marking patterns within nests may be
297 somewhat complex. Further research is needed to clarify hydrocarbon deposition mechanisms
298 and dynamics within nests and across time.

299 Finally, the chemical diversity among nests and among structures highlights the
300 existence of a colony signature. Indeed, chemical diversity among nests was high. As described
301 by Gévar et al. (2017), individual *V. velutina* hornets vary in their chemical signatures, and we

302 observed the same variability in the species' nests. Consequently, the chemical heterogeneity
303 observed among individuals could explain the heterogeneity seen in the nests themselves. This
304 result was somewhat surprising, however, because hornet populations in France are highly
305 related (Darrouzet et al. 2015) as the result of a genetic bottleneck (Arca et al. 2015). Previous
306 studies of invasive species all found that chemical variability was reduced in introduced
307 populations. Indeed, this is the case in the ants *Linepithema humile* (Brandt et al. 2009) and
308 *Wasmannia auropunctata* (Errard et al. 2005) as well as in the termite *Reticulitermes flavipes*
309 (Perdereau et al. 2010). This phenomenon could be related to the limited number of
310 introductions that have taken place. In the case of *V. velutina* in France, the population is inbred;
311 the heterogeneity in hydrocarbon profiles within nests could result from environmental and/or
312 epigenetic factors (Gévar et al. 2017). Additional research is clearly necessary to assess the
313 roles played by different factors in shaping nest chemical profiles.

314

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320

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583

584 **Legends**

585 **Table 1.** Identities and relative proportions of the hydrocarbons found on the three nest
586 structures: envelopes, combs, and pillars.

587 **Table 2.** Differences in hydrocarbon quantities among nest structures (Kruskal-Wallis chi-
588 squared test). Levels of 5 compounds differed between the envelopes and combs; levels of 10
589 compounds differed between the envelopes and pillars; and levels of 2 compounds differed
590 between the combs and pillars.

591 **Figure 1.** Results of the principal component analysis of the proportion of hydrocarbons found
592 on the three nest structures; the first two axes are shown. Three comparisons were performed:
593 envelopes vs. combs, envelopes vs. pillars, and combs vs. pillars.

594 **Figure 2.** Total hydrocarbon quantities found on the envelopes, combs, and pillars (mg of
595 hydrocarbons/gram of nest material).

596 **Figure 3.** Quantities of alkanes, branched alkanes, and alkenes found on the envelopes, combs,
597 and pillars (mg of hydrocarbons/gram of nest material).

598 **Figure 4.** Total hydrocarbon quantities (mg of hydrocarbons/gram of nest material) at the
599 comb's center, middle, and periphery.

600 **Figure 5.** Total hydrocarbon quantities (mg of hydrocarbons/gram of nest material) from the
601 top comb to the bottom comb within nests.

602

603 **Supplementary materials**

604 **Table S1.** Collection locations and dates for the nests used in the chemical analyses of nest
605 structures.

606 **Table S2.** Collection locations and dates for the nests used in the detailed chemical analyses of
607 the combs.

608 **Figure S1.** Chemical distances between the hydrocarbon profiles of the three nest structures:
609 envelopes, combs, and pillars. The among-nest chemical distances represent the chemical
610 diversity of the same structure across different nests; the among-structure chemical distances
611 represent the chemical diversity among nest structures within the same nest; and the within-
612 structure chemical distances represent the chemical diversity within a specific structure for a
613 given nest.

614 **Figure S2.** Chemical distances between hydrocarbon profiles for the comb center, middle, and
615 periphery. The within-zone chemical distances represent the distances among samples taken
616 from within the same comb zone; the between-zone chemical distances represent the distances
617 among samples taken from different comb zones.

618 **Figure S3.** Chemical distances between hydrocarbon profiles within combs from nest top to
619 bottom.

620

621 **Table S1**

Nest number	Latitude	Longitude	Collection date
1	47.401366	0.66336	07/13/2017
2	47.388264	0.827383	08/14/2017
3	47.413326	0.984407	08/17/2017
4	47.498614	0.57948	08/24/2017
5	47.543367	0.744155	10/06/2017
6	47.388264	0.827383	12/01/2017
7	47.388309	0.658258	07/29/2016
8	47.351861	0.66131	08/05/2016
9	47.394144	0.68484	08/29/2016
10	47.388264	0.827383	07/07/2015

622

623

624 **Table S2**

Nest Number	Latitude	Longitude	Height (cm)	Width (cm)	Comb number	Collection date
1	48.066152	-2.967055	73	41	10	20.02.2018
2	47.3404240	-1.526116	53	47	7	14.02.2018
3	47.3898439	0.7224790	44	40	7	20.03.2018

625

626

627 **Table 1**

RT (min)	Compound Identity	ECL	Envelopes		Combs		Pillars	
			%	+SD	%	+SD	%	+SD
23.8	n-C20	20	X	X	X	X	X	X
28.8	n-C23	23	3.57	0.56	1.92	0.11	2.62	0.13
31.5	C25 :1	24.72	0.68	0.11	0.76	0.10	0.66	0.04
31.9	n-C25	25	16.49	0.53	15.72	0.84	17.23	0.32
32.4	13-+11-MeC25	25.37	1.86	0.19	1.51	0.09	2.14	0.13
32.7	5-MeC25	25.47	0.36	0.11	0.33	0.03	0.35	0.04
33.1	3-MeC25	25.70	3.88	0.22	3.10	0.16	3.28	0.19
33.5	n-C26	26	1.72	0.05	1.59	0.13	1.60	0.09
34.1	12-+10-MeC26	26.31	0.55	0.14	0.45	0.04	0.50	0.03
34.7	C27 :1	26.75	4.30	0.35	5.25	0.46	4.94	0.22
34.8	C27 :1	26.83	1.14	0.14	1.07	0.10	0.92	0.06

35.1	n-C27	27	23.71	1.28	32.01	2.58	29.63	0.96
35.5	13-+11- +9MeC27	27.32	4.66	0.56	4.91	0.87	5.82	0.71
35.6	7,MeC27	27.37	0.90	0.23	0.80	0.06	0.87	0.08
35.9	11,15-diMeC27	27.64	4.04	0.52	2.77	0.27	2.65	0.16
36.1	3-MeC27	27.81	8.41	0.31	6.38	0.74	7.48	0.22
36.2	5,15-diMeC27	27.89	1.13	0.49	1.68	0.64	0.95	0.11
36.4	n-C28	28	3.00	150	0.79	0.07	0.87	0.08
36.7	diMe or triMeC27	28.13	1.05	0.23	1.02	0.16	0.97	0.12
36.9	12-MeC28	28.35	2.33	0.47	0.62	0.18	0.48	0.04
37.6	C29 :1	28.78	2.32	0.15	3.24	0.36	3.59	0.14
37.7	C29 :1	28.84	1.13	0.17	2.08	0.41	1.29	0.08
37.9	n-C29	29	5.39	0.39	5.80	0.66	5.43	0.25
38.4	13-+11-+9- MeC29	29.37	1.81	0.18	2.21	0.23	2.61	0.07
38.8	11,15-+11,17- diMeC29	29.68	1.80	0.26	1.37	0.32	1.06	0.15
39.7	4-MeC30	30.44	0.64	0.22	0.20	0.05	0.32	0.14
40.7	n-C31	31	3.32	1.05	2.39	0.37	1.69	0.13

628

629 **Table 2**

Compound	Structure comparison		
	Envelopes/Combs	Envelopes/Pillars	Combs/Pillars
n-C23	NS	NS	p < 0.01
C25 :1	NS	NS	NS
n-C25	NS	NS	NS
13-+11-MeC25	NS	p = 0.01	p < 0.01
5-MeC25	NS	NS	NS
3-MeC25	p = 0.01	p < 0.01	NS
n-C26	NS	NS	NS
12-+10-MeC26	NS	NS	NS
C27 :1	NS	p = 0.01	NS
C27 :1	NS	NS	NS
n-C27	p < 0.05	p < 0.01	NS
13-+11-+9MeC27	NS	NS	NS
7,MeC27	NS	NS	NS

11,15-diMeC27	NS	p < 0.05	NS
3-MeC27	p < 0.05	p = 0.01	NS
5,15-diMeC27	NS	NS	NS
n-C28	p < 0.01	NS	NS
diMe or triMeC27	NS	NS	NS
12-MeC28	p < 0.05	p < 0.01	NS
C29 :1	NS	p < 0.01	NS
C29 :1	NS	NS	NS
n-C29	NS	NS	NS
13-+11-+9-MeC29	NS	p < 0.01	NS
11,15-+11,17-diMeC29	NS	p = 0.05	NS
4-MeC30	NS	NS	NS
n-C31	NS	NS	NS

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