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1 **Designing a sex pheromone blend for attracting the yellow-legged hornet (*Vespa***
2 ***velutina*), a pest in its native and invasive ranges worldwide**

3

4 **Designing a sex pheromone blend for attracting the yellow-legged hornet**

5

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7

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21

22

23 **Abstract**

24 A subspecies of the Asian hornet *Vespa velutina*, *V. velutina nigrithorax* (*Vvn*), is
25 native to coastal China but has invaded several other parts of the world. It is a major
26 pest in both its native and invasive ranges. The subspecies *V. velutina auraria* (*Vva*) is
27 native to inland China, where it causes harm to local apiculture industries by preying
28 on honey bees. As part of a broader effort to develop effective attractants to control
29 the Asian hornet, we analyzed the species' sex pheromone compounds. We confirmed
30 that 4-oxo-octanoic acid (4-OOA) and 4-oxo-decanoic acid (4-ODA) are major
31 pheromone compounds in both subspecies, and we determined that 5-oxo-decanoic
32 acid (5-ODA) is an additional minor compound. We observed variability in the ratio
33 of pheromone compounds. However, using field bioassays, we were able to identify
34 an optimal blend for attracting *Vva* males in the subspecies' native range
35 (4-OOA:4-ODA:5-ODA in a ratio of 2:4:1). This blend also attracted males from both
36 native and invasive *Vvn* populations. Sex pheromone blends for attracting males could
37 be a useful general tool in efforts to control invasive hornet populations.

38

39 **Keywords:** hornet, invasive species, selective control, 4-oxo-octanoic acid,
40 4-oxo-decanoic acid, 5-oxo-decanoic acid

41

42

43 1 INTRODUCTION

44 At present, there is increased interest in developing pest control strategies that
45 have smaller environmental impacts with a view to limiting long-term population
46 declines. Programs for controlling insect pests employ various approaches: microbial
47 pesticides (e.g., bacteria, fungi, and viruses), beneficial insects (predators and
48 parasitoids), physiological disruptors (juvenile hormones and growth regulators),
49 naturally occurring insecticides (e.g., pyrethrum, derris, and neem), and
50 semiochemicals (e.g., pheromones and kairomones)¹. Some commonly used
51 semiochemicals are pheromones and allelochemicals. Pheromones and
52 allelochemicals are both groups of chemicals that are secreted or excreted by
53 individual organisms. Pheromones act as signals to other individuals of the same
54 species (e.g., sex pheromones), while allelochemicals convey information to
55 individuals of other species (e.g., kairomones and allomones). In the context of pest
56 control, pheromones hold great promise because they are generally non-toxic, species
57 specific, and harmless to non-target species. Thus, more and more control strategies
58 use pheromones to disrupt mating, implement mass trapping, and/or attract and kill
59 pests. Mating disruption has been particularly successful in controlling insect pests,
60 such as the tomato leafminer (*Tuta absoluta*)².

61 The yellow-legged hornet, *Vespa velutina* (Hymenoptera: Vespidae), is native to
62 eastern Asia³. The subspecies *Vespa velutina nigrithorax* Lepeletier, 1836 (*Vvn*), was
63 first observed in 2005 in southwestern France⁴. It rapidly spread across Europe to
64 Spain, Portugal, Italy, Switzerland, Germany, Belgium, the United Kingdom, and the

65 Netherlands⁵. It has also invaded South Korea and Japan^{6,7}. *Vvn* invasions have had
66 strong negative impacts on biodiversity, the economies of the afflicted countries, and
67 public health⁸. This subspecies is a voracious predator of wasps, flies, bumble bees,
68 and, most notably, honey bees. Furthermore, when its nests are disturbed, *Vvn* mounts
69 an aggressive response that can be fatal to humans⁹.

70 Pheromone-based control methods such as mass trapping or mating disruption
71 might be useful for controlling the expansion of invasive *Vvn* populations in Europe¹⁰.
72 However, little is known about the subspecies' sex pheromones. There is another *V.*
73 *velutina* subspecies found in Asia: *V. velutina auraria* (*Vva*). Archer¹¹ classified *Vva*
74 as its own species, *V. auraria* Smith, 1852, because its coloration differs from that of
75 *Vvn*. However, Nguyen et al.¹² did not find support for this taxonomic distinction and
76 instead showed that *V. auraria* was synonymous with *V. velutina*. Perrard et al.¹³
77 confirmed this synonymy. Two sex pheromone compounds have been identified in
78 *Vva* populations in China: 4-oxo-octanoic acid (4-OOA) and 4-oxo-decanoic acid
79 (4-ODA)¹⁴. Invasive *Vvn* populations in Europe appear to have similar sex pheromone
80 compounds¹⁵. This result is not surprising given that *Vva* and *Vvn* are very closely
81 related^{12,13}. Here, we describe the results of experiments aimed at developing
82 pheromone-based control methods that target *Vvn*. First, we analyzed the compounds
83 composing the sex pheromone produced by *Vva* in China (i.e., in hornets from native
84 populations) to identify any additional minor compounds. Second, we repeated the
85 process for *Vvn* in France (i.e., in hornets from an invasive population). We then
86 tested how well a range of sex pheromone blends attracted male hornets from native

87 *Vva* populations, native *Vvn* populations, and invasive *Vvn* populations.

88

89 **2 MATERIALS AND METHODS**

90 **2.1 Pheromone extraction and derivatization**

91 We analyzed more than 300 gynes sampled from hornet colonies in the wild. We
92 wanted to statistically assess the hornet's ratio of sex pheromone compounds, but not
93 all the gynes were producing all three compounds simultaneously. Some of them did
94 not produce, or very few, compounds. We discarded those last gynes, because they
95 could be too young to emit the three compounds, or for some other reasons still
96 unknown (see the discussion). We thus selected 30 gynes that were producing all three
97 compounds from 4 *Vva* colonies in Kunming (KMG; Yunnan Province, China) in
98 2017 (Table S1).

99 To confirm the identity of the compounds making up the hornet's sex pheromone,
100 we collected 22 gynes from 3 *Vva* colonies in Kunming (KMG; Yunnan Province,
101 China) and 24 gynes from 3 *Vvn* colonies in Indre et Loire (France) in 2019 (Table
102 S1). Gynes were identified by measuring mesoscutum width (> 4.5 mm)¹⁶.

103 When the crude acids were extracted, it was difficult to properly separate the
104 methyl esters and derivatives using the GC column available to us. We thus developed
105 a ring formation method to improve the separation process and better quantify
106 compound levels. We extracted the pheromones by rubbing each gyne's sixth
107 intersegmental sternal membrane with filter paper (strips measuring 4 mm by 15 mm).
108 The filter paper was then eluted with 20 μ L CH_2Cl_2 , and the extracts were evaporated

109 just to the point of dryness in clean 2.0 mL vials (Agilent) under nitrogen. We then
110 added 0.2 mg of NaBH₄ (20 μL of 10 mg NaBH₄ diluted in 1 mL of 1M NaOH) to the
111 vials. After 5 min, 30 μL of 1 M H₂SO₄ was slowly added to the mixture (i.e., in doses
112 of 1 μL over a period of 10 min) until bubbles were no longer observed. The resulting
113 acidic mixture was sealed up using a PTFE-lined cap and heated to 80 °C, which
114 resulted in spontaneous lactonization. We performed solid-phase microextraction
115 (SPME) at 80 °C for 20 min and collected the lactone products from the headspace
116 using 65-μm blue PDMS/DVB fibers (Supelco, CA).

117

118 **2.2 Chemical analyses**

119 In 2017, we performed gas chromatography-mass spectrometry (GC-MS) to
120 analyze the compounds found in derivatives obtained from the *Vva* sex pheromone.
121 For the analyses that took place in China, an HP-7890B-5975C GC-MS apparatus
122 (Agilent, Palo Alto CA, USA) at the Kunming Institute of Botany was used. We
123 employed splitless injection at 250 °C and an HP-5ms column (30 m × 250 μm × 0.25
124 μm film, Agilent); helium (1.2 mL/min) was the carrier gas. The temperature program
125 was as follows: an increase of 50°C/min for 2 min and then a climb of 10 °C/min to
126 280 °C over a 10-min period. The 70-eV electron impact ionization source was heated
127 to 230 °C, and the MS quadrupole was heated to 150 °C. The mass scanning range
128 was *m/z* 28.5–300; the threshold abundance was set to 10 to detect trace molecular
129 ions.

130 Also in 2017, we analyzed the ratio of sex pheromone components in *Vva* using

131 gas chromatography-flame-ionization detection (GC-FID). We utilized an HP-7890B
132 GC-FID apparatus (Agilent, Palo Alto CA, USA) with splitless injection at 250 °C.
133 An HP-5 column (30 m × 320 µm × 0.32 µm film, Agilent) was employed; nitrogen
134 (1.2 mL/min) was the carrier gas. The temperature program was as follows: 50 °C for
135 2 min; an increase of 10 °C/min to reach 230 °C; and an increase of 50 °C over a
136 1-min period to attain 280 °C.

137 In 2019, we performed GC-MS to characterize the compounds in the sex
138 pheromones of both native *Vva* and invasive *Vvn* (extracts from samples collected in
139 France). We used an HP-7890B-7000C GC-MS apparatus (Agilent, Palo Alto CA,
140 USA) with splitless injection at 250 °C. An HP-5 column (30 m × 320 µm × 0.25 µm
141 film, Agilent) was employed; helium (1.2 mL/min) was the carrier gas. The
142 temperature program was as follows: an increase of 50 °C/min from 70 °C to reach
143 120 °C; an increase of 1 °C/min to reach 135 °C; and an increase of 5 °C/min to attain
144 230 °C, at which the temperature remained for 10 min. The 70-eV electron impact
145 ionization source was heated to 230 °C, and the MS quadrupole was heated to 150 °C.
146 The mass scanning range was m/z 28–300; the threshold abundance was set to 10 to
147 detect trace molecular ions.

148 We obtained reference standards of *gamma*-octanoic lactone (GOL),
149 *gamma*-decanoic lactone (GDL), and *delta*-decanoic lactone (DDL) from TCI (Japan)
150 to synthesize the pheromone blends. The first two pheromone compounds, 4-OOA
151 (CAS No. 4316-44-3) and 4-ODA (CAS No. 4144-54-1), were synthesized from GOL
152 and GDL, respectively, using the general procedure described by Wen et al.¹⁴ The

153 third compound, 5-oxo-decanoic acid (5-ODA, CAS No. 624-01-1), was synthesized
154 from DDL via the same procedure.

155

156 **2.3 Field bioassays**

157 In the field, we identified *Vva* and *Vvn* colonies in China and *Vvn* colonies in
158 France. In China, more than 10 *Vva* colonies were identified at sites in Kunming
159 (KMG), Qujing (QJ), and the Xishuangbanna Tropical Botanical Garden (XTBG) in
160 the Yunnan Province. The colonies were all located within 2 km of the locations at
161 which the bioassays were conducted. In total, 12 *Vvn* colonies were identified in
162 Baiyunshan Park (BYS) in the Guangdong Province (n = 5 colonies) and in the
163 Longling Natural Reserve (LL) in the Guangxi Province (n = 7 colonies) (Table S2).
164 France is one of the European countries most affected by the yellow-legged hornet's
165 invasion. While no *Vvn* colonies were found in the areas where the bioassays were
166 conducted in France (Parc de la Perraudière [PP], Parc de la Gloriette [PG], and the
167 University of Tours [UT]), individual hornets and hornet colonies have been observed
168 in nearby areas. Peak swarming and testing occurred at the same solar time in France
169 and China.

170 In China, during swarming, male hornets patrol above the forest canopy, along
171 the edges of the open spaces around nests, forming what are called male congregation
172 areas (MCAs)¹⁴. In this study, MCAs were located 0.2–4 km from the colonies¹⁴.
173 Males from invasive *Vvn* populations in Europe have not been seen to display this
174 patrolling behavior, perhaps due to the limitations associated with the study locations.

175 Generally speaking, our bioassays had two stages that are described in greater
176 detail in the paragraphs below. During the first stage, we determined the most
177 effective pheromone blend created from the three compounds we had identified in
178 *Vva* via the ratio analysis. This process had two steps: 1) We optimized the ratio of
179 4-OOA to 4-ODA. 2) We added different amounts of 5-ODA to the optimal
180 combination of 4-OOA and 4-ODA to obtain the optimal blend of all three
181 compounds. During the second stage, we evaluated how well various pheromone
182 blends attracted native *Vva*, native *Vvn*, and invasive *Vvn* in the field.

183 More specifically, during the first stage, we explored how well different blends
184 attracted *Vva* males in China. Because we found that extracts contained variable
185 amounts of 5-ODA, we used a two-step process. First, we tested different blends of
186 4-OOA and 4-ODA (ratios: 0, 1:4, 1:3, 1:2, 1:1, and 2:1) for 9 days to identify the
187 most attractive compound blend. This work was performed in Kunming, Qujing, and
188 Xishuangbanna during the 2017 hornet mating season. Second, we added the third,
189 newly identified minor compound to the most attractive two-compound blend (ratio of
190 1:2). For three days, we tested different blends of 4-OOA, 4-ODA, and 5-ODA (ratios
191 = 0, 0:0:1, 1:2:0, 10:20:1, and 2:4:1) as well as an unbaited control of
192 dichloromethane solvent. This work took place in Kunming during the 2018 mating
193 season. We then conducted bioassays for 19 days using a range of blends (4-OOA:
194 4-ODA:5-ODA ratios = 0, 1:2:4, 1:2:3, 1:2:2, 1:2:1, 2:4:1) to find the optimal blend
195 for *Vva*. This work took place in Kunming and Xishuangbanna during the 2018
196 mating season.

197 During these bioassays, the following procedure was employed. Ten mg of a
198 given blend was placed on a strip of filter paper (4 mm by 15 mm). The strip and a
199 freshly freeze-killed male (i.e., visual dummy)¹⁴ were placed together on a sticky
200 board that served as a trap. During these trials, we placed the traps on tree trunks
201 located under MCAs. The distance between the MCAs and the traps was the height of
202 the canopy. In each set of trials, the traps were located 3 m apart; trap location was
203 randomly changed every half hour. The control trap contained a piece of filter paper
204 with 10 mg of solvent along with a visual dummy. From 11:00 to 13:00, we counted
205 the number of *Vva* males that were attracted to the traps. A hornet was considered to
206 show signs of attraction if it flew toward a trap in a zigzag pattern or if it hovered less
207 than 10 cm from a trap and stayed longer than 5 seconds. Any such hornets were
208 caught with a net. Hornets were sexed based on genital morphology and relative
209 antennal length.

210 During the second stage, we quantified the attractiveness of the optimal blend
211 identified above (4-OOA:4-ODA:5-ODA in a ratio of 2:4:1) to a broader sample of
212 native *Vva* males (sites: KMG and QJ) as well as to native *Vvn* males (sites: BYS and
213 LL) and invasive *Vvn* males (sites: TU, PP, and PG). Each trap contained a piece of
214 filter paper with 10 mg of the optimal blend along with a visual dummy. The control
215 trap contained a piece of filter paper with 10 mg of solvent along with a visual dummy.
216 Attraction was quantified as in stage 1. Trials were conducted from 11:00 to 13:00.
217 They took place in China during the 2018 mating seasons of *Vva* and *Vvn* for 18 and
218 22 days, respectively. They took place in France for 30 days during the 2019 mating

219 season of *Vvn*. During the trials in France, to understand the relationships between
220 climatic variables and the attraction patterns of *Vvn* males, ambient temperature,
221 relative humidity, light intensity, and wind speed were recorded using a multifunction
222 environment meter (DEM 900, Velleman, Belgium).

223

224 **2.4 Statistical analysis**

225 Compound quantities (mean \pm SE [ME]) were analyzed using t-tests ($\alpha = 0.05$).
226 The ratios of pheromone compounds were analyzed using χ^2 tests ($\alpha = 0.05$). One-way
227 ANOVAs were used to analyze site-related differences in the number of males
228 attracted by the different blends in stage 1 and the optimal blend in stage 2. Post-hoc
229 comparisons were conducted using Tukey's HSD method. The relationship between
230 the number of males displaying attraction and the climatic variables mentioned above
231 was analyzed using multivariate linear regression with stepwise model simplification.

232

233 **3 RESULTS**

234 **3.1 Pheromone analysis**

235 During the 2017 mating season, we hypothesized that 5-ODA formed part of the
236 yellow-legged hornet's sex pheromone. This prediction arose because, in the GC-MS
237 analyses, we observed that the peak for 4-ODA contained two even-electron ions (at
238 m/z 130 and m/z 112) characteristic of 5-ODA (Fig. 1A). We could not separate out
239 4-ODA and 5-ODA using the GC columns available to us; it is difficult to precisely
240 quantify the long-tailed peaks of the oxoacids obtained with nonpolar or semipolar

241 columns. We therefore used a derivatization method to confirm the presence of
242 5-ODA. GOL, GDL, and DDL are, respectively, cyclic derivatives of the hydroxy
243 acids obtained from 4-OOA, 4-ODA, and 5-ODA. These lactones were detected by
244 GC-MS after the oxoacids had been reduced and lactonized. For *Vva*, GOL and GDL
245 were observed in the derivatized sample, indicating that 4-OOA and 4-ODA were
246 present. When the GC traces of the derivatized extracts were examined, DDL was
247 found to be present as a minor compound; the base peak represented a diagnostic ion
248 (m/z 99) that confirmed that the lactone came from the alcohol produced by the
249 reduction of the 5-keto group and cyclization. This result showed that 5-ODA had
250 been present in the *Vva* samples (Fig. 1B). Similarly, GOL, GDL, and DDL were also
251 found in extracts obtained from *Vvn* in France (Fig. 1B).

252 The observed ratio of the three compounds varied dramatically (mean
253 4-OOA:4-ODA:5-ODA ratio: 3.8:2.8:1; χ^2 test: $df = 26$, $P < 0.001$). The same was
254 true for the 4-OOA:4-ODA ratio (χ^2 test: $df = 27$, $P < 0.001$). The amount of 5-ODA
255 in the 30 *Vva* gynes sampled in 2017 from 4 colonies in China was estimated to be
256 1.42 ± 0.12 μg per wasp (ME; t-test: $df = 29$, $P = 1.00$).

257

258 **3.2 Identification of the optimal pheromone blend using *Vespa velutina auraria***

259 The ratio of 4-OOA, 4-ODA, and 5-ODA varied greatly among gynes in the
260 initial analysis. We used a stepwise approach in the bioassays to identify the
261 pheromone blend most attractive to males. When we conducted the first bioassays
262 with *Vva* in China to determine the optimal ratio of 4-OOA to 4-ODA, we found that

263 the blends differed significantly in attractiveness (ANOVA: $F_{(5, 48)} = 64.64$, $P <$
264 0.0001)—the ratio of 1:2 drew in the most male hornets (HSD: $P < 0.05$) (Fig. 2Aa).
265 When we compared blends with a 4-OOA:4-ODA ratio of 1:2 that varied in their
266 amounts of 5-ODA, there were again significant differences in attractiveness
267 (ANOVA: $F_{(4, 10)} = 73.17$, $P < 0.0001$). The blend with the 4-OOA:4-ODA:5-ODA
268 ratio of 2:4:1 was significantly more attractive than the blend that was 100% 5-ODA
269 (HSD: $P < 0.0001$) or the blends that were 100% 4-OOA or 4-ODA (HSD: $P = 0.016$)
270 (Fig. 2Ab). Moreover, increasing the relative amount of 5-ODA did not further
271 enhance attractiveness (ANOVA: $F_{(4, 90)} = 1.88$, $P = 0.119$). We thus identified the
272 2:4:1 pheromone blend as the blend most attractive to *Vva* males.

273

274 **3.3 Testing the optimal blend's attractiveness to *Vespa velutina auraria* in China** 275 **and *Vespa velutina nigrithorax* in China and France**

276 The attractiveness of the optimal blend (4-OOA:4-ODA:5-ODA at a ratio of
277 2:4:1) was tested in the three populations (*Vva* in China, *Vvn* in China, and *Vvn* in
278 France). No males in any of the three populations were attracted by the blank with the
279 visual dummy. The numbers of *Vva* males attracted to the blend at the two study sites
280 in China were (ME) $N_{\text{KMG}} = 112.2 \pm 11.7$ and $N_{\text{QJ}} = 122.8 \pm 7.6$ per day. The optimal
281 blend attracted significantly larger numbers of *Vva* males at both KMG and QJ (Fig.
282 2B; ANOVA: $F_{(2, 17)} = 18.70$, $P < 0.001$). The numbers of *Vvn* males attracted to the
283 blend at the two study sites in China were (ME) $N_{\text{BYS}} = 93.7 \pm 13.5$ and $N_{\text{QJ}} = 106.4 \pm$
284 9.0 per day. The optimal blend attracted significantly larger numbers of *Vvn* males at

285 both KMG and QJ (Fig. 2B; ANOVA: $F_{(2, 10)} = 16.32, P < 0.001$). Compared to China,
286 in France, fewer *Vvn* males were attracted to the blend across all three sites (ME:
287 $N_{UT}=13.6 \pm 3.4, N_{PP}=10.3 \pm 3.8$, and $N_{PG}=7.0 \pm 1.5$). There was no significant effect of
288 the optimal blend on the number of males attracted at these sites (Fig. 2B; ANOVA:
289 $F_{(3, 10)} = 3.6, P > 0.05$).

290 In France, there was a relationship between the climatic variables and the
291 number of males attracted to the blend. Because the number of males attracted did not
292 differ across sites (ANOVA: $F_{(4, 10)} = 0.99, P = 0.41$), we pooled the data before
293 performing the analysis. Temperature and relative humidity had a significant effect on
294 the number of males attracted (ANOVA: $F_{(2, 8)} = 30.69, R^2 = 0.89, P < 0.001$). More
295 males were attracted (Fig. 3A) at higher temperatures (within the temperature range of
296 14°C – 26°C), and fewer males were attracted ($coeff = -0.26 \pm 0.097, t = -2.668, P =$
297 0.028 Fig. 3B) at higher humidity levels (within the RH range of 41–68%). However,
298 neither light intensity ($t = -0.677, P = 0.52$, Fig. S1A) nor wind speed ($t = -1.04,$
299 $P = 0.33$, Fig. S1B) had a significant effect.

300

301 4 DISCUSSION

302 In this study, we confirmed the presence of two previously identified sex
303 pheromone compounds, 4-OOA and 4-ODA, in *V. v. auraria*¹⁴ and *V. v. nigrithorax*¹⁵
304 gynes. We also identified a new, minor compound that was found in both subspecies:
305 5-ODA. The latter has a molecular weight of 186 and a density of 1.02 g/ml. It
306 dissolves well in polar or semi-polar solvents. To our knowledge, 5-ODA has never

307 been found in the pheromones of living organisms before. Using these three
308 compounds, we created an optimal blend that attracted *Vva* males in China and *Vvn*
309 males in both China (native population) and France (invasive population). The failure
310 to pick up on 5-ODA in previous research is likely due to its variable production
311 levels. Many gynes did not produce 5-ODA at all. In previous research conducted in
312 2016¹⁴, pheromone compounds were extracted from a small number of gynes; only
313 nine were used in the GC-MS analysis, and, unfortunately, they were not all
314 producing 5-ODA. To better characterize pheromone ratios in the wild, more gynes
315 were analyzed in 2017 and 2018. It was then that 5-ODA was discovered in *Vva*. In
316 2019, 5-ODA was detected in *Vvn* in France.

317 5-ODA was not found to co-occur with 4-OOA and 4-ODA in *Vva* in a previous
318 study¹⁴; neither were the three compounds observed together in *Vvn* in Europe¹⁵. In
319 both cases, proportions of the compounds varied. A variety of factors may be at play.
320 Gyne age (in days) could affect sex pheromone production. Mazomenos¹⁷ reported
321 that sex pheromone production began on the third day after emergence in virgin
322 *Dacus oleae* females and that production peaked at recurrent intervals of around 10
323 days. In *Sesamia nonagrioides*, sex pheromone production varied based on age,
324 mating status, and circadian rhythm¹⁸. It may also depend on environmental
325 conditions (e.g., rearing temperature)¹⁹. Han et al.²⁰ reported that, in *Carposina*
326 *sasakii*, sex pheromone production was mainly affected by age and circadian rhythm
327 in virgin females that had experienced the same environmental conditions. Teal et
328 al.²¹ hypothesized that, in *Heliothis virescens*, one compound was not detected in all

329 the samples because it was the biosynthetic precursor of a major compound and was
330 not actually part of the pheromone itself. In *Helicoverpa armigera*, the identities and
331 proportions of sex pheromone compounds varied among areas²². Taken together, this
332 body of research suggests that sex pheromone production may differ among
333 individuals, populations, and subspecies. Further studies are needed to elucidate how
334 biotic and/or abiotic factors influence sex pheromone composition in *V. velutina*. In
335 our study, broader scale sampling indicated that not all gynes were producing the
336 three compounds at the same time. The reasons for this pattern should be investigated.

337 Sex pheromone composition was similar between the native *Vva* population and
338 the invasive *Vvn* population. Apšegaitė and Skirkevičius²³ analyzed levels of
339 (*E*)-9-oxo-2-decenoic acid (9-ODA) in the queen pheromones of three *Apis mellifera*
340 subspecies. The compound was present in all three subspecies, and levels did not
341 differ among subspecies. There were, however, differences among individuals within
342 each subspecies. Recently, it was discovered that queens in native *V. simillima*
343 populations in Japan were carrying the sperm of male *V. velutina*, a non-native
344 sympatric species²⁴. There have not yet been any comparisons of sex pheromone
345 composition between the two species because *V. simillima*'s sex pheromone has yet to
346 be described. This finding in Japan showed that interspecific mating could occur
347 because phylogenetic proximity can lead to similarities in mating behavior and mating
348 phenology. The two subspecies examined here, *Vva* and *Vvn*, display similar mating
349 behaviors and mate during the same period (personal observation). They are also
350 partly sympatric in southwestern China¹³ and *Vva*'s sex pheromones can attract *Vvn*

351 males in the field. We have often observed that mating occurs between sympatric
352 populations of *Vva* and *Vvn* (personal observation).

353 Here, we created and tested a sex pheromone blend that mixed the minor
354 compound that we identified, 5-ODA, with the two major compounds, 4-OOA and
355 4-ODA, that had been previously identified¹⁴. Given compound volatility, the
356 compositional ratios of pheromones can easily be affected by temperature²⁵. Our
357 synthetic pheromone was applied to a cold medium, but real-life pheromone secretion
358 is carried out by a living, heat-generating hornet. This fact could explain the
359 variability in pheromone compound ratio, especially given that there is a two-carbon
360 difference between 4-OOA and 4-ODA²⁵.

361 In the bioassays in France, *Vvn* males were attracted to the optimal blend of the
362 three compounds. This result could have important implications since it suggests the
363 pheromone blend could be used to detect, monitor, and/or control *Vvn* in its invasive
364 range. For example, males could be trapped during the mating season (from
365 September to December), reducing mating success and thus could affect population
366 size. In the hornet life cycle, mated queens produce fertilized eggs that develop into
367 workers the following spring, whereas unmated queens produce unfertilized eggs that
368 develop into males²⁶. Workers play a crucial role in colony establishment and success,
369 so new colonies could face great difficulties if no workers are produced. Moreover,
370 decreasing the number of males would do more than just decrease the absolute
371 number of potential mates; it could also increase inbreeding, leading to low genetic
372 diversity and colony decline. Darrouzet et al.²⁷ found that the *Vvn* population in

373 France suffers from inbreeding and sometimes produces diploid males rather than
374 workers and gynes. The result has been a decrease in the number of workers and
375 gynes, and, consequently, in colony fitness. Moreover, if gynes mate with diploid
376 males, their offspring may be triploid (Darrouzet E, unpublished results) and are
377 likely to be sterile. These hypotheses need to be tested in future research. In any case,
378 removing males from the population via sex pheromone-baited traps could promote
379 inbreeding and potentially limit the spread of this invasive species into new areas of
380 Europe and Asia.

381 In our bioassays, we observed that *Vvn* males flew toward the visual dummy
382 using a zigzag pattern. They also hovered above the dummy without touching it. It
383 may be that short-range signals are also required to improve control efforts. For
384 example, males might expect to see the body of a gyne (i.e., with its visual cues)
385 and/or perceive gyne-specific cuticular hydrocarbons. Before an effective pheromone
386 trap can be developed to control invasive *Vvn* populations, it is necessary to clarify
387 this issue.

388 The optimal blend identified in the native population in China was tested in the
389 invasive population in France. *Vvn* males in France were attracted to our optimal sex
390 pheromone blend. However, fewer *Vvn* males were attracted to the blend in France
391 than in China. This result might stem from the more intensive control efforts taking
392 place in France, where a large number of nests have been destroyed⁸. It would not be
393 surprising if control campaigns had reduced the number of males. It is also possible
394 that male density differs geographically and that we performed our research in

395 low-density areas. A third possibility is that colonies in the invasive population in
396 France produce fewer males than colonies in the native population in China. Finally,
397 the optimal blend was identified using *Vva*; perhaps it was simply not as effective in
398 attracting *Vvn*. All these possibilities should be explored in future research.

399 The relationships between the climatic variables and the number of males
400 attracted to the pheromone blend provided some clues as to how males are attracted
401 by gynes. The effectiveness of sex pheromones is known to be related to release rate,
402 threshold concentration, physiology, and mating behavior²⁹. As a result, male
403 attraction to pheromone blends is also likely to be influenced by these factors. For
404 example, environmental temperatures significantly influenced the number of males
405 displaying attraction during the mating season in both China and France¹⁴.

406 In conclusion, our study demonstrated that *Vvn* males in France were attracted to
407 our optimal sex pheromone blend. This result is promising because it suggests that sex
408 pheromone blends could be a tool for detecting, monitoring, and controlling invasive
409 populations of *Vvn*. However, several biological, ethological, technical, and/or
410 economic factors must be explored before the pheromone blend we identified is used
411 at broader scales.

412

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418 Academy of Sciences.

419 **CONFLICT OF INTEREST DECLARATION**

420 The authors declare that they have no conflict of interest.

421

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493

494

495 **Table S1.** Description of the sites in China and France where yellow-legged hornet (*Vespa*
 496 *velutina*) gynes were sampled for sex pheromone analysis. The two focal subspecies were *Vespa*
 497 *velutina nigrithorax* (*Vvn*) and *Vespa velutina auraria* (*Vva*).

Country	Site	Geographical coordinates	Month-Year	Subspecies	Number of nests	Number of gynes
China	Kunming	25°14'18.2"N102° 74'73.5"E	Dec, 2017	<i>Vva</i>	4	30
	Kunming	25°14'18.2"N102° 74'73.5"E	Dec, 2019	<i>Vva</i>	3	22
France	Lignièrès de Touraine	47°29'77.2"N0°41' 84.4"E	Nov, 2019	<i>Vvn</i>	1	8
	Tours	47°23'43.4"N 0°41'08.4"E	Nov, 2019	<i>Vvn</i>	1	8
	Moutlouis	47°23'18.7"N 0°49'35.4"E	Nov, 2019	<i>Vvn</i>	1	8

498

499 **Table S2.** Description of the sites in China and France where the yellow-legged hornet (*Vespa*
 500 *velutina*) bioassays took place. The two focal subspecies were *Vespa velutina nigrithorax* (*Vvn*)
 501 and *Vespa velutina auraria* (*Vva*).

Country	Site	Geographical coordinates	Month-Year	Subspecies
China	Kunming	25°08'21.2"N	Oct and Nov, 2017	<i>Vva</i>
		102°44'22.4"E	Oct, 2018	
	Xishuangbanna	21°55'33.4"N	Nov and Dec,	<i>Vva</i>
		101°15'16.2"E	2017	
	Qujing	25°26'44.8"N	Oct, 2017	<i>Vva</i>
		103°45'21.7"E		
Baiyunshan Park	23°10'59.2"N 113°17'44.5"E	Oct, 2018	<i>Vvn</i>	
Longling Natural Reserve	24°54'01.2"N	Nov and Dec,	<i>Vvn</i>	
	105°08'23.2"E	2018		
France	University of Tours	47°21'14.0"N 0°42'15.8"E	Oct, 2018	<i>Vvn</i>
	Parc de la Perraudière	47°23'56.9"N 0°40'05.6"E	Oct and Nov, 2018	<i>Vvn</i>
	Parc de la Gloriette	47°22'01.3"N 0°40'14.5"E	Nov, 2018	<i>Vvn</i>

502

503

504 **Figure legends**

505

506 **Figure 1.** Results of the GC-MS analysis of the sex pheromone extracts and derivatives for *Vespa*
507 *velutina auraria* (*Vva*) in China. (A) Interpretation of the mass spectrometry results for
508 5-oxo-decanoic acid. The pathway for generating the ions characteristic of this compound (m/z
509 130 and m/z 112) is depicted in the box. (B) Derivatives generated by the sex pheromone
510 compounds in the extracts from *Vva* in China and *Vespa velutina nigrithorax* (*Vvn*) in France:
511 *gamma*-octanoic lactone (GOL), *gamma*-decanoic lactone (GDL), and *delta*-decanoic lactone
512 (DDL).

513

514 **Figure 2.** Results (mean values \pm standard errors) of the bioassays used to determine
515 the optimal blend of sex pheromone compounds in China (A), and tests of the selected
516 blend on male attraction in the native *Vva* population (*Vva*-N), native *Vvn* population
517 (*Vvn*-N), and invasive *Vvn* population (*Vvn*-I) (B). The number of *Vva* males attracted
518 by different blends of 4-OOA and 4-ODA (2Aa), or by different blends of 4-OOA,
519 4-ODA, and 5-ODA (2Ab). Differences in the letters or asterisk above the bars indicate
520 significant differences in the results (HSD: $P < 0.05$).

521

522 **Figure 3.** The effects of (A) ambient temperature, and (B) humidity. When there was a significant
523 relationship between two variables, a best-fit line is present on the graph.

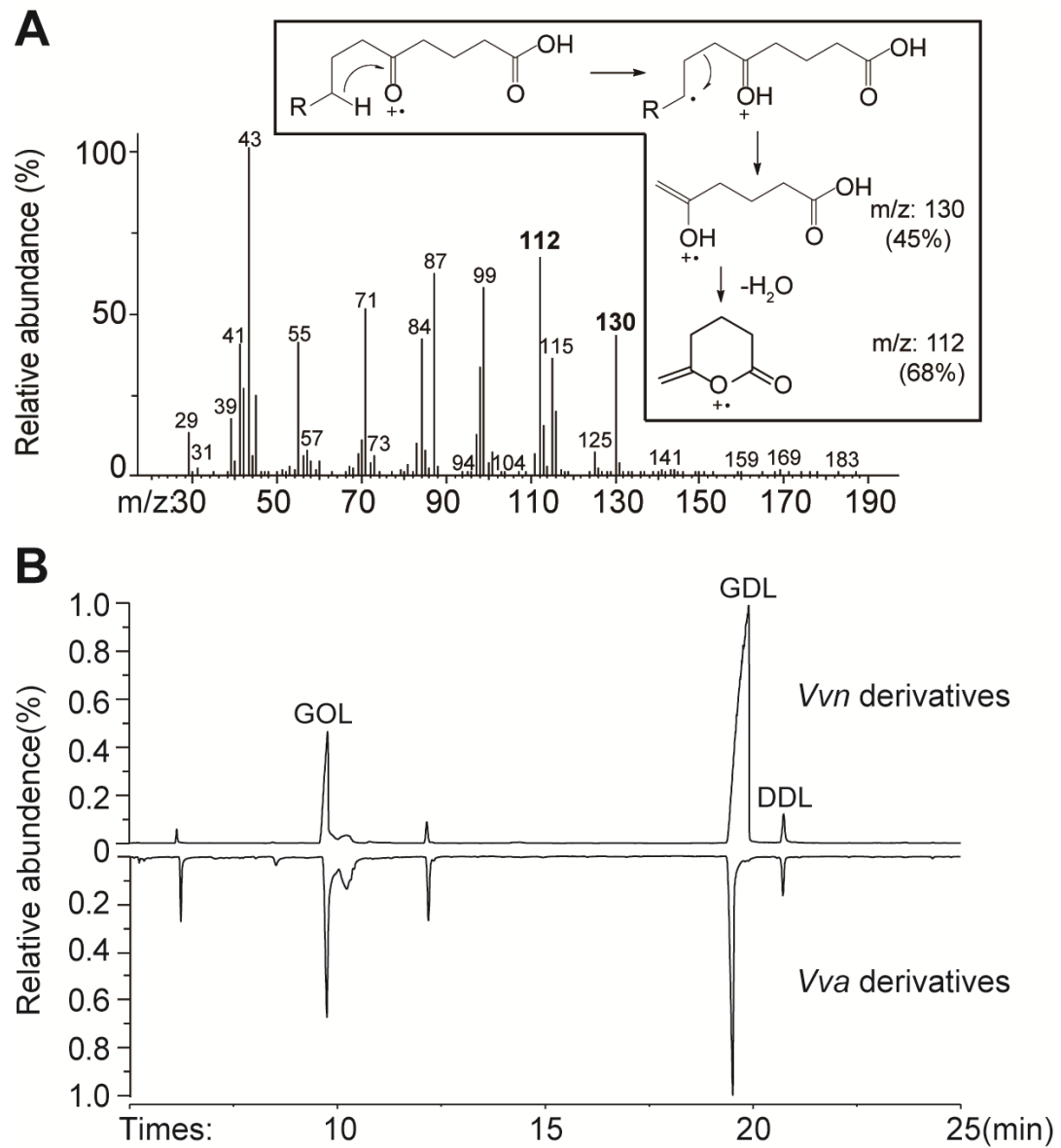
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525 **Figure S1.** The effects of (A) light intensity, and (B) wind speed. When there was a significant

526 relationship between two variables, a best-fit line is present on the graph.

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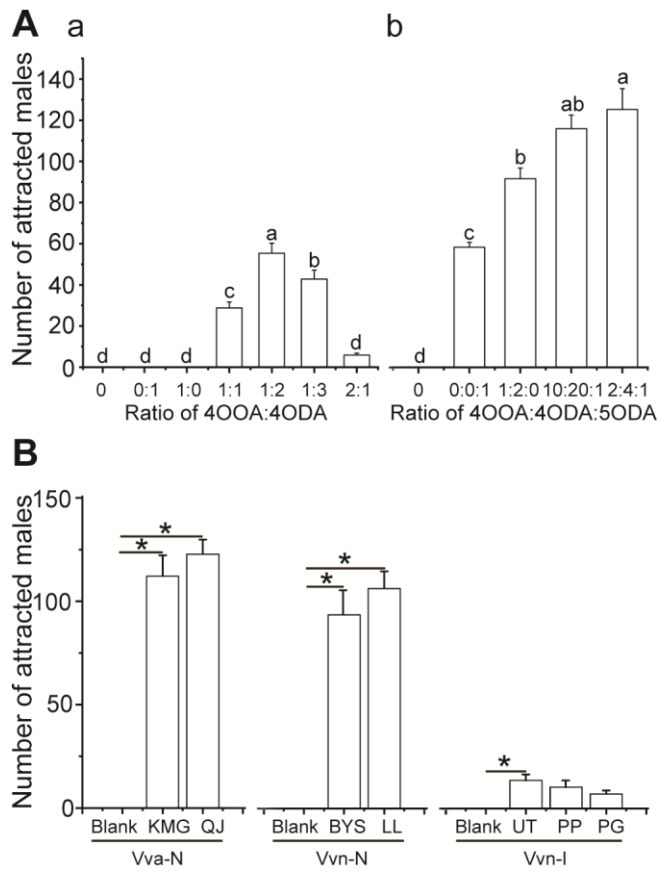
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534 Figure 2

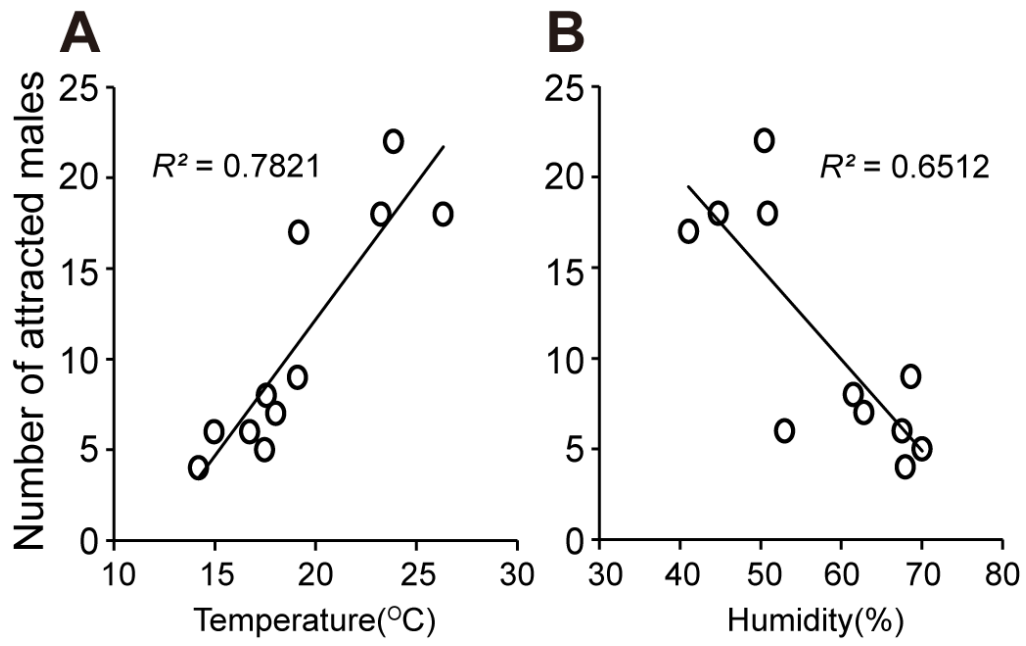


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538 Figure 3



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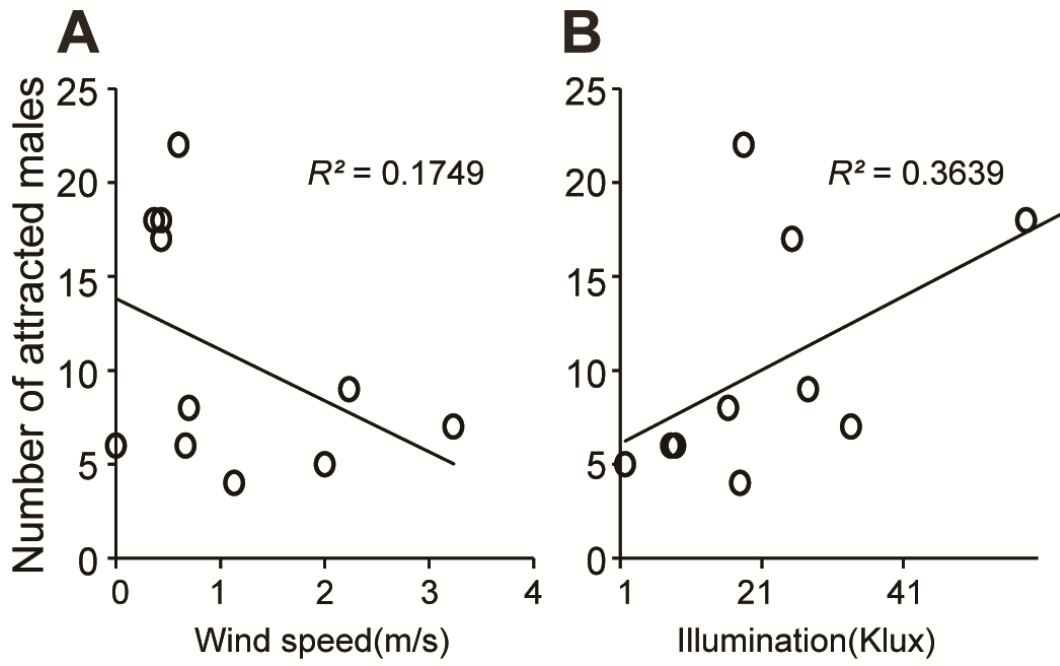
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545 Figure S1



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