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

Ya-Nan Cheng, Ping Wen, Ken Tan, Éric Darrouzet

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

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

Cheng Ya-Nan[1,2,3*], Wen Ping[1,4*], Tan Ken[1], Darrouzet Eric[2]

[1]CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan Province, 650223, China

[2]IRBI, UMR 7261 CNRS-Université de Tours. Avenue Monge, Parc Grandmont, 37200 Tours, France

[3]University of Chinese Academy of Sciences, Beijing, 100049, China

[4]RM104, Department of Entomology, University of California, Riverside, CA 92521, US

Correspondence:

eric.darrouzet@univ-tours.fr

kentan@xtbg.ac.cn

*Contributed equally to this manuscript

Abstract

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

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

1 INTRODUCTION

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

The yellow-legged hornet, *Vespa velutina* (Hymenoptera: Vespidae), is native to eastern Asia[3]. The subspecies *Vespa velutina nigrithorax* Lepeletier, 1836 (*Vvn*), was first observed in 2005 in southwestern France[4]. It rapidly spread across Europe to Spain, Portugal, Italy, Switzerland, Germany, Belgium, the United Kingdom, and the

Netherlands[5]. It has also invaded South Korea and Japan[6,7]. *Vvn* invasions have had strong negative impacts on biodiversity, the economies of the afflicted countries, and public health[8]. This subspecies is a voracious predator of wasps, flies, bumble bees, and, most notably, honey bees. Furthermore, when its nests are disturbed, *Vvn* mounts an aggressive response that can be fatal to humans[9].

Pheromone-based control methods such as mass trapping or mating disruption might be useful for controlling the expansion of invasive *Vvn* populations in Europe[10]. However, little is known about the subspecies' sex pheromones. There is another *V. velutina* subspecies found in Asia: *V. velutina auraria* (*Vva*). Archer[11] classified *Vva* as its own species, *V. auraria* Smith, 1852, because its coloration differs from that of *Vvn*. However, Nguyen et al.[12] did not find support for this taxonomic distinction and instead showed that *V. auraria* was synonymous with *V. velutina*. Perrard et al.[13] confirmed this synonymy. Two sex pheromone compounds have been identified in *Vva* populations in China: 4-oxo-octanoic acid (4-OOA) and 4-oxo-decanoic acid (4-ODA)[14]. Invasive *Vvn* populations in Europe appear to have similar sex pheromone compounds[15]. This result is not surprising given that *Vva* and *Vvn* are very closely related[12,13]. Here, we describe the results of experiments aimed at developing pheromone-based control methods that target *Vvn*. First, we analyzed the compounds composing the sex pheromone produced by *Vva* in China (i.e., in hornets from native populations) to identify any additional minor compounds. Second, we repeated the process for *Vvn* in France (i.e., in hornets from an invasive population). We then tested how well a range of sex pheromone blends attracted male hornets from native

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

2 MATERIALS AND METHODS

2.1 Pheromone extraction and derivatization

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

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

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

just to the point of dryness in clean 2.0 mL vials (Agilent) under nitrogen. We then added 0.2 mg of $NaBH_4$ (20 µL of 10 mg $NaBH_4$ diluted in 1 mL of 1M NaOH) to the vials. After 5 min, 30 µL of 1 M H_2SO_4 was slowly added to the mixture (i.e., in doses of 1 µL over a period of 10 min) until bubbles were no longer observed. The resulting acidic mixture was sealed up using a PTFE-lined cap and heated to 80 °C, which resulted in spontaneous lactonization. We performed solid-phase microextraction (SPME) at 80 °C for 20 min and collected the lactone products from the headspace using 65-µm blue PDMS/DVB fibers (Supelco, CA).

2.2 Chemical analyses

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

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

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

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

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

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

2.3 Field bioassays

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

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

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

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

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

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

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

2.4 Statistical analysis

Compound quantities (mean ± SE [ME]) were analyzed using t-tests (a = 0.95). The ratios of pheromone compounds were analyzed using χ^2 tests (a = 0.95). One-way ANOVAs were used to analyze site-related differences in the number of males attracted by the different blends in stage 1 and the optimal blend in stage 2. Post-hoc comparisons were conducted using Tukey's HSD method. The relationship between the number of males displaying attraction and the climatic variables mentioned above was analyzed using multivariate linear regression with stepwise model simplification.

3 RESULTS

3.1 Pheromone analysis

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

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

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

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

The ratio of 4-OOA, 4-ODA, and 5-ODA varied greatly among gynes in the initial analysis. We used a stepwise approach in the bioassays to identify the pheromone blend most attractive to males. When we conducted the first bioassays 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_{KMG} = 112.2 ± 11.7 and N_{QJ} = 122.8 ± 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_{BYS} = 93.7 ± 13.5 and N_{QJ} = 106.4 ±
284 9.0 per day. The optimal blend attracted significantly larger numbers of *Vvn* males at

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

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

4 DISCUSSION

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

been found in the pheromones of living organisms before. Using these three compounds, we created an optimal blend that attracted *Vva* males in China and *Vvn* males in both China (native population) and France (invasive population). The failure to pick up on 5-ODA in previous research is likely due to its variable production levels. Many gynes did not produce 5-ODA at all. In previous research conducted in 2016[14], pheromone compounds were extracted from a small number of gynes; only nine were used in the GC-MS analysis, and, unfortunately, they were not all producing 5-ODA. To better characterize pheromone ratios in the wild, more gynes were analyzed in 2017 and 2018. It was then that 5-ODA was discovered in *Vva*. In 2019, 5-ODA was detected in *Vvn* in France.

5-ODA was not found to co-occur with 4-OOA and 4-ODA in *Vva* in a previous study[14]; neither were the three compounds observed together in *Vvn* in Europe[15]. In both cases, proportions of the compounds varied. A variety of factors may be at play. Gyne age (in days) could affect sex pheromone production. Mazomenos[17] reported that sex pheromone production began on the third day after emergence in virgin *Dacus oleae* females and that production peaked at recurrent intervals of around 10 days. In *Sesamia nonagrioides*, sex pheromone production varied based on age, mating status, and circadian rhythm[18]. It may also depend on environmental conditions (e.g., rearing temperature)[19]. Han et al.[20] reported that, in *Carposina sasakii*, sex pheromone production was mainly affected by age and circadian rhythm in virgin females that had experienced the same environmental conditions. Teal et al.[21] hypothesized that, in *Heliothis virescens*, one compound was not detected in all

the samples because it was the biosynthetic precursor of a major compound and was not actually part of the pheromone itself. In *Helicoverpa armigera*, the identities and proportions of sex pheromone compounds varied among areas[22]. Taken together, this body of research suggests that sex pheromone production may differ among individuals, populations, and subspecies. Further studies are needed to elucidate how biotic and/or abiotic factors influence sex pheromone composition in *V. velutina*. In our study, broader scale sampling indicated that not all gynes were producing the three compounds at the same time. The reasons for this pattern should be investigated.

Sex pheromone composition was similar between the native *Vva* population and the invasive *Vvn* population. Apšegaitė and Skirkevičius[23] analyzed levels of (*E*)-9-oxo-2-decenoic acid (9-ODA) in the queen pheromones of three *Apis mellifera* subspecies. The compound was present in all three subspecies, and levels did not differ among subspecies. There were, however, differences among individuals within each subspecies. Recently, it was discovered that queens in native *V. simillima* populations in Japan were carrying the sperm of male *V. velutina*, a non-native sympatric species[24]. There have not yet been any comparisons of sex pheromone composition between the two species because *V. simillima*'s sex pheromone has yet to be described. This finding in Japan showed that interspecific mating could occur because phylogenetic proximity can lead to similarities in mating behavior and mating phenology. The two subspecies examined here, *Vva* and *Vvn*, display similar mating behaviors and mate during the same period (personal observation). They are also partly sympatric in southwestern China[13] 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 identified[14]. Given compound volatility, the
356 compositional ratios of pheromones can easily be affected by temperature[25]. 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-ODA[25].

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 males[26]. 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

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

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

The optimal blend identified in the native population in China was tested in the invasive population in France. *Vvn* males in France were attracted to our optimal sex pheromone blend. However, fewer *Vvn* males were attracted to the blend in France than in China. This result might stem from the more intensive control efforts taking place in France, where a large number of nests have been destroyed[8]. It would not be surprising if control campaigns had reduced the number of males. It is also possible that male density differs geographically and that we performed our research in

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

The relationships between the climatic variables and the number of males attracted to the pheromone blend provided some clues as to how males are attracted by gynes. The effectiveness of sex pheromones is known to be related to release rate, threshold concentration, physiology, and mating behavior[29]. As a result, male attraction to pheromone blends is also likely to be influenced by these factors. For example, environmental temperatures significantly influenced the number of males displaying attraction during the mating season in both China and France[14].

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

ACKNOWLEDGMENTS

We are grateful to L. Berville for helping on our statistics and to J. Pearce-Duvet for revising the manuscript. This study was supported by fundings from the regional government of Centre-Val de Loire (FRELON 2 project), and the Key Laboratory of

Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences.

CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflict of interest.

REFERENCES

1 Howse P, Stevens J and Jones OT, Insect pheromones and their use in pest management, Springer Science & Business Media, Berlin (2013).

2 Cocco A, Deliperi S and Delrio G, Control of *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae) in greenhouse tomato crops using the mating disruption technique. *J Appl Entomol* **137**:16-28 (2013).

3 Archer M, Vespine wasps of the world: behaviour, ecology & taxonomy of the Vespinae, Siri Scientific Press (2012).

4 Haxaire J, Bouguet JP and Tamisier JP, *Vespa velutina* Lepeletier, 1836, une redoutable nouveauté pour la faune de France (Hym., Vespidae). *Bull Soc Entomol Fr* **111**:194-194 (2006).

5 INPN. Le Frelon asiatique *Vespa velutina*. Inventaire National du Patrimoine Naturel, (http://frelonasiatique.mnhn.fr/home/)

6 Kim JK, Choi M and Moon TY, Occurrence of *Vespa velutina* Lepeletier from Korea, and a revised key for Korean Vespa species (Hymenoptera: Vespidae). *Entomological Research* **36**:112-115 (2006).

7 Takeuchi T, Takahashi R, Kiyoshi T, Nakamura M, Minoshima Y and Takahashi J, The origin and genetic diversity of the yellow-legged hornet, *Vespa velutina* introduced in Japan. *Insectes Soc* **64**:313-320 (2017).

8 Monceau K, Bonnard O and Thiéry D, *Vespa velutina*: a new invasive predator of honeybees in Europe. *J Pest Sci* **87**:1-16 (2014).

9 de Haro L, Labadie M, Chanseau P, Cabot C, Blanc-Brisset I, Penouil F and for Toxicovigilance NCC, Medical consequences of the Asian black hornet (*Vespa velutina*) invasion in Southwestern France. *Toxicon* **55**:650-652 (2010).

10 Turchi L and Derijard B, Options for the biological and physical control of *Vespa velutina nigrithorax* (Hym.: Vespidae) in Europe: A review. *J Appl Entomol* **142**:553-562 (2018).

11 Archer M, The number of species that can be recognized within the genus Vespa (Hym., Vespidae). *Entomol Mon Mag* **127**:161-164 (1991).

12 Nguyen LT, Saito F, Kojima J-i and Carpenter JM, Vespidae of Viet Nam (Insecta: Hymenoptera) 2. Taxonomic Notes on Vespinae. *Zool Sci* **23**:95-104 (2006).

13 Perrard A, Arca M, Rome Q, Muller F, Tan J, Bista S, Nugroho H, Baudoin R, Baylac M and Silvain J-F, Geographic variation of melanisation patterns in a hornet species: genetic differences, climatic pressures or aposematic constraints? *PloS one* **9**:e94162 (2014).

14 Wen P, Cheng YN, Dong SH, Wang ZW, Tan K and Nieh JC, The sex pheromone of a globally invasive honey bee predator, the Asian eusocial hornet, *Vespa velutina*. *Scientific reports* **7**:12956 (2017).
15 Cappa F, Cini A, Pepiciello I, Petrocelli I, Inghilesi AF, Anfora G, Dani FR, Bortolotti L, Wen P and Cervo R, Female volatiles as sex attractants in the invasive population of *Vespa velutina nigrithorax*. *J Insect Physiol* **119**:103952 (2019).
16 Pérez-de-Heredia I, Darrouzet E, Goldarazena A, Romón P and Iturrondobeitia JC, Differentiating between gynes and workers in the invasive hornet *Vespa velutina* (Hymenoptera, Vespidae) in Europe. *J Hymenoptera Res* **60**:119 (2017).
17 Mazomenos BE, Effect of age and mating on pheromone production in the female olive fruit fly, *Dacus oleae* (Gmel.). *J Insect Physiol* **30**:765-769 (1984).
18 Babilis N and Mazomenos B, Pheromone production in *Sesamia nonagrioides*: diel periodicity and effect of age and mating. *J Insect Physiol* **38**:561-564 (1992).
19 Ono T, Effect of rearing temperature on pheromone component ratio in potato tuberworm moth, *Phthorimaea operculella*, (Lepidoptera: Gelechiidae). *J Chem Ecol* **19**:71-81 (1993).
20 Han KS, Jung JK, Choi KH, Lee SW and Boo KS, Sex pheromone composition and male trapping of the peach fruit moth, *Carposina sasakii* (Matsumura) (Lepidoptera: Carposinidae) in Korea. *J Asia-Pacif Entomol* **3**:83-88 (2000).
21 Teal P, Tumlinson J and Heath R, Chemical and behavioral analyses of volatile sex pheromone components released by calling *Heliothis virescens* (F.) females (Lepidoptera: Noctuidae). *J Chem Ecol* **12**:107-126 (1986).
22 Wu D, Yan Y and Cui J, Sex pheromone components of *Helicoverpa armigera*: chemical analysis and field tests. *Insect Sci* **4**:350-356 (1997).
23 Apšegaitė V and Skirkevičius A, Content of (E)-9-oxo-2-decenoic acid in pheromones of honeybee (*Apis mellifera* L.) queens. *Pheromones* **6**:27-32 (1999).
24 Yamasaki K, Takahashi R, Harada R, Matsuo Y, Nakamura M and Takahashi J-i, Reproductive interference by alien hornet *Vespa velutina* threatens the native populations of *Vespa simillima* in Japan. *The Science of Nature* **106**:15 (2019).
25 Wen P, Death recognition by undertaker bees. bioRxiv (2020) doi: 10.1101/2020.03.05.978262.
26 Cook JM and Crozier RH, Sex determination and population biology in the Hymenoptera. *Trends Ecol Evol* **10**:281-286 (1995).
27 Darrouzet E, Gévar J, Guignard Q and Aron S, Production of early diploid males by European colonies of the invasive hornet *Vespa velutina nigrithorax*. *PLOS one* **10**:e0136680 (2015).
28 Cappa F, Cini A, Pepiciello I, Petrocelli I and Cervo R, Female body size, weight and fat storage rather than nestmateship determine male attraction in the invasive yellow-legged hornet *Vespa velutina nigrithorax*. *Ethol Ecol Evol* **31**:73-85 (2019).
29 Hirooka Y and Suwanai M, Role of Insect Sex Pheromone in Mating Behavior I.: Theoretical Consideration on Release and Diffusion of Sex Pheromone in the Air. *Applied Entomology Zoology* **11**:126-132 (1976).

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

Country	Site	Geographical coordinates	Month-Year	Subspecies	Number of nests	Number of gynes
China	Kunming	25º14'18.2"N102º 74'73.5"E	Dec, 2017	***Vva***	4	30
	Kunming	25º14'18.2"N102º 74'73.5"E	Dec, 2019	***Vva***	3	22
France	Lignières de Touraine	47º29'77.2"N0º41' 84.4"E	Nov, 2019	***Vvn***	1	8
	Tours	47º23'43.4"N 0º41'08.4"E	Nov, 2019	***Vvn***	1	8
	Moutlouis	47º23'18.7"N 0º49'35.4"E	Nov, 2019	***Vvn***	1	8

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

Country	Site	Geographical coordinates	Month-Year	Subspecies
China	Kunming	25º08'21.2"N 102º44'22.4"E	Oct and Nov, 2017 Oct, 2018	***Vva***
	Xishuangbanna	21º55'33.4"N 101º15'16.2"E	Nov and Dec, 2017	***Vva***
	Qujing	25º26'44.8"N 103º45'21.7"E	Oct, 2017	***Vva***
	Baiyunshan Park	23º10'59.2"N 113º17'44.5"E	Oct, 2018	***Vvn***
	Longling Natural Reserve	24º54'01.2"N 105º08'23.2"E	Nov and Dec, 2018	***Vvn***
France	University of Tours	47º21'14.0"N 0º42'15.8"E	Oct, 2018	***Vvn***
	Parc de la Perraudièce	47º23'56.9"N 0º40'05.6"E	Oct and Nov, 2018	***Vvn***
	Parc de la Gloriette	47º22'01.3"N 0º40'14.5"E	Nov, 2018	***Vvn***

Figure legends

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

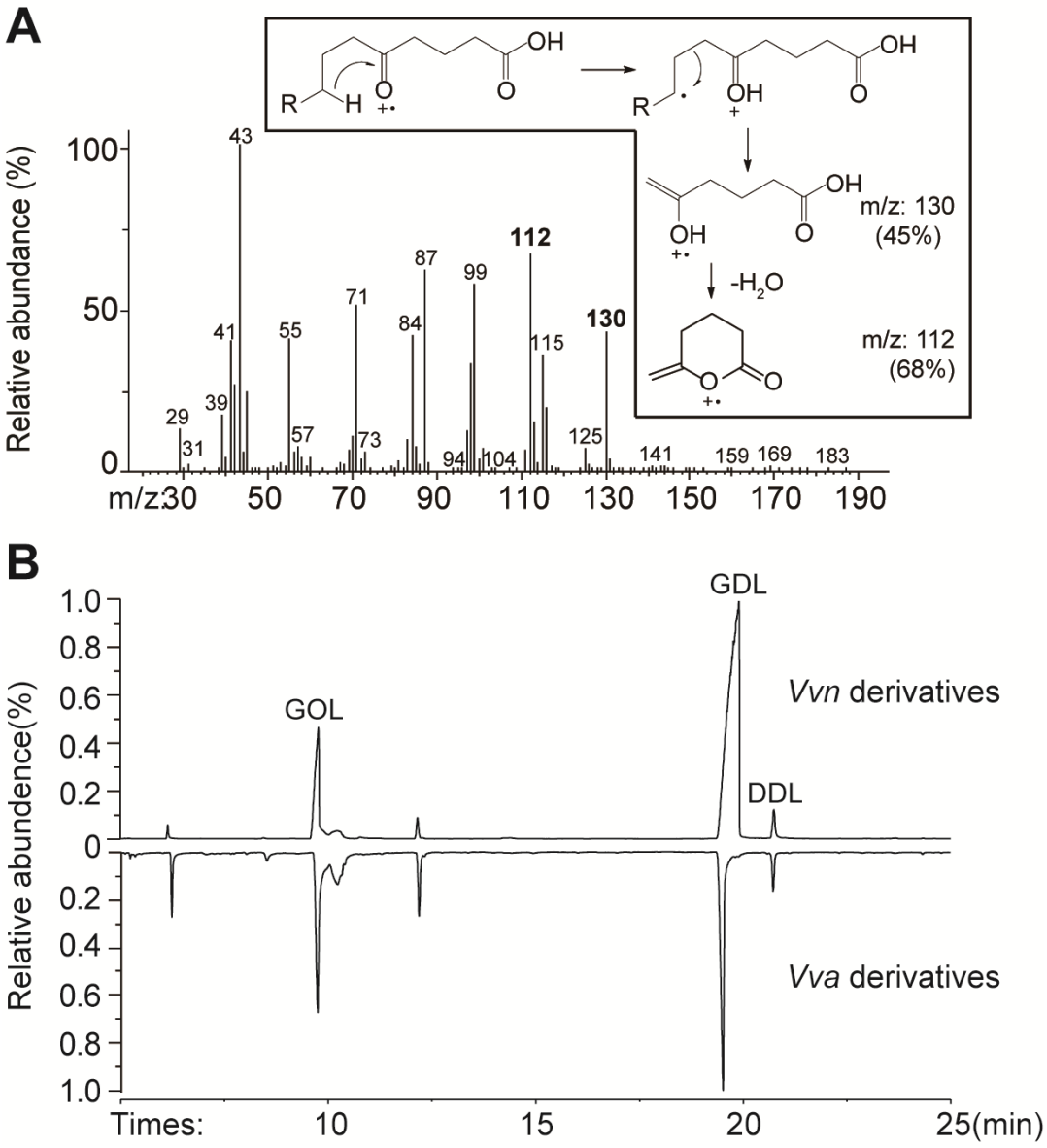
Figure 2. Results (mean values ± standard errors) of the bioassays used to determine the optimal blend of sex pheromone compounds in China (A), and tests of the selected blend on male attraction in the native *Vva* population (Vva-N), native *Vvn* population (Vvn-N), and invasive *Vvn* population (Vvn-I)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

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

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

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

529 Figure 1



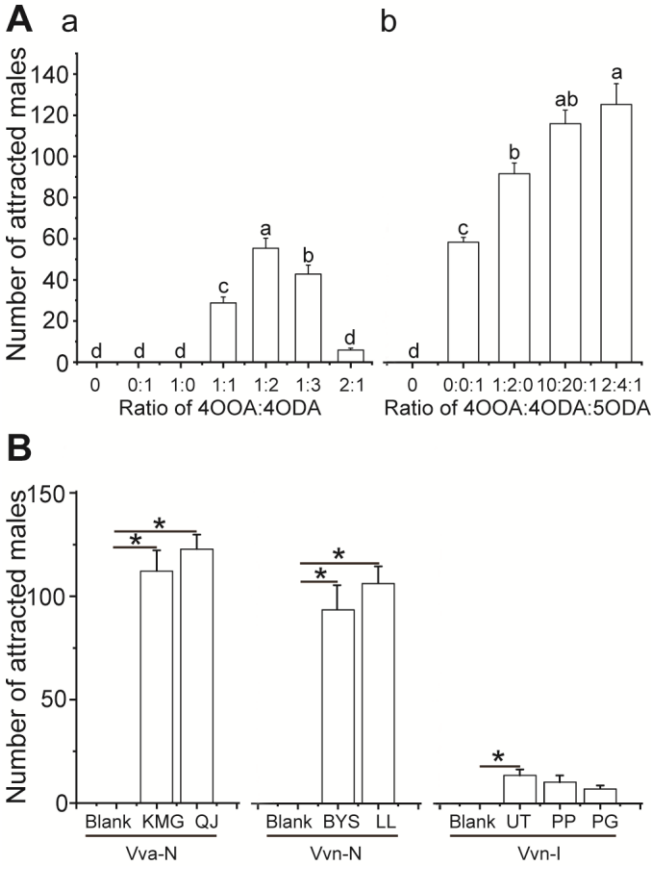
530

531

532

533

534 Figure 2

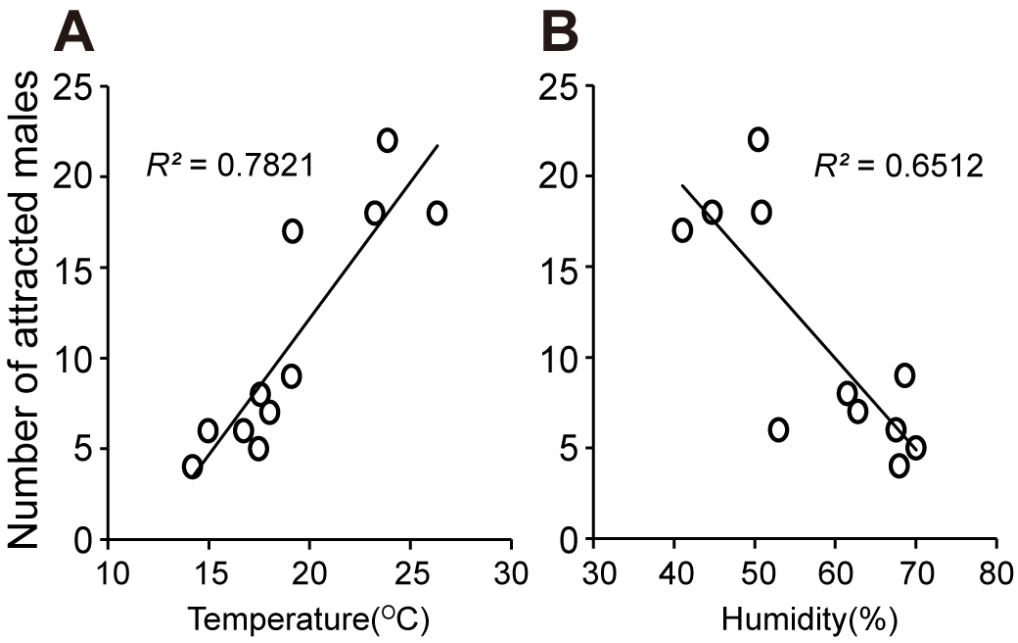


535

536

537

538 Figure 3



539

540

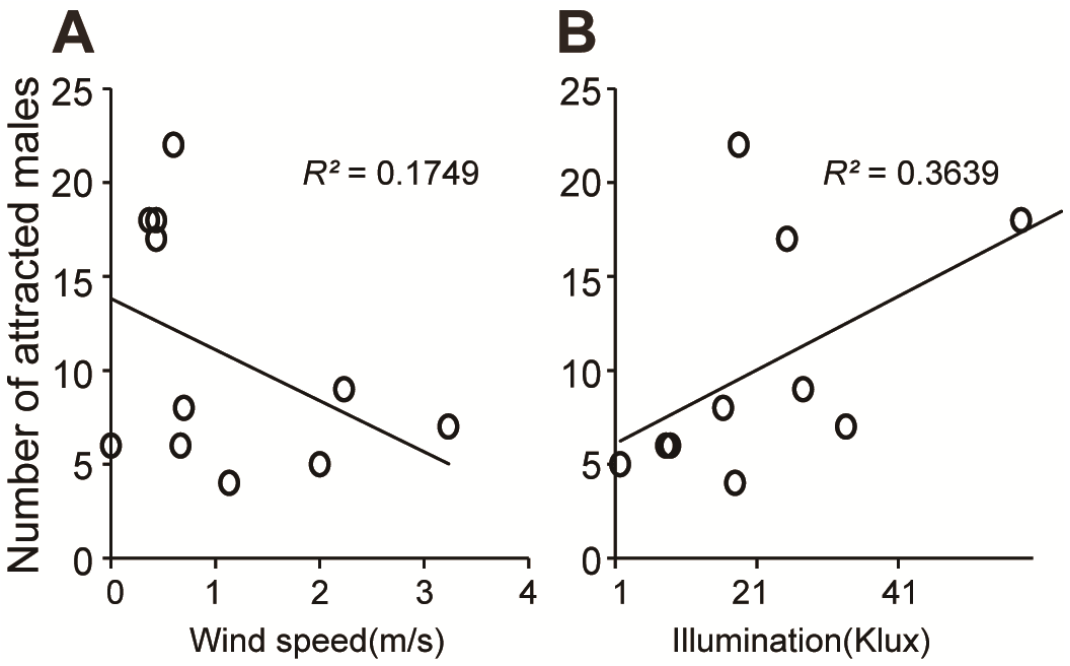
541

542

543

544

Figure S1


A
B
Number of attracted males
25
20
15
10
5
0
0
1
2
3
4
Wind speed(m/s)
R² = 0.1749
1
21
41
Illumination(Klux)
R² = 0.3639