

Correspondence

Identification of giant hornet *Vespa mandarinia* queen sex pheromone components

Shihao Dong^{1,2,5}, Aili Sun^{3,5},
Ken Tan^{1,2,*}, and James C. Nieh^{4,6,*}

The Vespidae is a diverse family of wasps and hornets that are formidable predators of insects, including social bees¹, and includes a number of invasive species². Recently, the world's largest hornet, *Vespa mandarinia* Smith (Hymenoptera: Vespidae), which occurs naturally in the Indomalayan region, has been found in Canada and the United States². Some simulations indicate that it could rapidly spread throughout Washington and Oregon in the western US, as well as some eastern parts of the country^{2,3}, threaten native bees and honeybees, and harm bee-pollinated crop production worth over \$100 million annually³. There is consequently an urgent need to learn more about *V. mandarinia*'s reproductive biology and to develop trapping methods to locate its nests and to control its reproduction. We identified *V. mandarinia* queen-produced sex pheromone from the 5th and 6th intersegmental sternal glands of virgin queens. The major active compounds were hexanoic acid, octanoic acid, and decanoic acid. When placed in field traps, the synthetic compounds and a queen-equivalent mixture rapidly attracted hundreds of males but no females or other species.

Invasive vespid species are difficult to eliminate because they are all eusocial, benefit from division of labor, have large colonies, and can have hidden nests¹. At present, trampling followed by manual nest removal is the primary control strategy for *V. mandarinia* but is laborious and depends mainly upon the visual detection of hornets. Sex pheromone traps are widely used to monitor harmful insect presence⁴, and *V. mandarinia* produces an

aggregation-sex pheromone that attracts males to nest entrances where they mate with virgin queens^{5,6}. However, the source and composition of this sex pheromone was unknown.

We collected sexually mature virgin queens and drones with insect nets as they emerged from three colonies in Yunnan, China. When we placed a queen inside a cage with males, the males immediately clustered around the queen and intensively antennated and tried to mate with her (Figure 1A). *Vespa* spp. have sternal glands on the 5th and 6th metasomal sternites⁷, and *Vespa velutina* queens produce sex pheromone in their 6th intersegmental sternal gland (Supplemental information). We therefore used clean filter paper strips to rub queen sternal intersegmental glands and immediately pinned one strip in a cage with 50 males. Only paper strips rubbed against the 5th and 6th intersegmental sternal gland surfaces attracted males. Males rapidly walked upon and antennated the strips and

sometimes tried to mount other males on the strip. These gland extracts were significantly more attractive than the clean control paper in all six trials (Figure 1B, $F_{2,49} = 5,681.35$, $P < 0.001$), and both gland extracts were equally attractive to males (Tukey HSD test, $P > 0.05$).

Next, we used solid phase microextraction and gas chromatography–mass spectrometry to identify the putative sex pheromone compounds from six queens. The major components and quantities (Figure 1C and Data S1) were hexanoic acid (mean \pm SD = $15.3 \pm 11.0 \mu\text{g}$ ^{5th gland} and $4.9 \pm 3.4 \mu\text{g}$ ^{6th gland}), octanoic acid ($38.3 \pm 25.4 \mu\text{g}$ ^{5th gland} and $6.8 \pm 4.0 \mu\text{g}$ ^{6th gland}), and decanoic acid ($12.3 \pm 6.7 \mu\text{g}$ ^{5th gland} and $7.4 \pm 2.5 \mu\text{g}$ ^{6th gland}). There were significant quantity differences among the compounds ($F_{2,27} = 3.62$, $P = 0.04$) and between glands ($F_{1,27} = 24.12$, $P < 0.0001$), but no significant interaction of compound \times gland ($F_{2,25} = 2.97$, $P = 0.07$, Figure S1A).

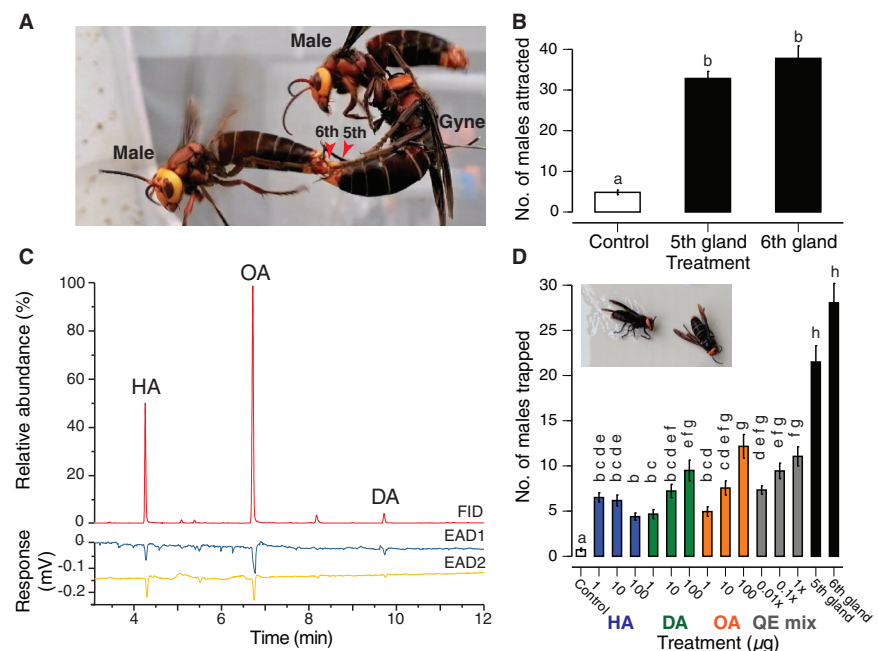


Figure 1. Identifying components of the *Vespa mandarinia* queen sex pheromone.

(A) Male mating with a tethered queen (the 5th and 6th intersegmental sternal glands of the queen are indicated with red arrows). (B) Males are attracted to surface extracts from these glands. (C) Queen sex pheromone (GC chromatogram) contains hexanoic acid (HA), octanoic acid (OA), and decanoic acid (DA). The antennae of males responded strongly to these major components (GC-EAD traces from two representative males). (D) Sticky traps baited with HA, DA, OA, QE mix (queen equivalent mixtures), and extracts of the 5th and 6th glands captured significantly more males in the field than control traps. The glandular extracts were the most attractive. Different letters (aligned to facilitate visualization of differences) indicate significant differences (Tukey HSD tests, $P < 0.05$). Means and standard errors are shown.



We used coupled gas chromatography electroantennogram detection and found that all three major components elicited strong responses from male antennae (Figure 1C). We then measured the responses of male antennae to pure synthetic compounds or a queen-equivalent mixture (Figure S1C). There was a significant effect of treatment type ($F_{16,160} = 16.99$, $P < 0.0001$), and all treatments elicited stronger responses than the solvent control (Tukey HSD test, $P < 0.05$). Antennal responses were similar across four orders of magnitude of treatment quantities. Male antennae showed significantly elevated responses, as compared to the control, even to 0.001x of the queen-equivalent mixture.

We then sought to determine whether the compounds we identified might be useful for trapping males in the field. We placed sticky trap arrays in the field in Anning, Yunnan, China and baited them with different treatments, each with a dummy hornet. From November to December of 2020, we tested the pure compounds and found a significant effect of treatment ($F_{9,168} = 13.77$, $P < 0.0001$), with 100 μ g of octanoic acid attracting the most males (Tukey HSD test, $P < 0.05$, Figure S1D). In total, we trapped 1,318 *V. mandarinia* males and no females over 36 h (2 h exposure per day). We repeated this experiment from October to November of 2021 and included tests of the queen-equivalent mixture and extracts from the 5th and 6th intersegmental glands. There was a significant effect of treatment ($F_{14,253} = 56.64$, $P < 0.0001$). Extracts of the 5th and 6th glands captured the most males, followed by mixtures of the synthetic compounds and octanoic and decanoic acids at the highest concentrations tested (Figure 1D). In total, we trapped 2,542 *V. mandarinia* males and no females over 90 h (5 h exposure per day). In both experiments, control traps collected very few males, suggesting that the dummy males were not inherently attractive. Because *V. mandarinia* drones mate near nests, we placed our traps 10–15 m from nests. Additional studies should be conducted to test the ability of these traps to capture males when located at greater distances from nests. The

traps were evidently species-specific: in both field seasons, traps were placed approximately 200–300 m from *Vespa velutina* colonies and we observed *V. velutina* flying near some traps, yet no *V. velutina* or any other insects were ever captured.

Hexanoic acid, octanoic acid, and decanoic acid are simple saturated aldehydes and have not previously been identified as components of hymenopteran sex pheromones. However, they are components of insect pheromones that attract congeners: ant trail pheromones⁸, locust aggregation pheromone⁹, and honeybee Nasonov pheromone¹⁰. The higher attraction of natural glandular extracts (Figure 1D) suggests a limitation of our study: other olfactory components, apart from the ones that we identified, likely play a role and deserve additional study. Queen cuticular lipids may increase attraction. However, 1x of the queen-equivalent mixture captured, on average, 16-fold more males than the control. Given the urgent need to monitor the spread of *V. mandarinia* in North America and to control its reproduction, we suggest that immediate testing of these sex pheromone components and their mixture would be beneficial, while studies to identify additional queen sex pheromone components are being carried out.

SUPPLEMENTAL INFORMATION

Supplemental information includes one figure, supplemental chromatograms (Data S1), supplemental results and discussion, experimental procedures, and supplemental references and can be found with this article online at <https://doi.org/10.1016/j.cub.2022.01.065>.

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AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualization and design of the experiments. S.D. and J.C.N. analyzed the paper and S.D., K.T., and J.C.N. contributed to the writing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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¹CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, 650000 Kunming, Yunnan, China. ²Center of Plant Ecology, Core Botanical Gardens, Chinese Academy of Sciences, 666303 Mengla, Yunnan, China. ³College of Plant Protection, Yunnan Agricultural University, 650201 Kunming, China. ⁴Division of Biological Sciences, Section of Ecology, Behavior, and Evolution, University of California San Diego, La Jolla, CA 92093, USA. ⁵Equal contribution. ⁶Lead contact. *E-mail: kentan@xtbg.ac.cn (K.T.); jnieh@ucsd.edu (J.C.N.)

SUPPLEMENTAL INFORMATION: Identification of giant hornet *Vespa mandarinia* queen sex pheromone components

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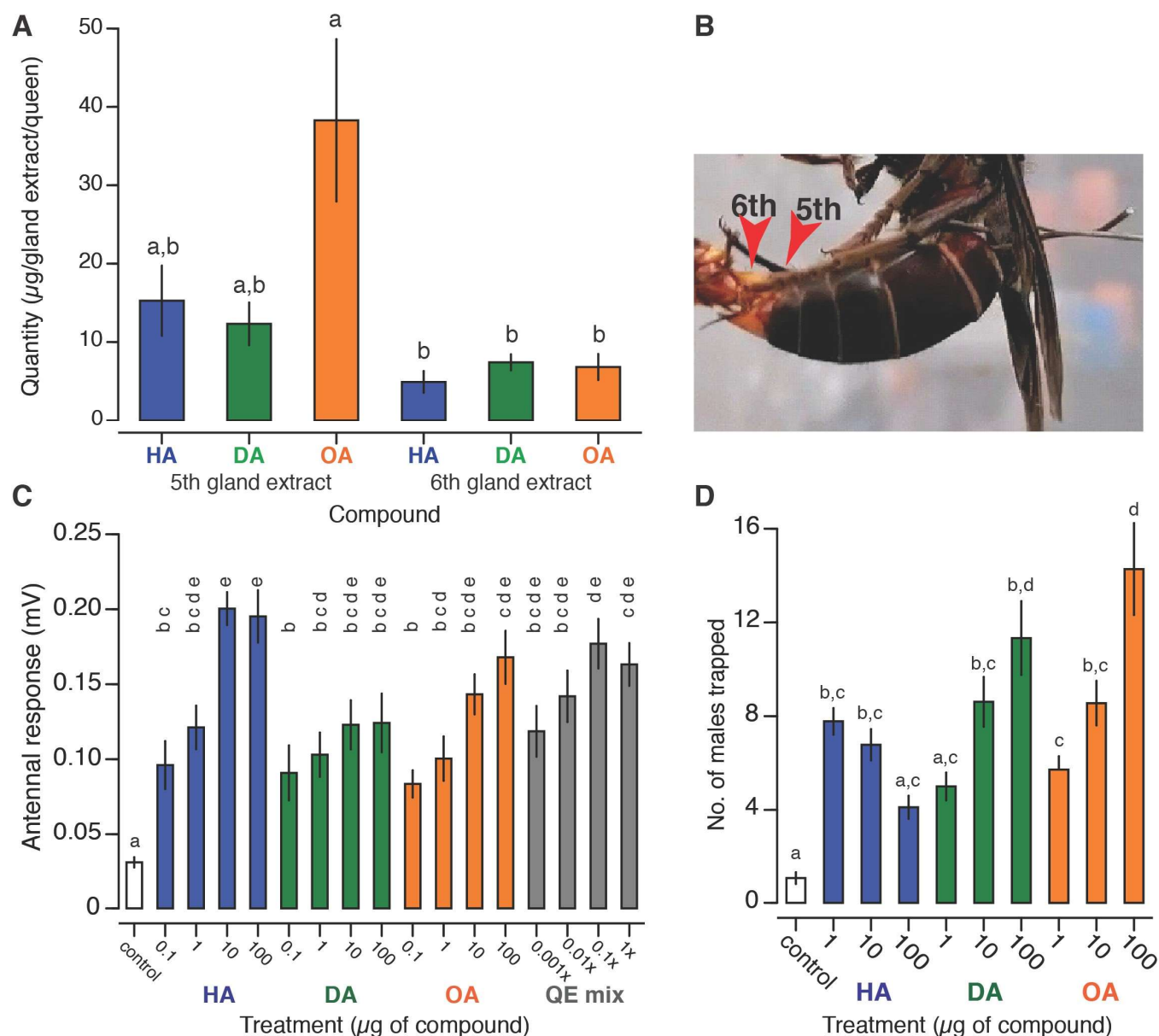


Figure S1. Quantification of female sex pheromone compounds, male antennal responses, and initial (2020) field trap data.

All plots show means and standard errors, and different letters indicate significant differences per plot (Tukey HSD test, $P < 0.05$). (A) Quantities of hexanoic acid (HA), decanoic acid (DA), and octanoic acid (OA) obtained from rubbing the 5th or 6th intersegmental sternal glands of virgin queens with a clean paper strip. (B) Gland locations. (C) Antennal responses of males to queen sex pheromone components. The synthetic

1x queen-equivalent (QE) mixture consisted of 20 µg HA, 20 µg DA, and 46 µg OA. All compound treatments elicited significantly higher amplitude antennal responses than the solvent control treatment. There was a general trend such that males had higher amplitude antennal responses (mV) when presented with larger amounts of each test compound and the QE mixture. However, within each compound type, the only significant differences were between 0.1 and 10 or 100 µg HA. For DA, OA, and the QE mixture, male antennae did not have significantly higher responses over quantities spanning four orders of magnitude. Male antennal responses to 0.001x QE were not significantly different from responses to 1x QE. (D) Results of the 2020 field trap bioassay experiment in which HA-, DA-, and OA-baited sticky traps captured significantly more males in the field than control traps.

SUPPLEMENTAL RESULTS & DISCUSSION

These experiments do not compare the attraction of males to a control compound such as nonanoic acid, but, in our preliminary trials, we tested *V. mandarinia* male attraction to two alarm pheromone compounds (2-pentanol and 3-methyl-1-butanol) produced by *V. mandarinia* queens. Males showed no attraction to these compounds. Similarly, *V. mandarinia* males were also not attracted to other compounds that could be considered controls, 4-oxo-octanoic acid and 4-oxo-decanoic acid, which are produced by *Vespa velutina* queens.

The 5 m spacing between traps may have been insufficient to prevent odor plumes between traps from interacting. However, if 5 m was insufficient, then we would expect to see an increase in the number of males attracted to a trap containing the control, a pure compound, or a synthetic mixture placed next to a natural gland extract trap, given that natural gland extracts were more attractive (Figure 1D). We did not observe such an effect.

We designed our field trap bioassays based upon the natural biology of *V. mandarinia*, in which males mate with virgin females near nests. The placement of our traps close to nests is a limitation of our study because it would be desirable to test the efficacy of these at distances far away from nests. Additional studies and optimization are needed. Given the urgency of finding invasive *V. mandarinia* nests, a potential strategy would be to place such traps near recently exterminated nests in addition to spacing them widely within an area known to be invaded.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Resource availability

Additional information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shihao Dong (dongshihao@xtbg.ac.cn).

Data and code availability

Data can be accessed at <https://doi.org/10.5281/zenodo.5831862>.

Methods details

Colonies and subject details

The mating season of *Vespa mandarinia* is from September to December in the southern and southwestern areas of Asia^{S1}. All virgin queens and males were therefore collected between 9:00 AM and 12:00 PM from September to November in 2020 and 2021 from natural *V. mandarinia* nests in Anning, Yunnan (25°07'22"N, 102°36'65"E). During hornet collection, average conditions were 25 ± 3 °C (mean ± 1 standard deviation) and 60% relative humidity. We used insect nets to capture drones and queens as they exited their nests and identified them based upon their distinctive appearances (Figure 1A). Drones and

queens were placed in separate wire mesh cages (30 cm x 30 cm x 30 cm) per colony and provided with 30% (w/w) sugar water in plastic syringes. Each cage was maintained at 25 °C and 60% relative humidity inside a dark incubator. Indoor experiments took place at the Southwest Biodiversity Research Centre, Chinese Academy of Sciences, China (25°1258'N, 102°7509'E). In total, we used hornets from six different *V. mandarinia* nests.

Preliminary cage bioassay

We tied a virgin queen to a rod with a soft wire wrapped around her thorax and placed her inside a cage (30 cm x 30 cm x 30 cm) with 50 males in it. In all cases, male activity rapidly increased, and they attempted to mate with her. The males attempted to mount her and tried to use their genital claspers to open her genital organ while continuously protruding their genitalia

Pheromone location bioassay

Based upon this response, we designed a pheromone localization bioassay using our previously published methods^{S2}. In brief, we used clean filter paper strips (4 mm × 15 mm) to rub each intersegmental sternal gland surface of a queen for 5 s. We then immediately used an insect pin to attach this strip to the bottom of a cage with 50 males. Based upon preliminary tests, only the strips rubbed against the 5th and 6th intersegmental sternal glands of queens attracted males, and we therefore focused on these two regions. The control consisted of a clean paper strip. Queen odors extracted by the strip were highly attractive to males, which walked excitedly on and rapidly antennated the strip. In some cases, males even tried to mount other males walking on the same strip. We therefore measured the attraction of males to a strip by counting the number of males that used their antennae to touch the strip within 5 min. We tested all three treatments (control, 5th, and 6th intersegmental sternal gland extracts) with one colony per day, alternating the order of treatment presentation to ensure that each all treatments equally occupied all possible order positions. Between each treatment trial, we waited 10 min. We replicated this on six separate days with each colony, using a total of three colonies and 150 males (50 males per colony).

Chemical analyses of odors

We used headspace solid phase micro-extraction (SPME) to determine the chemical composition of volatile compounds from the 5th and 6th intersegmental sternal gland surfaces. We used six queens, two per colony. We rubbed one of these surfaces with a paper strip, as in the bioassay, and immediately placed the strip into a clean 2 mL glass vial sealed with a PTFE cap containing a 0.2 mm diameter hole into which we inserted a blue SPME fiber (65 μm PDMS/DVB, Supelco, CA) to collect volatiles for 1 h. To identify the compounds, we used an HP 7890A-5975C gas chromatography-mass spectrometer (GC-MS). We initially used an HP-5 column (30 m × 320 μm × 0.25 μm; Agilent) with an oven ramp temperature of 50 °C for 2 min and then increased by 10 °C/min to 280 °C, where it was held for 10 min. However, after multiple repetitions with this HP-5 column, we were unable to obtain clear results, and so we switched to an HP-FFAP column (30 m × 320 μm × 0.25 μm, Agilent, US) with helium flowing at 1 mL/min as the carrier gas. We chose the HP-FFAP column based upon our prior study identifying the sex pheromone of *Vespa velutina*^{S2}. Correct column choice is essential for compound identification, and it is possible that using other columns could result in the identification additional compounds. This is a limitation of our study.

Pheromone samples were injected in splitless mode into an injection port heated to 250 °C. The oven ramp temperature was 150 °C for 2 min and then increased by 5 °C/min to 230 °C. Upon reaching 230 °C, it was held at this temperature for 10 min. The 70 eV electron impact ion source was heated to 230 °C and the MS quadrupole was heated to 150 °C. The scanned mass range was set as m/z 28.5 to 300 with a threshold abundance of 16 to detect trace molecular ions. Data were analyzed with Chemstation (Agilent Technologies) and AMDIS (NIST) software. Known structures were confirmed based upon their identical retention times and mass spectra to three authentic chemical standards: hexanoic acid (HA, CAS#142-62-1), octanoic acid (OA, CAS# 124-07-2), and decanoic acid (DA, CAS# 334-48-5) respectively from Macklin, Xianding, and Dibo (Shanghai, China, Data S1). We separately conducted these analyses with six different queens (two queens per colony from three colonies). For confirmation, we added 1 μL of each authentic

chemical standard to separate paper strips (one compound per strip) and then replicated our extraction technique by placing this strip into a clean 2 mL glass collection vial and using the SPME method and GC-MS settings described above.

Pheromone quantification

To quantify these compounds, we used an HP-7890B (Agilent, US) gas chromatograph (GC). We used paper strips to rub the 5th or 6th intersegmental sternal glands of queens^{S2}. We then positioned each paper strip in a separate clean glass vial, rapidly added 1 mL of ethyl ether, sealed the vial to obtain the extract, removed the paper strip after 1 h, and concentrated the extract to 10 μ L with nitrogen flowing at 1 L/min. We took 1 μ L of this concentrate for GC analysis. An HP-FFAP column (30 m \times 320 μ m \times 0.25 μ m, Agilent, US) was used with helium at 1 mL/min as the carrier gas. Pheromone samples were injected in splitless mode into an injection port heated to 240 °C. The outflow of the GC column was split into a flame ionization detector (FID) heated to 250 °C for compound identification. The oven ramp temperature was 150 °C for 2 min and then increased by 5 °C/min to 230 °C. The 230 °C temperature was then held for 10 min. To quantify the three components, we diluted the three compounds in ethyl ether to concentrations of 1 μ g/ μ L, 10 μ g/ μ L, 50 μ g/ μ L, and 100 μ g/ μ L and injected 1 μ L of each concentration for GC analysis. We created GC-FID external standard curves based upon the relationships between peak areas and injection volume. We then quantified each component (Figure S1A) by measuring its peak area. We separately conducted these analyses with six different queens (two from each of our three colonies).

Responses of male antennae to odors

We used coupled Gas Chromatography-Electroantennogram Detection (GC-EAD) to determine if *V. mandarinia* male antennae would respond to the putative queen sex pheromone^{S2}. We used a paper strip to rub the 5th or 6th intersegmental sternal glands as described above and immediately placed each strip into different clean 2 mL glass vials for 1 h with a SPME fiber (see above). We then placed the SPME fiber in a splitless injection port at 240 °C in a HP-7890B GC (Agilent, US). An HP-FFAP column (30 m \times 320 μ m \times 0.25 μ m, Agilent, US) was used with helium as the carrier gas. The oven temperature was 150 °C for 2 min and then increased by 5 °C/min to 230 °C. Half of the outflow of the GC column was split into a flame ionization detector heated to 250 °C for compound identification while the other half was connected to an EAD system with a 40 cm long transfer line heated to 230 °C. An HP-34465A digital multimeter (Keysight) controlled by BenchVue software (Keysight) running on a PC was used to record antennal responses to the stimuli from the GC column.

To obtain a male for testing, we carefully captured it from a cage. Our preliminary tests found no differences in the responses of left or right antennae, and thus we randomly choose a left or right antenna, excised it at its base, and placed it between glass electrodes filled with insect Ringer's solution. Each antenna was placed 1 cm away from the outlet of a PTFE tube (1 cm inner diameter, 15 cm long) that provided the SPME extracts with clean (500 mL active charcoal filtered) and wet air (distilled water, 90% RH) flowing at 15 mL/s. In total, we used 10 males from three different colonies: 3, 3, and 4 from colonies 1, 2, and 3, respectively. Typical responses are shown in Figure 1C.

We next examined the dose-dependent antennal responses of males to the three authentic chemical standards and a blend designed to match the proportions of these compounds in natural queen pheromone (Figure S1C). The individual components and the blend were diluted and presented in 1 μ L of diethyl ether (Aladdin, CN), which is highly volatile and does not elicit EAG responses after being allowed to evaporate for 15 s. For the individual compounds, we tested the following quantities: 0 μ g (solvent control consisting only of diethyl ether), 0.1 μ g, 1 μ g, 10 μ g, and 100 μ g. Each antenna was tested with all compounds and the synthetic mixture. We randomly selected which compound to test first and then increased the concentration tested for HA, OA, and DA. Because both 5th and 6th intersegmental sternal glands were highly attractive to males, we calculated 1x queen equivalent (QE) as the total amount that we extracted, on average per compound, from *both* the 5th and 6th sternal glands (20 μ g HA, 20 μ g DA, and 46 μ g OA, a ratio of 1:1:2.3). The 1x QE mixture, therefore contained 86 μ g of test compounds. We tested different

quantities of this QE mixture based upon the following proportions: 0.001x, 0.01x, 0.1x, and 1x QE mixture.

The EAG recording system was identical to the one described in Wen *et al.*^{S2}. The antennal preparation was placed 1 cm away from the outlet of an odor pipette (1 cm inner diameter, 15 cm long) that provided the test odor by combining a clean and wet continuous air flow (15 mL/s, 90% relative humidity) and a pre-filtered and wet pulsed air flow (5 mL/s, 90% relative humidity). The test odor stimulus was presented for 3 s. For each preparation, we gently removed one male at a time from a cage, cut off a randomly chosen antenna (left or right, see above), and mounted it between a glass reference electrode and a recording electrode, both filled with insect Ringer's solution. We waited for 30 s between stimulations to provide enough recovery time and then provided a 3 s stimulus^{S2}. We measured the baseline-peak amplitude (mV)^{S2}. We added 1 μ L of each test compound (diluted to the appropriate concentration) to a paper strip (4 mm x 15 mm) and allowed the solvent to evaporate for 15 s before placing the strip into a clean odor-delivery pipette (2 mL, 4 mm inner diameter). We used 11 males from three different colonies: 4, 4, and 3 from colonies 4, 5, and 6, respectively.

Field trap bioassay with sex pheromone components (2020)

We conducted field bioassays to determine if the synthetic queen sex pheromone components could capture males. We used sticky traps (33.7 cm x 21.7cm) baited with different treatments. Each trap contained one quantity of one compound (HA, DA, or OA) or the control (solvent blank). We applied 1 μ L of each treatment to a clean filter paper strip (15 mm x 4 mm) placed inside an uncapped clean glass vial (1.5 mL) and positioned in the center of a sticky trap with a freshly killed male (a visual dummy that, as a male, did not have queen sex pheromones). We used the visual dummy as a proxy for the visual appearance of a queen based upon our prior work^{S2}. In our preliminary trials, we observed males approaching the traps with the test compounds but not landing on the trap. However, the addition of the dummy male encouraged males to land. It is not clear if the odor of the dummy facilitated landing. Future tests on the importance of the visual stimulus of the dummy, male odors, or both should be conducted.

We diluted the synthetic compounds in diethyl ether. Based upon our pheromone quantification and the EAG response of drones to different odor doses, we presented the single compounds in the following quantities: 1 μ g, 10 μ g, or 100 μ g per trap (Figure S1D). Mating occurs near *V. mandarinia* nest entrances^{S1}, and we therefore placed a semicircular array of 10 sticky traps, each with a different treatment and spaced 5 m apart, 10 m from each three different colonies (approximately 3 km apart) from November to December in 2020. Each day, we set out the traps for 2 h (10 am to 12 pm) and then removed them to count the number of hornets trapped. We tested only one *V. mandarinia* colony per day and replicated each bioassay six times per colony.

Field trap bioassay with sex pheromone components and a blend (2021)

From October to November of 2021, we replicated this experiment with additional treatments and three different colonies. In this experiment, all treatments were applied to paper strips that were then directly pinned to the dummy. No glass vials were used. In addition to the treatments used in the 2020 experiment (see above), we tested 1x QE of synthetic pheromone (see above) applied to a paper strip and pinned to the dummy or a paper strip rubbed against either the 5th or 6th intersegmental glands of queens (see above) and pinned to the dummy. We placed a semicircular array of 15 sticky traps, each with a different treatment and spaced 5 m apart, 15 m from each colony (increasing the trap-to-colony distance from the 2020 experiment to accommodate the larger number of traps per colony). We set out the traps for 5 h (9 AM to 2 PM) and then removed them to count the number of hornets trapped. We tested only one *V. mandarinia* colony per day. Each colony was approximately 3 km from the neighboring colony, and we replicated each bioassay six times per colony. Results are shown in Figure 1D.

Statistical analyses

In our pheromone location bioassay, we used a univariate repeated measures analysis, with colony as the repeated measure. The number of males visiting the paper strip was the dependent variable and type of

glandular extract was the independent variable. We used Tukey's honestly significant difference (HSD) test to make all pairwise corrected comparisons.

To analyze differences between the quantities of the three main compounds, we log-transformed quantities and used mixed models (REML algorithm) with queen identity and colony as random effects and gland location, compound name, and the interaction of gland location x compound name as fixed effects. We ran the full model with the interaction and then excluded the interaction because it was not significant ($P>0.05$). We used Tukey's HSD test to make all pairwise corrected comparisons.

To analyze the responses of male antennae (mV) to the compounds, we used a univariate repeated measures analysis with male as the repeated measure because we used only one antenna per male, and each antenna was tested with all three compounds found in queen sex pheromone. We log-transformed antennal responses for a better model fit and used Tukey's HSD test to make all pairwise corrected comparisons.

For the field trap bioassays, we also ran a univariate repeated measures analysis with colony as the repeated measure because each array of traps was placed nearby a specific colony. The log-transformed number of males in each trap was the dependent variable and the treatment type was the independent variable. We used Tukey's HSD test to make all pairwise corrected comparisons. All tests were conducted with JMP Pro 16 software.

Author identification (name, ORCID, email)

Shihao Dong, 0000-0002-9247-6553, dongshihao@xtbg.ac.cn

Aili Sun, 0000-0003-4634-1292, sunaili12345@126.com

Ken Tan, 0000-0002-0928-1561, kentan@xtbg.ac.cn

James C. Nieh, 0000-0001-6237-0726, jnieh@ucsd.edu

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