Title

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Journal

Journal of Experimental Biology, 220(4)

ISSN

0022-0949

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Publication Date

2017-02-15

DOI

10.1242/jeb.148783

Peer reviewed

J Exp Biol Advance Online Articles. First posted online on 6 December 2016 as doi:10.1242/jeb.148783
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.148783

Poison and alarm: The Asian hornet *Vespa velutina* uses sting venom volatiles as alarm pheromone

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Journal of Experimental Biology • Advance article

Abstract: In colonial organisms, alarm pheromones can provide a key fitness advantage by enhancing colony defense and warning of danger. Learning which species use alarm pheromone and the key compounds involved therefore enhances our understanding of how this important signal has evolved. However, our knowledge of alarm pheromones is more limited in the social wasps and hornets as compared to the social bees and ants. *Vespa velutina* is an economically important and widespread hornet predator that attacks humans and honey bees. This species is native to Asia and has now invaded Europe. Despite growing interest in *V. velutina*, it was unknown if it possessed an alarm pheromone. We show that these hornets use sting venom as an alarm pheromone. Sting venom volatiles were strongly attractive to hornet workers and triggered attacks. Two major venom fractions, consisting of monoketones and diketones, also elicited attack. We used GC-EAD and identified 13 known and three unknown aliphatic ketones and alcohols in venom that elicited conspicuous hornet antennal activity. Two of the unknown compounds may be an undecen-2-one and an undecene-2,10-dinone. Three major compounds (heptan-2-one nonan-2-one, and undecan-2-one) triggered attacks, but only nonan-2-one did so at biologically relevant levels (10 hornet equivalents). Nonan-2-one thus deserves particular attention. However, the key alarm releasers for *V. velutina* remain to be identified. Such identification will help to illuminate the evolution and function of alarm compounds in hornets.

Keywords: *Vespa velutina*, sting venom, alarm pheromone, aliphatic ketones, hornet aggression, undecen-2-one, undecene-2,10-dinone

Introduction

Pheromones are important information agents and help to regulate colony behaviour in social insects, such as honey bees (Slessor *et al.* 2005), ants (Hölldobler 1995), termites (Wen *et al.* 2014) and wasps (Bruschini *et al.* 2010; Turillazzi and Bruschini, 2010). Alarm pheromones can play dual roles, by activating nest defence and serving as a warning that allows foragers to avoid dangerous sites. For example, honeybee alarm pheromone can attract guards to the nest entrance for nest defence (Boch & Shearer 1971; Roubik 1989) and repel foragers from foraging sites with predators (Li *et al.* 2014; Wang *et al.* 2016). In social wasps and hornets, nest guards can also release alarm pheromones to recruit nest defenders (Bruschini *et al.* 2008). Hornet alarm pheromones can likewise be used to mark foraging sites (Ono *et al.* 2003). However, less is known about the pheromones involved in hornet defence as compared to bee and ant alarm pheromones.

The diversity of alarm pheromone usage and glandular sources in social hornets and wasps provides key variation that can be used to understand the evolution of alarm pheromones in social insects. Determining the character states is therefore important. What species use alarm pheromones, what is the source, and what chemical components are involved? The ritualization hypothesis predicts that chemical weapons can become associated with attack or defense and thereby evolve into alarm pheromones. In fact, sting venom appears to be a primary source of alarm pheromone. *Polistes dominulus* (Bruschini *et al.* 2006; Landolt 1998), *Vespula squamosa* (Heath & Landolt 1988; Landolt *et al.* 1995), *Vespa crabro* (Veith *et al.* 1984), *Vespa mandarinia* (Ono *et al.* 2003), and *Vespa simillima xanthoptera* (Ono *et al.* 2003) provide examples. In these species, volatile alarm pheromones are released when sting venom is exuded by the stinger or via stinging (Ali & Morgan 1990; Downing 1991; Jeanne 1981; Landolt & Akre 1979). In *Vespula* spp., mandibular glands may provide alarm pheromones (Reed & Landolt 2000), as they do in the stingless bees (Schorkopf *et al*. 2009). However, like some bumble bees species (Maschwitz, 1967), a few hornet and wasp species appear to lack alarm pheromone (Landolt *et al.* 1998).

In most social insects studied to date, alarm pheromones consist of multi-component blends (Bruschini *et al.* 2010; Turillazzi and Bruschini, 2010; Hölldobler 1995; Slessor *et al.*

2005; Wen *et al.* 2014). Hornet alarm pheromones also contain multiple components, some of which are known to elicit alarm behaviour. It is useful to consider the functions of these different components. Some compounds may act synergistically with components that elicit alarm or serve different functions (Bruschini *et al.* 2010). Components that trigger alarm behaviour include N-3-methylbutylacetamide in *Vespula squamosa* (Heath & Landolt 1988; Landolt *et al.* 1995), 2-methyl-3-butene-2-ol in *Vespa crabro* (Veith *et al.* 1984), 2-Pentanol, 3-methyl-1-butanol, 1-methylbutyl 3-methylbutanoate in *V. mandarinia*, and *V. simillima xanthoptera* (Ono *et al.* 2003), nonan-2-one in *V. orientalis* (Saslavasky *et al.* 1973), and amides and ketones in *Dolichovespula maculata* (Jimenez *et al.* 2016). However, the functions of other components remain unclear. For example, the roles of venom volatiles such as tridecane, pentadecane, pentadecene and undecane in seven Stenogastrinae species (Dani *et al.* 1998) are unknown, as are the functions of alkanes, monounsaturated alkenes and 2-alcohols in one Polistinae species (Sledge *et al.* 1999). Some compounds, particularly minor ones, may not have a clear adaptive value but could arise as metabolic by-products.

To learn more about hornet alarm pheromones, we focused on *Vespa velutina* Lepeletier, 1836, a common honey bee predator (Tan *et al.*, 2007, 2016) and pest in Asia (Liu *et al.* 2016; de Haro *et al.* 2010) and a species that has recently invaded parts of Europe, to the detriment of the European honey bee, *Apis mellifera* (Rortais *et al.* 2010; Villemant *et al.* 2006). *Vespa velutina* appears to be a fairly derived species within the genus *Vespa* (Perrard et al. 2013). Initial observations led us to believe that *V*. *velutina* has an alarm pheromone. When alerted near the nest, guards would exit to search for the disturbance. Once a guard found the intruder, it attempted to sting or exuded venom from its stinger. As a result, multiple hornets were immediately alerted and began attacking the target. Our goals were therefore to identify the source of the alarm pheromone, determine its behavioural effects on hornet workers, chemically analyse the pheromone, use electroantennography to identify active components, and test if these components can elicit attacks.

Materials and methods

Hornet and alarm behaviour

We used 15 *Vespa velutina* colonies from two sites separated by over 100 km: Kunming Botanical Garden (KBG) in Kunming, China (N25.44°, E105.37°) and a site in Wuding (WD), China (N25.94°, E104.27°). Some experiments required dissecting hornets, but *V. velutina* is not an endangered species. In fact, it is invasive (Rortais *et al.* 2010; Villemant *et al.* 2006), and, in some areas, is considered a harmful pest (de Haro *et al.* 2010). All studies were carried out in compliance with relevant provincial and national guidelines. Sample sizes for each experiment are summarized in Table 2.

Bioassays

In preliminary observations, we noted that *V. velutina* hornets disturbed at their nests would extend their stingers. A droplet of exuded venom was sometimes visible on these stingers. Subsequently, guards exited the nest in search of the disturbance. We therefore tested for the alarming effect of sting venom by observing hornet responses to different quantities (quantities) of sting gland extract applied to a target. We define an attack as a hornet landing on the filter paper and showing a sting posture by bending its abdomen.

We pipetted 0 (hexane-only control), 0.01, 0.1, and 1.0 gland equivalents onto a filter paper (5 mm x 15 mm) placed behind a dry *Quercus acutissima* leaf hanging on a tree branch 30 cm from the nest entrance. Each wasp has one sting gland and thus one gland = 1.0 wasp equivalents (eq). Immediately after adding the treatment, we began a 3-min trial during which we counted the total number of hornets attacking the leaf. Only one quantity was used per trial. We conducted three trials per colony per quantity and ran one trial per colony per day. Each day, decided upon the order of quantity presentation, following a pseudo-random pattern that interspersed the different quantities but ensured that each quantity was tested the same number of times. We used a new leaf for each trial. We video recorded each trial.

Chromatography naturally separated the venom compounds into two fractions, monoketones and diketones. In separate bioassays, we therefore tested if monoketones or

diketones would elicit alarm behaviour. We used micro scale silica chromatography to separate these pheromone components. A glass capillary tube (1.2 mm inner diameter, 110 mm long) was filled with 55 mg 400 mesh silica gel (Haiyang, Qingdao). The extracts were concentrated down to 20 µl (see above) and added to the silica column, which was then successively washed with 55 µl hexane, 260 µl ethyl acetate/hexane and 260 µl ethyl acetate/hexane (1:1, v/v). Fraction 1 (monoketones) eluted out at the sixth tube (130 to 195 µl 4:1 acetate/hexane v/v), and fraction 2 (diketones) eluted out at the ninth tube (1:1 acetate/hexane v/v). The components in each fraction were confirmed with GC analysis. Fraction 1 contained monoketones and fraction 2 contained undeca-2,10-dinone (identified by comparison with an authentic standard) and an unknown undecene-2,10-dinone (Table 2).

We also video recorded hornet responses to these different fractions. We added 4 eq of the test fraction (or an equivalent volume of pure solvent in control trials) to a piece of clean filter paper placed <1 cm behind a leaf and presented the sample 30 cm from the nest entrance for 3 min, as in the whole venom bioassay. We measured aggression by counting the total number of hornets that tried to attack the sample. We conducted one trial per colony per day (detailed sample sizes in Table 1).

We tested hornet responses to four of the major or most volatile identified venom volatiles (heptan-2-one (most volatile), non-8-en-2-one (most volatile), undecan-2-one (major component), and nonan-2-one (major component). We used the same 3-min bioassay as above, but tested hornet aggression responses to 0, 10, 100, 1000, and 10000 ng of pure synthetic standards. We conducted one trial per colony per day (detailed sample sizes in Table 1).

Pheromone extraction

We extracted volatile pheromones with solid phase microextraction (SPME). After comparison of fibres, we selected a 65 µm PDMS/DVB blue fibre (Supelco, CA) because it rapidly adsorbed the most volatiles. Using clean glass 5 ml vials, we collected the headspace volatiles of (1) attacked workers from three different nests or (2) a dissected and crushed

worker venom gland for 30 min (samples sizes given in Table 1). To collect alarm volatiles from a living worker, we gently caught it with a cotton sieve in front of its nest. We then briefly cold anesthetized it on ice for 2 min and transferred it into a clean collection vial that we immediately capped. After it revived, we penetrated the PTFE lined cap with a needle and used this needle to disturb the hornet by lightly touching (without piercing) the hornet's thorax a total of 10 times over 30 s. During this process, the hornet exhibited alarm behaviour and began to exude venom from the tip of its stinger. The hornet was then confined to the bottom of vial with the needle, and we introduced the SPME fibre in a sleeve through the cap. We thereby only obtained volatiles. The fibre had no direct contact with the needle, the hornet, cap, or vial walls. For chemical analysis, each SPME fibre was desorbed in the GC injection port at 250 °C.

In addition, we extracted pheromone from venom gland contents for use in some of the bioassays. Workers were anesthetized in a freezer and then their venom sacs were dissected out and extracted with hexane. We placed 10 glands in 100 µl of hexane in a clean glass vial. After 2 h, the solvent and two washes of 50 µl of hexane were transferred to a 250 µl micro vial insert tube. The extract was concentrated by 10X to a final volume of 20 µl with a gentle nitrogen flow for all bioassays, compound identification, and compound quantification. All extracts were kept at -20 °C until use.

Chemical standards

We purchased commercially available heptan-2-one (CAS 110-43-0, Sigma-Aldrich, Shanghai, China), nonan-2-one (CAS 821-55-6, J&K, Beijing, China), undecan-2-one (CAS 112-12-9), and other reagents (TCI (Tokyo, Japan). The Non-8-en-2-one was synthesized by condensation of 6-bromo-hexene and ethyl acetoacetate in the presence of sodium ethoxide followed by hydrolyzation and decarboxylation. The heptan-2,6-dinone was synthesized via condensation of dibromomethane and ethyl acetoacetate in the presence of sodium ethoxide followed by hydrolyzation and decarboxylation (low yield but detectable for GC-MS identification). Undeca-2,10-dinone was synthesized in the same manner using 1,5-dibromopentane. All synthetic compounds were purified with silica gel chromatography.

Gas Chromatography coupled with Mass Spectrometry (GC-MS) analyses

SPME extracts and derivatives were analysed with GC-MS, using an HP 7890A-5975C (Agilent, US) with an HP-5ms capillary column (30 m × 250 µm × 0.25 µm, Agilent, US). The carrier gas was helium flowing at 37 cm/s. The oven ramp was set as 50 °C for 2 min, followed by 5 °C/min and then 280 °C for 10 min. For the quadrupole mass spectrometry, a 70 eV EI ion source was used at 230°C. The mass range scanned consisted of *m/z* ratios 28.5 – 300 at a rate of 2 × 4 scan/s. The detection abundance threshold was set to 10. Data were analysed using Chemstation software (Agilent Technologies, US) and AMDIS (NIST).

Compound identification

We used micro-scale derivatization to narrow down the number of potential compounds by determining possible functional groups in the unknown GC-EAD active compounds. To determine if unknown GC-MS peaks with a mass-to-charge ratio (*m/z*) of 43 were acetic esters, we hydrolyzed 20 µl of supernatant from a pooled extract (10 glands in hexane) by adding 20 µl of 0.1 M NaOH and stirring for 30 min. The organic layer was then chemically analysed.

We used $NaBH_4$ reduction to confirm the existence of ketone groups (Attygalle 1998): 20 µl of 0.5 M $NaBH_4$/NaOH solution was added to the supernatant obtained from a different extract of 10 glands. This mixture was neutralized with 0.5 mol/L HCl, stirred for 30 min, and then analysed with GC.

We used Pt catalysed reduction to determine if there were rings or olefinic bonds in the compounds. Approximately 0.1 mg of Pt/C catalyst was added to a 10-gland extract in hexane. The extract was stirred under hydrogen for 30 min. After removal of the Pt/C particles by filtration, the solvent was subjected to chemical analysis (Attygalle 1998).

GC-FID analysis

SPME extracts, solvent extracts and derivatives were analysed using GC with a Flame Ionization Detector (FID). We used an HP-7890B GC (Agilent, US) with FID and splitless injection at 250 °C. For GC-FID analysis, an HP-5 column (30 m×320 µm×0.25 µm, Agilent, US) was used with nitrogen flowing at 37 cm/s as carrier gas. The oven ramp was set to 50 °C for

2 min, then 10 °C/min to 280 °C for 5 min. We used GC-FID quantity-response standard curves to quantify each known compound in a venom gland against pure synthetic standards. For quantification, we used extracts from 15 foragers from three colonies (five foragers per colony). We calculated the linear retention index (LRI) using retention times of C8-C15 n-alkanes analysed under the same GC and GC-MS conditions.

Electrophysiological analysis

We used GC coupled to electroantennographic detection (GC-EAD) to measure the electrophysiological olfactory responses of hornet antennae to volatile sting venom compounds collected by SPME. We used the same instruments and protocol as Wang et al. (2016). In brief, a custom EAD system was coupled to the HP7890B GC. GC conditions were the same as for the GC-FID analysis. An HP-34465A digital multi meter (Keysight, US) controlled by BenchVue software (Keysight, US) running on a PC was used to record antennal responses. For electroantennogram (EAG) analysis, the odour preparation was delivered to the antennal preparation with a custom stimulus controller (Wang *et al.* 2016).

For GC-EAD and EAG, we followed the same capture method used to analyse alarm volatiles (see above) and detached one antenna per hornet (left or right, randomly chosen) at its base with iris scissors. The distal end of the antennae was cut open with scissors to improve signal strength and both ends were mounted between two glass electrodes filled with insect Ringer's solution. The antennal preparation was positioned in a clean and wet air flow (40 cm/s, room temperature, relativity humidity >95%) conducting the odours from the GC column outlet or an odour pipette, as appropriate. Sample sizes for the GC-EAD and the EAG experiments are given in Table 1. We tested the following compounds: heptan-2-one, undecan-2-one, nonan-2-one, and non-8-en-2-one.

Statistics

To determine the effect of venom quantity and venom fractions on the number of hornets that attacked the target and to analyse the effect of identified GC-EAD-active compounds on hornet attacks, we used a Repeated-Measures General Linear Model (GLM)

with a Poisson distribution, Log link, Maximum Likelihood estimation and an overdispersion parameter. Colony was the repeated measure. We used Dunnett's test to make comparisons corrected for Type I error between bee responses to the blank control and the different compound quantities.

To test for the independence of attacks (whether each attacker added additional alarm pheromone), we ran a Univariate Repeated-Measures Analysis of Variance of the number of attackers with time (attacks per minute) and colony as factors. If alarm pheromone accumulated during attacks, there should be a significant increase in attacks over the 3 min trial to venom fractions that elicited attacks. The results (see below) suggested that attackers did not add alarm pheromone to our stationary target, perhaps because it did not fight back or struggle like a living target. Each attack appeared to be largely independent of prior attacks.

In all models, we included colony as an effect and used post-hoc Likelihood-Ratio contrast tests. For multiple tests run on the same data, we used Bonferroni corrections, reporting results as significant only if $P<\text{alpha}_{\text{Bonferroni}}$. All analyses were conducted with JMP Pro 12.0.1.

Results

The venom gland is the source of volatiles released by attacked workers and higher quantities elicited more attacks

In chemical analyses, all volatiles from attacked workers were identical to those from dissected venom glands (Fig. 1A). We identified the same 16 major compound peaks (Fig. 1A, Table 2) in the volatiles of all nine attacked hornets. All 15 samples (each a separate GC-MS run) of hornet venom volatiles contained these identical peaks. The venom gland is therefore the source of the alarm pheromone.

Venom extract significantly attracted hornets (quantity effect: L-R $\chi^2_3=92.19$, $P<0.0001$) in all colonies, but some colonies had stronger responses (colony effect: L-R $\chi^2_2=10.01$, $P=0.01$). All quantities $\geqslant$0.01 venom gland equivalents (eq) attracted more hornets than the control (contrast tests, L-R $\chi^2_1\geqslant13.61$, $P\leqslant0.0002<\text{alpha}_{\text{Bonferroni}}=0.017$, Fig. 1B).

Chemical identification

Chemical analysis of the venom of *V. velutina* workers revealed 16 major compounds of which 13 elicited reproducible antennal (GC-EAD) responses (Fig. 3, Table 2). Known structures were confirmed with authentic chemical standards.

Unknown major GC-EAD active peaks *10* and *15* were further analysed via MS interpretation and derivatization (Fig. 2). After hydrolyzation, peaks *10* and *15* were unchanged. Thus, these compounds did not contain ester structures. Both peaks disappeared after $NaBH_4$ and Pt-catalysed reduction, indicating the presence of ketone and olefinic structures.

The compound corresponding to peak *10* had a mass to charge ratio of *m/z* 168. The ratio of its isotope peak at *m/z* 169 was 12.20%, indicating a formula of $C_{11}H_{22}O$, with a ring double bond (RDB) value of 2. A base peak with *m/z* 43 resulted from the loss of $CH_3C{=}O^+$. The existence of 2-ketone groups with γ-H was suggested by *m/z* 58 resulting from McLafferty rearrangement. Thus, the compound is probably an undecen-2-one.

The compound corresponding to peak *15* had *m/z* 182, indicating a formula of $C_{11}H_{22}O_2$ with a RDB value of 3. This compound had a base peak with *m/z* 43, and a characteristic ion with *m/z* 58 from McLafferty rearrangement, indicating 2-ketone groups with γ-H. Because there were two oxygen atoms, we hypothesize that this compound has two 2-ketone groups and an olefinic double bond. Thus, peak *15* likely corresponds to an undecene-2,10-dinone. The peak areas of undecan-2-one (peak *13*) and undecane-2,10-dinone (peak *16*) increased after Pt-catalysed reduction, suggesting the presence of ketones.

Venom monoketones and diketones elicited attacks

The venom ketones can be separated into two fractions consisting primarily of monoketones (fraction 1) and diketones (fraction 2, Fig 4A). There was an overall effect of treatment type on the number of attacks that a target received (L-R χ^2_3=86.60, P<0.0001, Fig. 4B). There was a significant effect of colony (L-R χ^2_2=106.80, P<0.0001) because some colonies had stronger responses. However, for each colony, the overall response pattern was consistent. All fractions and their combination received significantly more attacks than the control (contrast tests, L-R $\chi^2_1 \geqslant 48.90$, P<<0.00001< $alpha_{Bonferroni}$=0.017, Fig. 4B).

Each attacking hornet could potentially deposit additional alarm pheromone on the target. If so, then the number of attacks should increase over time on each fraction and the combination of both fractions. However, the number of attacks per minute did not increase over the 3 min trial (no effect of time for all fractions separately or in combination: $F_{2,22} \geq 2.75$, $P \geq 0.09$).

Hornets had similar EAG responses to four identified compounds but these did not elicit aggression

Hornet antennae did not respond strongly to four major identified compounds that we tested (heptan-2-one, undecan-2-one, nonan-2-one, and non-8-en-2-one). The EAG response difference threshold, the lowest quantity that elicited a statistically different response from exposure to the blank control was 1000 ng for all these compounds (Dunnett's test, $p<0.05$, Fig. 5A). With one exception, 1000 ng is far greater than the quantity released by a single hornet venom gland, suggesting a low biologically-relevant sensitivity. However, nonan-2-one does occur at 852 ng/hornet (Table 2).

There was a significant effect of compound type (L-R $\chi^2_3=18.51$, $P=0.0003$), colony (L-R $\chi^2_2=86.00$, $P<0.0001$, but similar trends when colonies examined separately), quantity ($\chi^2_4=54.39$, $P<0.0001$), and the interaction compound type*quantity (L-R $\chi^2_{12}=27.73$, $P=0.006$) on the number of attacking hornets (Fig. 5B). For non-8-en-2-one there were no significant contrasts (L-R $\chi^2_1=0.70$, $P=0.40$). However, contrast tests revealed a quantity effect in heptan-2-one (0 vs. 10^4 ng, L-R $\chi^2_1=6.87$, $P=0.009$), nonan-2-one (0 vs. 10^4 ng, L-R $\chi^2_1=8.19$, $P=0.004$) and undecan-2-one (0 vs. 10^4 ng, L-R $\chi^2_1=13.39$, $P=0.0003$ and 0 vs. 10^3 ng, L-R $\chi^2_1=6.07$, $P=0.01$). For all of these contrast tests, $\text{alpha}_{\text{bonferroni}}=0.025$. Thus, these four compounds, presented in isolation, did not elicit strong attack response because only levels far greater than those that found in one venom gland elicited attacks.

Discussion

Alarm pheromones play a key role in social insects, but their function in social wasps and hornets remains poorly understood as compared to other social insects. We provide the first evidence that *V. velutina*, a widespread and invasive species uses an alarm pheromone.

Our analyses of volatiles produced by alarmed hornets revealed that this alarm pheromone is produced by the sting gland, and increasing quantities of sting gland extract increased aggressive attacks. We then used GC-MS analysis and authentic standards and identified 13 of the 16 major compounds found in these volatiles, all ketones. Using micro-scale derivatization, we narrowed the possibilities for two compounds and hypothesize that they are a type of undecen-2-one and a type of undecene-2,10-dinone. Worker hornets had antennal responses to four of the identified compounds (non-8-en-2-one, heptan-2-one, nonan-2-one, and undecan-2-one) and exhibited aggression to three of these compounds (heptan-2-one, nonan-2-one, and undecan-2-one). The alarm pheromone fractions that we tested (monoketones, diketones, and their combination) all significantly elevated attacks (Fig. 2B). Thus, as in other Vespidae species (Akre *et al.* 1982; Ali & Morgan 1990; Downing 1991; Landolt & Akre 1979; Ono *et al.* 2003), ketones are important components of hornet and wasp alarm pheromones, which are commonly volatile venom components.

Two of the major components (non-8-en-2-one and nonan-2-one) that we tested did not elicit strong responses at biologically relevant doses (one hornet equivalent). However, nonan-2-one did elicit a significant antennal response at 1000 ng (Fig. 5A) and occurred at 852 ng/sting gland. Nonan-2-one appeared to trigger more hornet attacks at 1000 ng, but this was not significantly different from responses to the blank control. There was a clear trend with increasing quantities, but only 10,000 ng significantly elevated attacks (10 hornet equivalents, Fig. 5B). Thus further studies of nonan-2-one, the remaining 12 compounds, and combinations of these may narrow down the key attack releasers. Synergistic interactions between compounds may be important.

Saslavasky *et al.* (1973) reported that multiple ketones would elicit strong alarm responses in *Vespa orientalis*, an Asian species related to *V. velutina*. Recently, Jimenez *et al.* (2016) demonstrated that ketones in *Dolichovespula maculata* venom also acted as an alarm pheromone, with some ketones eliciting attacks directed at a target, much as we observed. With respect to the *V. velutina* ketone, nonan-2-one, we observed significantly increased attacks at 10 eq, which is similar to the effects observed by Jimenez et al. (2016) with natural venom or candidate synthetic ketones tested at 5 eq (5 venom sac extract equivalents). We also tested lower concentrations of natural venom extracts and found exceptionally high

sensitivity, with hornets attacking the target significantly more often at only 0.01 eq (Fig. 1B).

Because we used a single target in our assays, not multiple ones (Jimenez *et al.* 2016), we cannot distinguish between targeting (spatially localized) versus the general alarming (not tightly localized) effects of individual compounds. However, given the relatively low behavioural responses of hornets to most of our pure synthetic compounds, even to nonan-2-one, as compared to natural venom, further investigations of the remaining 12 venom compounds are warranted. It is possible that a less abundant component in venom (Table 2) could elicit the observed responses to 0.01 eq of natural venom. Alternatively, a combination of compounds may be required.

Recent findings suggest that persistent (lower vapour pressure) components in honey bee venom pheromone (sting alarm pheromone) play an important role because they provide longer-lasting information (Wang *et al.* 2016). For *V. velutina*, the dimethylnonen-2-one (peak *9*), the unknown undecen-2-one (peak *10*), and the unknown undecene-2,10-dinone (peak *15*) are therefore potentially components that can mark attack targets. These three compounds have lower vapour pressure and can persist to mark a predator or indicate danger.

Our results contribute to a growing body of evidence that alarm pheromones in social insects are closely linked to toxic or venomous components, as predicted by the evolutionary ritualization hypothesis. Alarm pheromones should easily evolve from volatiles associated with defensive chemical weapons, like venoms, because these are associated with attack and defence by receivers and because this information enhances colony fitness by rallying nestmates to attack. An unresolved evolutionary question is the function of the complex volatile blends that we see today. In some cases, these mixes may be metabolic “spandrels”, by-products of a common metabolic pathway. Multiple compounds may also provide more reliable information in noisy chemical environments. Finally, individual components may be selected based upon volatility to provide information through time. A more complete understanding of the functions of individual components and mixtures is required, though it is interesting that the story of venom and alarm is largely parallel in ants, bees, and wasps.

Acknowledgements

This work was supported by the Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, and the CAS 135 program (XTBGT01) of Chinese Academy of Science, China National Research Fund (31260585) to Ken Tan. We thank the Central Laboratory at Xishuangbanna Tropical Botanical Garden for their assistance in chemical analysis.

Author Contributions

PW and KT conceived of and designed the experiments. YC, PW and SD performed the experiments. PW and JN analysed the data. PW, KT, and JN contributed reagents, materials or analysis tools. PW, YC, KT and JN wrote the paper.

Data accessibility

All data are accessible via Dryad at the following URL: http://dx.doi.org/10.5061/dryad.p5pj6.

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Table 1. Sample sizes of experiments (KBG = Kunming Botanical Garden, WD = Wuding). In total, we used 15 different colonies over two years at two different locations separated by over 100 km.

Experiment	Sites	Year	No. of colonies	No. of hornets used per trial	No. of replicates per colony	Total No. of individual hornets
HS-SPME-GC of alarmed worker	KBG	2016	3	1	3	9
HS-SPME-GC of sting gland	KBG	2015	3	1	5	17
HS-SPME-GC-MS of sting gland	KBGWD	20152016	1 KBG in 2015, 1 WD in 2015 and 1 KBG in 2016	1	3	9
GC-EAD of sting glands	KBG	2015	3	2 (one for obtaining extract & one for obtaining an antenna)	3	18
EAG of STDs	KBG	2016	3	6 per sample	6 per sample	108
Bioassay of extracts, fractions and synthetic chemical standards (STD)	WD, KBG	2015, 2016	1 in KBG, 2 in WD for Nonanone, Nonenone STD, 3 in WD 2015 for undecanone heptanone STD, extract and fraction samples	Colony activity level assayed	9 per sample tested	594 hornet responses (colony treated as unit of replication)
Microscale chemistry of extracts	WD, KBG	2015, 2016	>6 colonies, for 4 micro-reactions	10	3	120

Table 2. The known and GC-EAD active compounds in hornet alarm pheromone. We show the Linear Retention Index (LRI). The antennal response of hornets (GC-EAD) is given as "+" (response) or "-" (no response). For quantification, we used 17 hornets from three colonies (5-6 hornets per colony) and conducted a separate GC-MS analysis per hornet. Samples were not pooled. We conducted detailed GC-EAD tests and bioassays of hornet aggression in response to the compounds shown in bold (Fig. 5).

Peaks	LRI	Structure	GC-EAD	Quantities from SPME (ng/insect)
1	**889**	**Heptan-2-one**	**+**	**31.3 ± 4.1**
2	898	Heptan-2-ol	+	10.8 ± 1.5
3	993	Heptan-2,6-dinone	+	5.6 ± 0.8
4	1069	Acetophenone	+	30.3 ± 5.9
5	**1086**	**Non-8-en-2-one**	**+**	**75.2±11.3**
6	**1097**	**Nonan-2-one**	**+**	**852.0 ± 141.7**
7	1102	Nonan-2-ol	+	145.0 ± 20.0
8	1155	Unknown $C_{10}H_{20}O$	+	--------
9[a]	1231	4,8-Dimethylnon-7-en-2-one	+	173.3 ± 36.5
10[a]	1279	Unknown undecen-2-one	+	170.3 ± 27.8
11	1284	Undecen-6-one	-	86.6 ± 11.4
12	1289	Unknown undecen-2-one	+	7.7 ± 1.0
13	**1298**	**Undecan-2-one**	**+**	**178.4 ± 30.9**
14	1302	Undecan-2-ol	-	33.5 ± 4.1
15[a]	1466	Unknown undecene-2,10-dinone	+	126.0 ± 23.0
16	1485	Undecane-2,10-dinone	-	31.3 ± 4.1

[a]Unknown C11 compounds were quantified based upon the ratios of their molecular weights to Undecan-2-one, using the internal standard method.

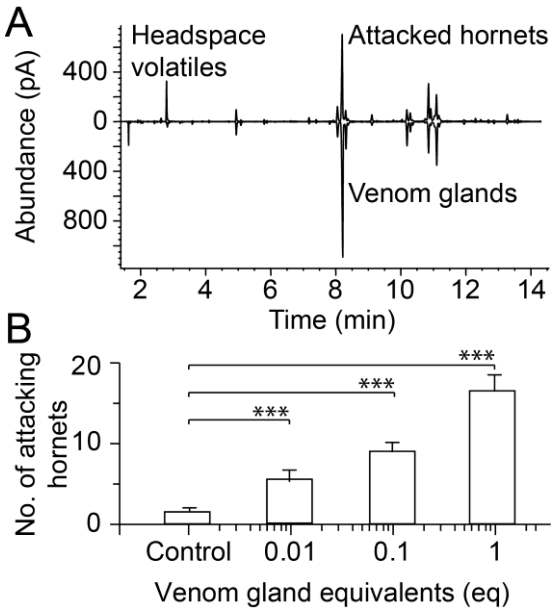

Figure 1. The venom gland is the source of alarm pheromone and higher quantities of gland extracts elicited more attacks. (A) The headspace volatiles of alarmed hornets correspond to volatiles released by their venom glands. Representative chromatograms (HS-SPME-GC) are shown. (B) Hornets attacks increased with higher quantities of venom and were significantly higher than for the control, even at 0.01 venom gland equivalents (eq). Means and standard errors are shown. Contrast test results are given ($P \leqslant 0.0002$***).

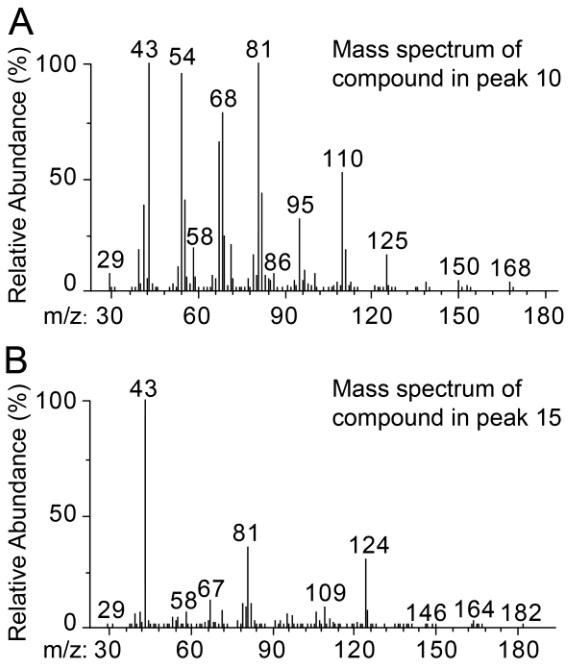

Figure 2. The mass spectra of the unknown GC-EAD active compounds in (A) peak 10 (unknown undecen-2-one) and (B) in peak 15 (unknown undecene-2,10-dinone).

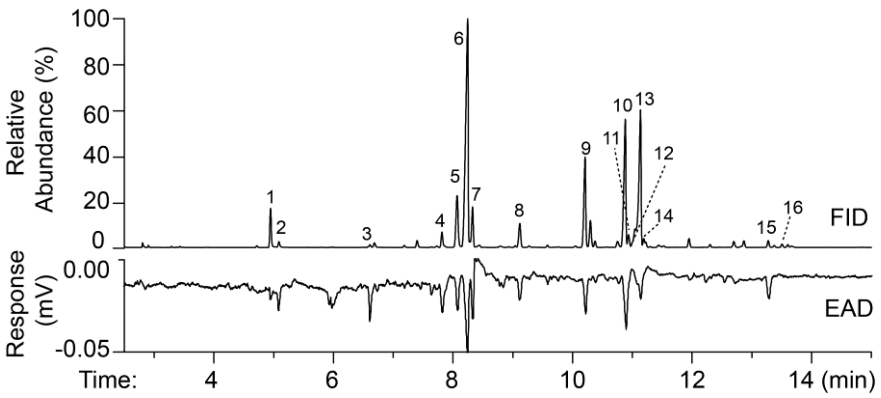

Figure 3. Typical venom headspace SPME extract (un-numbered peaks are impurities) of *V.* velutina worker and its antennal responses to these compounds. The FID plot shows the chemical components and the GC-EAD plot shows the antennal neural responses.

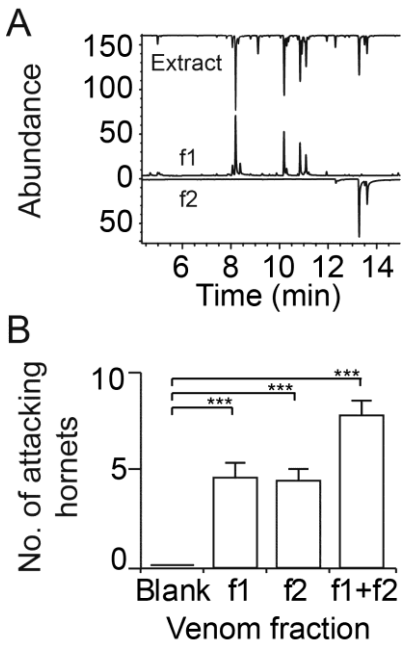

Figure 4. Hornet venom fractions (f1=monoketones, f2=diketones) elicited attacks and increased the number of alarmed hornets on the nest when tested at four venom gland equivalents (eq). (A) GC profile of the fractions showing the complete extract (top), f1 (middle), and f2) (bottom). (B) Each fraction and their combination significantly increased attacks. Results of contrast tests are shown (P<0.0001***). Bar graphs show means and standard errors. Per treatment, we conducted three trials with three colonies (nine trials per treatment).

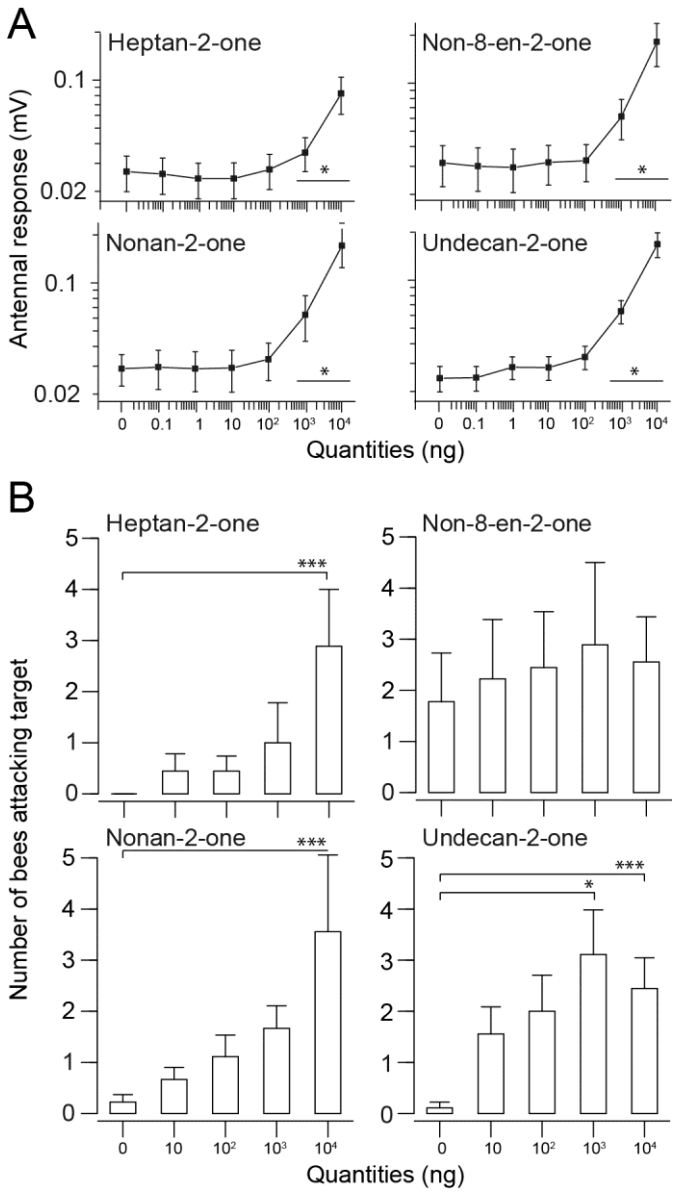

Figure 5. Hornets responded to four major identified venom volatiles. (A) Antennal recordings (EAG) of hornets responding the compounds (24 hornets, six from each of four colonies). The starred line indicates quantities that differed significantly from the control (Dunnett's tests, $p<0.05$). (B) Attack responses of hornets to the compounds. Only heptan-2-one, nonan-2-one, and undecan-2-one elicited significantly more attacks as compared to controls, but only at high levels much greater than those found in one sting gland (one hornet equivalent). Significant differences are indicated (L-R contrast tests, *P=0.01, ***$P\leqslant$0.009). Means and standard errors are shown in all plots.