Insect science

Insect Science (2024) 0, 1–8, DOI 10.1111/1744-7917.13448

ORIGINAL ARTICLE

Identification of alarm pheromone components of the southern giant Asian hornet, *Vespa soror*, a major pest of honey bees

Shihao Dong[1,#], Aili Sun[1,#], Tao Lin[1], Jianjun Li[1], Gaoying Gu[1], James C. Nieh[2] and Ken Tan[1]

[1]*CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, China and* [2]*Division of Biological Sciences, Section of Ecology, Behavior, and Evolution, University of California San Diego, La Jolla, CA, USA*

Abstract The rise of biological invasions threatens biodiversity and food security, with the vespid family, including *Vespa soror*, being of particular concern. Our study focused on the alarm pheromone components of *V. soror*. By using gas chromatography-mass spectrometry (GC-MS) chemical analyses, electroantennograms, and field bioassays, we identified 5 compounds—2-pentanol, 3-methyl-1-butanol, 2-heptanol, 2-nonanol (2-N), and isopentyl acetate (IPA)—in hornet sting venom that elicited defensive behavior from hornets. IPA and 2-N also serve as alarm pheromone components in multiple honey bee species that are important prey for *V. soror*. This shared chemical signaling may allow cross-detection by each species on the other's alarm cues. While it should be advantageous for bees to detect *V. soror* alarm pheromone, the benefits to *V. soror* of using IPA and 2-N are unclear. *V. soror* may manipulate bee behavior, potentially distracting defenders, because they mark victim bee colonies by rubbing their abdomens, which contain their sting glands, at bee hive entrances. Our findings pose new evolutionary questions about the role of manipulation in the arms races.

Key words alarm pheromone; aggressive responses; collective defense; convergent evolution; venom

Introduction

Biological invasions pose significant challenges to biodiversity and food production in the 21st century. Among the invasive social insects, the vespid family is notable because at least 9 species have proven detrimental ecological effects (Wilson Rankin, 2021). For example, *Vespa velutina* Lepeletier, has expanded throughout Europe since 2004, and this expansion has significantly harmed managed honey bee populations and increased apiary maintenance expenses in countries such as Spain, France, Italy, and the UK (Requier *et al.*, 2019; Barbet-Massin *et al.*, 2020; Angulo *et al.*, 2021; Otis *et al.*, 2023).

The recent identification of *Vespa mandarinia* (the northern giant hornet) and *Vespa soror* (the southern giant hornet) in Canada and the United States has raised concerns due to its capacity to prey on honey bees and the risks that it poses to human safety (Zhu *et al.*, 2020). Although attention has chiefly focused on *V. mandarinia*, in the spring of 2019, a *V. soror* gyne was collected at the harbor in Vancouver, British Columbia (Bass *et al.*,

Correspondence: James C. Nieh, School of Biological Sciences, Department of Ecology, Behavior, and Evolution, University of California San Diego, La Jolla, CA 92093, USA. Email: jnieh@ucsd.edu; Ken Tan, CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, 650000, China. Email: kentan@xtbg.ac.cn

[#]These authors contributed equally to this work.

2022). Thus, *V. soror* is also potentially invasive (Otis *et al.*, 2023). In its native habitats, *V. soror* is found in southern China and subtropical regions of Southeast Asia (Perrard *et al.*, 2013) where it is a formidable predator of insects such as honey bees because these hornets are social insects that can recruit nestmates to their food sources (Fig. S1) (Mattila *et al.*, 2020). Once a scout hornet finds a honey bee colony, it can rub its gaster on the entrance, likely depositing pheromones secreted by its sternal glands (van der Vecht and Richards' glands) (Mattila *et al.*, 2021b). The recruited hornets then grab and kill the defending bees, decimating their colonies (Fig. S2, Video S1) (Mattila *et al.*, 2020). In Vietnam, *Apis cerana* can repel *V. soror* by foraging for animal feces and applying it in spots around their nest entrances (Mattila *et al.*, 2020). Moderate to high levels of fecal spotting decreased hornet recruitment and significantly lowered the likelihood of hornets landing on bee nests and marking them with hornet recruitment pheromone (Mattila *et al.*, 2020). Additionally, *A. cerana* workers can produce antipredator vibroacoustic signals that warn their nestmates of attacks by these hornets (Mattila *et al.*, 2021a). Defending *A. cerana* workers in Kunming, China, could detect *V. velutina* sting alarm pheromone (based on electroantennogram data and bioassays) and responded by forming a defensive heat ball to disable or kill the hornet (Dong *et al.*, 2018). This olfactory eavesdropping provides insight into coevolutionary arms races based on signal interception. However, whether *V. soror* alarm pheromone can elicit a similar response in sympatric *A. cerana* requires first identifying this pheromone.

In Vespidae species, defensive behavior is typically triggered by alarm pheromones. Although the alarm pheromone of *V. soror* was previously unknown, Ono et al., (2003) reported that the alarm compounds of *V. mandarinia* contain venom-derived volatile compounds, including 2-pentanol (2-P), 3-methyl-1-butanol (3-M-1-B), and 1-methylbutyl 3-methylbutanoate (1-M3-MB). *V. soror* and *V. mandarinia* are closely related (Perrard *et al.*, 2013; Mattila *et al.*, 2022), and thus *V. soror* may also employ similar sting alarm pheromones. We therefore chemically analyzed the sting venom volatiles of *V. soror* and used electroantennography and field bioassays to identify some of the principal active components of *V. soror* alarm pheromone.

Materials and methods

Location

V. soror is plentiful from June to October in the southern and southeast regions of Asia. We used insect nets to collect workers from 3 natural *V. soror* nests in Anning, Yunnan, China as they departed their nest entrances (Fig. S3A) on sunny days in September and October 2021. The collected workers (60 hornets total, 20 hornets per nest) were then placed in 3 separate wire mesh cages (15 cm × 15 cm × 15 cm) and provided with 30% (w/w) sugar water in plastic syringes. Cages were placed inside an incubator kept at 25°C with a relative humidity of 60%. Chemical analyses were conducted at the Xinan Center of Biodiversity in Kunming, Yunnan, China.

Chemical identification of alarm pheromone

V. soror is morphologically and behaviorally similar to its better-known sister species, the northern giant hornet *V. mandarinia*. Because *V. mandarinia* alarm pheromone derives from its sting venom volatiles (Ono *et al.*, 2003), we used headspace solid phase micro-extraction (SPME) to determine the chemical composition of volatile compounds from *V. soror* sting venom. We analyzed the venom of 5 *V. soror* workers per colony from 3 colonies (a total of 15 workers). First, a glass tube with 30% (w/w) sugar water was placed through an opening in the wire mesh cage to attract a hornet. After the hornet climbed into the glass tube to feed, we plugged the opening with a stopper and placed it in a freezer (MDF-U5412N, Panasonic) at −40°C for 1 h. The worker was then taken out and its sting was inserted into a capillary tube (1.2 mm inner diameter, 110 mm long) chosen because the tube diameter was slightly larger than the sting diameter. We then thawed out the worker at room temperature and applied gentle manual pressure to cause the sting venom to flow out and be collected by the capillary tube (Fig. S3B). Once the sting venom ceased to flow, we considered it to be fully extracted. We then used a ceramic scribe column cutter (Agilent) to cut down the capillary containing the sting venom, which was immediately placed into a clean 2 mL glass vial sealed with a polytetrafluoroethylene (PTFE) cap containing a 0.2 mm diameter hole. The vial was shaken by hand 10 times to allow the sting venom to flow to the bottom. A blue SPME fiber (65 μm polydimethylsiloxane / divinylbenzene, Supelco, CA, USA) was inserted through the hole in the cap and used to collect volatiles for 1 h. We used an HP 7890A-5975C gas chromatography-mass spectrometry (GC-MS) system to identify the volatile compounds. We utilized a HP-FFAP column (30 m × 320 μm × 0.25 μm, Agilent, USA) with helium as the carrier gas flowing at a rate of 1 mL/min. The injection port was set at 240°C, and pheromone samples were injected in splitless mode into the injection port. The oven of the GC-MS was initially set at 50°C for 2 min, with the temperature rising by 10°C

Table 1 The major volatile components of the sting venom of *Vespa soror*.

Peak	Chemical	Quantity (μg/hornet)	Source	Chemical identifier	Significant differences
1	2-pentanol	11.37 ± 2.68	Meryer, Shanghai	CAS# 6032-29-7	bc
2	3-methyl-1-butanol	68.87 ± 17.44	Meryer, Shanghai	CAS# 123-51-3	a
3	isopentyl acetate	5.51 ± 2.53	Macklin, Shanghai	CAS# 123-92-2	c
4	1-methylbutyl 3-methylbutanoate	16.63 ± 7.97	Kamel, Sichuan	CAS# 117421-34-8	b
5	2-heptanol	10.2 ± 9.14	Macklin, Shanghai	CAS# 543-49-7	bc
6	2-nonanol	9.26 ± 6.25	Macklin, Shanghai	CAS# 628-99-9	c

For quantification, we utilized 15 hornets from 3 colonies (5 hornets per colony) and performed a different gas chromatography – mass spectrometry / gas chromatography analysis for each hornet. We used external standards to measure the quantity of compounds per hornet and list the calculated quantities per hornet. In the "Significant differences" column, different letters indicate significantly different quantities per hornet (Tukey's Honestly Significant Difference test, $P < 0.05$).

per minute until it reached 230°C, where it was held for 10 min. A 70 eV electron impact ionization on source was utilized at 230°C for quadrupole mass spectrometry. The data were analyzed with Chemstation software from Agilent Technologies and with AMDIS from the National Institute of Standards and Technology The compounds were confirmed by comparing retention times and mass spectra to chemical standards (see Table 1 for sources of the standards and Fig. S4 for the mass spectra and chromatograms). For confirmation, 0.1 μL of each chemical standard was added to a clean 2 mL glass vial, and the SPME method and GC-MS settings described above were used (Fig. S4).

Pheromone quantification

We determined the mean mass of venom per hornet by extracting this venom via the capillary method (see above) from 15 different hornet workers. We measured the mass of each hornet before and after the venom extraction and also performed dissections to verify that our extraction method had obtained all the venom from the venom glands.

Separately, with a different set of hornets, we quantified the volatile compounds found in their sting venom with a HP-7890B Gas Chromatograph (GC) from Agilent, USA. We used the method described above to collect venom from *V. soror* workers, but used a standard Agilent autosampler syringe to take 1/10 of the venom collected per hornet for GC analysis. We used a HP-FFAP column (30 m in length, 320 μm in diameter, and 0.25 μm in film thickness, Agilent, USA), with helium serving as the carrier gas flowing at a rate of 1 mL/min. Sting venom samples were injected into an injection port in splitless mode. The outflow from the GC column was then subsequently split into a flame ionization detector (FID) also heated to 240°C for compound identification. We began the GC-MS oven temperature (which contained the column) at 50°C for 2 min and then increased it by 10°C per minute until it reached 230°C and maintained at 230°C for 10 min. To quantify these components, we created diluted solutions of our chemical standards in ethyl acetate at concentrations of 1, 10, 50, and 100 μg/μL. This range includes the high concentrations that were based on the large quantities of compounds identified in the large volume of hornet sting venom (see Results).

We injected 1 μL of each concentration of each chemical standard for GC analysis. External standard curves for the GC-FID were created by plotting the relationships between peak areas and injection volumes. Using these curves, we quantified each venom component by measuring its respective peak area. The sting venom from 15 different workers (1 analysis per worker with 5 workers from each of the 3 colonies) was analyzed. To calculate the total mass of each compound per worker, we multiplied by 10 because we sampled 1/10 of the venom per worker.

Responses of worker antennae to odors

We used coupled gas chromatography-electroantennogram detection (GC-EAD) to identify alarm pheromone components (Wang *et al.*, 2016). We employed an SPME fiber to collect sting venom volatiles from *V. soror* workers (methods in Wang *et al.*, 2016). The SPME fiber was then placed in a splitless injection port at 240°C in an HP-7890B GC (Agilent, USA). A HP-FFAP column (30 m $\times$ 320 μm $\times$ 0.25 μm, Agilent, USA) was used with helium as the carrier gas. The oven temperature was initially set to 50°C for 2 min and then ramped up at a rate of 10°C per minute until it reached

230°C. The GC column's effluent was divided, with 1 portion directed to a FID set at 240°C for compound identification, and the other portion linked to an EAD system through a 40 cm long heated transfer line at 230°C. A HP-34465A digital multimeter (Key Sight) was used to record the responses of the antennae to the stimuli from the GC column. The multimeter was controlled by BenchVue software (Key Sight) running on a PC (Cheng *et al.*, 2017).

To measure antennal responses, we carefully captured a worker hornet from a cage using the glass tube method described above. Initial tests did not reveal any indication of variations in the responses between the left and right antennae. As a result, we chose a left or right antenna at random, cut it at its base, and placed it between glass electrodes filled with insect Ringer's solution. Every antenna was placed at a distance of 1 cm from the opening of a 15 cm long PTFE tube with a 1 cm inner diameter, from which the SPME extracts were delivered. The tube combined air from two sources. Clean air was pumped through a 500 mL glass chamber filled with activated charcoal and combined with humidified air bubbled through a separate 500 mL glass chamber filled with distilled water (90% humidity). These two air streams were combined to create a continuous airflow of 15 mL/s (Cheng *et al.*, 2017). We performed these tests with 9 workers from three different colonies (three workers per colony).

We next examined the antennal responses of workers to six chemical standards and a blend designed to match the proportions of these compounds found in 1 hornet equivalent of natural alarm pheromone. We tested the blank (ethyl acetate) first and then haphazardly selected the presentation order of the test compounds, ensuring that each antenna was exposed to all test compounds. We tested the following chemicals at concentrations equivalent to 1 hornet (Table 1): 2-P (11.4 μg in 1 μL ethyl acetate), 3-M-1-B (68.9 μg in 1 μL ethyl acetate), 1-M3-MB (16.6 μg in 1 μL ethyl acetate), 2-heptanol (2-H) (10.2 μg in 1 μL ethyl acetate), 2-nonanol (2-N) (9.3 μg in 1 μL ethyl acetate), isopentyl acetate (IPA) (5.5 μg in 1 μL ethyl acetate), and a mixture of these compounds (totaling 121.8 μg in 1 μL of ethyl acetate). Each test compound was applied to a paper strip (4 mm × 15 mm) and rapidly placed into a clean odor-delivery pipette (2 mL, 4 mm inner diameter). Each antenna (a total of 18 antennae from 18 different workers, six workers per colony) was tested with all compounds and the synthetic mixture. Each odor presentation lasted for 3 s, with a 40 s recovery time between odor presentations. We measured the baseline-peak amplitude (mV). We performed these tests with 18 workers from three different colonies (six workers per colony).

Field bioassays

Finally, we tested the effects of our hypothesized alarm pheromone components and their 1-hornet equivalent blend on hornet colonies. Due to the sensitivity of *V. soror* to human presence near their nest, we took precautions to avoid inducing alarm behavior during our experiments. Instead of approaching their nest directly, we used a clean, 5 m long bamboo rod to deliver the test compounds, each at the quantity of 1 sting equivalent. Each test compound (2-P, 3-M-1-B, 2-H, 2-N, IPA, 1-M3-MB or their mixture in natural proportions, with ethyl acetate as solvent control) was carefully pipetted onto a clean, new filter paper measuring 5 mm × 20 mm that was attached to the tip of the rod. To maintain a safe distance from the nest, we used a micropipette to withdraw the test compound from a glass vial at a location approximately 20 m from the *V. soror* nest and then used wax to seal the pipette tip. To limit potential evaporation, we then rapidly moved to a location 5 m from the *V. soror* nest, where we pipetted out the test compound onto the filter paper attached to the end of the rod. We then immediately placed the filter paper approximately 30 cm from the hornet colony nest entrance. Each test trial lasted for 2 min. We then waited for 10 min, a period that was sufficient for the alarmed colony to calm down, and repeated this test with the next test compound. We therefore tested 7 different treatments per colony per day. With each of our 3 colonies, we conducted 6 replicates, each conducted on a different day. For each replicate, we pseudorandomly changed the order of the test compounds being presented.

We used a Sony™ HDR-PJ790 camera to video record the nest area for each test compound trial. Previous studies have suggested that the primary function of alarm pheromones is to reinforce individual guarding behavior or induce searching activities at the hive entrance. Based on our observations, alarmed hornets exhibited excited flight patterns around the nest and rushed toward the paper target (Video S2). This behavior matched what we observed when humans, a predator of hornets, approached the nest. To quantify the alarm response, we carefully reviewed the recorded videos and counted the total number of alarmed hornets that landed on the paper target. Video reviewers were blind to the compounds being tested.

Statistics

We used JMP Pro v16 software and employed univariate repeated measures models to analyze our electroantennogram (EAG) data (individual hornet as the unit of

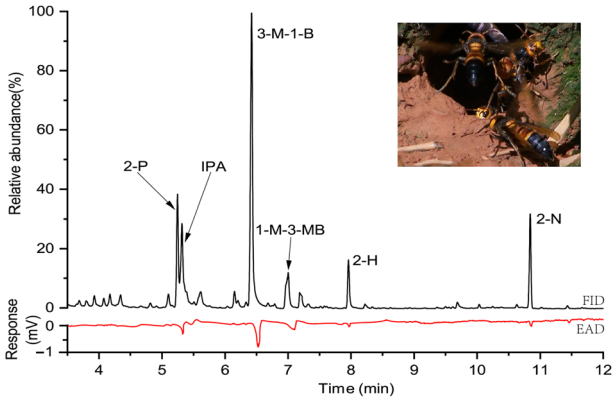

Fig. 1 Typical venom headspace solid phase micro-extraction (SPME) extract of a *Vespa soror* worker (flame ionization detector [FID], upper trace) and its antennal responses to these compounds (electroantennogram detection, lower trace). The inset image shows the entrance of a *V. soror* colony. The 6 major compounds are 2-pentanol (2-P), isopentyl acetate (IPA), 3-methyl-1-butanol (3-M-1-B), 1-methylbutyl 3-methylbutanoate (1-M3-MB), 2-heptanol (2-H), and 2-nonanol (2-N).

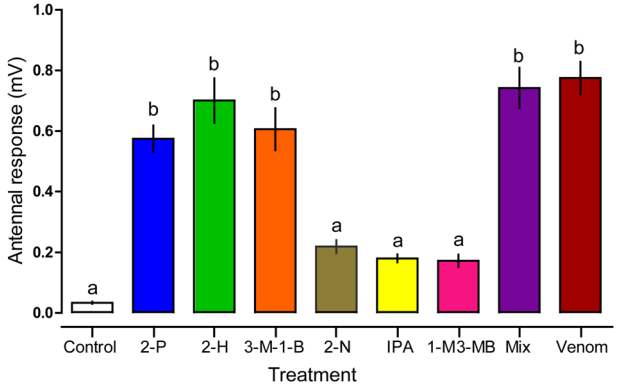

Fig. 2 Worker antennae of *Vespa soror* exhibit different responses to the tested compounds: 2-pentanol (2-P), 2-heptanol (2-H), 3-methyl-1-butanol (3-M-1-B), 2-nonanol (2-N), isopentyl acetate (IPA), 1-methylbutyl 3-methylbutanoate (1-M3-MB), a mixture (2-P, 2-H, 3-M-1-B, 2-N, IPA, and 1-M3-MB in natural proportions, see Table 1), and natural sting venom. All compounds were tested at the average quantities found in 1 hornet equivalent of venom. Different letters indicate significant differences (Tukey's Honestly Significant Difference [HSD] test, $P < 0.05$).

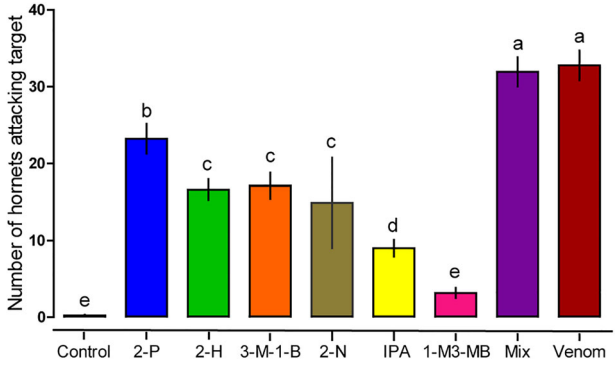

Fig. 3 The number of *Vespa soror* workers attacking a target with the specified test compounds at 1 hornet equivalent: 2-pentanol (2-P), 2-heptanol (2-H), 3-methyl-1-butanol (3-M-1-B), 2-nonanol (2-N), isopentyl acetate (IPA), 1-methylbutyl 3-methylbutanoate (1-M3-MB), a mixture (2-P, 2-H, 3-M-1-B, 2-N, IPA, and 1-M3-MB in natural proportions, see Table 1), and natural sting venom. Different letters indicate significant differences (Tukey's Honestly Significant Difference [HSD] test, $P < 0.05$).

replication) and our bioassay data (colony as the unit of replication). We use Tukey's Honestly Significant Difference (HSD) tests to make all pairwise comparisons corrected for Type I statistical error (false positives) and report mean ± 1 standard deviation.

Results

Each worker produced 20.7 ± 5.5 mg of sting venom ($N = 15$ workers). The main venom components identified were 3-M-1-B, 1-M3-MB, 2-P, 2-H, 2-N, and IPA (see Table 1 for the quantities). These compounds differed significantly in their quantities ($F_{5,70} = 97.18$, Tukey HSD test, $P < 0.0001$), and 3-M-1-B was the most abundant.

By using coupled GC-EAD, we found that all six of these components elicited responses from worker antennae (Fig. 1). We further compared the responses of workers' antennae ($N = 18$, six workers per colony) to pure synthetic compounds (Fig. 2). A significant effect of treatment type was observed ($F_{8,148} = 39.12$, Tukey HSD test, $P < 0.0001$), with 2-P, 3-M-1-B, 2-H, the synthetic mixture of chemicals, and sting venom (all at 1-hornet equivalents) eliciting significantly stronger responses as compared to the control (Tukey HSD test, $P < 0.05$).

In field bioassays, we found a significant effect of treatment ($F_{8,151} = 100.96$, Tukey HSD test, $P < 0.0001$). Except for 1-M3-MB, all individual chemicals elicited significantly stronger responses compared to the control (Tukey HSD test, $P < 0.05$). The mixture of synthetic alarm pheromone compounds and natural sting venom caused the most intense alarm behavior, and the level of alarm elicited by the synthetic mixture and natural sting venom was not significantly different (Fig. 3, Tukey HSD test, $P < 0.05$).

Discussion

The venom of *V. soror* is a major source of its alarm pheromone and consists of at least five active compounds: 2-P, 3-M-1-B, 2-H, 2-N, and IPA. Previous studies have shown that *V. velutina* uses volatile compounds found in its sting venom, specifically heptan-2-one, nonan-2-one, and undecane-2-one, as alarm pheromones (Cheng *et al.*, 2017). In *V. mandarinia*, 2-P has been identified as the primary active alarm component, while 3-M-1-B and 1-M3-MB work synergistically with it (Ono *et al.*, 2003). Research has demonstrated that 3-M-1-B, a component that *V. mandarinia* uses to mark victim *A. cerana* colonies, caused *A. cerana* workers to exit their colony and become more active (McClenaghan *et al.*, 2019).

Our EAG detection results showed that 2-H, 2-P, and 3-M-1-B elicited strong responses, suggesting their importance as the principal alarm components for *V. soror*. However, field bioassays revealed that, in addition to these compounds, 2-N and IPA also play important roles in the alarm behavior of *V. soror*. We note that 2-H, 2-N, and IPA have not been found in other Vespidae species. We found that 3-M-1-B was the most abundant sting venom component and elicited strong hornet antennal responses. Nonetheless, in our field bioassays, 2-P elicited a stronger alarm response than any other individual component, including 3-M-1-B, although it was less abundant (Table 1). This result is consistent with Ono *et al.* (2003) who showed that 2-P is the main active alarm component in *V. mandarinia*. These results therefore contribute to our understanding of the chemistry of sting venom alarm pheromone in *Vespa* species. However, additional studies are needed to determine if other glands also provide components of *V. soror* alarm pheromone. The use of current analytical methods to analyze and reanalyze the alarm pheromones of multiple *Vespa* species could also be revealing.

An intriguing result was the discovery that IPA is a key aggression-eliciting component in *V. soror* alarm pheromone, as it is in all honey bee species in which alarm pheromones have been chemically identified (Wang *et al.*, 2016; Wen *et al.*, 2017). In addition, our results show that *V. soror* and *Apis* species also share 3-M-1-B (multiple honey bee species, Wang *et al.*, 2016) and 2-N (found in *Apis mellifera*) (Allan *et al.*, 1987) in their alarm pheromones. These components elicit different behaviors. Wager and Breed (2000) found that IPA increased defensive flight activity, recruited guard bees, and attracted them to attack a moving target. Although 3-M-1-B is found in *A. mellifera* sting alarm pheromone, it did not elicit any of these effects. In field experiments, Li *et al.* (2014) reported that *Apis dorsata* foragers avoided multiple sting alarm pheromone compounds found in *A. dorsata* sting pheromone, but did not avoid inflorescences that had 3-M-1-B placed on them, even though this compound is found in *A. dorsata* alarm pheromone. Thus, the function of 3-M-1-B in bee sting alarm pheromone is unclear. However, 2-N decreased flight activity and increased the recruitment of *Apis mellifera* guard bees (Wager & Breed, 2000). Collins and Blum (1982) also reported that 2-N elicited strong responses from *A. mellifera* workers in terms of increasing movements, decreasing time to react, the initial intensity of the reactions, the total duration of the reactions, and the number of bees engaged in Nasanov fanning by the end of the bioassay.

The presence in a predator and its prey of the same alarm pheromone components (IPA, 3-M-1-B, and 2-N) is surprising. The use of identical compounds in different species may be coincidental. For example, Johnson *et al.* (1985) discovered that the main alarm pheromone components of the stingless bee, *Trigona silvestriana*, are 2-N and 2-H. The pupae of *A. mellifera* honey bees also produce 2-P, although its function in pupae is unclear (Haber *et al.*, 2019). However, the use of the same compounds in the alarm pheromones of a hornet and its bee prey opens up the possibility of interspecific alarm communication and signal exploitation. For example, when *V. soror* hornets attack *A. cerana* colonies, they are heat-balled by the bees (Mattila *et al.*, 2021a), which likely release bee sting alarm pheromone, while the hornets also likely produce sting alarm pheromone, as demonstrated when *V. velutina* attacks *A. cerana* (Dong *et al.*, 2018). In the defensive honey bee and offensive hornet melee that results, the role of these shared compounds is likely complex. Bees should benefit by eavesdropping on hornet alarm pheromone, as shown in *A. cerana* detecting *V. velutina* sting alarm pheromone (Dong *et al.*, 2018). However, it is unclear how *V. soror* could benefit from alarm components shared with bees. Could they be manipulating their honey bee prey? *V. soror* hornets mark the honey bee colony entrance with compounds that attract other hornets. In turn, honey bees overmarking these odors with animal dung may serve to mask hornet pheromone marks (Mattila *et al.*, 2020). We suggest that this manipulation hypothesis is worth exploring, particularly because it is counterintuitive for a predator and its prey to share components of a chemical signal that likely evolved, separately, for the benefit of each species.

Acknowledgments

This work was supported by the CAS Key Laboratory of Tropical Forest Ecology, the 14th Five-Year

Plan of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (E3ZKFF3B), Innovation Project of Caiyun Postdoctoral Program (E4YN021B06), National Natural Science Foundation of China (No. 32322051) and the Yunnan Revitalization Talents Support Plan (XDYC-QNRC-2023-0566). We are grateful to the Gard Otis and the anonymous reviewer for their suggestions, which have significantly improved this manuscript. Dr. Fei Li (Service center for experimental biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences) assisted with the GC-MS analysis.

Disclosure

The authors declare they have no conflicts of interest with respect to the contents of this article.

Data Availability Statement

The data used in this study is available at https://doi.org/10.5281/zenodo.12789544.

References

Allan, S.A., Slessor, K.N., Winston, M.L. and King, G.G.S. (1987) The influence of age and task specialization on the production and perception of honey bee pheromones. *Journal of Insect Physiology*, 33, 917–922.

Angulo, E., Ballesteros-Mejia, L., Novoa, A., Duboscq-Carra, V.G., Diagne, C. and Courchamp, F. (2021) Economic costs of invasive alien species in Spain. *NeoBiota*, 67, 267–297.

Barbet-Massin, M., Salles, J.M. and Courchamp, F. (2020) The economic cost of control of the invasive yellow-legged Asian hornet. *NeoBiota*, 55, 11–25.

Bass, A., Needham, K. and Bennett, A.M. (2022) First record of *Vespa crabro* Linnaeus (Hymenoptera: Vespidae) in western North America with a review of recorded species of *Vespa* Linnaeus in Canada. *Zootaxa*, 5154, 305–318.

Cheng, Y.N., Wen, P., Dong, S.H., Tan, K. and Nieh, J.C. (2017) Poison and alarm: the Asian hornet *Vespa velutina* uses sting venom volatiles as an alarm pheromone. *Journal of Experimental Biology*, 220, 645–651.

Collins, A.M. and Blum, M.S. (1982) Bioassay of compounds derived from the honeybee sting. *Journal of Chemical Ecology*, 8, 463–470.

Dong, S., Wen, P., Zhang, Q., Wang, Y., Cheng, Y., Tan, K. *et al.* (2018) Olfactory eavesdropping of predator alarm pheromone by sympatric but not allopatric prey. *Animal Behaviour*, 141, 115–125.

Haber, M., Mishyna, M., Martinez, J.J.I. and Benjamin, O. (2019) Edible larvae and pupae of honey bee (*Apis mellifera*): odor and nutritional characterization as a function of diet. *Food Chemistry*, 292, 197–203.

Johnson, L.K., Haynes, L.W., Carlson, M.A., Fortnum, H.A. and Gorgas, D.L. (1985) Alarm substances of the stingless bee, *Trigona silvestriana*. *Journal of Chemical Ecology*, 11, 409–416.

Li, J., Wang, Z., Tan, K., Qu, Y. and Nieh, J.C. (2014) Effects of natural and synthetic alarm pheromone and individual pheromone components on foraging behavior of the giant Asian honey bee, *Apis dorsata*. *Journal of Experimental Biology*, 217, 3512–3518.

Mattila, H.R., Kernen, H.G., Otis, G.W., Nguyen, L.T.P., Pham, H.D., Knight, O.M. *et al.* (2021a) Giant hornet (*Vespa soror*) attacks trigger frenetic antipredator signalling in honeybee (*Apis cerana*) colonies. *Royal Society Open Science*, 8, 211215.

Mattila, H.R., Otis, G.W., Billen, J., Nguyen, L.T.P. and Shimano, S. (2022) Comparison of the external morphology of the sternal glands for hornets in the genus *Vespa*. *Biology*, 11, 245.

Mattila, H.R., Otis, G.W., Nguyen, L.T.P., Pham, H.D., Knight, O.M. and Phan, N.T. (2020) Honey bees (*Apis cerana*) use animal feces as a tool to defend colonies against group attack by giant hornets (*Vespa soror*). *PLoS ONE*, 15, e0242668.

Mattila, H.R., Shimano, S., Otis, G.W., Nguyen, L.T.P., Maul, E.R. and Billen, J. (2021b) Linking the morphology of sternal glands to rubbing behavior by *Vespa soror* (Hymenoptera: Vespidae) workers during recruitment for group predation. *Annals of the Entomological Society of America*, 115, 202–216.

McClenaghan, B., Schlaf, M., Geddes, M., Mazza, J., Pitman, G., McCallum, K. *et al.* (2019) Behavioral responses of honey bees, *Apis cerana* and *Apis mellifera*, to *Vespa mandarinia* marking and alarm pheromones. *Journal of Apicultural Research*, 58, 141–148.

Ono, M., Terabe, H., Hori, H. and Sasaki, M. (2003) Components of giant hornet alarm pheromone. *Nature*, 424, 637–638.

Otis, G.W., Taylor, B.A. and Mattila, H.R. (2023) Invasion potential of hornets (Hymenoptera: Vespidae: *Vespa* spp.). *Frontiers in Insect Science*, 3, 1145158.

Perrard, A., Pickett, K., Villemant, C., Kojima, J.I. and Carpenter, J.M. (2013) Phylogeny of hornets: a total evidence approach (Hymenoptera, Vespidae, Vespinae, *Vespa*). *Journal of Hymenoptera Research*, 32, 1–15.

Requier, F., Rome, Q., Chiron, G., Decante, D., Marion, S., Menard, M. *et al.* (2019) Predation of the invasive Asian hornet affects foraging activity and survival probability of honey bees in Western Europe. *Journal of Pest Science*, 92, 567–578.

Wager, B.R. and Breed, M.D. (2000) Does honey bee sting alarm pheromone give orientation information to defensive

bees? *Annals of the Entomological Society of America*, 93, 1329–1332.

Wang, Z.W., Wen, P., Qu, Y.F., Dong, S.H., Li, J.J., Tan, K. *et al.* (2016) Bees eavesdrop upon informative and persistent signal compounds in alarm pheromones. *Scientific Reports*, 6, 25693.

Wen, P., Cheng, Y.N., Dong, S.H., Wang, Z.W., Tan, K. and Nieh, J.C. (2017) The sex pheromone of a globally invasive honey bee predator, the Asian eusocial hornet, *Vespa velutina*. *Scientific Reports*, 7, 12956.

Wilson Rankin, E.E. (2021) Emerging patterns in social wasp invasions. *Current Opinion in Insect Science*, 46, 72–77.

Zhu, G., Gutierrez Illan, J., Looney, C. and Crowder, D.W. (2020) Assessing the ecological niche and invasion potential of the Asian giant hornet. *Proceedings of the National Academy of Sciences USA*, 117, 24646–24648.

Manuscript received July 22, 2024
Final version received August 16, 2024
Accepted August 20, 2024

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Multiple *Vespa soror* workers that have been recruited to the entrance of an *Apis cerana* colony.

Fig. S2 A *Vespa soror* worker approaches *Apis mellifera* workers near the entrance of their bee colony.

Fig. S3 (A) Entrance of a *Vespa soror* nestbox. A tube was held at the entrance to capture live worker hornets. (B) We used a capillary tube to collect venom from each hornet's sting.

Fig. S4 A chromatogram showing the 6 major peaks in *Vespa soror* worker natural alarm pheromone and a chromatogram of the corresponding synthetic compounds.

Video S1 Multiple *Vespa soror* workers being recruited to the entrance of an *Apis cerana* colony.

Video S2 Typical example of how *Vespa soror* workers behaved when presented with sting pheromone extracts on a filter paper for the field bioassay.

