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Female volatiles as sex attractants in the invasive population of *Vespa velutina nigrithorax*

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ABSTRACT

Due to its huge invasion potential and specialization in honeybee predation, the invasive hornet *Vespa velutina nigrithorax* represents a high-concern species under both an ecological and economical perspective. In light of the development of specific odorant attractants to be used in sustainable control strategies, we carried out both behavioural assays and chemical analyses to investigate the possibility that, in the invasive population of *V. velutina nigrithorax*, reproductive females emit volatile pheromones to attract males, as demonstrated in a Chinese non-invasive population. We focused on the secretions produced by sternal and venom glands; because of the volatility and complexity of their composition, both of them could potentially allow an attraction and a species-specific response, decreasing therefore non-target species by-catches. Results of chemical analyses and behavioural assays showed that venom volatiles, although population-specific, are unlikely candidates as male attractants since they do not differ in composition or in quantity between reproductive females and workers and do not attract males. Conversely, sternal gland secretion differs between female castes for the presence of some ketoacids exclusive of gynes already reported as sex pheromones for the non-invasive subspecies *V. velutina auraria*. Despite such a difference, males are attracted by the sternal gland secretion of both workers and gynes. These results provide a first step to understand the reproductive biology of *V. velutina nigrithorax* in its invasive range and to develop effective and sustainable management strategies for the species.

1. Introduction

Biological invasions are widely recognised as a major component of human-driven global environment change and the second most important threat to biodiversity (DAISIE, 2009). Invasive alien species (IAS) can alter native species communities and the services they provide that support ecosystem function (Chapin et al., 2000; Mooney and Cleland, 2001; Vanbergen et al., 2018) with noticeable consequences on human activities and environmental conservation (Pejchar and Mooney, 2009; Walther et al., 2009; Pyšek and Richardson, 2010; Simberloff et al., 2013).

Social insects are among the most successful invaders worldwide due to their reproductive strategies and social habit (Moller, 1996; McQuillan and Hingston, 1999; Holway et al., 2002; Dafni et al., 2010; Beggs et al., 2011; Lester and Beggs, 2019). One invasive social hymenopteran species, which has driven particular attention in recent years, is the yellow-legged or Asian hornet, *Vespa velutina nigrithorax* Lepeletier (Monceau et al., 2014). This hornet was introduced in Europe from China in 2004, in South West France (Villemant et al., 2006). *Vespa velutina nigrithorax* appears to have a heavy impact on pollinators and beekeeping, being a specialized and efficient predator of bees (Shah and Shah, 1991; Tan et al., 2007; Monceau et al., 2014). Moreover, it represents a potential competitor for the native hornet species *Vespa crabro* Linnaeus, due to their overlapping ecological niches (Monceau et al., 2015; Cini et al., 2018). Finally, the yellow-legged hornet also represents a risk for human health due to its venomous sting and habit

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to nest in urban environment (Choi et al., 2012; Liu et al., 2015). The spread of the yellow-legged hornet across Europe continues and effective management policies sustainable on the long term are still lacking and urgently required (Monceau and Thiéry, 2017). At present, the strategies to face the invasion rely primarily on the ability to find and destroy the nests before the colony size reaches full maturity with the production of new reproductives, but such method has encountered several limitations (Monceau et al., 2014; Monceau and Thiéry, 2017).

Mass trapping with non-specific attractants has also been employed to catch spring queens for population regulation and workers to reduce local predatory pressure at apiaries (Monceau and Thiéry, 2017; Rome et al., 2011a), but the technique it is highly controversial because of the relatively low capture yields and the often considerable side effects on the native entomofauna (Dauphin and Thomas, 2009; Haxaire and Villemant, 2010; Rome et al., 2011b; Monceau et al., 2012, 2014). Moreover, there is a lack of evidence of the efficiency of such method to regulate the yellow-legged hornet (Monceau and Thiéry, 2017).

A more promising route to follow would be a selective trapping strategy (Monceau and Thiéry, 2017), which should target specific life stages of the invader, such as reproductive individuals (i.e. sexuals), using species-specific attractants (i.e. pheromones) (McNeil, 1991; Baker and Heath, 2004). Intensive trapping of sexuals (males and gynes, i.e. reproductive females) via pheromones could hold the potential to locally reduce mating partners, therefore reducing female mating success and, in turn, colony density in the following season.

Sex attractants are good candidates for selective trapping of insect pests (Monceau and Thiéry, 2017), since they are species-specific, active at very low concentrations and in the vast majority they do not harm other species (for a review see Witzgall et al., 2010). Sex attractants often target males, which represent the sex more involved in mate-searching behaviours (Darwin, 1871; Parker, 1978; Andersson and Simmons, 2006). However, the limited knowledge about both the glandular sources and the chemical nature of sex pheromones in social hymenopterans and in particular in social wasps has been limiting the development of strategies for selective trapping of sexuals to control the populations of invasive social wasps (Lester and Beggs, 2019)

Experimental evidence in other social Vespidae show that males are attracted by putative pheromones derived from nests (Ono and Sasaki, 1987), receptive females (Sandeman, 1938; Batra, 1980; Keeping et al., 1986, Reed and Landolt, 1990a,b; Cappa et al., 2013, 2014; Derstine et al., 2017) or also from other males, which have been in contact with receptive females (Thomas, 1960; Ross, 1983; Keeping et al., 1986). The first demonstration of vespine males attracted by female sex pheromones was reported for *Vespula squamosa* Drury in a wind tunnel (Reed and Landolt, 1990b). More recently, both field and lab trials carried out in New Zealand on the invasive *Vespula vulgaris* Linnaeus showed that females release volatiles, albeit unidentified, which attract conspecific males (Brown et al., 2014). Also, in the European hornet, *V. crabro*, males are attracted by caged reproductive gynes or workers treated with gyne extracts, suggesting the presence of sexual attractants (Spiewok et al., 2006). Finally, a sex pheromone has just been discovered for an Asian subspecies of the yellow-legged hornet, *V. velutina auraria*, distributed in the Chinese province of Yunnan (Nguyen et al., 2006; Takeuchi et al., 2017; Wen et al., 2017). Virgin gynes of this subspecies produce, from the sixth intersegmental sternal glands of their abdomens, a mixture of volatile compounds, where 4-oxo-octanoic acid (4-OOA) and 4-oxo-decanoic acid (4-ODA) are active as male-attracting sex pheromone (Wen et al., 2017).

Based on these premises, it seems plausible that also in the invasive population of the subspecies *V. velutina nigrithorax* there could be a similar chemical signal emitted by reproductive females. Nonetheless, it is known that differences in pheromones among different populations often occur (Lanier et al., 1980; Hansson et al., 1990; Rollmann et al., 2000); thus, our work represents the first effort to investigate potential attractants of males in the invasive population of *V. velutina nigrithorax*.

Several lines of indirect evidence suggest that mating in the invasive range of *V. velutina nigrithorax* could happen inside the nest: firstly, the vast majority of future queens are already mated when leaving the nest (Poidatz et al., 2017), secondly, aggregations of courting males have not been observed in the field (Monceau et al., 2014) differently from the Yunnan subspecies (Wen et al., 2017), and finally, the European population shows a high level of diploid males and inbreeding (Darrouzet et al., 2015).

Venom volatiles contained in the venom reservoir of social wasps and hornets could also in principle be suitable candidates for the role of sex attractants (Post and Jeanne, 1983), since hymenopteran sting originated as an ovipositor and venom glands might still contain compounds used as sex-pheromones in wasps; moreover, the blend is usually species-specific (Dani et al., 1998; Bruschini et al., 2006) and, due to their low molecular weight, their range of action could spread far from the source. Although they do not appear to be involved in male attraction in the Yunnan population (Wen et al., 2017), their implication as pheromones, albeit in different contexts (i.e. alarm pheromones; Bruschini et al., 2010), has been recently demonstrated also for *V. velutina* (Cheng et al., 2016; Thiéry et al., 2018) and a synergic role alongside the sternal gland pheromone in mate attraction could be hypothesized.

Here, we assess if also in the invasive population of *V. velutina nigrithorax*, reproductive females release sex pheromones either contained in the sternal gland secretion, as it occurs in the Asian sub-species, or in the venom volatile mixture, as it has been suggested for other Vespidae species. We carried out behavioural tests and chemical analyses on gland secretions on both female phenotypes (i.e. workers and gynes) since males, to obtain successful mating and therefore fitness, should bias their mating attempts toward reproductive gynes. Male biased choice towards the reproductive phenotype has been shown in other social wasps, such as *Polistes*, where it seems to be driven by chemical cues (Cappa et al., 2013; Beani et al., 2014). If putative sternal glands or venom pheromones are responsible for male attraction towards reproductive females, we could expect qualitative and/or quantitative differences in their composition between gynes and workers and a differential male attractiveness by different female castes.

2. Materials and methods

2.1. Sample collection

V. velutina nigrithorax males and females originated from laboratory reared combs belonging to 6 different field colonies located at least 9 km apart. Combs were collected in the Liguria region (Italy) in Autumn 2016 and 2017 and then transferred to the laboratory to be maintained under standard conditions (natural daylight cycle, 25 °C) in closed cages. Newly emerged adults were daily sexed and transferred into separate male and female exclusive glass cages (size 15 × 15 × 15 cm). Hornets were fed *ad libitum* with water and sugar until behavioural bioassays or chemical analyses. This procedure guaranteed for male and female virginity. Males used in behavioural assays were aged between 10 and 20 days post-emergence, in order to have sexually mature males (Poidatz et al., 2017; Cappa et al., 2019). Adult females used to extract venom and sternal gland secretion were older than one week to ensure a full development of their glands (Turillazzi and Bruschini, 2010).

In order to assess female phenotypes (gyne or worker), since body size is largely overlapping between castes in *V. velutina nigrithorax* (Rome et al., 2015; Cappa et al., 2019), both weight and fat storage were considered. The presence of abundant fat bodies is a clear character of reproductive females in temperate social vespids where only, or mainly, gynes overwinter (Strassmann et al., 1984; Markiewicz and O'Donnell, 2001; Toth et al., 2009; Cappa et al., 2013, 2019). Adult females used as source of chemical stimuli in behavioural test and chemical analyses killed by freezing and kept at −20 °C. Frozen specimens were then thawed for a couple of minutes at room temperature,

weighted on a KERN PCB 350-3 scale and then dissected under a Wild M5A stereomicroscope and the presence and abundance of fat bodies was assessed (Monceau et al., 2013, Rome et al., 2015; Cappa et al., 2019). Females of uncertain caste assignment (large females with scarce fat storage or smaller females with fat bodies) were discharged.

2.2. Venom reservoir content collection

Two different pools of venom from gynes (N = 28; 18 from one colony, 10 from a second one) and workers (34 workers (originating from 4 colonies; at least 4 workers per colony) were prepared to be used as stimuli in behavioural assays. Since hornets tend to release and even spray venom when captured, pools were preferred with respect to single specimen extract in order to minimize differences among the presented chemical stimuli. The sting apparatus of each hornet was dissected, the tip of the sting was inserted into a 10 µl capillary tube and the venom reservoir gently squeezed with a glass slide, to force the reservoir content into the capillary (Bruschini et al., 2008). The venom amount was recorded for each individual and then stored in a 250 µl glass conical insert placed in a 2 ml glass vial. Venom vials were kept on ice during extraction and then immediately frozen at −20 °C until analyses. Venom pool volumes were resuspended in dichloromethane (DCM) according to the mean reservoir equivalent for the two castes (ca 1 µl for workers and 0.6 µl for gynes): 30 µl of workers' venom in 270 µl DCM and 17.8 µl of gynes' venom in 280 µl DCM; the total volume of each solution was sufficient for around 30 behavioural trials (see below), each corresponding to one hornet reservoir equivalent.

For the chemical analyses intended to assess possible differences between castes (Bruschini et al. 2008), the venom of each individual (4 workers and 5 gynes coming from two colonies, at least two individuals per colony) was separately stored.

2.3. Sternal gland secretion collection

The secretion of the sternal glands for behavioural assays was collected according procedure used by Wen et al. (2017): for each female we rubbed the sixth intersegmental sternal gland surface, identified as the source of the sex pheromone in the Yunnan *V. velutina* subspecies (Wen et al., 2017), with a pentane-washed 0.3 cm^2 filter paper. Under the stereoscope, the secretion appears as a clearly visible whitish waxy secretion more abundant in gynes than in workers. After collecting the secretion, we dissected each female in order to assess their phenotypes (gyne or worker) as described above. Each filter paper containing the secretion (gynes, N = 23; workers, N = 23, all females came from the same colony) was immediately used as chemical stimulus in the behavioural assays.

For the chemical analyses, we analysed both the secretion of single specimens (3 gynes and 4 workers) and the pooled secretion of four specimens after derivatization of the oxyacids possibly present (Wen et al., 2017). Secretion of sternal glands from single specimens was sampled by gently rubbing on the sixth intersegmental sternal gland surface of frozen specimens (gynes, N = 3; workers, N = 4; all hornets came from the same colony) a 50/30 µm thick Divynilbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) Solid Phase Microextraction Fibre (SPME). Samples containing the pooled secretion of four individuals (pool N = 2 both for workers and for gynes) were prepared by collecting the secretion with the tip of an insect pin and transferring it into 2 ml glass vials kept on ice. Sampling was performed under a Wild M5A stereomicroscope.

2.4. Behavioural assays

We assessed male attraction toward potential sex pheromones by comparing male response towards different odour stimuli. Focal male groups, consisting of five nestmate males, were transferred to a transparent plastic experimental cage (20x13x12 cm). Cages had a round 6 cm diameter steel mesh at the centre of the top roof covered with a 7 cm diameter Petri dish inner plate. After 15 min of habituation, the stimulus was introduced under the plate over the mesh and the trial started. Focal males were presented with different stimuli in three odour attraction experiments, one for venom volatiles, one for sixth intersegmental sternal glands secretion, and one for the synthetic sternal gland pheromone (a mixture of 4-OOA and 4-ODA; Wen et al., 2017), and their behavioural response was evaluated. Control in the different bioassays consisted of blank solvent in the two bioassays testing venom volatiles and synthetic pheromone mixture or no stimulus in the bioassay testing sternal gland secretion.

Trials lasted three minutes, a timeframe also used by Cheng et al. (2016) for testing behavioural effects of venom volatiles on *V. velutina* workers. Male behaviour was recorded by an observer blind to the nature of the presented stimulus, every 30 s, by counting the number of individuals showing the following behaviours: walking, flying, grooming, ventilating or antennating under the stimulus at each check. The level of male antennation under the stimulus in particular was considered as a proxy of male interest towards the perceived stimulus, while the other observed behaviours (walking, flying, grooming, ventilating) were considered as indicators of male activity. After three minutes the stimulus was removed. Trials were performed at an interval of 20 min during the central hours of the day (11 AM–3 PM) when males were more active as occurs in other temperate wasp males (Beani, 1996; Spiewok et al., 2006; *pers. obs.*). Each group was tested in a random order with the different stimuli for each experiment. After each trial, each group of males was transferred to a new cage to avoid biases due to potential odour persistence. The new cage was provided with a sugar cube that was removed from the cage one minute before the starting of the following trial.

In the bioassays testing venom volatiles attraction, focal male groups (N = 26) were presented with one of four stimuli: a blank control (0.5 cm^2 pentane-washed filter paper), a solvent control dichloromethane (10 µl) absorbed on a 0.5 cm^2 pentane-washed filter paper, the venom reservoir content of workers (1 worker average reservoir equivalent, ca 1 µl), and the venom reservoir content of gynes (1 gyne average reservoir equivalent ca 0.6 µl each). Both venom stimuli were suspended into 10 µl of dichloromethane and absorbed on a 0.5 cm^2 pentane-washed filter paper.

For the sternal gland secretion bioassay, we adopted a simplified protocol since we aimed to confirm its putative role as sex attractant source previously demonstrated in the Yunnan *V. velutina* population (Wen et al., 2017). Thus, we presented focal groups of males (N = 23) with the sixth intersegmental sternal gland secretion of a gyne (N = 23) or a worker (N = 23) on a 0.3 cm^2 pentane-washed filter paper (for the secretion collection see above) and a clean pentane-washed 0.3 cm^2 filter paper as control. The three stimuli (gyne secretion, worker secretion and control) were sequentially presented in a random order.

Finally, to test the synthetic pheromone attractiveness, we diluted in dichloromethane a mixture of 4-OOA and 4-ODA in the same ratio of 0.78 used by Wen et al. (2017) in their field bioassays to reach the concentration of 14 µg/µl. Focal groups of males (N = 18) were presented with 2 µl of the diluted mixture corresponding to a hornet equivalent (28 µg of synthetic pheromone mixture, Wen et al. 2017) on a 0.3 cm^2 pentane-washed filter paper, and 2 µl of the dichloromethane solvent as control. Behavioural data were analyzed using PAST 3.20 (Hammer et al., 2001). Differences in observed behaviours in response to different stimuli were compared with Friedman test for in the two bioassays testing venom volatiles and sternal gland secretion and with Wilcoxon test in the bioassay testing male response to the synthetic pheromone mixture. Post hoc tests (Wilcoxon signed-ranks tests) were used to assess whether, and where, a significant difference existed between pairs of treatments, using the Bonferroni correction for multiple comparisons with a P value of less than α/number of comparisons considered significant. Different males were used in the different bioassays testing venom volatiles, sternal gland secretion and synthetic

pheromone mixture.

2.5. Chemical analyses

2.5.1. Venom volatiles

Volatile components of venom from individual hornets (5 gynes and 4 workers, see above) were sampled by means of headspace solid phase micro-extraction (HS-SPME) (Moneti et al., 1997) and analysed by gas chromatography–mass spectrometry (GC–MS). The headspace in the vials containing the venom of each specimen was sampled for 10 min at 45 °C in a block heater using a fibre coated with a 100 μm polydimethylsiloxane (PDMS) phase (Supelco, Inc., Bellefonte, PA, USA). The HS-SPME is a selective extraction method and the use of a non-polar extraction phase (PDMS) may underestimate the presence or abundance of more polar compounds; however, preliminary trials with a more polar phase (polyacrilate, 85 μm), did not give substantially different chromatographic profiles. Analytes were then desorbed in the injection port of a Hewlett Packard (Palo Alto, CA, USA) 5890 gas chromatograph connected to a Hewlett Packard 5971 quadrupole mass spectrometer operating in electronic ionization (EI) at 70 eV. A Rtx-5MS column (Restek, Bellefonte, PA, USA) was used (30 m, 0.25 mm, 0.5 μm film thickness). The injection port temperature was set at 250 °C and the transfer line at 280 °C; carrier gas was helium (at 12 psi head pressure). The oven temperature was initially set at 45 °C, maintained for 3 min and then increased by 20 °C/min to 200 °C. This temperature was maintained for 14.25 min. Mass spectra were compared with mass spectral electronic libraries (Wiley 275, NIST 2.0), and if not reported in the electronic libraries, they were searched in Pherobase (online Database of Insect Pheromones and Semiochemicals, www.pherobase.com) and in literature through chemical databases (Beilstein Commander 2000; Scifinder Scholar 2004). A mixture of n-alkanes ranging from C9 to C20 was injected under the same conditions in order to calculate the linear retention index of the analytes. Based on the mass spectrum, one peak was putatively identified as citronellyl acetate, a compound previously undescribed for the species. To confirm the identification, citronellyl acetate was chemically synthesized, sampled in HS-SPME and analysed in GC–MS alongside another two samples consisting of the venom volatiles pooled from three *V. velutina* hornets and the venom from the reservoir of a single individual.

For each venom total ion chromatogram (TIC), the peak area of each compound was expressed as a percentage of the total area given by all the compounds present. As some compounds were represented in only a few samples, we only considered compounds if they met the three following criteria: relative abundance within individual profile greater than 1%, the compound was present in at least half of the samples of gynes or workers, the compound was present in at least one third of all samples. The compounds selected according to these criteria accounted on average for 94.2 and 95.4% of the total amount of compounds at the individual level.

To evaluate overall differences in relative composition of volatiles profile between phenotypes we performed a non-metric multidimensional scaling (hereafter nMDS) using all selected compounds and Euclidean distances. We also performed K-means clustering to evaluate the possibility to identify two clusters corresponding to the two phenotypes. This descriptive approach was confirmed by statistically comparing within-phenotype (for both gynes and workers) and between-phenotype chemical distances (calculated as Euclidean distances) with a Kruskal Wallis test. To understand if any of the compounds was differentially expressed between the two phenotypes we compared relative amount of each single compound between the two phenotypes with a Mann Whitney test. Chemical data were analyzed using SPSS 20.0 (IBM, 2011).

2.5.2. Synthesis

Citronellyl acetate was prepared through Fisher esterification by reacting citronellol and acetic acid in the presence of sulfuric acid sulfonic acid at 60 °C for 24 h. Reaction mixture was injected on the same instrument reported above and the spectrum of the obtained ester was successfully compared with those reported in spectral libraries for citronellyl acetate. Retention time and spectrum were also compared with those of the compound putatively identified as citronellyl acetate in the venom analysed under the same conditions.

2.5.3. Sternal gland secretion

SPME fibers used to sample the secretion from single specimens were desorbed for three minutes in the injection port (250 °C) of a 7820 GC-5977B MSD Agilent Technologies instrument. One μl of a 700 ng/μl dichloromethane solution containing the two synthetic ketoacids (4-OOA and 4-ODA, 1:2) identified as putative sex pheromones in the sternal gland secretion (Wen et al. 2017) was injected as reference standards.

To improve chromatography of the several fatty acids observed in the single specimen analysis, samples prepared from the secretion of more hornets, were silanized and the results compared with the silanized standards of 4-oxo-octanoic and 4-oxo-decanoic acids. Twenty μl of a 99:1 solution of N-methyl-N-(trimethylsilyl)trifluoroacetamide and chlorotrimethylsilane were added to the sample vials that were maintained at 60 °C for 30 min. One μl of each derivatized sample was analysed on the same instrument as described above.

Ten μl of the standard solution of ketoacids, were inserted into a 2 ml vials and derivatized as for sternal gland secretion, after solvent was evaporated under a gentle nitrogen stream. In all the analyses the injection port was set at 280 °C. Oven was equipped with an Agilent Technologies 19091S-433UI capillary column (30 m × 0.250 mm, 0.25 μm phase thickness, 95% polydimethylsiloxane, 5% phenyl); helium was used as carrier gas (1 ml/min). The oven temperature ran from 45 °C (maintained for 2 min) to 150 °C at 7 °C/min and then raised to 310 °C at 15 °C/min; final temperature was maintained for 5.33 min. Masses were acquired in the range from 50 to 550 *m/z*. SPME samples were analysed splitless, while liquid samples were analysed in split mode (1:10).

3. Results

3.1. Behavioural assays

Antennation score was not different among treatments in bioassays testing venom volatiles (Friedman test, chi-square = 0.083, p = 0.951, N = 26 replicates for treatments) (Fig. 1a). The possibility that low antennation score were due to male inactivity can be discarded, as males were equally active among different trials (Friedman test, chi-square = 3.473, d.f.3, p = 0.310, N = 26 replicates for treatments) and were moving in the cages (on average 2.66 ± 0.23 out of five focal males in each group were seen walking or flying every observation check).

Antennation scores differed among treatments in bioassays testing sternal gland secretions (Friedman test, chi-square = 5.152, df = 2, p = 0.021, N = 23) (Fig. 1b). Males showed a higher interest towards the sternal gland secretion of reproductive gynes with respect to blank controls (gynes vs control: Wilcoxon post-hoc comparisons, W = 114, N = 23, p = 0.001, Bonferroni-adjusted α = 0.017). Males appeared to respond also to the secretion of workers since there was no difference in the antennation rate towards the sternal gland secretion of the two castes (Wilcoxon post-hoc comparisons, W = 83 N = 23, p = 0.453). However, the male interest towards the secretion of workers was somehow lower compared to the gynes' secretion since the difference in antennation rate between workers' secretion and controls was not significant after the Bonferroni correction (Wilcoxon post-hoc comparisons, W = 67,5, N = 23, p = 0.023, Bonferroni-adjusted α = 0.017) (Fig. 1b). In the trials, males did not show a different rate of activity (i.e. number of males walking, flying, ventilating or grooming at each check) (Friedman test, chi-square = 3.413, df = 2, p = 0.148, N = 23)

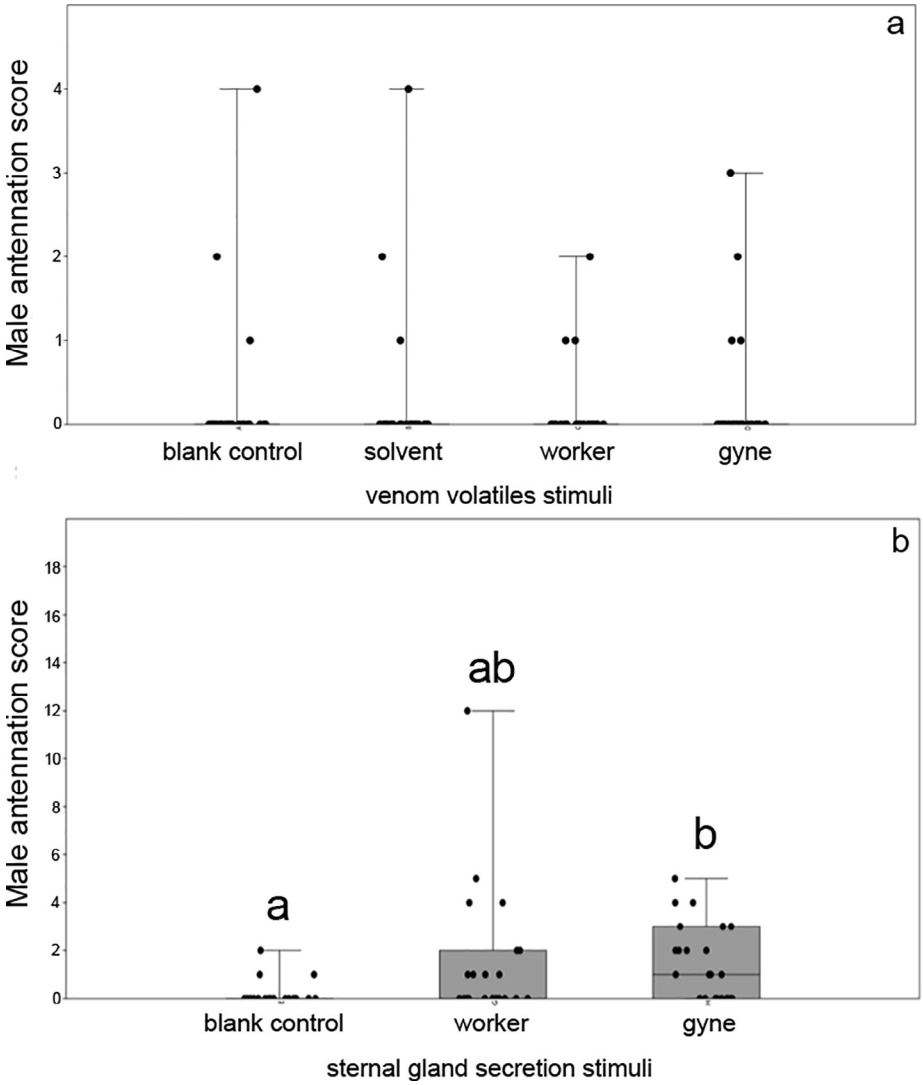

Fig. 1. Behavioural results from bioassays testing the antennation rate of male Vespa velutina nigrithorax presented with venom volatiles (a) and sternal gland secretions (b) extracted from conspecific females.

and there was no difference in the number of males in proximity of the stimulus (i.e. males on the 6 cm diameter steel mesh underneath the stimulus) at every check (Friedman test, chi-square = 3.283, df = 2, p = 0.156, N = 23), which means that despite being active and in the stimulus proximity, only the secretion elicited a higher antennation response in males.

In the trials testing the synthetic pheromone attractiveness there were no differences either in the antennation score (Wilcoxon test, W = 4, N = 18, p = 0.564) or in the male activity rate (Wilcoxon test, W = 97,5, N = 18, p = 0.335) and in the stimulus proximity (Wilcoxon Signed-Rank test, W = 64, N = 18, p = 0.484) compared to the solvent control. The only difference observed was in the rate of male ventilation, which increased when the synthetic pheromone mixture was presented compared to controls (Wilcoxon test, W = 96.5, N = 18, p = 0.037).

3.2. Venom volatiles differences between castes

Twelve major peaks were found in the chromatograms of venom volatiles (Fig. 2). The mass spectra of 5 peaks (1, 2, 3, 5, 9) fitted with aliphatic 2-ketones, according to the presence of a strong ion at *m/z* 58 expected on the base of McLafferty rearrangement. Peaks 2 and 9 corresponded respectively to 2-nonanone and 2-undecanone. Peak 4 was an unidentified compound whose molecular weight matches with the formula $C_{10}H_{20}O$. The spectrum of peak 1 corresponded to 8-nonen-2-one, which coeluted with an unidentified compound. Peak 5 was identified as 4,8-dimethylnon-7-en-2-one. All these compounds have already been reported by Cheng et al. (2016) and Thiéry et al. (2018) respectively for the native and French invasive populations. Spectra of peaks 6, 8, 10 and 11 corresponded to acetates of aliphatic alcohols, which, on the contrary have not been previously reported. On the basis of mass spectrum, retention index and comparison with a standard, peak 10 was identified as citronellyl acetate; surprisingly, this peak, which is the second for intensity in our chromatograms, has not been reported before. Some samples also presented geranyl acetate. Only peaks 2, 5, 7, 8, 9, 10, 12 were considered in the comparison between the worker and the gyne secretion.

Chemical composition of venom volatiles did not differ between workers and gynes (Fig. 2). No compound was found to be exclusively present in one caste. nMDS showed that chemical composition of the two castes is clearly overlapping (nMDS, Euclidean distances, stress = 0.0, r2: axe 1:0.966; axe 2:0.050). K-mean identified two clusters, which were not correspondent to the two phenotypes, misclassifying more than half of the individuals (uncorrect classification: gynes = 60%, workers = 50%). Chemical distance within group (worker-worker or gyne-gyne) was not different that chemical distance

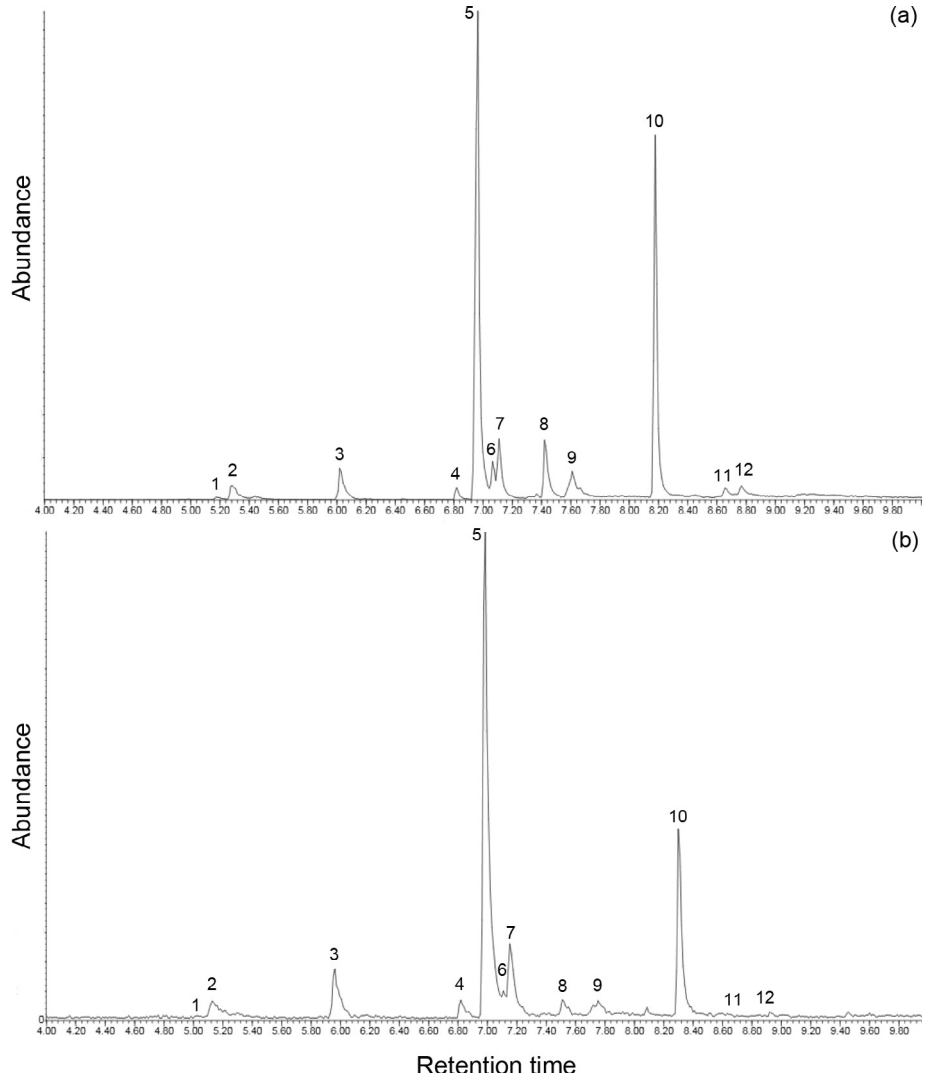

Fig. 2. Total ion chromatogram of venom volatiles extracted from *Vespa velutina nigrithorax* gynes (a) and workers (b). Numbers correspond to compounds quoted in the text.

between groups (worker-gyne) (Euclidean Distances, Kruskal Wallis test, chi-square = 0.914, p = 0.633 $N_1 = 6$, $N_2 = 10$, $N_3 = 20$). Lack of difference was also confirmed at the level of individual peaks: none of the 11 major peaks of the venom volatiles profile was differently expressed between the two castes.

3.3. Sternal glands differences between castes

Sternal gland secretion was found to differ between gynes and workers for the presence of 4-OOA and 4-ODA, which gave broad peaks in the total ion chromatograms of the gynes, similarly to the results obtained in the non-invasive Chinese subspecies (Wen et al., 2017), but absent in the workers. In all samples, broad peaks corresponding to long-chained fatty acids and several peaks given by cuticular hydrocarbons (CHCs) were also observed; the main hydrocarbons peaks corresponding to alkenes and linear and methyl-branched alkanes containing 25–29 carbon atoms. Silanization of the standard compounds and of the samples confirmed that 4-OOA and 4-ODA were present in gynes but not in workers (Fig. 3). Traces of 4-oxo-hexanoic acids were also observed in both gynes and workers.

4. Discussion

Our results show that, at least in laboratory bioassays, males respond to the sternal glands secretion of female hornets, corroborating the hypothesis that they release male-attracting compounds as in the different Asian sub-species *V. velutina auraria* (Ono and Sasaki, 1987; Wen et al., 2017). However only a non-specific response (increased rate of male ventilation behaviour) was recorded when the synthetic pheromone mixture used by Wen et al. (2017) in their field bioassays was presented. Conversely, no sign of male interest was observed in the trials testing the effect of venom volatiles.

We showed that the venom volatile blend did not differ between female castes. The comparison of our results with those reported for different populations of the same species has shown that our venom volatile samples mainly contained ketones, already reported for *V. velutina* both in its native (Cheng et al., 2016) and invasive range (Thiéry et al., 2018) and known for *Vespa orientalis* Linnaeus where they elicit the alarm behaviour (Saslavasky et al., 1973).

Interestingly our samples also contained some previously unreported compounds, mainly aliphatic acetates; in particular we found that citronellyl acetate, known for *V. crabro* (Wheeler et al., 1983), was among the most abundant compounds.

Sexual attraction via putative sex pheromones has been found in many social Vespidae species (Sandeman, 1938; Thomas, 1960; Batra, 1980; Ross, 1983; Keeping et al., 1986; Ono and Sasaki, 1987; Reed and Landolt, 1990a,b; Spiewok et al., 2006; Brown et al., 2014; Derstine et al., 2017), but, so far, the only identified volatile molecules active as

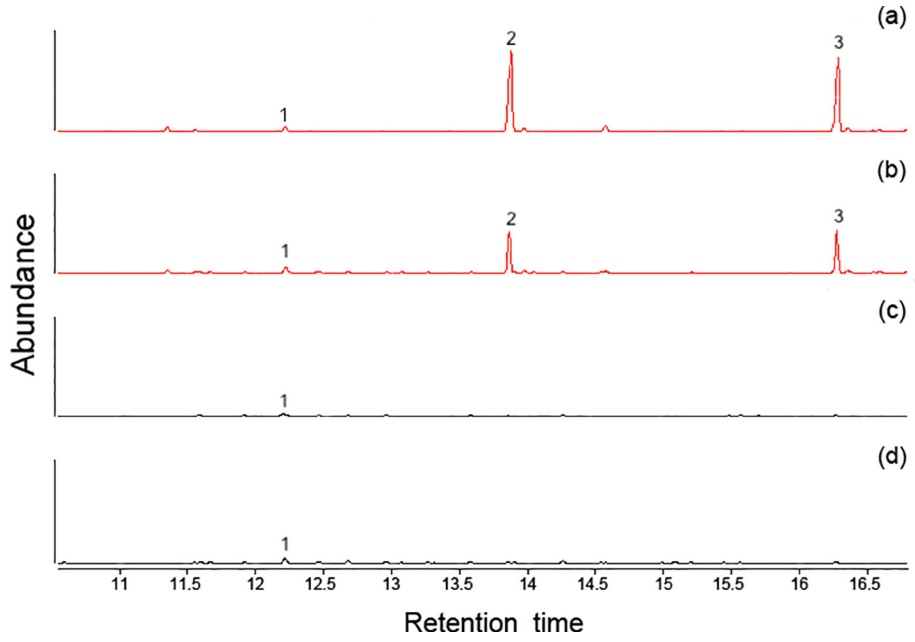

Fig. 3. Total ion chromatogram of sternal gland secretion extracted from *Vespa velutina nigrithorax* gynes and workers after silanization. Peaks 1, 2 and 3 correspond to trimethylsilyl esters of 4-oxo-hexanoic acid, 4-oxo-octanoic acid, and 4-oxo-decanoic acid, respectively.

sex pheromone are the two oxyacids (4-oxo-octanoic acid and 4-oxo-decanoic acid) in the sternal gland secretion of virgin gynes of *V. velutina auraria* (Wen et al., 2017). Our behavioural trials testing such secretion suggest the presence of male attractants also in the invasive population of *V. velutina nigrithorax*. Males clearly responded to the secretion of reproductive gynes, while their response towards the secretion of workers was less evident, not significantly differing from that of controls. The male response to the workers' secretion, albeit to a lesser degree and despite the absence of 4-OOA and 4-ODA, may be due to the fact that other compounds, mainly fatty acids and cuticular hydrocarbons (CHCs), also present in the secretion, could exert an effect on males at the short distance tested in our bioassays. Males, however, should be able to discriminate between reproductive gynes and sterile workers to avoid mating with a partner that will not guarantee a return in terms of fitness (O'Donnell, 1998) as recently demonstrated for *V. velutina nigrithorax* males, more attracted to larger females with more abundant fat storages, reliable indicators of reproductive caste (Cappa et al., 2019). To carry out such a discrimination between gynes and workers, males could rely on compositional differences in the sternal gland secretion (i.e. presence of 4-OOA and 4-ODA) and on the different behaviour of females of the two castes. In fact, only virgin gynes leave their nests to attract males at specific sites in the field in the non-invasive sub-species *V. velutina auraria* (Wen et al., 2017), therefore, mature males in search of mates could be actively enticed by gynes emitting the sex pheromone away from the nest. Once encountering a potential partner, males could also use short-range chemical cues, such as CHCs to discriminate between female castes (Ferveur, 2005; Gévar et al., 2017).

On the other hand, based on the very low genetic diversity and high level of male diploidy as a consequence of inbreeding (Cook, 1993) inside *V. velutina nigrithorax* colonies in the European invasive range (Monceau et al., 2014; Darrouzet et al., 2015; Granato et al., 2019), it has been proposed that males could also mate inside the nests with their relative gynes. In this case, they would not need long-range attractants to find their mates and may rely only on CHCs as chemical cues to identify receptive females. Such a hypothesis seems corroborated by the fact that: (i) males did not show any preference for non-nestmate receptive females over nestmate ones (Cappa et al., 2019); (ii) CHCs of invasive *V. velutina nigrithorax* differ according to caste and gender (Gévar et al., 2017). Such evidences may appear in contrast with the results of the present study suggesting the presence of a sex pheromone-based mate attraction system similar to that described in the Yunnan sub-species (Wen et al., 2017). However, mate search might likely consist of a first step of long-distance male attraction via sex pheromone emitted by gynes, followed by a female caste assessment by males based on short-range chemical cues (CHCs). Furthermore, we could also hypothesize a certain degree of plasticity in the species nuptial system. In the new environment of the invasive range, both receptive females and males may adopt a different strategy compared to native populations or close-related non-invasive sub-species, and mate inside the nest instead of leaving it in search of a potential partner.

Finally, the lack of male oriented-attraction towards the synthetic pheromone mixture may be due to some constraints of different experimental set-up we adopted with respect to the bioassays carried out by Wen et al. (2017) in the Yunnan sub-species. Our bioassays were carried out under laboratory conditions and this might have influenced the sexual behaviour of tested individuals, which might require further triggers that we were not able to reproduce in the laboratory. If this was the case, however, we might have expected a similar responsiveness (or not responsiveness) to both the extracted pheromone in the form of the female sixth intersegmental sternal gland secretion and synthetic pheromone. An alternative explanation is that, even though used in a concentration comparable to a hornet equivalent, the volatile synthetic mixture could quickly saturate the cage space inducing only an apparently unspecific non-directional response in the form of an increased rate of male ventilation. Further research should aim at investigating the actual attractiveness of both the extracted and the synthetic pheromones in the field to evaluate if also in the invasive range receptive females and males of the yellow-legged hornet congregate at specific sites away from the nests or if they just use short-range cues (CHCs) to find a suitable mate inside their natal nests.

Despite the potential influence of laboratory setting, we believe that our results provide a first step to understand the reproductive biology of *V. velutina nigrithorax* in its invasive range and to develop effective and sustainable management strategies for the species.

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Authors contributions

FC, AC, IP and RC conceived and designed the research. FC, AC, IP, IP, AFI and RC collected the data. FC, AC and FRD analysed the data. FC and AC wrote the manuscript. LB, GA, PW and RC provided material, facilities and reagents. All authors read and approved the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2019.103952.

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