Entomologia Generalis, Vol. 38 (2018), Issue 2, 145–156 **Article**
Published in print December 2018

An alarm pheromone in the venom gland of *Vespa velutina*: evidence revisited from the european invasive population

Denis Thiéry[1,*], Olivier Bonnard[1], Laurent Riquier[2], Gilles de Revel[2] and Karine Monceau[3]

[1] UMR INRA 1065 Santé et Agroécologie du Vignoble, INRA, ISVV, BSA, F-33883 Villenave d'Ornon, France
[2] USC 1366 Œnologie, ISVV, Université de Bordeaux, INRA, F-33883 Villenave d'Ornon, France
[3] UMR CNRS 7372 Centre d'Etudes Biologiques de Chizé, Université de la Rochelle, 79360 Villiers-en-bois, France
* Corresponding author: denis.thiery@inra.fr

With 1 figure and 3 tables

Abstract: Alarm pheromones are major communication signals in animals and major semiochemicals in the colony organisation of social insects. We investigated the composition of venom in *Vespa velutina*, an invasive hornet species accidentally introduced into Europe a decade ago. Crushed venom glands were applied to seven wild *V. velutina* nests and induced aggressive responses of workers in all the colonies tested. Then, solvent extracts or headspace Solid Phase Micro Extraction (SPME) were analysed and quantified by gas chromatography coupled with either flame ionization detector or a mass spectrometer, and checked retention times to Kovats indices and Linear retention indices (LRI). We compared our results to those obtained in the same species from its native area in a previous study. Nonan-2-one and the 4, 8-dimethylnon-7-en-2-one were found in large amount in the venom gland. Five other molecules including the heptan-2-one, non-8-en-2-one, undecan-2-one and two unknown molecules were also found. Similar compounds were globally found, however, one unknown compound was present in our European population but absent in native area ones and should be identify. Although pheromones are thought to be species-specific, our results suggest that their composition may vary within species due to population history, thus questioning its value as a taxonomic tool. Although our results are preliminary, we also recommend carefully considering the origin of the *Vespa velutina* individuals chosen to develop pheromone-based methods (attractants or mating disruption) for pest management.

Keywords: biological invasion, chemical communication, intraspecific variability, pheromone, Vespidae

20181213-092554
C7285/16634/936351CC

© 2018 E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, Germany www.schweizerbart.de
DOI: 10.1127/entomologia/2018/0719 0171-8177/18/0719 $ 3.00

1 Introduction

Alarm pheromones are major communication signals in animals (Wertheim et al. 2005), and major semiochemicals in the colony organization of social insects (e.g. wasps, bees, ants). They have long been studied (Blum 1969, Shorey 1973, Pasteels et al. 1983, Hölldobler & Wilson 2008, Bruschini et al. 2010) as they often trigger aggressive and defence behaviours to ensure colony survival and are made of a cocktail of molecules that elicit the different behaviours (recruitment, dispersal, aggregation, aggressiveness) (Maschwitz 1964, Billen & Morgan 1998, Alaux et al. 2010). In European honeybees for instance, isopentylacetate (IPA) is the major active molecule in an alarm blend involving several other molecules that also cause honeybee recruitment for defence (Breed et al. 2004). To maximise the transmission of the signal, alarm pheromones should be highly volatile to (i) quickly recruit nestmates to defend the colony and (ii) have a limited time persistence to avoid detrimental long-lasting reaction. Most importantly, by definition pheromones are species-specific, even if alarm ones may concern several species like in aphids (Guerrieri & Digilio 2008), they can thus be used as taxonomic tool to discriminate species including for example the volatile compound of venom gland in Aculeata (Bruschini et al. 2007).

Vespidae are part of the Aculeata and alarm pheromone has been studied in several species including *Polistes* sp. (*P. dominula* Christ., *P. gallicus* L., *P. nimphus* Christ., *P. sulcifer* Zimmer.: Bruschini et al. 2007, Bruschini & Cervo 2011), *Vespula* sp. (the southern yellow jacket *V. squamosal* Drur.: Landolt & Heath 1987, Heath & Landolt 1988, Landolt et al. 1999), *Polybia* sp. (*P. occidentalis* Olivier: Jeanne 1981, London & Jeanne 1996), *Dolichovespula* sp. (*D. maculata* L., *D. saxonica* Fab.: Maschwitz 1984, Jimenez et al. 2016), *Mischocyttarus* sp. (*M. immarginatus* Rich.: London & Jeanne 1996) and *Vespa* sp. (*V. crabro* L., *V. mandarinia* Smith, *V. orientalis* L., *V. velutina* Lepeletier: Saslavasky et al. 1973, Veith et al. 1984, Ono et al. 2003, Cheng et al. 2017). Identifying molecules that are responsible for aggressive insect reactions is, in some cases, of major interest especially in food flavouring or cosmetics. Unexpected attacks on human have been previously reported by the giant Japanese hornet *Vespa mandarinia* (Ono et al. 2003). Such molecules can also be used for push-pull pest management strategies (Cook et al. 2007). Therefore, identifying alarm pheromones, especially in social insect pests, is of major concern.

The Asian yellow-legged hornet, *Vespa velutina*, is an invasive hornet species accidentally introduced into Europe via container boat shipment followed by truck transport close to Agen (South West France) in or just before 2004 (Arca et al. 2015). From its introduction site in South West France, it has spread through mainland and has now reached Spain (López et al. 2011), Portugal (Grosso-Silva & Maia 2012), Germany (Witt 2015), Italy (Bertolino et al. 2016), Mallorca (Leza et al. 2018), and more recently Alderney and Jersey, and United Kingdom (Budge et al. 2017, Kennedy et al. 2018). The last invaded countries are Belgium, a nest being destroyed at ca. 5 km from the French border, and Switzerland where an individual was seen near the border (west of Geneva). This hornet species is mostly known for its damage on honeybee hives as it feeds its larvae in preying on honey-

20181213-092554
C7285/16634/936351CC

bees. In the current pollinator decline (Goulson et al. 2015), *V. velutina* is an important additional stressor for bee colonies and has been recognized by the European Union as harmful, being listed among the 37 invasive species of major concern (EU Regulation 1143/2014).

Recently, the first evidence for an alarm pheromone and the composition of volatile molecules from venom gland were published by Cheng et al. (2017). Their study was undertaken in the native range of *V. velutina*. We realized a similar study to identify volatile venom compounds from the invasive range to compare with the populations from native area. The effect of these volatile compounds was tested on seven different *V. velutina* colonies reared in laboratory to describe the behavioural reaction elicited. Then, the composition of the volatile part of the venom gland was characterized by gas chromatography–mass spectrometry (GC-MS) and the quantification of the major compounds realized by Gas Chromatography with Flame Ionization Detector (GC-FID).

2 Materials and methods

2.1 Nest collection, caging and rearing

Seven nests of *V. velutina* were collected at different periods and from different locations (see Table 1 for details and nest abbreviation) and then placed into laboratory rearing cages following Monceau et al. (2013) who studied the nest defence behaviour in this species. Briefly, the nests were removed either early in the morning or in the evening. Once in the laboratory, the nests were placed at 4° C for 2 h to anesthetize the hornets and then transfer to the cage within 5 minutes at room temperature. Hornets

Table 1. Characteristics of the nests involved in the experiment.

Nest ID	FL	ST	PO	LP	MA	SA	PE
Location	Floirac	St Symphorien	Pompignac	Le Pout	Martillac	Sanguinet	Pessac
GPS coordinate	-	-	44°51’ 23.61”N 0°25’ 44.63”W	44°48’ 28.7”N 0°21’ 39.9”W	44°43’ 35.7”N 0°33’ 07.7”W	44°28’ 50.8”N 1°04’ 55.5”W	44°47’ 41.56”N 0°38’ 13.51”W
Date of caging	22/08/ 2012	23/05/ 2013	02/07/ 2013	19/06/ 2014	23/07/ 2014	07/08/ 2014	15/06/ 2016
At caging							
Number of queen	1	1	1	1	1	1	1
Number of workers	36	7	5	15	> 30	> 30	> 30
Number of males	11	0	1	0	0	0	0
Eggs and larvae	yes	yes	yes	yes	yes	yes	yes

20181213-092554
C7285/16634/936351CC

were fed with honey or blackcurrant syrup as sources of carbohydrates for adults and commercial wet cat food (11% proteins, 78% water) for larvae. Food and water were provided ad libitum at the bottom of the cage in a mobile drawer that could be closed by a stainless steel plate to securely manipulate the delivery of food, water and sample odours.

2.2 Bioassay design

The effect of venom volatile compounds was tested by exposing the hornet colonies to crushed venom glands directly in the cage placed at 21° C and red light similar to Couto et al. (2014). For each assay, two Petri dishes (90 mm diameter) were exposed to the hornets: i) a control with only paper filter (Whatman™, Grade 1, 70 mm diameter) and ii) paper filter with 10 crushed venom glands, placed in the mobile drawer. To obtain venom glands for each assay, *V. velutina* workers were captured in our experimental apiary located at INRA Bordeaux-Aquitaine, France (GPS: N 44° 47927.050 W 0° 34938.350) and thus originated from different colonies as those tested to avoid a potential effect of relatedness. These hornets were stored at −20° C until dissection. Immediately before experiment, ten venom glands from ten hornets were removed and freshly placed on a filter paper within the Petri dish and gently crushed with a glass stirrer previously washed with pure hexane. The Petri dish was immediately sealed and placed inside the mobile drawer of the cage with the control sample. Before closing and locking the drawer, the top of the Petri dishes was removed, the drawer was sealed and the stainless steel plate removed to deliver the samples. Each assay lasted five minutes (beginning when the steel plate was removed) and the observations made by a single experimenter (OB). The number of visits on each Petri dish as well as the time to the first visit on the venom sample was recorded. For FL, ST, and PO nests (see Table 1), eight, five and three replicates respectively of the experiment were realized over 15, 10 and three days respectively. For LP, MA, SA and PE nests (see Table 1), the experiment was performed once.

2.3 Pheromone analyses

2.3.1 Extractions

Two different types of extraction were performed: i) a solid-phase microextraction (SPME) was undertake prior to chromatography and mass spectrometry analysis and ii) a solvent extraction of whole glands for a more accurate molecule quantification confirmation of SPME chromatograms. Extractions and all glass washes were done in pure hexane (SDS, Villeurbanne, France, 99% purity for traces analysis).

Solid-Phase Microextraction (SPME) sampling of venom volatile molecules

In order to determine the profile of the volatile compounds present in the venom, extraction by SPME was carried out with 65 µm PDMS/DVB blue fibres (Agilent Technologies, Santa Clara, CA, USA). Ten venom glands and 10 glass microbeads

20181213-092554
C7285/16634/936351CC

(2 mm diameter, previously washed with pure hexane) were placed in a 1.5 ml Eppendorf tube (polypropylene first grade) closed hermetically and then crushed through high-speed shaking for 30 s (QIAGEN TissueLyser). Then, the samples were centrifuged for 3 minutes at 4° C and the SPME fibre was exposed 1cm above the liquid during 30 min at 35° C. The SPME fibres were classically previously conditioned for 30 min at 250° C before exposure to the glands. Ten replicates were carried out, and profiles were compared to a control (SPME fibres exposed to 10 glass microbeads in an Eppendorf tube).

Solvent extractions

Solvent extraction was performed to confirm the SPME profiles and to calculate the concentrations of each constituent of the venom gland. Ten venom glands were placed in a clean brown glass vial with 200 µL of hexane and 2 µL internal standard (trans-3-nonen-2-one, 100 ng.µL^{-1}). After stirring with a rotor, the extraction time was 12 hours at room temperature. Ten replicates were done.

2.3.2 Chemical and semi-quantitative analyses of venom volatile components

To ensure that our results were comparable to those obtained by Cheng et al. (2017), we followed the same analytical methodology.

Gas chromatography (GC) coupled with mass spectrometry (MS) analyses (GC-MS)

SPME extracts and solvent extracts were analysed with GC-MS following Cheng et al. (2017). Briefly, analyses were carried on using an HP 7820A-5977E (Agilent Technologies, Santa Clara, CA, USA) with an HP-5ms capillary column 30 m × 250 µm × 0.25 µm (Agilent Technologies, Santa Clara, CA, USA). The carrier gas was helium flowing at 37 cm.s^{-1}. The oven ramp was set as 50° C for 2 min, followed by 5° C/min end then 280° C for 10 min. The electronic impact ion source was used at 230° C. The mass range scanned consisted of m/z ratios 20 – 350 at a rate of 4.3 scan.s^{-1}. Data were analysed using Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA) with the international database NIST and compared to chemical standards.

Gas Chromatography with Flame Ionization Detector (GC – FID)

SPME fibres and solvent extracts were analysed using GC–FID following Cheng et al. (2017). HP5890 with Flame Ionization Detector (Agilent Technologies, Santa Clara, CA, USA) was used associated with split less injection at 250° C and a HP-5ms column (30 m × 250 µm × 0.25 µm, Agilent Technologies, Santa Clara, CA, USA) was used with hydrogen as carrier gas flowing set at 74 cm.s^{-1}. The oven ramp was set to 50° C for 2 min, then 10° C.min^{-1} to 280° C for 5 min. We used GC-FID quantity-response standard curves to quantify each known compound in 10 venom glands against pure synthetic standards. For quantification, we used 10 extracts of 10 venom glands from foragers caught in the INRA apiary. Linear retention index (LRI) and Kovats retention indices were defined by reference to retention times of C8–C15 n-alkanes under the same GC and GC-MS conditions.

20181213-092554
C7285/16634/936351CC

Table 2. Summary of the behavioural experiment for each nest including the number of trials, the number of hits on the control and venom items and the latency to the first hit on the venom item (mean ± standard deviation).

Nests	Number of trials	Number of hits (mean ± SD)		Latency to first hit (mean ± SD) (in s)
		Control	Venom	
FL	8	0.75±1.03	13.62±7.40	29.37±15.91
ST	5	0.80±1.30	10.20±5.93	22.40±10.97
PO	3	0.00±0.00	6.00±2.65	40.00±34.64
LP	1	1	49	34
MA	1	0	26	15
SA	1	0	24	22
PE	2	1.00±0.00	28.00 ± 14.14	12.50 ± 3.54

Chemicals standards

The amount of specific venom gland constituents, authentic standards of the main compound were quantified by GC-FID calibration curves at increasing doses 20, 50, 100 and 200 ng.µl^{-1}. All chemical standards were purchased as mass spectrometry standards (> 99.5% purity): Heptan-2-one, Acetophenone, Nonan-2-one, Nonan-2-ol, Undecan-2-one (Sigma Aldrich, France), 4,8-Dimethyl non-7-en-2-one (Molport, Riga, Latvia).

2.4 Statistical analyses

To obtain an overall value of the reaction of the hornet, we used Cliff's δ with its 95% confidence interval. The magnitude of the differences between control and venom items was based on thresholds proposed by Romano et al. (2006): $\delta < 0.147$: negligible differences, $\delta < 0.33$: small differences, $\delta < 0.474$: medium differences and $\delta \geq 0.474$: large differences. Statistics were done with R software (v.3.1.2, R Core Team 2014) implemented with effsize package (Torchiano 2014) for Cliff's δ computing.

3 Results

The venom items elicited a rapid (range 15 to 40 s) and stronger response than the control item in all the seven colonies (Cliff's $\delta = 0.99$ and 95% CI = [0.95, 0.99], Table 2). Several molecules have been identified in the venom glands of the invasive populations of *V. velutina* (Table 3, Fig. 1). All major compounds belonged to ketone family. The nonan-2-one (peak #6) and the 4,8-dimethylnon-7-en-2-one (peak #9) were the most abundant molecules in the venom (Table 3). Five other molecules were also well represented including the heptan-2-one (peak #1), the non-8-en-2-one

20181213-092554
C7285/16634/936351CC

Table 3. Molecules identified in *Vespa velutina* venom gland from invasive populations. Quantification is based on 10 extracts (solvent extracts) of 10 venom glands (mean ± SD). Peak numbers (#1 to 16) are designated to correspond to those identified by Cheng et al. (2017). LRI: Linear Retention Index.

Peak #	Structure	LRI	French workers (mean ± SD, ng/insect)
1	heptan-2-one	888	8.0±2.9
2	heptan-2-ol		Not measured
3	heptan-2,6-dinone		Not measured
4	acetophenone	1063	3.5±1.8
5	non-8-en-2-one	1087	207.9±58.5
6	nonan-2-one	1089	1624.7±261.7
7	nonan-2-ol	1100	11.8±3.5
8	unknown C10H20O		Not measured
9	4,8-dimethyl-7-nonen-2-one	1227	326.1±121.0
10	unknown undecen-2-one		Not measured
11	undecen-6-one		Not measured
12	unknown undecen-2-one		Not measured
13	undecan-2-one	1293	40.1±8.6
14	undecan-2-ol		Not measured
15	unknown undecen-2,10-dione		Not measured
16	undecane-2,10-dinone		Not measured
-	unidentified molecule		Not measured

(peak #5), the undecan-2-one (peak #13) and two unknown molecules (peak #10 and unidentified).

4 Discussion

Alarm pheromones are critical in social insects for their implication in colony defence and thus survival (review in Bruschini et al. 2010), as previously shown in *Vespa* sp. (Saslavasky et al. 1973, Veith et al. 1984, Ono et al. 2003). In *V. velutina*, venom glands produce volatile compounds that elicit the attack of the targets when colonies are exposed to them (Cheng et al. 2017). From this point of view, our results are clearly consistent with those obtained in previous studies. Nevertheless, our results concerning the volatile molecules presented in the venom differ from the study by Cheng et al. (2017) while obtained from the same species with the same methodology.

20181213-092554
C7285/16634/936351CC

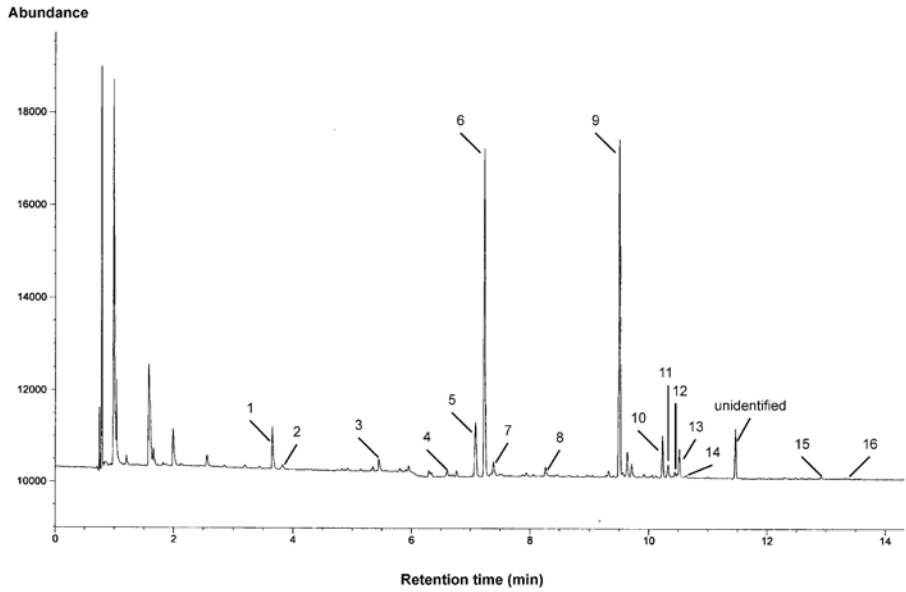

Fig. 1. Headspace SPME extract from *V. velutina* worker venom gland based on GC-FID. Peak numbers (#1 to 16) are designated to correspond to those identified by Cheng et al. (2017), see Table 3 for details. Un-numbered peaks are impurities.

Roughly, five molecules seem to be commonly observed in the venom of *V. velutina*: the heptan-2-one, the nonan-2-one, the 4,8-dimethylnon-7-en-2-one, the undecan-2-one and an unknown undecen-2-one. All these molecules are ketones that are known to elicit alarm at different levels in *V. orientalis* (Saslavski et al. 1973). The undecan-2-one has been also detected in *Polistes* sp. (Bruschini et al. 2007) and *Dolichovespula maculata* (Jimenez et al. 2016). However, in this latter study, it did not elicit any behavioural reaction. So, these molecules are potentially common in Vespidae venom. Similarly to Cheng et al. (2017), the major volatile compound was the nonan-2-one but we found at least two fold more in our samples as compared to them. Then, the abundance of the other compounds was not similar between the venom from the native and the invasive hornets. Indeed, we found that the second most abundant compound was the 4,8-dimethylnon-7-en-2-one that could be similar between studies considering standard deviation but the third and fourth places in Cheng et al. (2017) correspond to molecules that were slightly represented in our samples. Instead, we found that the non-8-en-2-one was also largely abundant in our sample, ca. three fold more concentrated in our case.

In their study, Cheng et al. (2017) performed electroantennography analyses for four molecules including the nonan-2-one and the non-8-en-2-one. They found that these molecules trigger an Electroantennogram (EAG) response but not up to a bio-

20181213-092554
C7285/16634/936351CC

logically significance at least at the concentration corresponding to one native hornet equivalent. Significant antennal response was reach for ca. 1000 ng in their study. Considering that the abundance of the nonan-2-one for one hornet equivalent in our study is above this value (1624.7 ± 261.7 ng), a reaction should be elicited with a single individual. Two hypotheses could be formulated: i) the individuals within the invasive range are either less sensitive to pheromone thus higher concentration are required to elicit aggressive/defensive behaviours or ii) the individuals within the invasive range are as sensitive to these molecules so only few individuals are required to elicit a response of the colony. The same experiment should be performed on invasive *V. velutina* to test these hypotheses. Preliminary tests show that the nonan-2-one (concentration 10^4 ng) alone does not elicit any attacks but alert when presented to encaged nest (O. Bonnard, pers. com.). It is also possible that molecules only elicit strong response when presented in blends such as alarm pheromone in honeybees (Breed et al. 2004).

Finally, our results differ qualitatively (an unknown molecule observed) and quantitatively from previous findings by Cheng et al. (2017). This may rely on the origin of the individuals and colonies involved in the experiment. Indeed, Cheng et al. (2017) performed the experiments on native populations of *V. velutina* while we worked on the European invasive population. One major question that is addressed here is, in fact, how different these chemical blends are. The literature on alarm pheromones even restricted to Vespidae show a great diversity among species (review in Bruschini et al. 2010) that suggests that alarm signals have evolved several times independently. The present study suggests that diversity is also present within species while it has been previously considered as a potential taxonomic tool (Bruschini et al. 2007), which implies that *V. velutina* invasive population has evolved another composition of alarm pheromone. Cheng et al. (2017) have sampled their hornets in the western part of China, in Yunnan. Population genetic study reveals that the invasive population in Europe originated from eastern China (Zhejiang and Jiangsu provinces most probably) and was distinct from Yunnan populations (Arca et al. 2015). These genetic differences may reflect independent evolution due to isolation by the distance for instance or evolutionary lineages as reflected by the differences in haplotype distribution in China (the haplotypes found in Zhejiang and Jiangsu provinces are not present in Yunnan). Therefore, one could imagine that differentiation may also occur in the chemicals that are produced.

These preliminary results are of fundamental importance in regard to the development of efficient pest management strategy. In the case of *V. velutina*, one promising strategy in the regulation would be the use of pheromones either for mating disruption or mass trapping (Monceau et al. 2012, Monceau & Thiéry, 2017, see also Monceau et al. 2014 for a review). However, caution must be taken to the populations on which pheromone based methods would be developed. An interesting perspective would be to assess the difference between castes and the potential effect of the last peak molecule as a sex pheromone (after the identification of this unknown formulation) that could act on mature males because some of them could act as sex pheromone. Indeed, recent work by Couto et al. (2016) suggests that sexual pheromones may play a crucial role in this species mating behaviour.

20181213-092554
C7285/16634/936351CC

Acknowledgments: Author contribution: DT and KM: work design, writing, data analysis, OB: behavioural experiments, chemical analysis and LR and G de R: chemical analysis. This work only benefits from INRA core funding. An important part of the GC MS analysis was kindly performed in the Analytical Platerform of the Institut des Sciences de la Vigne et du Vin (ISVV) funded by The Comité Interprofessionnel des Vins de Bordeaux (CIVB) and the Region Aquitaine.

References

Alaux, C., Maisonnasse, A. & Le Conte, Y. (2010): Pheromones in a superorganism: from gene to social regulation. – Vitamins and Hormones 83: 401–423.

Arca, M., Mougel, F., Guillemaud, T., Dupas, S., Rome, Q., Perrard, A., Muller, F., Fossoud, A., Capdevielle-Dulac, C., Torres-Leguizamon, M., Chen, X.X., Tan, J.L., Jung, C., Villemant, C., Arnold, G. & Silvain, J.-F. (2015): Reconstructing the invasion and the demographic history of the yellow-legged hornet, *Vespa velutina*, in Europe. – Biological Invasions 17: 2357–2371.

Bertolino, S., Lioy, S., Laurino, D., Manino, A. & Porporato, M. (2016): Spread of the invasive yellow-legged hornet *Vespa velutina* (Hymenoptera: Vespidae) in Italy. – Applied Entomology and Zoology 51: 589–597.

Billen, J. & Morgan, E.D. (1998): Pheromone communication in social insects: sources and secretions. – In: Vander Meer, R.K., Breed, M.D., Espelie, K.E. & Winston, M.L. (eds): Pheromone Communication in Social Insects, 159–192, Westview Press, Boulder.

Blum, M.S. (1969): Alarm pheromones. – Annual Review of Entomology 14: 57–80.

Breed, M.D., Guzmán-Novoa, E. & Hunt, G.J. (2004): Defensive behaviour of honey bees: organization, genetics, and comparisons with other bees. – Annual Review of Entomology 49: 271–298.

Bruschini, C. & Cervo, R. (2011): Venom volatiles of the paper wasp social parasite *Polistes sulcifer* elicit intra-colonial aggression on the nest of the host species *Polistes dominulus*. – Insectes Sociaux 58: 383–390.

Bruschini, C., Cervo, R., Dani, F.R. & Turillazzi, S. (2007): Can venom volatiles be a taxonomic tool for *Polistes* wasps (Hymenoptera, Vespidae)? – Journal of Zoological Systematics and Evolutionary Research 45: 202–205.

Bruschini, C., Cervo, R. & Turillazzi, S. (2010): Pheromones in social wasps. – Vitamins and Hormones 83: 447–492.

Budge, G.E., Hodgetts, J., Jones, E.P., Ostojá -Starzewski, J.C., Hall, J., Tomkies, V., Semmence, N., Brown, M., Walefield, M. & Stainton, K. (2017) The invasion, provenance and diversity of *Vespa velutina* Lepeletier (Hymenoptera: Vespidae) in Great Britain. – PLoS ONE 12: e0185172.

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

Cook, S.M., Khan, Z.R. & Pickett, J.A. (2007): The use of push-pull strategies in integrated pest management. – Annual Review of Entomology 52: 375–400.

Couto, A., Monceau, K., Bonnard, O., Thiéry, D. & Sandoz, J.-C. (2014): Olfactory attraction of the hornet *Vespa velutina* to honeybee colony odors and pheromones. – PLoS ONE 9: e115943.

Couto, A., Lapeyre, B., Thiéry, D. & Sandoz, J.-C. (2016): Olfactory pathway of the hornet *Vespa velutina*: new insights into the evolution of the hymenopteran antennal lobe. – Journal of Comparative Neurology 524: 2335–2359.

Goulson, D., Nicholls, E., Botias, C. & Rotheray, E.L. (2015): Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. – Science 347: 1435–1444.

20181213-092554
C7285/16634/936351CC

Grosso-Silva, J.M. & Maia, M. (2012): *Vespa velutina* Lepeletier, 1836 (Hymenoptera, Vespidae), new species for Portugal. – Arquivos Entomolóxicos 6: 53–54.

Guerrieri, E. & Digilio, M.C. (2008): Aphid-plant interactions: a review. – Journal of Plant Interactions 3: 223–232.

Heath, R.R. & Landolt, P.J. (1988): The isolation, identification and synthesis of the alarm pheromone of *Vespula squamosa* (Drury) (Hymenoptera: Vespidae) and associated behaviour. – Experientia 44: 82–83.

Hölldobler, B. & Wilson, E.O. (2008): The Superorganism. – WW Norton & Company, New York.

Jeanne, R.L. (1981). Alarm recruitment, attack behaviour, and the role of the alarm pheromone in *Polybia occidentalis* (Hymenoptera: Vespidae). – Behavioral Ecology and Sociobiology 9:143–148.

Jimenez, S.I., Gries, R., Zhai, H., Derstine, N., Mccann, S. & Gries, G. (2016): Evidence for a nest defense pheromone in bald-faced hornets, *Dolichovespula maculata*, and identification of components. – Journal of Chemical Ecology 42: 414–424.

Kennedy, P.J., Ford, S.M., Poidatz, J., Thiéry, D. & Osborne, J.L. (2018): Searching for nests of the invasive Asian hornet (*Vespa velutina*) using radio-telemetry. – Nature Communications Biology 1: 88, DOI: 10.1038/s42003-018-0092-9.

Landolt, P.J. & Heath, R.R. (1987): Alarm pheromone behaviour of *Vespula squamosa* (Hymenoptera: Vespidae). – Florida Entomologist 70: 222–225.

Landolt, P.J., Reed, H.C. & Heath, R.R. (1999): An alarm pheromone from heads of worker *Vespula squamosa* (Hymenoptera: Vespidae). – Florida Entomologist 82: 356–359.

Landolt, P.J., Heath, R.R., Reed, H.C. & Manning, K. (1995): Pheromonal mediation of alarm in the eastern yellowjacket (Hymenoptera: Vespidae). – Florida Entomologist 78: 101–108.

Leza, M., Miranda, M.A. & Colomar, V. (2018): First detection of *Vespa velutina nigrithorax* (Hymenoptera: Vespidae) in the Balearic Islands (Western Mediterranean): a challenging study case. – Biological Invasions 20: 1643–1649.

London, K.B. & Jeanne, R.L. (1996): Alarm in a wasp-wasp nesting association: do members signal cross-specifically? – Insectes Sociaux 43: 211–215.

López, S., Gonzáles, M. & Goldarazena, A. (2011): *Vespa velutina* Lepeletier, 1836 (Hymenoptera: Vespidae): first records in Iberian Peninsula. – Bulletin OEPP/EPPO Bulletin 41: 439–441.

Maschwitz, U.W. (1964): Alarm substances and alarm behaviour in social hymenoptera. – Nature 204: 324–327.

Monceau, K., Bonnard, O. & Thiéry, D. (2013): Relationship between the age of *Vespa velutina* workers and their defensive behaviour established from colonies maintained in the laboratory. – Insectes Sociaux 60: 437–444.

Monceau, K., Bonnard, O. & Thiéry, D. (2012): Chasing the queens of the alien predator of honeybees: a water drop in the invasiveness ocean. – Open Journal of Ecology 2: 183–191.

Monceau, K., Bonnard, O. & Thiéry, D. (2014): *Vespa velutina*: a new invasive predator of honeybees in Europe. – Journal of Pest Science 87: 1–16.

Monceau, K. & Thiéry, D. (2017): *Vespa velutina* nest distribution at a local scale: An 8-year survey of the invasive honeybee predator. – Insect Science 24: 663–674.

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

Pasteels, J.M., Grégoire, J.C. & Rowell-Rahier, M. (1983): The chemical ecology of defense in arthropods. – Annual Review of Entomology 28: 263–289.

R Core Team (2016): R: A language and environment for statistical computing. – R Foundation for Statistical Computing, Vienna, Austria.

Romano, J., Kromrey, J.D., Coraggio, J. & Skowronek, J. (2006): Appropriate statistics for ordinal level data: Should we really be using t-test and Cohen's d for evaluating group differences on the NSSE and other surveys? – Annual Meeting of the Florida Association of Institutional Research, 1–3.

Saslavasky, H., Ishay, J. & Ikan, R. (1973): Alarm substances as toxicants of oriental hornet, *Vespa orientalis*. – Life Sciences 12: 135–144.

20181213-092554
C7285/16634/936351CC

Shorey, H.H. (1973): Behavioral responses to insect pheromones. – Annual Review of Entomology 18: 349–380.
Torchiano, M. (2014): effsize: Efficient effect size computation. – R package version 0.5.2.
Veith, H.J., Koeniger, N. & Maschwitz, U. (1984): 2-Methyl-3-butene-2-ol, a major component of the alarm pheromone of the hornet *Vespa crabro*. – Naturwissenschaften 71: 328–329.
Wertheim, B., van Baalen, E.J.A., Dicke, M. & Vet, L.E.M. (2005): Pheromone-mediated aggregation in nonsocial arthropods: an evolutionary ecological perspective. – Annual Review of Entomology 50: 321–46.
Witt, R. (2015): Erstfund eines Nestes der Asiatischen Hornisse *Vespa velutina* Lepeletier, 1838 in Deutschland und Details zum Nestbau (Hymenoptera, Vespinae). – Ampulex 7: 42–53.

Manuscript received: 10 July 2018
Revision requested: 3 September 208
Revised version received: 12 September 2018
Accepted: 13 September 2018

20181213-092554
C7285/16634/936351CC