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► **To cite this version:**

Laurence Berville, Christophe Lucas, Mélissa Haouzi, Ali Khalil, Jérémy Gévar, et al.. Chemical profiles of venom glands in queens, foundresses, pre-wintering gynes, and workers in the hornet *Vespa velutina nigrithorax*. *Comptes Rendus. Chimie*, 2023, pp.1-16. 10.5802/crchim.228 . hal-04018659

**HAL Id: hal-04018659**

**<https://hal.science/hal-04018659v1>**

Submitted on 7 Mar 2023

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**Chemical profiles of venom glands in queens, foundresses, pre-wintering gynes, and workers in the hornet  
*Vespa velutina nigrithorax***

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1 **Abstract** - Invasive species are permanently modifying the distribution and diversity of native species worldwide.  
2 For nearly two decades, a hornet, *Vespa velutina nigrithorax* (Hymenoptera: Vespidae), has been spreading in  
3 Europe. Due to its marked invasiveness, this yellow-legged hornet is of great economic and ecological concern,  
4 mainly because of the damage it causes to insects in general and bees in particular. Current management methods  
5 are sparse and ineffective. Naturally produced by insects, semio-chemicals have been proposed as integrated  
6 management tools in this context, either for disruption or mass trapping, as an alternative tool to conventional non-  
7 selective traps. Here, we focused on the venom gland, which produces the alarm pheromone. In previous studies,  
8 individuals showed marked diversity in their chemical profiles. However, to successfully conduct targeted  
9 pheromone-based trapping, the hornet's chemical ecology must be thoroughly characterized. Therefore, it was  
10 necessary to better understand the chemical composition of the alarm pheromone of not only workers but also  
11 other *V. v. nigrithorax* females. First, we evaluated the differences in venom gland profiles between the four types  
12 of females: queens, foundresses, pre-winter gynes, and workers. Next, we experimentally explored the venom  
13 gland profiles of *V. velutina nigrithorax* workers and pre-wintering gynes by *in vivo* and *in vitro* approaches. We  
14 found 13 new compounds in the venom gland, of which 9 were identified (chain lengths: C<sub>8</sub> to C<sub>12</sub>). Two  
15 compounds were found exclusively in reproductive females. Profiles differed among pre-wintering gynes,  
16 foundresses, and queens but not between pre-wintering gynes and workers. This result indicates that the chemical  
17 signature of the female venom gland changes over the course of life history: from pre-wintering gynes to  
18 foundresses to queens.

19

20

21 **Keywords** - Yellow-legged hornet, Venom gland, Invasive species, Chemical communication, Vespidae

## DECLARATIONS

**Funding:** This study received funding from the Centre-Val de Loire region, in the form of French regional grants for our FRELON and FRELON 2 projects, as well as from Veto-pharma and the French administrative department of La Manche. The TQ-MS was funded by the region Centre-Val de Loire (APR-IA 2012).

**Availability of data and materials:** The datasets generated during the current study are available from the corresponding author upon reasonable request.

**Ethics approval:** The study does not require ethics approval since the yellow-legged hornet is classified as an invasive species; all research was carried out in compliance with the relevant national guidelines.

**Conflict of interest:** The authors declare no competing interests.

**Code availability:** Not applicable.

**Authors' contributions:** The experiments and data collection were carried out by LB, JG, MH, and ED. The chemical assays were carried out by CL, AGB, LB, and AK. Formal Analysis: LB. The first draft of the manuscript was written by LB. The manuscript was revised and edited by LB, CL, AGB, and ED. Funding was obtained by ED. All the authors read the manuscript and gave their approval for submission. Conceptualization & Project Administration: ED.

**Consent to participate:** Not applicable.

**Consent for publication:** All the authors have approved the content of this paper and have agreed to the “special Issue MediatEC: Chemical Mediation in the Environment” Comptes Rendus de Chimie submission policies. This manuscript has not been published previously and is not under consideration for publication elsewhere.

## INTRODUCTION

Invasive species are increasingly affecting the Earth's ecosystems [1]. They are permanently modifying the distribution and diversity of native species worldwide [2] at a variety of biological scales and with differing degrees of severity [3]. In many different habitats, social insects are highly successful invaders because of their excellent ability to disperse, compete for resources, mount effective defenses, reproduce at high rates, and exploit a broad range of habitats and diets [4]. Native to China, the invasive yellow-legged hornet, *Vespa velutina nigrithorax* (Lepeletier 1836) [5], was accidentally introduced into southwestern France around 2004 [6]. It is a hardy, adaptable species whose flexible dietary requirements have allowed it to invade and establish itself within a variety of habitats [7]. *Vespa v. nigrithorax* has successfully expanded its range and is now found throughout most of France [8] as well as in several other European countries [9–13]. Ecosystems invaded by this hornet are impacted in various ways since *V. v. nigrithorax* preys upon several insect and arthropod taxa [14], including the domestic honey bee, *Apis mellifera* [15]. Finally, *V. v. nigrithorax* presents human health risks as hornets can aggressively spray liquid in people's eyes [16]; also, their stings are painful and sometimes even fatal [17,18]. Consequently, medical practitioners and researchers are increasingly seeking information on *V. v. nigrithorax* venom for diagnostic and treatment purposes [17,19]. The hornet's introduction into Europe led to calls for control efforts, which have largely focused on passively trapping adults using homemade or commercial poison baits [20]. Unfortunately, this approach has done little to limit *V. v. nigrithorax* population sizes [20,21]. Furthermore, because they lack specificity, these traps can have significant ecological impacts on a wide range of non-target species [22,23]. As a result, work is currently underway [20] to develop new species-specific systems that are based on pheromones [24–27].

Chemical signals are an essential component of animal communication systems [28,29] and mediate a variety of behaviors, including social organization. In insects, long-distance signals play an important role in intra- and interspecific communication [29] and elicit species-specific responses. Such communication provides fitness benefits by enhancing colony cohesion, defense, and alarm systems. There is a long history of research on chemical signals (e.g., pheromones) in social insects (e.g., ants, bees, hornets, and termites); these signals often trigger different behaviors (recruitment, dispersal, aggressiveness, or aggregation) that can affect colony survival [30,31]. Thus, a promising pest control approach is to develop species-specific attractants (e.g., trap bait) or repellents that target specific life stages. Alarm pheromones are of key importance because they are deployed when colonies are threatened: they prompt nestmate recruitment and defense behaviors [32,33]. They can also be used by workers to signal danger in areas farther away from the nest [34]. The most effectively disseminated signals are made up of highly volatile compounds that act quickly and then dissipate, limiting further responses. Most alarm pheromones in social insects are multicomponent blends [24,35,36] that are generally involved in both offensive and defensive reactions by workers. The venom gland tends to be the primary source of alarm pheromones [33,37], although interspecific variation exists. Thus, during aggressive interactions, alarm signals are released at the same time as venom [38]. Such is the case in several wasp and hornet species, including *Polistes dominulus* [39], *Vespula squamosa* [40], *Vespa crabro* [41], *Vespa mandarinia* [37], and *Vespa simillima xanthoptera* [37].

The first study conducted on alarm pheromones in a subspecies of the yellow-legged hornet characterized the volatile compounds found in worker venom glands in a native *V. velutina auraria* population in China [42]. A second study [43] did the same in an invasive *V. v. nigrithorax* population in France. In both cases, researchers identified some venom gland compounds that elicited hornet attacks when colonies were threatened. When the alarm pheromones were released, they attracted other workers and encouraged stinging behavior. However,

43 compound abundance differed between the two subspecies. Next, researchers explored the chemical signals  
44 associated with caste and species' reproductive status using four colonies in an invasive population in Italy; the  
45 study looked at compounds in volatile venom gland mixtures in gynes and workers [27]. Twelve major compounds  
46 were observed, which contrasted with the 16 and 17 compounds observed previously ([42,43] respectively). Five  
47 of these 12 compounds had been seen in the first two studies. However, others had never been reported before,  
48 including 4 unknown acetates of aliphatic alcohols and citronellyl acetate, which had the second highest abundance  
49 [27]. Finally, work on an invasive population in Spain observed 8 compounds [26], collected *in vivo* by SPME  
50 from seven hornets, including 3 previously seen ketones [27,42,43]. However, in this most recent study, individuals  
51 displayed marked diversity in their chemical profiles since 2 of the 8 compounds were found in only one hornet  
52 and 3 others in fewer than 3 hornets [26]; only one compound occurred across all the profiles. There is clearly a  
53 need for further research given this range of results. First, a better understanding of the chemical composition of  
54 *V. v. nigrithorax*'s alarm pheromone is needed. Second, since gynes, foundresses, and queens alike use alarm  
55 pheromones, we also must clarify associations between hornet caste and alarm pheromone composition. However,  
56 in the latter three cases, the volatile compounds involved remain poorly characterized. Here, we hypothesized that  
57 females from different castes and/or with different tasks might differ in their venom gland profiles.

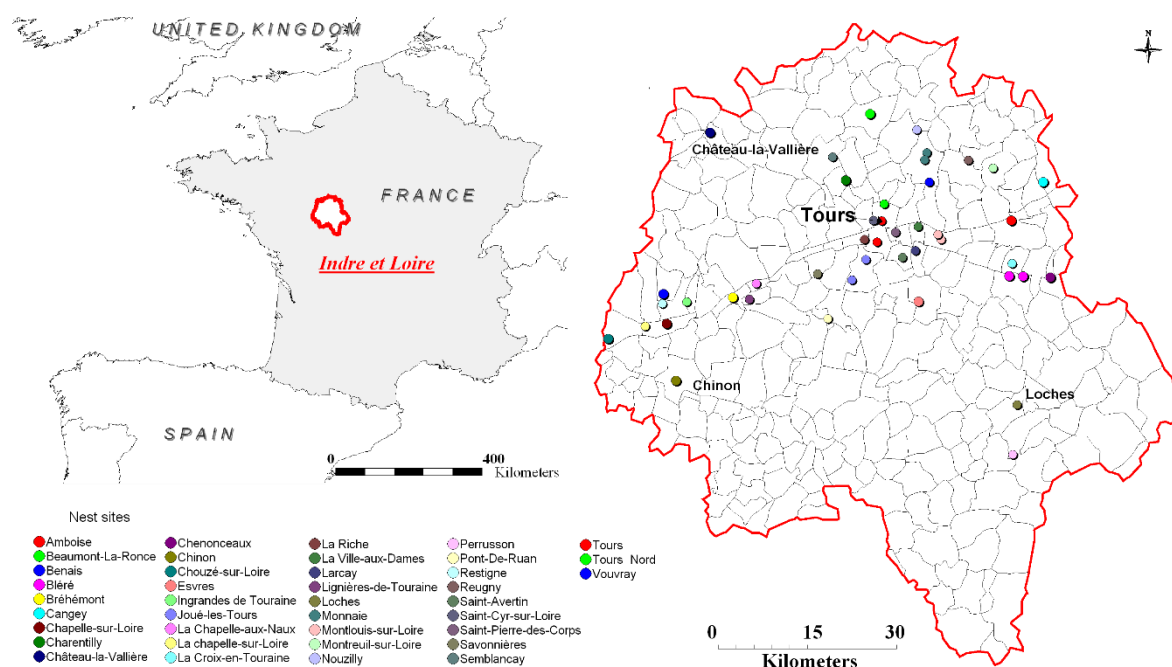
58         This study thus had two goals. First, we assessed differences in venom gland profiles among the four  
59 types of females: queens, foundresses, pre-wintering gynes, and workers. Second, we experimentally explored  
60 venom gland profiles of *V. velutina nigrithorax* workers and pre-wintering gynes via an *in vivo* approach  
61 (collection of volatiles, solid-phase microextraction [SPME]) and an *in vitro* approach (liquid extraction of  
62 compounds from the venom gland).

63  
64

## MATERIALS AND METHODS

### Study sites and hornet sampling.

Hornet colonies were collected at 44 different sites in the administrative department of Indre-et-Loire, which is part of the Centre-Val-de-Loire region of France (Fig. 1). The yellow-legged hornet is classified as an invasive species, and all research was carried out in compliance with relevant national guidelines. To determine hornet caste (worker vs. reproductive), mass and wing spacing were measured [44]. The reproductives were either pre-wintering gynes (females without nest), foundresses (mated females with small nest and some eggs), or queens (mated females with workers). To characterize the venom gland's compounds, hornets of each caste were stored at  $-80^{\circ}\text{C}$  until they could be dissected. For the second experiment, individuals from a given colony were placed in a mesh breeder box and fed an *ad libitum* mixture of honey and water, which was replenished daily. The hornets underwent SPME within three days of collection.



**Fig. 1:** *Vespa velutina nigrithorax* collection sites

### Analysis of volatile compounds.

To characterize the venom gland's compounds, hornets ( $n = 75$  workers, 40 pre-wintering gynes, 30 foundresses, and 30 queens) were dissected under a binocular microscope. Within each group, a set of 5 undamaged venom sacs ( $n = 15, 8, 6,$  and  $6$  replicates for the workers, pre-wintering gynes, foundresses, and queens, respectively) was placed in a vial containing heptane (1 mL). The vials were then stored at  $-20^{\circ}\text{C}$ . Glands were perforated right before performing the gas chromatography-mass spectrometry (GC-MS) analysis; the vials were vortexed at 300 rpm for 1 min. Gland extract (2  $\mu\text{L}$ ) from each vial were analyzed using a gas chromatograph (Agilent Technologies 7890B) coupled to a mass spectrometer (Agilent Technologies 7000C GC/MS Triple Quad, Les Ulis, France) equipped with an HP-5 capillary column (Agilent Technology, USA; 30 m x 250  $\mu\text{m}$  x 0.25  $\mu\text{m}$ ) and using a Gerstel MPS autosampler (electron impact at 70 eV). Helium was the carrier gas (flow rate = 2.3 mL/min). Injectors were used in splitless mode (splitless time = 2 min) at a constant temperature of  $250^{\circ}\text{C}$ . The

92 oven temperature was set to ramp up from 50°C to 200°C at a rate of 8°C/min and then from 200°C to 315°C at a  
93 rate of 5°C/min (held constant for 5 min). Masses were scanned between 15 and 550 amu at 0.1 scan/s. Immediately  
94 before the analysis, 10 µL of internal standard (*n*-eicosane at 10<sup>-3</sup> g/mL diluted in heptane) was added to each  
95 sample.

96 To identify and quantify the compounds released by workers or pre-wintering gynes in response to stress,  
97 two experimental stimuli were assessed. The experiments were conducted with sets of four live co-workers or four  
98 live pre-wintering gynes. The hornets were cold anesthetized (-18°C for 10 min) and were subsequently placed in  
99 a clean 350mL glass vial at room temperature (19-21°C for 10 min). The hornets were still able to move but not  
100 to fly. Each hornet was exposed once to either a stressed hornet group (SH, replicates: pre-wintering gynes n = 4;  
101 workers n = 8) or a wounded hornet group (WH, replicates: pre-wintering gynes n = 4; workers n = 8). The SH  
102 treatment consisted of gently shaking the hornet group inside the glass vial. The WH treatment consisted of  
103 applying pressure to a hornet's thorax using metal forceps; wounded hornets were then resealed before closing the  
104 glass vial. We assumed that the WH treatment induced a higher stress level than the SH treatment. To extract the  
105 resulting volatile compounds, a 40-min procedure was carried out using red solid-phase microextraction (SPME)  
106 fibers coated with polydimethylsiloxane (PDMS, 100 µm; Supelco). Immediately after the treatments, the fibers  
107 were placed within a metal wire to prevent any physical contact with the hornets. First, for each caste, two fibers  
108 were desorbed, and the resulting compounds were identified using the temperature program and GC-MS procedure  
109 described above. Second, individual fibers representing different castes and treatment groups were desorbed  
110 (WH<sub>workers</sub> n = 8; SH<sub>workers</sub> n = 8; WH<sub>gynes</sub> n = 4; SH<sub>gynes</sub> n = 4) using a GC system (CPG Agilent Technologies  
111 7820A) equipped with a flame-ionization detector (FID) and a capillary column (HP-5 Agilent Technology, Santa  
112 Clara, USA; 30 m × 0.32 mm × 0.25 µm); helium served as the carrier gas (1.7 ml/min). The temperature program  
113 was the same as above. The relative area of each compound was calculated based on the proportions represented  
114 by each peak within the chromatogram using ChemStation (v. 04.02, Agilent).

115

#### 116 *Compound identification.*

117 The data were analyzed using MassHunter (v. B.07.00; Agilent) and AMDIS (v. 2.0g; NIST, 2011).  
118 Compounds were identified based on their mass spectra, which were interpreted via fragmentation analyses [45].  
119 The results were then compared to published spectra and/or confirmed using commercial standards (Table 1)  
120 purchased from different suppliers (Sigma-Aldrich, Saint-Louis, Missouri, USA; GreenPharma, Orléans, France;  
121 and ChemSpace, Monmouth Junction, New Jersey, USA). The molecules 4,8-dimethyl-7-nonen-2-one and 4,8-  
122 dimethyl-1,7-nonadiene were synthesized by Synthenova (Hérouville-Saint-Clair, France). Kovats retention index  
123 (KRI) values were calculated based on the retention times of C8-C20 n-alkane standards (Fluka, 94234) that were  
124 analyzed under the same set of GC and GC-MS conditions.

125

Chemical compound name, identifiers and properties										Compound identifications		
ID	Molecular		weight (g/mol)	CAS	Major fragments		Kovats Retention Index		Vespa velutina sub-species	in this study		previous studies
	name	formula			(m/z)	from literature	measured	methods		id		
1	Heptan-2-one	C <sub>7</sub> H <sub>14</sub> O	114	110-43-0	43,58,71,27	888	-	a & n	-	-	1	
2	Heptan-2-ol	C <sub>7</sub> H <sub>16</sub> O	116	543-49-7	45,27,43	[877-924]	-	a & n	-	-	1	
3	2,6-Heptanedione	C <sub>7</sub> H <sub>12</sub> O <sub>2</sub>	128	13505-34-5	43,39,82,58	-	-	a & n	-	-	1	
4	Acetophenone	C <sub>8</sub> H <sub>8</sub> O	120	98-86-2	105, 77, 43	[1026-1091]	1032	a & n	+Δε•	a	1	
5	8-Nonen-2-one	C <sub>9</sub> H <sub>16</sub> O	140	5009-32-5	67, 43, 82	[1042-1055]	1045	a & n	+Δε•	b	1, 2	
6	Nonan-2-one	C <sub>9</sub> H <sub>18</sub> O	142	821-55-6	58, 43, 71	[1046-1094]	1052	a & n	+Δε•	c	1, 2, 3	
7	Nonanal	C <sub>9</sub> H <sub>18</sub> O	142	124-19-6	57,41,43,56	1099	-	n	-	-	3	
8	Nonan-2-ol	C <sub>9</sub> H <sub>20</sub> O	144	628-99-9	69, 55, 56, 57	[1072-1110]	1062	a & n	+Δε•	d	1	
9	Isoamyl isovalerate	C <sub>10</sub> H <sub>20</sub> O <sub>2</sub>	172	659-70-1	70, 85, 43, 57	[1080-1110]	1065	n	+Δε•	e	-	
10	Unknown 1 (cyclohexanol)	C <sub>10</sub> H <sub>20</sub> O	156	-	43, 71, 95, 138	-	1117	a & n	•	f	1, 2, 3?	
11	Decanal	C <sub>10</sub> H <sub>20</sub> O	156	112-31-2	43,41,57,55	1200	-	n	-	-	3	
12	Isoamyl senecioate	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	170	56922-73-7	83, 100, 55	1146	1145	n	+ε•	g	-	
(!)	4,8-dimethyl-1,7-nonadiene	C <sub>11</sub> H <sub>20</sub>	152	62108-28-5	41,69,55,68	1343	1149	n	Δ	-	3	
13	X-C12.1	C <sub>12</sub> H <sub>24</sub>	-	-	55, 83, 70, 56	-	1152	n	•	h	-	
14	Unknown 2	-	-	-	44, 71, 95, 91	-	1181	n	•	i	-	
15	4,8-dimethyl-7-nonen-2-one	C <sub>11</sub> H <sub>20</sub> O	168	3664-64-0	95, 110, 85, 69	-	1188	a & n	+Δε•	j	1, 2, 3	
16	2-Nonanyl acetate	C <sub>11</sub> H <sub>22</sub> O <sub>2</sub>	186	14936-66-4	43, 87, 55	[1186-1221]	1195	n	+ε•	k	1, 2, 3?	
17	β-Citronellol, methyl ether	C <sub>11</sub> H <sub>22</sub> O	170	55915-70-3	82, 85, 81, 69	-	1220	a & n	Δ•	l	1, 2, 3?	
18	2-Phenethyl acetate	C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>	164	103-45-7	82, 85, 81, 109	[1221-1279]	1219	n	+ε•	m	-	
19	X-Undecen-2-one	C <sub>11</sub> H <sub>20</sub> O	-	-	43, 81, 118, 68	-	1234	a & n	• ×	n	1	
20	X-Undecen-6-one	C <sub>11</sub> H <sub>22</sub> O	-	-	43, 71, 99, 58	-	1242	a & n	• ×	o	1	
21	X'-Undecen-2-one	C <sub>11</sub> H <sub>20</sub> O	-	-	44, 69, 97	-	1243	a & n	• ×	p	1	
22	Undecan-2-one	C <sub>11</sub> H <sub>22</sub> O	170	112-12-9	58, 43, 71, 59	[1247-1342]	1249	a & n	+Δε•	q	1, 2, 3	
23	Undecan-2-ol	C <sub>11</sub> H <sub>24</sub> O	172	1653-30-1	45,83,55,69	1302	-	a & n	Δ	-	1	
24	Citronellyl acetate	C <sub>12</sub> H <sub>22</sub> O <sub>2</sub>	198	150-84-5	81, 95, 69, 123	[1314-1360]	1305	n	+ε•	r	2	
25	Unknown 3 *	C <sub>11</sub> H <sub>20</sub>	-	-	96, 109, 82, 81	-	1310	n	•	s	-	
26	Geranyl acetate	C <sub>12</sub> H <sub>20</sub> O <sub>2</sub>	196	105-87-3	69, 68, 41, 43	[1340-1409]	1336	n	+ε•	t	2	
26'	Geranyl acetone	C <sub>13</sub> H <sub>22</sub> O	194	3796-70-1	43,69,41,151	1432	-	n	-	-	3	
27	Decane-2,9-dione	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	170	16538-91-3	43, 58, 71, 113	-	1339	n	+ε•	u	-	
28	Unknown 4	-	-	-	110, 81, 68, 43	-	1370	n	•	v	-	
29	Isopentyl benzoate	C <sub>12</sub> H <sub>16</sub> O <sub>2</sub>	192	94-46-2	105, 70, 123, 55	[1402-1436]	1394	n	+ε•	w	-	
30	3-Methyl-3-butenyl benzoate	C <sub>12</sub> H <sub>14</sub> O <sub>2</sub>	190	5205-12-9	105, 68, 77	[1412-1421]	1403	n	Δ•	x	-	
31	X-Undecene-2,10-dione	C <sub>11</sub> H <sub>18</sub> O <sub>2</sub>	-	-	43, 81, 124	-	1416	a & n	• ×	y	1	
32	Undecane-2,10-dione	C <sub>11</sub> H <sub>20</sub> O <sub>2</sub>	184	18667-24-8	43, 71, 109, 58	-	1485	a & n	+Δε•	z	1	
33	α-Farnesene	C <sub>15</sub> H <sub>24</sub>	204	502-61-4	41,93,69,55	1494	-	n	-	-	3	

CAS: Chemical Abstracts Service  
The major *m/z* fragments are sorted based on their occurrence frequency (highest to lowest).  
X: corresponds to the putative position of the double bond for each compound  
(!) standard of the molecule suggested by ⑤  
\* Molecule mass spectra of the unknown compound (Fig.3A)

Compound identification methodologies:  
+ Literature and/or published spectra (PubChem)  
Δ Standard  
ε Mass spectral data bases (NIST)  
• Fragmentation analyses

a: *Vespa velutina auraria*  
n: *Vespa velutina nigrithorax*

Molecules:

a to z in this study, see table 2  
① in Cheng et al. 2016 and Thierry et al. 2018  
② in Cappa et al. 2019  
③ in Rodriguez-Flores et al. 2021  
①, ②, ③? not named in articles, postulated based on profile sighting

127

128 **Table 1:** Compounds found in venom glands of *V. velutina* subspecies. ① Peaks present in both *V. v. auraria* [42]  
129 and *V. v. nigrithorax* [43]. Peaks present in *V. v. nigrithorax* populations in ② Italy [27] and ③ Spain [26]. All  
130 the compounds observed in this study (a to z) underwent MS analysis (•) and were identified by comparing their  
131 mass spectra with those in the NIST 2017 database (ε) and/or in the literature (+); final validation was performed  
132 using available standards (Δ). The major *m/z* fragments are sorted based on their occurrence frequency (highest to  
133 lowest). The Kovats retention index values were calculated and obtained from PubChem and the literature.  
134 Chemical Abstracts Service (CAS) of the American Chemical Society. (!) compound (Fig. 3B) identified by  
135 Rodríguez-Flores (2021) on the basis of the HS-SPME chromatogram and mentioned as eluting after undecan-2-  
136 one. However, its MS spectra, retention time, and KRI value differed from those of the 4,8-dimethyl-1,7-nonadiene  
137 standard. \* Mass spectra of unknown compound s (Fig. 3A) isolated from the venom gland (Fig. 3). n: *Vespa*  
138 *velutina nigrithorax*. a: *Vespa velutina auraria*.

139

140

141 *Statistical analyses.*

142 Venom gland profile composition was analyzed using multivariate principal component analysis (PCA)  
143 performed in Statistica (v. 10; Statsoft). The analysis used the percentage representation of identified compounds  
144 that occurred above a minimum threshold of 0.5%. The worker and gyne profiles obtained in the stress experiment  
145 were explored using another PCA, which employed the percentage representation of all the compounds collected



146 via PDMS-SPME. To assess profile-based grouping patterns, we conducted a K-means cluster analysis [46].  
147 Because the number of groups is unknown ahead of time, the choice of initial group number is somewhat arbitrary.  
148 To optimize this number, we used the Caliński-Harabasz index [47].

149

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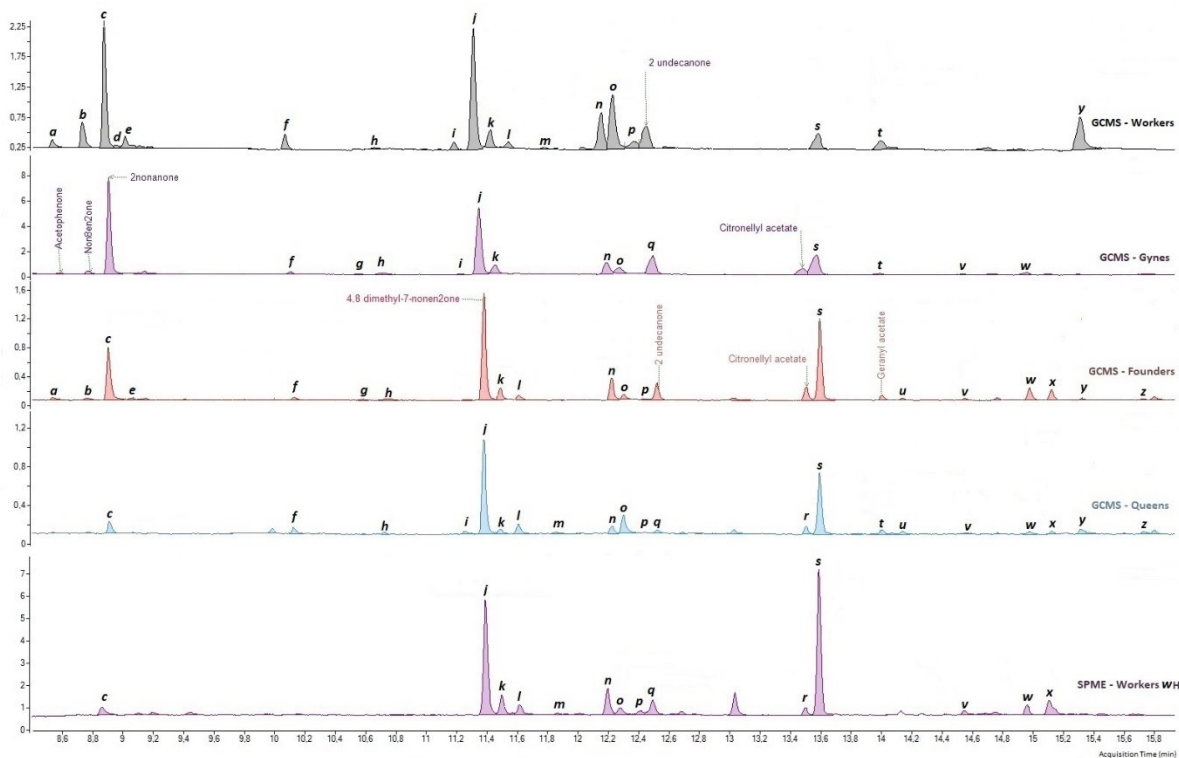
## RESULTS

152

### 153 Identification of venom gland compounds

154 The venom gland profiles contained 38 compounds with chain lengths ranging from 8 to 12 carbons (Fig.  
155 2, Tables 1 & 2). Of these, 26 occurred at levels higher than the 0.5% threshold (Table 2). This group included 11  
156 ketones, 8 esters, 4 alcohols, and 3 unknown molecules. The MS spectrum were investigated, and the identities of  
157 the compounds were confirmed using chemical standards (Table 1). The unknown compounds were subject to MS  
158 interpretation. The third unknown compound (s, Table 2, Fig. 3A) was thought to be 4,8-dimethyl-1,7-nonadiene  
159 (Fig. 3B, Table 1). Indeed, this compound was identified [26] based on the HS-SPME chromatogram. However,  
160 its MS spectra, retention time, and KRI value differed from those of the 4,8-dimethyl-1,7-nonadiene standard (Fig.  
161 3). The MS spectra for compound s (Fig. 3A) and citronellyl acetate (compound r) shared some similarities.  
162 However, they differed in the presence of a fragment at  $m/z$  152 fragment and a trace of  $m/z$  170. Furthermore,  
163 compared to the citronellyl acetate, compound s had higher peaks at  $m/z$  137 (instead of at  $m/z$  138),  $m/z$  96, and  
164  $m/z$  109. Unfortunately, spectra for the citronellyl family remain quite rare, limiting more detailed comparisons.  
165 Additionally, we found no other commercial compounds with more similar spectra, thus we were not able to  
166 confirm our hypothesis.

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169 **Fig. 2:** Chemical profiles of hornet workers, gynes, foundresses, and queens (unit of analysis = pools of five venom  
 170 glands). The letters refer to the different compounds observed (Table 2).  
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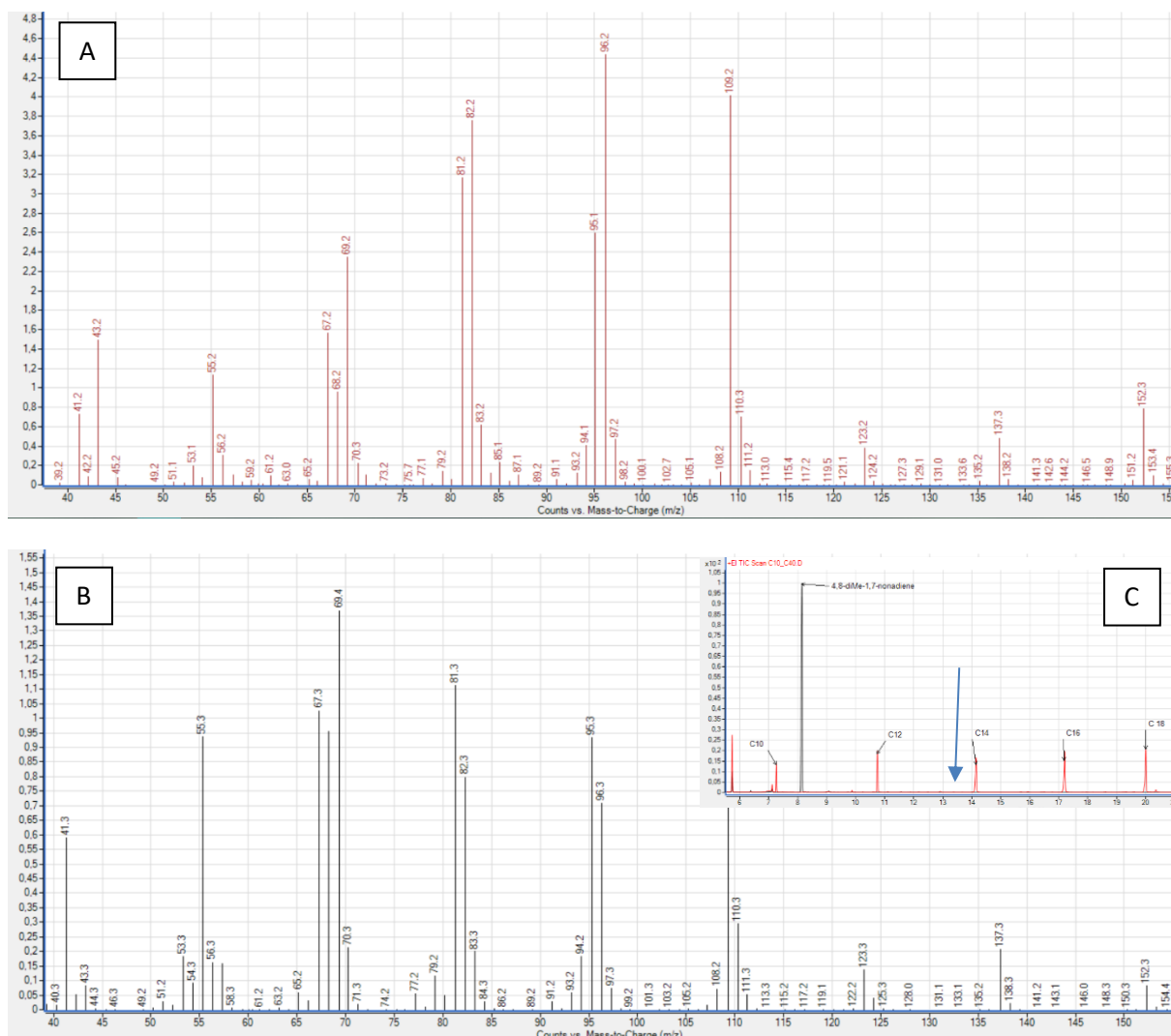
Name:	VENOM GLAND 's profile				SPME analysis			
	Queens	Foundresses	Workers	Prewintering gynes	Workers		Pre-wintering gynes	
					SH	WH	SH	WH
Acetophenone <b>a</b>	1.7 ± 1	0.4 ± 0.3	0.2 ± 0.2	8.10-3 ± 1.10-3	- - -	- - -	- - -	- - -
8-Nonen-2-one <b>b</b>	0.4 ± 0.3	0.8 ± 0.2	3.7 ± 0.9	3.2 ± 0.5	1.1 ± 0.9	1.4 ± 0.4	3.1 ± 2.5	1.1 ± 1.05
Nonan-2-one <b>c</b>	3.4 ± 0.9	16.4 ± 2.8	18.2 ± 3.2	14.2 ± 3.7	7.7 ± 3.8	10.9 ± 3.8	3.3 ± 2.2	6.3 ± 2.6
Nonan-2-ol <b>d</b>	0.3 ± 0.3	0.4 ± 0.2	1.6 ± 0.8	0.8 ± 0.9	1.2 ± 0.7	1.5 ± 0.5	1.8 ± 1.1	0.6 ± 0.3
Isoamyl isovalerate <b>e</b>	0.1 ± 0.1	0.8 ± 0.2	2.0 ± 0.9	2.5 ± 0.8	1.2 ± 1.3	0.8 ± 0.5	0.4 ± 0.5	1.2 ± 0.9
Unknown 1 (cyclohexanol) <b>f</b>	3 ± 0.4	0.7 ± 0.1	1.3 ± 0.9	0.8 ± 0.7	- - -	- - -	- - -	- - -
Isoamyl senecioate <b>g</b>	0.6 ± 0.5	0.2 ± 0.1	0 ± 0	0.1 ± 0.1	- - -	- - -	- - -	- - -
X-C12:1 <b>h</b>	0.4 ± 0.4	0.4 ± 0.1	0.7 ± 0.9	0.8 ± 1.1	- - -	- - -	- - -	- - -
Unknown 2 <b>i</b>	1.4 ± 0.3	0.2 ± 0.1	0.8 ± 0.6	0.5 ± 0.5	- - -	- - -	- - -	- - -
4,8-dimethyl-7-nonen-2-one <b>j</b>	28.3 ± 5.7	21.8 ± 2.9	23.3 ± 5.3	24.6 ± 3.1	34.4 ± 4.8	38.2 ± 3.1	37.7 ± 7.6	32.5 ± 5.5
2-Nonanyl acetate <b>k</b>	1 ± 0.6	3.7 ± 0.5	3.4 ± 1.1	4 ± 0.8	5.6 ± 1.4	6.7 ± 1.6	6.7 ± 1.2	5.2 ± 1.1
β-Citronellol, methyl ether <b>l</b>	4.5 ± 2.1	1.0 ± 0.4	1.2 ± 0.8	1.7 ± 0.8	4.1 ± 0.6	3.5 ± 1.1	5.7 ± 2.1	3.8 ± 0.8
2-Phenethyl acetate <b>m</b>	0 ± 0	0.4 ± 0.2	0 ± 0	0.2 ± 0.3	- - -	- - -	- - -	- - -
X-Undecen-2-one <b>n</b>	2.6 ± 0.2	5.5 ± 0.4	9.3 ± 2.9	5.8 ± 1.4	7.1 ± 2.4	7.5 ± 2.8	4.9 ± 1.2	5.7 ± 2.4
X'-Undecen-6-one <b>o</b>	6.7 ± 1.8	2.3 ± 0.7	8.5 ± 2	11.5 ± 2.0	1.3 ± 0.8	0.9 ± 1.3	1.5 ± 1.3	3.5 ± 0.7
X''-Undecen-2-one <b>p</b>	0.2 ± 0.3	0.2 ± 0.1	1.2 ± 0.9	1.5 ± 0.5	1.5 ± 0.5	1.3 ± 0.3	0.3 ± 0.2	1.5 ± 0.4
Undecan-2-one <b>q</b>	2.4 ± 1	6.1 ± 1	7.7 ± 1.6	7.7 ± 2.0	6.9 ± 2.9	8.1 ± 3.1	2.7 ± 0.4	5 ± 1.4
Citronellyl acetate <b>r</b>	1.6 ± 0.8	5.9 ± 2	0.5 ± 0.5	0.7 ± 0.6	5.8 ± 3.8	1.8 ± 1.1	7.9 ± 3.3	11.3 ± 4.9
Unknown 3 <b>s</b>	23 ± 5	15.8 ± 2.2	5.2 ± 1.6	7.2 ± 2.6	21.9 ± 6.1	17.3 ± 5.6	23.7 ± 10	22.4 ± 5.9
Geranyl acetate <b>t</b>	1.2 ± 0.5	1.7 ± 0.4	0.1 ± 0.1	0.1 ± 0.2	- - -	- - -	- - -	- - -
Decane-2,9-dione <b>u</b>	0.2 ± 0.2	0.1 ± 0.2	0.8 ± 0.6	0.6 ± 0.4	- - -	- - -	- - -	- - -
Unknown 4 <b>v</b>	0.2 ± 0.2	0.5 ± 0.1	1.2 ± 0.7	1 ± 0.3	- - -	- - -	- - -	- - -
Isopentyl benzoate <b>w</b>	1.3 ± 0.1	2.4 ± 1	0.0 ± 0	0.3 ± 0.4	- - -	- - -	- - -	- - -
3-Methyl-3-butenyl benzoate <b>x</b>	1.2 ± 0.3	2.0 ± 0.8	0.2 ± 0.2	0.4 ± 0.6	- - -	- - -	- - -	- - -
X-Undecene-2,10-dione <b>y</b>	2 ± 0.6	0.8 ± 0.4	4.5 ± 2.1	4.7 ± 1.8	- - -	- - -	- - -	- - -
Undecane-2,10-dione <b>z</b>	1.6 ± 0.5	0.5 ± 0.3	1.3 ± 0.8	0.4 ± 0.5	- - -	- - -	- - -	- - -

172  
 173 **Table 2:** Chemical composition of the venom glands of *V. v. nigrithorax* queens, foundresses, workers, and pre-  
 174 winterring gynes (percentages ± standard error), which were obtained either via solvent- or fiber-based extraction.  
 175 Experimental treatments: SH, stressed hornet; WH, wounded hornet.

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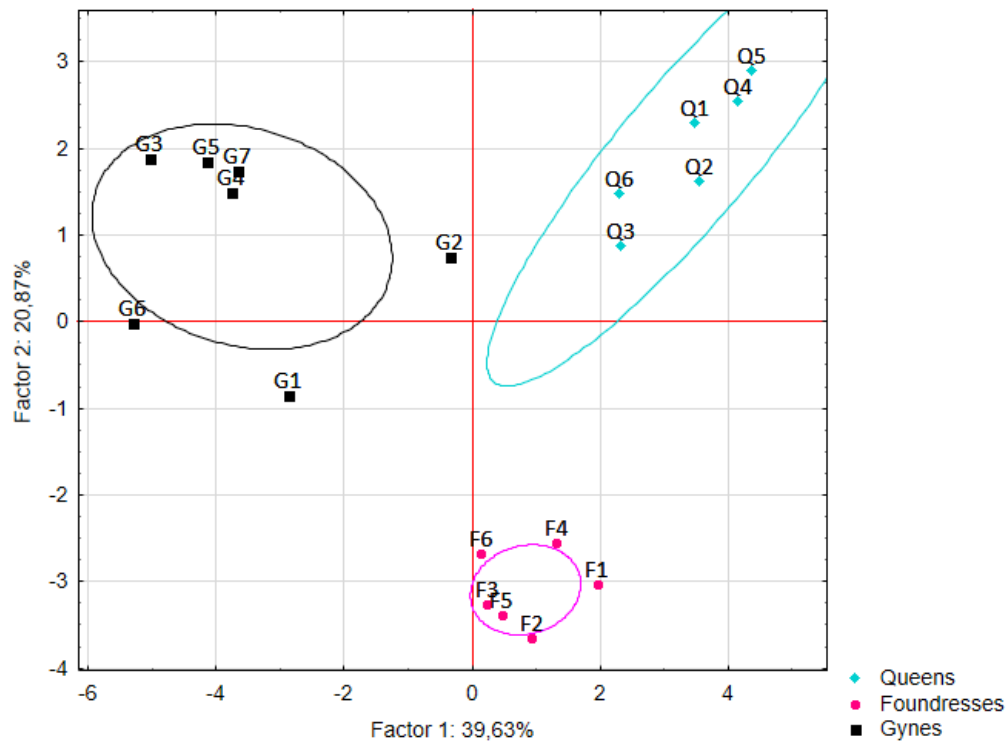
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183 **Fig 3:** (A) Mass spectra for unknown compound s (Kovats retention index = 1310), isolated from the venom gland  
184 of *V. v. nigrithorax*; (B) Mass spectra for the 4,8-dimethyl-1,7-nonadiene standard (Kovats retention index =  
185 1149); (C) combined GC-MS profiles for C10-C18 and 4,8-dimethyl-1,7-nonadiene. The blue arrow represents  
186 the molecule s location (RT), in the gland profile.

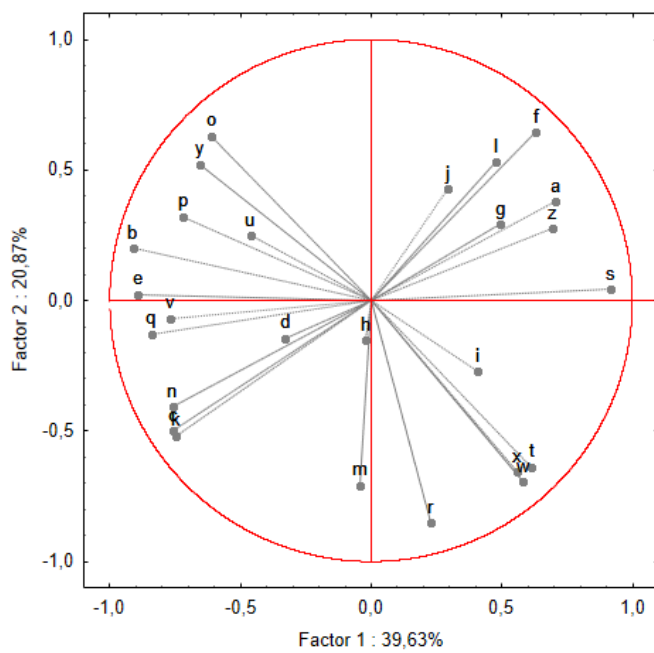
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188 While the 26 compounds were all present in the pre-wintering gynes and foundresses, 3 were absent from  
189 the workers (**g**, **m**, and **w**; Table 2), and 1 was absent from the queens (**m**; Table 2). In the queens and foundresses,  
190 two compounds, 4,8-dimethyl-7-nonen-2-one (**j**) and compound **s**, accounted for more than 58% and 42% of the  
191 overall profile, respectively. In the workers and pre-wintering gynes, the two most common compounds were 4,8-  
192 dimethyl-7-nonen-2-one (**j**) and nonan-2-one (**c**); they accounted for 43% and 42% of the overall profile,  
193 respectively.

194           PCAs were carried out on these 26 compounds. Two rounds of analysis were performed. The first  
195 explored differences among pre-wintering gynes, foundresses, and queens (n = 7, 6, and 6 pools, respectively, Fig.  
196 4). The first and second axes accounted for 40% and 21% of the total variance, respectively, and revealed a marked  
197 separation among the three groups. This clustering was confirmed by the K-means analysis, in which the  
198 foundresses and queens were assigned to distinct groups. Only one pre-wintering gyne (G2) was incorrectly placed  
199 in the queens' group. The queens stood apart from the other two groups based on the presence of three compounds:  
200 Acetophenone (**a**) and two unknown compounds (**f** and **s**). The foundresses formed a distinct group based on the  
201 presence of four compounds: citronellyl acetate (**r**), geranyl acetate (**t**), isopentyl benzoate (**w**), and isoprenyl  
202 benzoate (**x**, *i.e.*, 3-methyl-3-butenyl benzoate). The second round of analysis added profile information for the  
203 workers (n = 15 pools) (Fig. 5). No differences were seen between workers and pre-wintering gynes, a result  
204 confirmed by the K-means analysis resulting in no group separation.

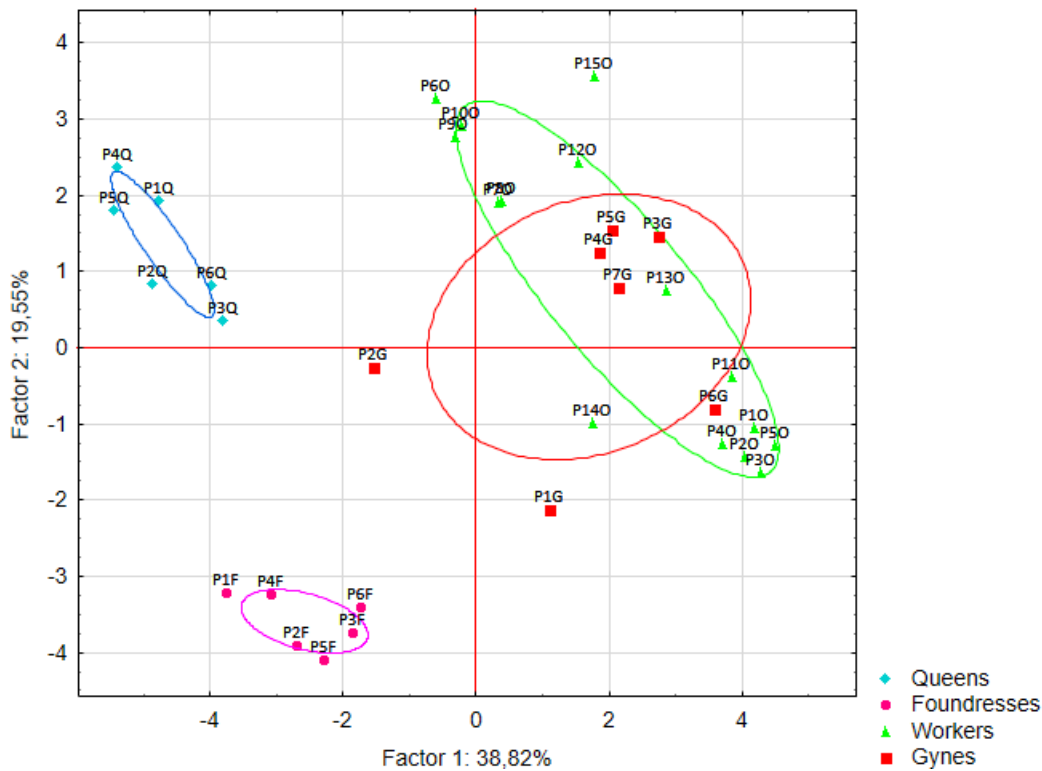


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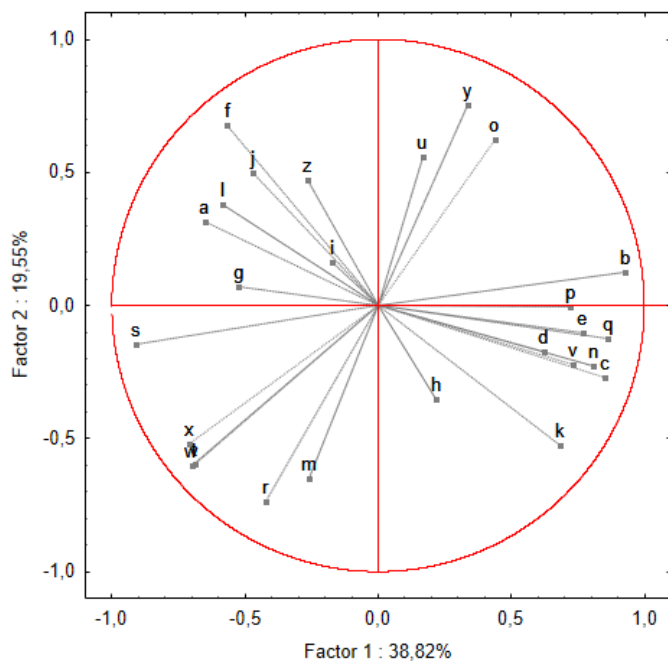


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207 B

208 **Fig. 4:** (A) Principal component analysis results and (B) correlation circle plot for the venom gland  
 209 profiles of queens, foundresses, and pre-wintering gynes. Q: queens (n = 6), F: foundresses (n = 6), and G: pre-  
 210 wintering gynes (n = 7). The ellipses correspond to the 95% confidence interval of the data. Compounds found  
 211 closer to the circumference of the circle convey greater amounts of information.  
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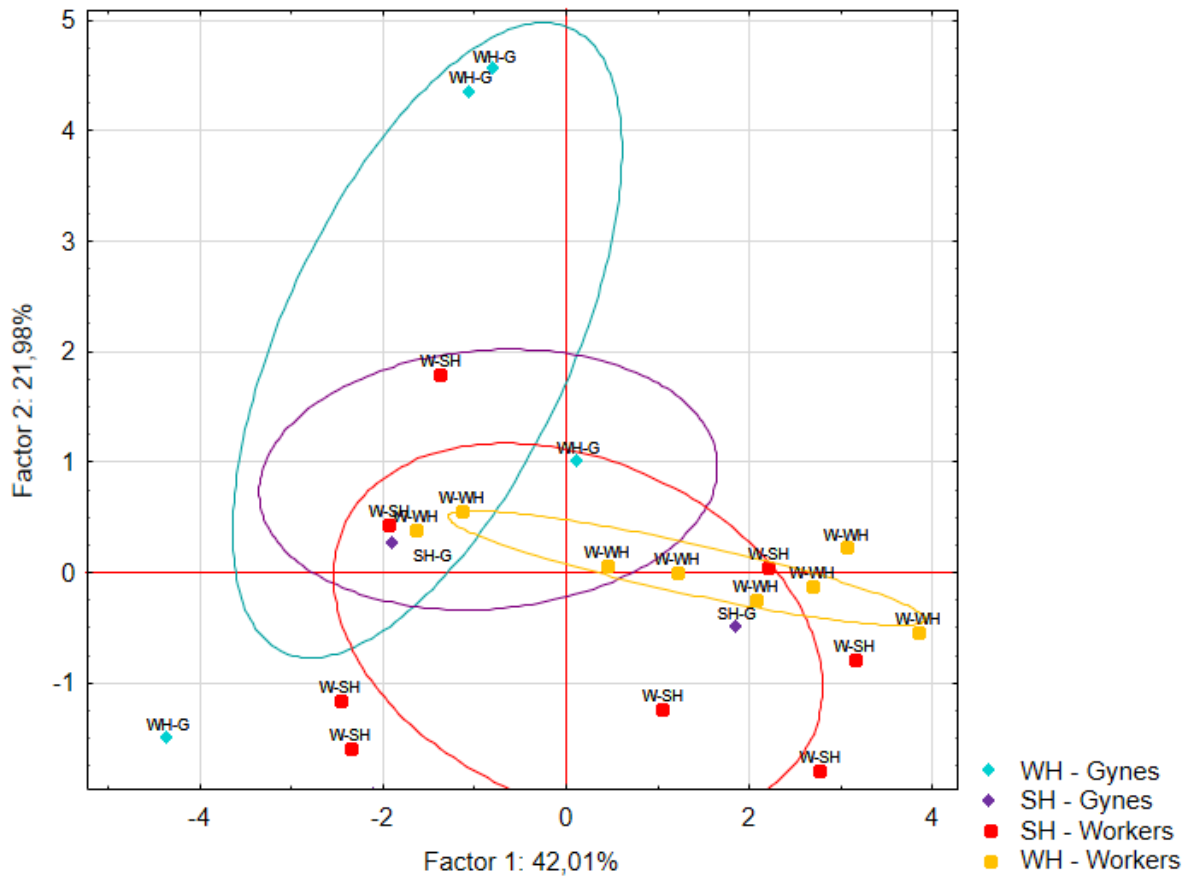
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216 **Fig. 5:** (A) Principal component analysis and (B) correlation circle plot for the venom gland profiles of  
 217 queens, foundresses, workers, and pre-wintering gynes. Q: queens (n = 6), F: foundresses (n = 6), W: workers (n  
 218 = 15), and G: gynes (n = 7). The ellipses indicate the 95% confidence interval of the data.

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220 The fiber-based extractions revealed that the workers and pre-wintering gynes released 13 compounds  
 221 under stressful conditions (SH and WH). All the compounds were present in the solvent extract obtained from the  
 222 venom glands (Table 2). Two compounds accounted for more than 50% of the total profile: 4,8-dimethyl-7-nonen-

223 2-one (j) and the unknown compound s. A PCA was carried out on the profiles of the workers (n = 16 pools) and  
 224 pre-wintering gynes (n = 8 pools) subject to the SH and WH treatments. The first and second axes accounted for  
 225 42% and 22% of the total variance, respectively. The analysis did not show any clear differences based on caste  
 226 or treatment (Fig. 6).  
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 230 **Fig. 6:** Principal component analysis of the 13 compounds identified via PDMS-SPME after workers and pre-  
 231 wintering gynes were experimentally stressed (SH treatment, G: n = 4 and W: n = 8) or wounded (WH treatment,  
 232 G: n = 4 and W: n = 8). The ellipses indicate the 95% confidence interval of the data.

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## DISCUSSION

As insects evolved to form organized societies, it became essential for them to develop communication systems for sharing diverse messages [48]. Eusocial insects most commonly employ chemical communication [49], and it can be challenging to study the cues and signal patterns involved in different behaviors (e.g., recruitment, dispersal, aggressiveness, or aggregation) [30,35,50]. Chemical signals, like pheromones, can rapidly and efficiently disseminate information and are used by all eusocial insects [51,52]. Volatile pheromones are common and may convey information across great distances [33,52,53]. Previous studies identified 19 compounds with chain lengths ranging between 7 and 12 carbons in workers of both *V. v. auraria* and *V. v. nigrithorax* [26,27,42,43]. The majority were ketones, the most phylogenetically widespread class of alarm pheromones [52]. In the study presented here, 9 new compounds were identified among the 13 compounds found, which displayed the same range of chain lengths as in previous research (Table 1). To our knowledge, this is the first time that three of them ( $\beta$ -citronellol methyl ether [l], 2-nonanyl acetate [k], and 2,9-decanedione [u]; Table 2) could be identified as potential semiochemicals or as molecules involved in toxic actions. Other compounds that we observed are known to have communication functions in Hymenoptera. Geranyl acetate (t) is used by three *Bombus* species [54], and citronellyl acetate (r), isoamyl isovalerate (e), and isoamyl senecioate (g) occur in the European hornet *Vespa crabro* [55]. Undecan-2-one (q) occurs in *Dolichovespula maculate* [56] and *Polistes* species [39]. Nonan-2-one (c), 4,8-dimethyl-7-nonen-2-one (j), and undecan-2-one (q) are known to elicit different levels of alarm in *Vespa orientalis* [57]. Thus, these compounds may be common in Vespidae. Some compounds have also been found in other insects. For example, phenethyl acetate (m) is an attractant in *Diachasmimorpha longicaudata* [58] and is part of a pheromone released by the milkweed bug *Neocoryphus bicrucis* [59]. Others, such as isopentyl benzoate (w) and isoprenyl benzoate (x), are volatiles in plants [60,61]. The presence of undecan-2-ol, in contrast to previous research in *V. v. auraria* [34] and *V. v. nigrithorax* [57], was not observed. Since a reference sample of undecan-2-ol was also injected, using the same method, and present in the profile, we are confident that the absence of this compound does not reflect a dehydration or thermolysis artifact in our GC-MS injector.

This same previous research [42,43] discovered that four compounds were common in the venom glands of *V. velutina* workers: nonan-2-one (c), 4,8-dimethyl-7-nonen-2-one (j), X-undecen-2-one (n), and undecan-2-one (q). In our study, however, the main compound observed in all castes was 4,8-dimethyl-7-nonen-2-one (21–28 %, liquid extraction), not nonan-2-one. Nonan-2-one was the second most common compound in workers, pre-wintering gynes, and foundresses but not in queens (relative representation: 4%). Instead, the second and third most common compounds in queens were compound s (23%, Fig 3) and undecen-6-one (o), respectively. In Cheng et al. (2017), *Vespa v. auraria* collected did not display the peak for compound s. Nevertheless, compound s might correspond to the unidentified peak placed after the citronellyl acetate in both *Vespa v. auraria* (seen by [62]) and *V. v. nigrithorax* (seen by [43]). Additionally, compound quantity differed between our work here and that of Cheng et al. (2017). For example, in *V. v. auraria*, Cheng et al. (2017) found that X-undecen-2-one (n) and undecan-2-one (q) were the second and third most common compounds, respectively; in our study, however, they were present at far lower levels (2.6–9.3%, liquid extraction). In short, our results differ quantitatively and qualitatively from those of both Cheng et al. (2017) and Thiéry et al. (2018).



276           Considering that undecan-2-ol was absent from our results and that we observed a different set of common  
277 compounds (i.e., like citronellyl acetate in the French and Spanish hornet populations), it could be that the hornet's  
278 chemical blends are changing at the population scale, as has been seen in some ants [63–65]. Whether such shifts  
279 between and within subspecies could arise from isolation by distance or difference in evolutionary lineages is a  
280 question that merits further exploration.

281           In the venom gland profiles, there were clear differences among the gynes, queens, and foundresses. This  
282 result indicates that the chemical signature of the female venom gland changes over the course of life history: from  
283 pre-wintering gynes (females without nest) to foundresses (mated females with small nest and some eggs) to  
284 queens (mated females with workers). However, no differences between the pre-wintering gynes and workers  
285 profiles were observed. This result shows that pre-wintering gynes start off with profiles similar to those of workers  
286 but that those profiles then change over time. The most obvious change could be seen in compound **s**: its mean  
287 relative levels were 5% in workers versus 7, 15, and 23% in pre-wintering gynes, foundresses, and queens,  
288 respectively. Unfortunately, no match was found for compound **s** in any of the available databases. However, this  
289 compound did have a fragmentation spectrum highly similar to that of citronellyl acetate (**r**): fragments 137 and  
290 152 were present, and fragment 138 was almost completely absent.

291           The differences among the females raise questions about the hornet's chemical ecology. The blend  
292 observed could act as an alarm pheromone for pre-wintering gynes, which occur in their colony of origin and can  
293 play a defensive role alongside workers. However, the queens displayed a different compound ratio, which could  
294 mean that the blend had a more queen-specific function. Indeed, this compound ratio could serve as a specific  
295 signal from the queen, potentially informing the colony's workers that their queen has a problem or is in danger.  
296 Alternatively, it could act as a signal of queen presence and health. Indeed, research on wasps, bees, and ants has  
297 shown that reproductives may chemically communicate their fertility [66]. Consequently, it could be that  
298 compound **s** is a signal generated by mature egg-laying queens. Further study is needed to explore this hypothesis  
299 in *V. v. nigrithorax*.

300           Previous studies identified 12 compounds in the crushed venom glands of workers (*V. v. nigrithorax*;  
301 [43]) and in living hornets (*V. v. auraria*; [42]) using blue PDMS fibers (Supleco). Here, we used SPME and liquid  
302 extracts to characterize the compounds released by the venom glands of *V. velutina* hornets as a result of  
303 experimental stress or wounding. 13 out of 26 compounds in this study were identified in living hornets using red  
304 PDMS fibers (Supleco). We found 4,8-dimethyl-7-nonen-2-one (32–38%; **j**) to be the most common compound,  
305 followed by compound **s**. The results were unaffected by either caste (worker *vs.* pre-wintering gyne) or treatment.  
306 When stressed, both groups of hornets emitted an alarm signal that was the same as the one emitted when facing  
307 a life-threatening event. Eight of the compounds associated with this signal had been previously observed in both  
308 *V. v. auraria* and *V. v. nigrithorax* [42,43]. Recent work by Rodriguez-Flores et al. (2021) identified 8 compounds  
309 in queens (n = 4) and workers (n = 3) using HS-SPME/GC-MS [26]. Among them were nonane-2-one, undecan-  
310 2-one, and 4,8-dimethyl-7-nonen-2-one, which have been seen in all studies on the hornet's chemical ecology; in  
311 contrast, nonanal, decanal, geranyl acetone, and 4,8-dimethyl-1,7-nonadiene were not seen [26]. The differences  
312 between our results and previous research could be explained by SPME fiber type, collection materials, and/or  
313 chromatography methods and machine systems.

314           This study represents an important step towards developing pheromone-based traps. Indeed, the next step  
315 is to identify all the components of *V. v. nigrithorax* pheromones, including compound **s**, which might be important  
316 in queen signaling. A useful approach could be to assess the effects of each major compound, first individually

317 and then in proportionally accurate blends. Indeed, certain compounds may only elicit a strong response when they  
318 occur in blends, as is the case with alarm pheromones in honey bees [67] or sex pheromones in moths [68].  
319 Moreover, the precise compound ratio of blends could be of major importance. It is also essential to test the effects  
320 of any target compounds on bees. Given that alarm pheromones are produced during both defensive and offensive  
321 situations, they could also serve as a kairomone for the hornet's prey. Indeed, bees may detect certain components  
322 of hornet alarm pheromones and modify their behavior accordingly.

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### 329 **Acknowledgments**

330 Thanks to Rémi De La Burgade for the advice and access to the chemistry platform. This study received funding  
331 from the Centre-Val de Loire region, in the form of French regional grants for our FRELON and FRELON 2  
332 projects, as well as from Veto-pharma and the French administrative department of La Manche. We are grateful  
333 to everyone who helped locate the hornets' nests. We would like to thank the chemical ecology platform of the  
334 IRBI (UMR7261 CNRS, University of Tours) for the services and facilities. Our thanks also go out to Ignacio  
335 Ruiz for the help in the field and Jessica Pearce for the English-language editing.

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