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.

Keywords: Asian hornet; sex pheromone; venom volatiles; invasive species; pest management.

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 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 (Rome et al., 2011a; Monceau and Thiéry, 2017), 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 et al., 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

81 has been limiting the development of strategies for selective trapping of sexuals to control the
82 populations of invasive social wasps (Lester and Beggs, 2019)

83 Experimental evidence in other social Vespidae show that males are attracted by putative
84 pheromones derived from nests (Ono et al., 1985; Ono and Sasaki, 1987), receptive females
85 (Sandeman, 1938; Batra, 1980; Keeping et al., 1986, Reed and Landolt, 1990a, b; Cappa et al., 2013,
86 2014; Derstine et al., 2017) or also from other males, which have been in contact with receptive
87 females (Thomas, 1960; Ross, 1983; Keeping et al., 1986). The first demonstration of vespine males
88 attracted by female sex pheromones was reported for *Vespula squamosa* Drury in a wind tunnel (Reed
89 and Landolt, 1990b). More recently, both field and lab trials carried out in New Zealand on the
90 invasive *Vespula vulgaris* Linnaeus showed that females release volatiles, albeit unidentified, which
91 attract conspecific males (Brown et al., 2014). Also, in the European hornet, *V. crabro*, males are
92 attracted by caged reproductive gynes or workers treated with gyne extracts, suggesting the presence
93 of sexual attractants (Spiewok et al., 2006). Finally, a sex pheromone has just been discovered for an
94 Asian subspecies of the yellow-legged hornet, *V. velutina auraria*, distributed in the Chinese province
95 of Yunnan (Nguyen et al., 2006; Takeuchi et al., 2017; Wen et al., 2017). Virgin gynes of this
96 subspecies produce, from the sixth intersegmental sternal glands of their abdomens, a mixture of
97 volatile compounds, where 4-oxo-octanoic acid (4-OOA) and 4-oxo-decanoic acid (4-ODA) are
98 active as male-attracting sex pheromone (Wen et al., 2017).

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

105 Several lines of indirect evidence suggest that mating in the invasive range of *V. velutina*
106 *nigrithorax* could happen inside the nest: firstly, the vast majority of future queens are already mated

107 when leaving the nest (Poidatz et al., 2017), secondly, aggregations of courting males have not been
108 observed in the field (Monceau et al., 2014) differently from the Yunnan subspecies (Wen et al.,
109 2017), and finally, the European population shows a high level of diploid males and inbreeding
110 (Darrouzet et al., 2015).

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

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

132

133 **Materials and Methods**

134 *Sample collection*

135 *V. velutina nigrithorax* males and females originated from laboratory reared combs belonging
136 to 6 different field colonies located at least 9 km apart. Combs were collected in the Liguria region
137 (Italy) in Autumn 2016 and 2017 and then transferred to the laboratory to be maintained under
138 standard conditions (natural daylight cycle, 25° C) in closed cages. Newly emerged adults were daily
139 sexed and transferred into separate male and female exclusive glass cages (size 15x15x15 cm).
140 Hornets were fed *ad libitum* with water and sugar until behavioural bioassays or chemical analyses.
141 This procedure guaranteed for male and female virginity. Males used in behavioural assays were aged
142 between 10 and 20 days post-emergence, in order to have sexually mature males (Poidatz et al., 2017;
143 Cappa et al., 2019). Adult females used to extract venom and sternal gland secretion were older than
144 one week to ensure a full development of their glands (Turillazzi and Bruschini, 2010).

145 In order to assess female phenotypes (gyne or worker), since body size is largely overlapping
146 between castes in *V. velutina nigrithorax* (Rome et al., 2015; Cappa et al., 2019), both weight and fat
147 storage were considered. The presence of abundant fat bodies is a clear character of reproductive
148 females in temperate social vespids where only, or mainly, gynes overwinter (Strassmann et al., 1984;
149 Markiewicz and O'Donnell, 2001; Toth et al., 2009; Cappa et al., 2013; 2019). Adult females used
150 as source of chemical stimuli in behavioural test and chemical analyses killed by freezing and kept at
151 -20°C. Frozen specimens were then thawed for a couple of minutes at room temperature, weighted
152 on a KERN PCB 350-3 scale and then dissected under a Wild M5A stereomicroscope and the
153 presence and abundance of fat bodies was assessed (Monceau et al., 2013, Rome et al., 2015; Cappa
154 et al., 2019). Females of uncertain caste assignment (large females with scarce fat storage or smaller
155 females with fat bodies) were discharged.

156

157 *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.

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 cm² 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

183 filter paper containing the secretion (gynes, N=23; workers, N=23, all females came from the same
184 colony) was immediately used as chemical stimulus in the behavioural assays.
185 For the chemical analyses, we analysed both the secretion of single specimens (3 gynes and 4
186 workers) and the pooled secretion of four specimens after derivatization of the oxyacids possibly
187 present (Wen et al., 2017). Secretion of sternal glands from single specimens was sampled by gently
188 rubbing on the sixth intersegmental sternal gland surface of frozen specimens (gynes, N=3; workers,
189 N=4; all hornets came from the same colony) a 50/30 μm thick
190 Divynilbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) Solid Phase Microextraction
191 Fibre (SPME). Samples containing the pooled secretion of four individuals (pool N=2 both for
192 workers and for gynes) were prepared by collecting the secretion with the tip of an insect pin and
193 transferring it into 2 ml glass vials kept on ice. Sampling was performed under a Wild M5A
194 stereomicroscope.

195

196 *Behavioural assays*

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

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

222 In the bioassays testing venom volatiles attraction, focal male groups (N=26) were presented
223 with one of four stimuli: a blank control (0.5 cm² pentane-washed filter paper), a solvent control
224 dichloromethane (10 µl) absorbed on a 0.5 cm² pentane-washed filter paper, the venom reservoir
225 content of workers (1 worker average reservoir equivalent, ca 1 µl), and the venom reservoir content
226 of gynes (1 gyne average reservoir equivalent ca 0.6 µl each). Both venom stimuli were suspended
227 into 10 µl of dichloromethane and absorbed on a 0.5 cm² pentane-washed filter paper.

228 For the sternal gland secretion bioassay, we adopted a simplified protocol since we aimed to
229 confirm its putative role as sex attractant source previously demonstrated in the Yunnan *V. velutina*
230 population (Wen et al., 2017). Thus, we presented focal groups of males (N=23) with the sixth
231 intersegmental sternal gland secretion of a gyne (N=23) or a worker (N=23) on a 0.3 cm² pentane-
232 washed filter paper (for the secretion collection see above) and a clean pentane-washed 0.3 cm² filter

233 paper as control. The three stimuli (gyne secretion, worker secretion and control) were sequentially
234 presented in a random order.

235 Finally, to test the synthetic pheromone attractiveness, we diluted in dichloromethane a
236 mixture of 4-OOA and 4-ODA in the same ratio of 0.78 used by Wen et al. (2017) in their field
237 bioassays to reach the concentration of 14 µg/µl. Focal groups of males (N=18) were presented with
238 2 µl of the diluted mixture corresponding to a hornet equivalent (28 µg of synthetic pheromone
239 mixture, Wen et al. 2017) on a 0.3 cm² pentane-washed filter paper, and 2 µl of the dichloromethane
240 solvent as control. Behavioural data were analyzed using PAST 3.20 (Hammer et al., 2001).
241 Differences in observed behaviours in response to different stimuli were compared with Friedman
242 test for in the two bioassays testing venom volatiles and sternal gland secretion and with Wilcoxon
243 test in the bioassay testing male response to the synthetic pheromone mixture. Post hoc tests
244 (Wilcoxon signed-ranks tests) were used to assess whether, and where, a significant difference existed
245 between pairs of treatments, using the Bonferroni correction for multiple comparisons with a *P* value
246 of less than α /number of comparisons considered significant. Different males were used in the
247 different bioassays testing venom volatiles, sternal gland secretion and synthetic pheromone mixture.

248

249 *Chemical Analyses*

250 *Venom volatiles*

251 Volatile components of venom from individual hornets (5 gynes and 4 workers, see above)
252 were sampled by means of headspace solid phase micro-extraction (HS-SPME) (Moneti et al., 1997)
253 and analysed by gas chromatography–mass spectrometry (GC–MS). The headspace in the vials
254 containing the venom of each specimen was sampled for 10 min at 45 °C in a block heater using a
255 fibre coated with a 100 µm polydimethylsiloxane (PDMS) phase (Supelco, Inc., Bellefonte, PA,
256 USA). The HS-SPME is a selective extraction method and the use of a non-polar extraction phase
257 (PDMS) may underestimate the presence or abundance of more polar compounds; however,
258 preliminary trials with a more polar phase (polyacrilate, 85 µm), did not give substantially different

259 chromatographic profiles. Analytes were then desorbed in the injection port of a Hewlett Packard
260 (Palo Alto, CA, USA) 5890 gas chromatograph connected to a Hewlett Packard 5971 quadrupole
261 mass spectrometer operating in electronic ionization (EI) at 70 eV. A Rtx-5MS column (Restek,
262 Bellefonte, PA, USA) was used (30 m, 0.25 mm, 0.5 μ m film thickness). The injection port
263 temperature was set at 250 °C and the transfer line at 280 °C; carrier gas was helium (at 12 psi head
264 pressure). The oven temperature was initially set at 45 °C, maintained for 3 min and then increased
265 by 20 °C/min to 200 °C. This temperature was maintained for 14.25 min. Mass spectra were compared
266 with mass spectral electronic libraries (Wiley 275, NIST 2.0), and if not reported in the electronic
267 libraries, they were searched in Pherobase (online Database of Insect Pheromones and
268 Semiochemicals, www.pherobase.com) and in literature through chemical databases (Beilstein
269 Commander 2000; Scifinder Scholar 2004). A mixture of n-alkanes ranging from C9 to C20 was
270 injected under the same conditions in order to calculate the linear retention index of the analytes.
271 Based on the mass spectrum, one peak was putatively identified as citronellyl acetate, a compound
272 previously undescribed for the species. To confirm the identification, citronellyl acetate was
273 chemically synthesized, sampled in HS-SPME and analysed in GC-MS alongside another two
274 samples consisting of the venom volatiles pooled from three *V. velutina* hornets and the venom from
275 the reservoir of a single individual.

276 For each venom total ion chromatogram (TIC), the peak area of each compound was expressed
277 as a percentage of the total area given by all the compounds present. As some compounds were
278 represented in only a few samples, we only considered compounds if they met the three following
279 criteria: relative abundance within individual profile greater than 1%, the compound was present in
280 at least half of the samples of gynes or workers, the compound was present in at least one third of all
281 samples. The compounds selected according to these criteria accounted on average for 94.2 and 95.4
282 % of the total amount of compounds at the individual level.

283 To evaluate overall differences in relative composition of volatiles profile between
284 phenotypes we performed a non-metric multidimensional scaling (hereafter nMDS) using all selected

285 compounds and Euclidean distances. We also performed K-means clustering to evaluate the
286 possibility to identify two clusters corresponding to the two phenotypes. This descriptive approach
287 was confirmed by statistically comparing within-phenotype (for both gynes and workers) and
288 between-phenotype chemical distances (calculated as Euclidean distances) with a Kruskal Wallis
289 test. To understand if any of the compounds was differentially expressed between the two phenotypes
290 we compared relative amount of each single compound between the two phenotypes with a Mann
291 Whitney test. Chemical data were analyzed using SPSS 20.0 (IBM, 2011).

292

293 *Synthesis*

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

300

301 *Sternal gland secretion*

302 SPME fibers used to sample the secretion from single specimens were desorbed for three
303 minutes in the injection port (250 °C) of a 7820 GC-5977B MSD Agilent Technologies instrument.
304 One µl of a 700 ng/µl dichloromethane solution containing the two synthetic ketoacids (4-OOA and
305 4-ODA, 1:2) identified as putative sex pheromones in the sternal gland secretion (Wen et al. 2017)
306 was injected as reference standards.

307 To improve chromatography of the several fatty acids observed in the single specimen analysis,
308 samples prepared from the secretion of more hornets, were silanized and the results compared with
309 the silanized standards of 4-oxo-octanoic and 4-oxo-decanoic acids. Twenty µl of a 99:1 solution of
310 N-methyl-N-(trimethylsilyl) trifluoroacetamide and chlorotrimethylsilane were added to the sample

311 vials that were maintained at 60 °C for 30 min. One µl of each derivatized sample was analysed on
312 the same instrument as described above.

313 Ten µl of the standard solution of ketoacids, were inserted into a 2 ml vials and derivatized as
314 for sternal gland secretion, after solvent was evaporated under a gentle nitrogen stream. In all the
315 analyses the injection port was set at 280 °C. Oven was equipped with an Agilent Technologies
316 19091S-433UI capillary column (30 m x 0.250 mm, 0.25 µm phase thickness, 95%
317 polydimethylsiloxane, 5% phenyl); helium was used as carrier gas (1 ml/min). The oven temperature
318 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;
319 final temperature was maintained for 5.33 min. Masses were acquired in the range from 50 to 550
320 m/z. SPME samples were analysed splitless, while liquid samples were analysed in split mode (1:10).

321

322 **Results**

323 *Behavioural assays*

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

330 Antennation scores differed among treatments in bioassays testing sternal gland secretions
331 (Friedman test, chi-square=5.152, df=2, p=0.021, N=23) (Fig. 1b). Males showed a higher interest
332 towards the sternal gland secretion of reproductive gynes with respect to blank controls (gynes vs
333 control: Wilcoxon post-hoc comparisons, W=114, N=23, p=0.001, Bonferroni-adjusted α =0.017).
334 Males appeared to respond also to the secretion of workers since there was no difference in the
335 antennation rate towards the sternal gland secretion of the two castes (Wilcoxon post-hoc
336 comparisons, W=83 N=23, p=0.453). However, the male interest towards the secretion of workers

337 was somehow lower compared to the gynes' secretion since the difference in antennation rate between
338 workers' secretion and controls was not significant after the Bonferroni correction (Wilcoxon post-
339 hoc comparisons, $W=67,5$, $N=23$, $p=0.023$, Bonferroni-adjusted $\alpha=0.017$) (Fig. 1b). In the trials,
340 males did not show a different rate of activity (i.e. number of males walking, flying, ventilating or
341 grooming at each check) (Friedman test, chi-square=3.413, $df=2$, $p=0.148$, $N=23$) and there was no
342 difference in the number of males in proximity of the stimulus (i.e. males on the 6 cm diameter steel
343 mesh underneath the stimulus) at every check (Friedman test, chi-square=3.283, $df=2$, $p=0.156$,
344 $N=23$), which means that despite being active and in the stimulus proximity, only the secretion
345 elicited a higher antennation response in males.

346 In the trials testing the synthetic pheromone attractiveness there were no differences either in
347 the antennation score (Wilcoxon test, $W=4$, $N=18$, $p=0.564$) or in the male activity rate (Wilcoxon
348 test, $W=97,5$, $N=18$, $p=0.335$) and in the stimulus proximity (Wilcoxon Signed-Rank test, $W=64$,
349 $N=18$, $p=0.484$) compared to the solvent control. The only difference observed was in the rate of male
350 ventilation, which increased when the synthetic pheromone mixture was presented compared to
351 controls (Wilcoxon test, $W=96.5$, $N=18$, $p=0.037$).

352

353 *Venom volatiles differences between castes*

354 Twelve major peaks were found in the chromatograms of venom volatiles (Fig. 2). The mass spectra
355 of 5 peaks (1, 2, 3, 5, 9) fitted with aliphatic 2-ketones, according to the presence of a strong ion at
356 m/z 58 expected on the base of McLafferty rearrangement. Peaks 2 and 9 corresponded respectively
357 to 2-nonanone and 2-undecanone. Peak 4 was an unidentified compound whose molecular weight
358 matches with the formula $C_{10}H_{20}O$. The spectrum of peak 1 corresponded to 8-nonen-2-one, which
359 coeluted with an unidentified compound. Peak 5 was identified as 4,8-dimethylnon-7-en-2-one. All
360 these compounds have already been reported by Cheng et al. (2016) and Thiery et al. (2018)
361 respectively for the native and French invasive populations. Spectra of peaks 6, 8, 10 and 11
362 corresponded to acetates of aliphatic alcohols, which, on the contrary have not been previously

363 reported. On the basis of mass spectrum, retention index and comparison with a standard, peak 10
364 was identified as citronellyl acetate; surprisingly, this peak, which is the second for intensity in our
365 chromatograms, has not been reported before. Some samples also presented geranyl acetate. Only
366 peaks 2, 5, 7, 8, 9, 10, 12 were considered in the comparison between the worker and the gyne
367 secretion.

368 Chemical composition of venom volatiles did not differ between workers and gynes (Fig. 2). No
369 compound was found to be exclusively present in one caste. nMDS showed that chemical composition
370 of the two castes is clearly overlapping (nMDS, Euclidean distances, stress=0.0, r²: axe 1:0.966; axe
371 2:0.050). K-mean identified two clusters, which were not correspondent to the two phenotypes,
372 misclassifying more than half of the individuals (uncorrect classification: gynes=60%,
373 workers=50%). Chemical distance within group (worker-worker or gyne-gyne) was not different that
374 chemical distance between groups (worker-gyne) (Euclidean Distances, Kruskal Wallis test, chi-
375 square=0.914, p=0.633 N₁=6, N₂=10, N₃=20). Lack of difference was also confirmed at the level of
376 individual peaks: none of the 11 major peaks of the venom volatiles profile was differently expressed
377 between the two castes.

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379 *Sternal glands differences between castes*

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

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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 (Thiery et al., 2018) and known for *Vespa orientalis* Linnaeus where they elicit the alarm behaviour (Saslavsky 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; Ono et al., 1985; 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 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), 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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468

469 **Authors contributions**

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

473

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479

480 **Declarations of interest:** none

481

482 **Ethical approval**

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

485

486 **References**

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745 Legends
 746

747 Fig. 1. Behavioural results from bioassays testing the antennation rate of male *Vespa velutina*
 748 *nigrithorax* presented with venom volatiles (a) and sternal gland secretions (b) extracted from
 749 conspecific females.
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751

752 Fig. 2. Total ion chromatogram of venom volatiles extracted from *Vespa velutina nigrithorax* gynes
 753 (a) and workers (b). Numbers correspond to compounds quoted in the text.
 754

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