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Female body size, weight and fat storage rather than nestmateship determine male attraction in the invasive yellow-legged hornet *Vespa velutina nigrithorax*

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In the early stage of the invasion process, alien species may face costs linked to pioneer effect due to genetic bottleneck, drift and the consequential inbreeding depression. Thus, introduced species that show an immediate exponential growth in their invasive population should have some mechanisms to reduce such costs minimizing the chance of inbreeding or allowing them to cope with them. The yellow-legged hornet *Vespa velutina nigrithorax* has been spotted in France in 2004; since then, the species has been invading Europe with a relentless pace. In their native range, males and reproductive females of a Chinese non-invasive sub-species of *V. velutina* seem to leave their nests to search for unrelated partners. However, previous studies showed a low genetic diversity and a high rate of diploid males in colonies of the invasive population, suggesting that mating could occur inside nests, where males should be able to discriminate between reproductive gynes and sterile workers. Here, we used laboratory behavioural assays to investigate the mating preferences of yellow-legged hornet males from the recent invasive population in Italy. We assessed the importance of nestmateship and female morpho-physiological traits, likely indicators of caste, in determining male attraction towards potential partners. Our results demonstrate that males are more attracted to bigger females with more abundant fat storage, good indicators of female reproductive caste in wasps, regardless of nestmateship. Our findings represent a first step in understanding the reproductive biology of *V. velutina nigrithorax* in its invasive range, providing a framework for future research in the field to prevent or reduce the species expansion.

28 KEY WORDS: invasive alien species, nestmate recognition, reproductive biology, Asian hornet.

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32

33 Running head: Male reproductive attraction in Asian hornets

34

35 Highlights

- 36 - We investigated mating preferences of invasive hornet males under laboratory standard conditions
- 37 - Males attraction was influenced by female caste, with more attraction toward reproductive females
- 38 - Unexpectedly, males were not more attracted by non-nestmate females than nestmate ones.
- 39 - Our results might explain the high level of inbreeding found in invasive populations of *V. velutina*

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INTRODUCTION

42 Invasive species and their management have represented a major issue in recent years
43 (Chapin et al. 2000; Lowe et al. 2000; Mooney & Cleland 2001). As the volume of global trade has
44 expanded enormously in the last decades, so has the risk of invasive alien species (IAS) reaching
45 new regions with dramatic consequences in terms of biodiversity, ecosystem services' loss and
46 economic or public health issues (Lonsdale 1999; Mooney & Cleland 2001; Levine & D'antonio
47 2003; Lockwood et al. 2005; Pejchar & Mooney 2009). Thus, it is important to understand the
48 processes that promote and accompany the spread of introduced IAS to find sustainable
49 management solutions (Sakai et al. 2001; Lee 2002; Allendorf & Lundquist 2003; Mehta et al.
50 2007; Pyšek & Richardson 2010).

51 The invasion process can start with either a single introduction or multiple introductions
52 over time of a scant number of individuals able to endure sudden habitat transitions (Lee 2002).

53 Because of their low number, introduced individuals might likely face reproduction via inbreeding,
54 which could strongly affect their fitness by allowing for the expression of deleterious recessive
55 alleles and decreasing allelic diversity within the gene pool in the emerging population (i.e.
56 inbreeding depression) (Fonseca et al. 2000; Tsutsui et al. 2000; Rasner et al. 2004). Thus, in order
57 to be successful in their invasion, IAS should present effective mechanisms to either avoid or
58 minimize inbreeding, or be able to cope with its costs (Sakai et al. 2001; Lee 2002; Frakham 2005).
59 Unfortunately, the mating behaviour in IAS pioneer populations is relatively understudied, as
60 research often focuses on other aspects of the invasion process such as population genetics related
61 to the rapid spread or IAS impact in the invaded range (Sakai et al. 2001; Lee 2002; Davidson et al.
62 2011).

63 Eusocial Hymenoptera are especially successful IAS because of their reproductive strategies
64 and social habit (Moller 1996; Hingston & McQuillan 1999; Chapman & Bourke 2001; Holway et
65 al. 2002; Paini & Dale Roberts 2005; Inoue et al. 2008; Beggs et al. 2011). A single mated queen
66 can found a new colony and its social organization, with a considerable number of sterile workers
67 engaged in rearing and defending the immature brood and the production of a high number of
68 reproductive individuals each season, can favour the rapid establishment of a new population
69 (Moller 1996; Holway et al. 2002; Beggs et al. 2011).

70 However, small colonizing populations may face difficulties due to the production of
71 genetic 'misfits' known as diploid males (Whiting 1933; Liebert et al. 2010), which pose fitness
72 costs to the colony because they consume resources without contributing to colony productivity,
73 and are functionally sterile (Liebert et al. 2004). Diploid males are produced because of the
74 Hymenopteran complementary sex determination (CSD) mechanism, which requires heterozygosity
75 at sex-determining loci to produce normal diploid females (Crozier 1971; van Wilgenburg et al.
76 2006). Homozygosity at these loci results in diploid males instead of females (Whiting 1933, 1943).
77 Individuals in an incipient invasive population may at first have limited or no access to unrelated
78 partners and the fitness costs of not mating is likely to be greater than producing a reduced worker

79 brood alongside diploid males (Liebert et al. 2010). Thus, for individuals may be advantageous to
80 have a low acceptance threshold for inbreeding avoidance when choosing their potential mates
81 (Starks et al. 1998a, 1998b; Liebert et al. 2010) and this characteristic could contribute to the
82 success of invasive species.

83 Among the IAS which have recently invaded Europe, one causing major concern is
84 undoubtedly the Asian yellow-legged hornet, *Vespa velutina nigrithorax* (Monceau et al. 2014).
85 The alien hornet was accidentally introduced into Europe from China probably in a single event
86 (Arca 2012), and was first observed in 2004 in South-West France (Rortais et al. 2010; Monceau et
87 al. 2014). The species poses serious concerns both from the ecological and economic point of view,
88 with its impact on both wild and managed pollinators populations as well as on native competitive
89 species (Shah & Shah 1991; Tan et al. 2007; Gallai et al. 2009; Villemant et al. 2011; Cini et al.
90 2018), and for public health, since the hornet is adapted to nest in urban environments (Choi et al.
91 2012) and its sting can induce severe allergic reactions, resulting in organ failure and death (Choi et
92 al. 2012; Liu et al. 2015). Thus, it is of crucial importance to know the biology of the invader in
93 order to adopt the most successful strategies for managing it with the lowest impact on native
94 species communities (Mehta et al. 2007; Beggs et al. 2011).

95 To date, however, scarce information is available on the reproductive behaviour of *V.*
96 *velutina nigrithorax* both in its native and invasive range (Monceau et al. 2014). In the invasive
97 populations it has been hypothesized that mating could occur inside the nest because of the high
98 rate of diploid males and inbreeding in the European population (Monceau et al. 2014; Darrouzet et
99 al. 2015). Moreover, aggregations of courting males, observed in other Vespinae species (Matsuura
100 & Yamane 1990; Ross & Carpenter 1991; Wen et al. 2017) have never been described in the
101 invasive population of *V. velutina nigrithorax* (Monceau et al. 2014; Darrouzet et al. 2015),
102 although males of a different Asian subspecies of *V. velutina* distributed in Yunnan are attracted in
103 the field by a volatile sex pheromone produced by the sixth intersegmental sternal glands of virgin
104 gynes (i. e. reproductive females) (Wen et al. 2017).

105 Here, we use behavioural bioassays in the laboratory to investigate the first phase of the
106 reproductive behaviour of the invasive *V. velutina nigrithorax*, by focusing on the male attraction
107 towards females to assess the potential effect of nestmateship and physiological features (weight,
108 body size, fat stores) linked to caste in the male search for a potential partner. Reproductive gynes
109 start to emerge in Autumn, after the emergence of males (Monceau et al. 2014; Darrouzet et al.
110 2015; Rome et al. 2015), while workers are still present inside the nest. Thus, males are likely to
111 encounter and interact with female nestmates of both castes inside their colonies. Since mating with
112 a related nestmate female may be detrimental for males and the two female caste greatly differ in
113 their reproductive potential, with only reproductive gynes providing males with a return in terms of
114 fitness, we might expect *V. velutina nigrothorax* males to prefer non-nestmate females over
115 nestmates and to distinguish between female castes preferring bigger reproductive gynes compared
116 to sterile workers (Liebert et al. 2010; Cappa et al. 2013; Beani et al. 2014).

117 Given the recent arrival and rapid spread of the species starting from a very low number of
118 individuals (Arca 2012), disentangling the variables influencing the mating behaviour of the
119 yellow-legged hornet might provide useful insights into the mechanisms regulating reproduction in
120 an alien species in the first phases of the invasion process, which, hopefully, might help in
121 preventing or reducing its spread.

122

123 MATERIALS AND METHODS

124 *Sample collection*

125 *V. velutina nigrithorax* males and females issued from laboratory reared combs belonging to
126 four different field colonies located at least 9 Km apart. Combs were collected in Liguria region in
127 Autumn 2015 and 2016. Combs were transferred to the laboratory and maintained under standard
128 laboratory condition (natural daylight cycle, 25 °C) in closed cages. Everyday newly emerged
129 adults were sexed and transferred into separate male and females exclusive glass cages (size 15 ×
130 15 × 15 cm) where they were fed with ad libitum water and sugar until behavioural tests. Each cage

131 contained a comb fragment (around 20 empty cells) from the hornets' nest of origin to allow the
132 newly emerged individuals to acquire the chemical cues and form a template for nestmate
133 recognition as occurs in other social wasps (Gamboa et al. 1986; Signorotti et al. 2014). Daily
134 removal of newly emerged hornets guaranteed male and female virginity as well as knowledge of
135 adult age.

136

137 *Behavioural assays*

138 We assessed male attraction toward females by comparing interest of groups of focal males
139 towards different stimuli: (a) nestmate female, (b) non-nestmate female, (c) non-nestmate male as
140 control. Focal male groups were transferred to a transparent plastic experimental cage (size 20 × 13
141 × 12 cm). Cages had a round Ø 6 cm steel mesh (1.5 × 1.5 mm) at the centre of the top roof, 3 cm
142 from the cage sides. Groups consisted of five nestmate males, aged between 10 and 20 days, in
143 order to have sexually mature males (see Poidatz et al. 2017). After 10 min of habituation, the
144 stimulus, i.e. female or male hornet, was introduced in a Ø 2.5 × 5 cm plastic tube over the mesh
145 and the trial started. Stimuli were free to move within the tube during the trial and mesh density (1.5
146 × 1.5 mm) allowed male antennae to stick through and directly come into contact with the stimulus
147 body on the other side of the screen. Focal males were presented with one of three stimuli (a, b or
148 c) and their antennation rate under the stimulus was evaluated. We chose antennation as a clear sign
149 of male interest towards the presented stimulus (Cappa et al. 2013). All the individuals used as
150 stimuli (N = 56: 15 males, 41 females) were apparently healthy when tested. Males were used from
151 1 to 3 times; females used from 1 to 5 times.

152 As an index of male interest towards the different stimuli, we recorded the number of males
153 antennating in the Ø 2.5 cm circle of wire mesh under the stimulus every minute for 10 min. An
154 (antennation score, AS: total number of males observed antennating during the 10-min trial as the
155 sum of antennating males at each of the 10 checks per trial; AS score could range from 0, no male
156 antennating during checks, to 50, all five males antennating at each check). After 10 min the

157 stimulus was removed. Trials were performed during the central hours of the day (11 am—3 pm)
158 when males were more active, in October, when both workers and reproductive gynes are present in
159 the nests (Monceau et al. 2014; Rome et al. 2015). Overall we tested 29 five-male groups,
160 belonging to 3 colonies, for a total of 172 trials of 10 min. Each group was tested in a random order
161 with all the stimuli.

162

163 *Female caste assessment*

164 After behavioural trials, females used as stimuli (N = 41) were weighted on KERN PCB
165 350-3 scale, dissected under a Wild M5A stereomicroscope and the presence of fat bodies was
166 assessed. In *V. velutina* female caste is hard to recognize and morphological differences between
167 castes have been expressed in various ways (Rome et al. 2015; Pérez-de-Heredia et al. 2017). Some
168 authors have reported differences linked to the capability of gynes to overwinter using female
169 weight, both wet and dry, and fat storage to distinguish between workers and gynes (Monceau et al.
170 2014; Rome et al. 2015). Also the mesoscutum width (MW), from tegula to tegula. is often used as
171 parameter to discriminate castes in some Vespidae species (Noll et al. 2004; Felippotti et al. 2009;
172 Felippotti et al. 2010; Pérez-de-Heredia et al. 2017). Given the relative degree of uncertainty linked
173 to each single measure, we decided to combine different parameters (see below) by using weight
174 and fat storage alongside MW (index of overall linear size) of each female stimulus as potential
175 indicators of caste (Noll & Zucchi 2002; Ohl & Thiele 2007; Beani et al. 2011; Pérez-de-Heredia et
176 al. 2017).

177

178 *Statistical analysis*

179 In order to account for the non-independence of data we used a generalized estimating
180 equations (GEE) approach, which extends the generalized linear model in order to allow the
181 analysis of correlated observations, such as occurs for clustered data (Burton et al 1998). Moreover

182 GEE are robust even when the error structure model is unknown and/or misspecified, and are more
183 relaxed on distributional assumptions (Overall & Tonidandel 2004; Hubbard et al. 2010). For both
184 GEEs, we selected the final model on the basis of the “quasi-likelihood under the independence
185 model” criterion QIC, by choosing the model parameters that provided the smallest QIC (Pan
186 2001).

187 We first assessed whether male were more attracted by females (i.e. higher number of
188 antennations below female stimulus) than by male stimuli. We did it by using a GEE with the
189 following parameters: antennation score as dependent variable; Negative binomial distribution with
190 log link function; independent working correlation matrix; fixed effect: stimulus sex; subject effect:
191 focal male group, stimulus identity, and nest trial (repeated trials for the different focal male
192 groups) as intra-subject effect.

193 We then focused only on trials with female stimuli to assess the importance of individual
194 weight, mesoscutum width, presence/absence of fat bodies and nestmateship (i.e. whether the
195 stimulus came from the same colony of the focal males). As individual weight, mesoscutum width,
196 presence/absence of fat bodies were positively correlated (Pearson correlation test, Weight X
197 Mesoscutum width: $r = 0.862$, $P < 0.001$, $n = 37$; Weight X Fat body: $r = 0.786$, $P < 0.001$, $n = 37$;
198 Mesoscutum width X Fat body: $r = 0.724$, $P < 0.001$, $n = 37$; Fig. 1) we used principal component
199 analysis to create a single predictor explaining most variance of the three original individual
200 features (86.1% of variance explained, Kaiser-Meyer-Olkin Measure of Sampling Adequacy =
201 0.727) retaining the first principal components (hereafter female physical condition, FPC). Weight,
202 mesoscutum width, presence/absence of fat bodies had loadings of 0.953, 0.930, 0.899
203 respectively). We used the following model parameters in GEE: antennation score as dependent
204 variable; Negative binomial distribution with log link function; independent working correlation
205 matrix; fixed effect: female physical condition, nestmateship and the interaction between
206 nestmateship and female physical condition; subject effect: focal male group, stimulus identity and

207 nest trial as intra-subject effect. All analyses used a model-based estimator and a type III analysis.
208 Statistical Analyses were performed in SPSS 20.0 (SPSS 2011).

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RESULTS

211 Overall antennation response was rather low, with at least one antennation present only in
212 the 34 % of the tests. Antennation score was higher when the stimulus was female than when it was
213 a male (Wald $\chi^2 = 21.111$, $df = 1$, $P < 0.001$, estimated marginal means \pm SE: Female stimuli 2.212
214 ± 0.220 ; male stimuli: 0.292 ± 0.125). When considering only trials with female stimuli,
215 antennation score was significantly affected by female physical condition (Wald $\chi^2 = 69.329$, $df = 1$,
216 $P = 0.002$) with heavier, larger females with fat bodies evoking a higher antennation response (Fig.
217 2). Neither nestmateship nor the interaction between nestmateship and female physical condition
218 were significant (respectively Wald $\chi^2 = 1.688$, $df = 1$, $P = 0.194$; Wald $\chi^2 = 2.206$, $df = 1$, $P =$
219 0.137).

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DISCUSSION

222 Our results demonstrated that males respond differentially to the presented stimuli showing
223 a higher interest towards those females which could provide the highest potential return in terms of
224 fitness, regardless of nestmateship.

225 As expected, males in our laboratory bioassays were more attracted to females than males,
226 preferring bigger heavier females, with more abundant fat storage, compared to smaller ones.
227 Vespinae wasps usually show morphological differences between female castes, with reproductive
228 females being larger than workers (Jeanne & Suryanarayanan 2011). However, not all species
229 present the same degree of caste differentiation. In the genus *Vespa* some species, such as *Vespa*
230 *mandarina*, *V. affinis*, *V. crabro* or *V. simillima*, have castes characterized by clear size separation.
231 By contrast, hornets like *V. tropica* and *V. analis*, show an overlap of caste sizes (Matsuura &
232 Yamane 1990). Also in *V. velutina nigrithorax*, female caste is hard to recognize (Pérez-de-Heredia

233 et al. 2017), however, as in other temperate social wasps (Spradberry 1973; Strassmann et al 1984;
234 O'Donnell 1998; Toth et al. 2009; Perrard et al. 2012) reproductive gynes, that are the only females
235 surviving winter, are usually bigger and show more abundant fat bodies, whereas smaller workers,
236 that do not overwinter, have very scarce or absent fat bodies. Female body size, weight and fat
237 storage appear therefore as reliable indicators of female caste (Strassmann et al 1984; Hunt et al.
238 2007, 2010; Cervo et al. 2008; Toth et al. 2009). Thus, the observed male preference for bigger
239 heavier females, with more abundant fat storages, might due to the fact that such females are the
240 more likely to survive the winter diapause and found a new colony the following season, as in other
241 Vespidae species (Hunt et al. 2007, 2010; Cervo et al. 2008), and we may expect males to have
242 evolved accurate systems to detect the most suitable partners. Moreover, it seems likely that
243 reproductive females could actively signal their condition, while there might be a lack of male-
244 attractive signal production by non-reproductive workers (Wen et al. 2017); thus, variability in
245 female signal production may underlies the difference in male interest response towards the two
246 female castes.

247 Due to the conditions of our laboratory set-up, we were able to highlight mainly short-range
248 attractiveness. The observed male attraction towards putative reproductive females could be due to
249 short-range chemical cues, such as cuticular hydrocarbons (CHCs), which can be perceived at
250 distances of few cm (Ferveur 2005; Saïd et al. 2005). It is possible that males walking around the
251 cage could sense the cuticular blend of receptive females inside the tubes through the separating
252 mesh. Indeed, CHCs of *V. velutina nigrithorax* differ according to caste and sex (Gévar et al. 2017).
253 Also in the European hornet, *Vespa crabro*, males are attracted by caged reproductive gynes or
254 workers treated with gynes extracts, suggesting the cuticular alkenes and methyl-branched
255 compounds, different between the two castes, as short-range sexual attractants (Spiewok et al.
256 2006).

257 Contrary to expectations, our males did not show any preference for non-nestmate females
258 over nestmates ones. This lack of preference together with the alleged intra-nest mating could

259 explain the very low genetic diversity and high level of male diploidy observed inside *V. velutina*
260 *nigrithorax* colonies in the European invasive range (Monceau et al. 2014; Darrouzet et al. 2015). If
261 mating takes place, at least in part, inside the nest, males may rely on CHCs as chemical cues to
262 identify receptive females. Such hypothesis is corroborated by the fact that yellow-legged hornet
263 males present an olfactory subsystem for CHCs perception similar to females (Couto et al. 2017).
264 However, also long-range attractants, such as the putative sex pheromone recently identified in the
265 other *V. velutina* Asian subspecies (Wen et al. 2017), could be at work in the attraction process. In
266 fact, males might leave the nest in order to find receptive gynes attracting them away from their
267 nests via sex pheromones (Wen et al. 2017). Thus, males would not need to discriminate female
268 caste and nestmateship in order to find the most suitable partner. Although we did not observed any
269 clear sign of immediate attraction towards the preferred females, which could be expected in the
270 case of rapidly spreading volatile sex attractants, it is possible that our laboratory setting may have
271 influenced the results. Indeed, both female and male hornets in our behavioural assays emerged in
272 the lab and they did not experience field conditions. This might have had an impact on their sexual
273 behaviour, which might require other triggers that we were not able to reproduce under laboratory
274 conditions, especially if males leave the nest in search for receptive females emitting sex
275 pheromones (Wen et al. 2017). Finally, inbreeding avoidance could also be the result of a separate
276 decision that occurs after attraction. Males might be attracted via a long-distance sex pheromone,
277 but then copulation could be inhibited by nestmate chemical or behavioural cues upon the initiation
278 of mating behaviour, therefore avoiding inbreeding. Although males were able to contact the body
279 of the stimuli through the mesh, they could not directly interact with them, it is therefore possible
280 that behavioural interactions between the two potential partners might prevent or favour successful
281 copulations (e.g. females might actively reject mating attempts from nestmate males).

282 Overall, our findings deepen the knowledge on the reproductive behaviour of this invasive
283 species investigating for the first time the extent of male sexual preference by *V. velutina*
284 *nigrithorax* males. Our results show that males from an invasive population of *V. velutina*

285 *nigrithorax* do not seem capable of nestmate recognition in a sexual context. We might hypothesize
286 that in the invasive population the chemical distance among nests' signature mixtures, although
287 present (Gévar et al. 2017) may be less marked than in the native population because of the high
288 rate of inbreeding, not providing males with the cues needed for nestmate discrimination. Such
289 hypothesis, however, is unlikely, since the chemical signature of the invasive population of *V.*
290 *velutina nigrithorax* is quite heterogeneous with each colony, caste, and gender characterized by a
291 specific CHC profile (Gévar et al. 2017). Alternatively males could have not evolved the ability to
292 recognize nestmates when choosing a mate in their invasive range: in native populations, nestmate
293 recognition at natural mating sites may not be of great importance due to a lower probability of
294 encountering relatives in the field where females seem to attract males from a distance via sex
295 pheromones (Wen et al. 2017). However, the absence of inbreeding avoidance by males and the
296 consequent costs could be counterbalanced by the relative degree of polyandry recorded in *V.*
297 *velutina nigrothorax* queens, which could compensate the low genetic diversity due to the single
298 introduction event and inbreeding inside nests (Arca 2012; Monceau et al. 2014). Comparative
299 studies examining acceptance thresholds for mating with relatives and chemical signature
300 differences among colonies across native and invasive populations would help illuminate whether
301 colonizing populations do indeed shift their mate acceptance thresholds in a new environment.
302 Overall, our findings, although limited by the laboratory settings, give new insights in the
303 understanding of the largely unknown reproductive biology of the yellow-legged hornet in its
304 invasive range. Further research should aim at investigating male attraction also in the field, even if
305 the task may not be easy since current management practice call for an early detection and
306 destruction of nests.

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ACKNOWLEDGEMENTS

309 The authors greatly thank Fabrizio Zagni and Nuccio Lanteri for their help in collecting hornet
310 colonies in the field.

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FUNDING

Financial support was provided by the project ALIEM (Action pour Limiter les risques de diffusion des espèces Introduites Envahissantes en Méditerranée), funded by the ‘Programma Interreg Italia-Francia Marittimo 2014-2020. La cooperazione al cuore del Mediterraneo’, axe II. A. Cini was funded by a Marie Skłodowska-Curie Action, grant 539176.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

ETHICAL STANDARD

All procedures involving any experimental animals were performed in compliance with local animal welfare laws.

AUTHOR CONTRIBUTION

F. Cappa, A. Cini, I. Pepiciello and R. Cervo conceived and designed research. A. Cini, F. Cappa, I. Pepiciello and I. Petrocelli performed the assays and collected the data. A. Cini analyzed the data. F. Cappa and A. Cini wrote the manuscript. All authors read and approved the manuscript. F. Cappa, A. Cini and I. Pepiciello contributed equally to this work.

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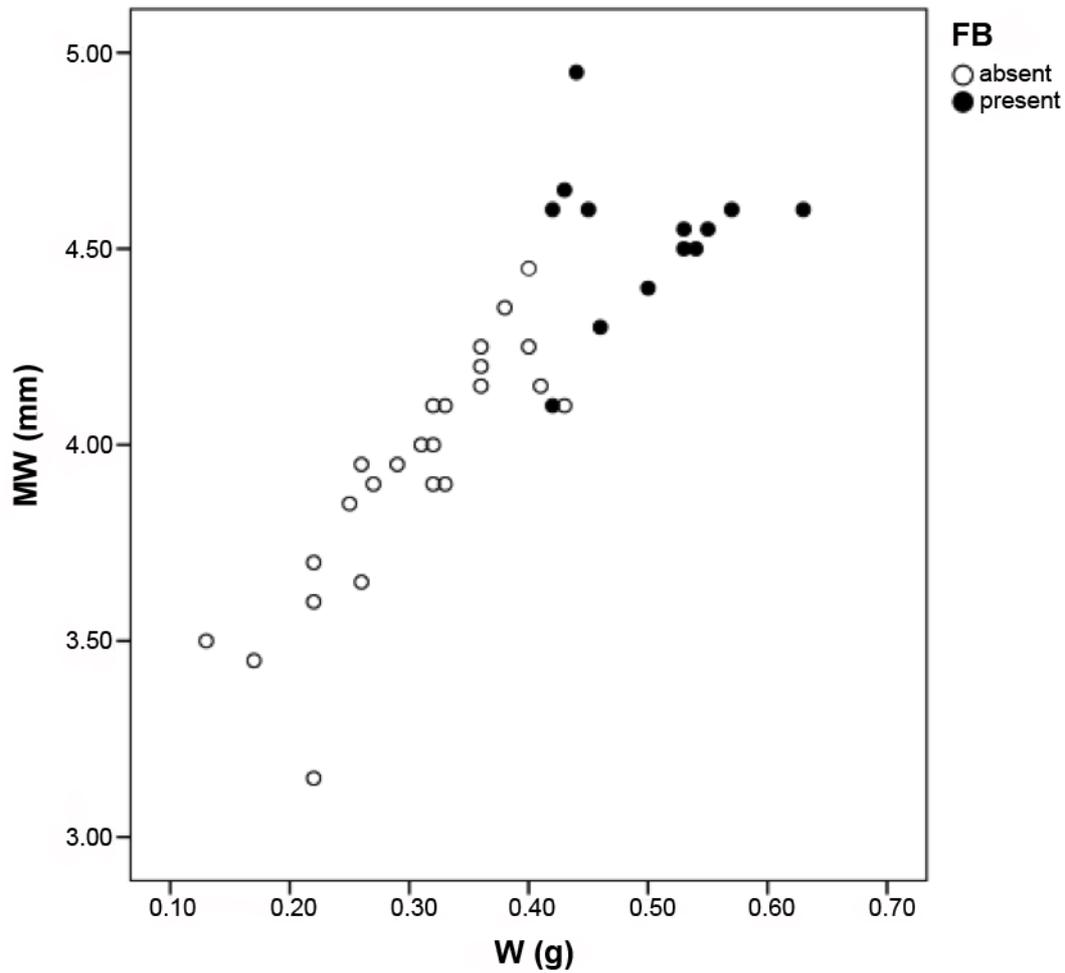
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595 Fig. 1. — Correlation between weight (W), presence versus absence of fat bodies (FB) and
596 mesoscutum width (MW) in female hornets used as stimuli.

597

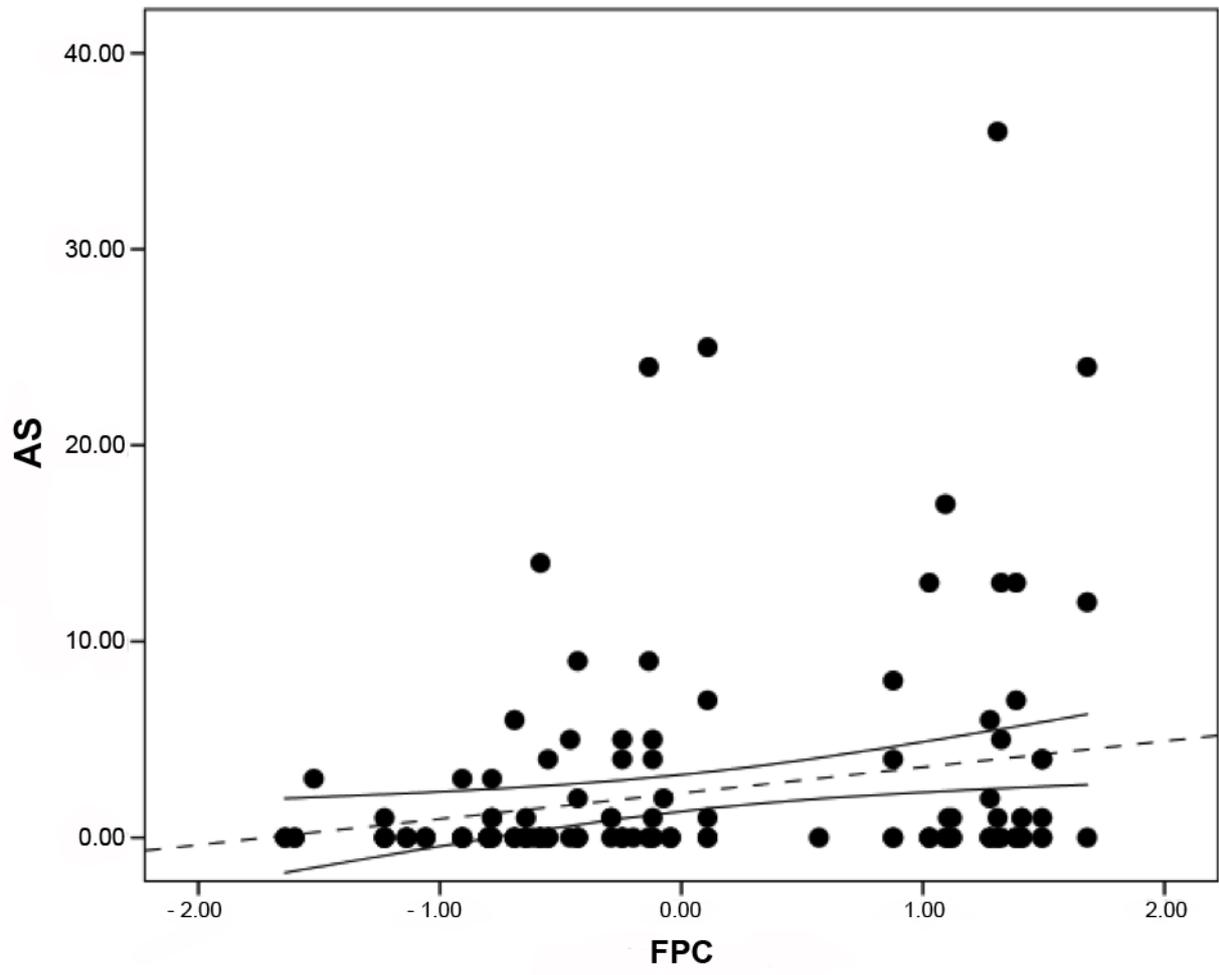
598 Fig. 2. — Differences in antennation score (AS) by focal males according to the physical condition
599 (defined as in the text, higher values of FPC means heavier, larger females with more developed fat
600 bodies) of the females presented as stimuli. Solid and dotted lines respectively represent linear
601 regression line and 95% confidence interval.

602



603

604 Fig.1



605

606 Fig.2