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1 Cuticular hydrocarbons as cues of sex and health condition in *Polistes dominula* wasps

2

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12

13 ABSTRACT

14 In the paper wasp *Polistes dominula*, cuticular hydrocarbons play a critical role
15 to acquire information regarding conspecific individuals. However, the relationship
16 between cuticular hydrocarbons, health status and male sexually selected traits is poorly
17 investigated. In this study, we characterized the cuticular hydrocarbon profile of adult
18 male and female wasps, infected or not by the strepsipteran endoparasite *Xenos*
19 *vesparum*, to assess whether the chemical signature provides information about sex and
20 health status (parasite infection). Moreover, we tested whether the chemical profile
21 reflects male quality as measured via morphological and behavioural (sexually
22 selected) traits at leks. Our results showed that males and females had similar total
23 amount of CHCs, quantitatively different profiles and, to a lesser extent, sex specific
24 chemical compounds. Cuticular profiles were influenced by the strepsipteran infection,

25 and the effect was stronger in females (the primary host) than in males, according to
26 the physiological castration of female but not of male hosts. Regarding territorial and
27 non-territorial males, no significant difference emerged in their chemical profiles.
28 Furthermore, sex-dimorphic visual signals (size, shape and asymmetry of abdominal
29 yellow spots) were related to the behavioural displays of territorial males. We
30 hypothesize that cuticular hydrocarbons are potential multi-role cues to assess sex and
31 health status in male and female wasps, in synergy with visual signals and territorial
32 performance in signaling male quality

33 KEY WORDS: cuticular hydrocarbons, *Polistes dominula*, *Xenos vesparum*, sexually
34 selected traits, sexual signals, territorial behaviour, sex differences

35 INTRODUCTION

36 Sex pheromones are chemical substances capable to elicit behaviours related to
37 sex recognition, mate-finding and mate-selection in insects, including social insects of
38 both sexes (Landolt et al. 1998; Ayasse et al. 2001; Wen et al. 2017). In primitively
39 eusocial *Polistes* paper wasps (Hymenoptera, Vespidae), semiochemicals used in
40 communication among conspecifics are typically multicomponent blends derived from
41 numerous exocrine tissues, including the cuticle, which is “essentially a glandular layer”
42 (Wheeler 1892, quoted in Jeanne 1996). Cuticular hydrocarbons (CHCs) were
43 identified as “surface pheromones” involved in male-female interactions by Post and
44 Jeanne (1984). CHCs are synthesized by the oenocytes and play a dual role, as a barrier
45 against dehydration and as semiochemicals (Chung and Carroll 2015). Among the wide
46 variety of semiochemicals (Keeling et al. 2004), CHCs allow the recognition of species,
47 colony, physiological status and age in *Polistes* (Singer 1998; Lorenzi et al. 1996;
48 Howard and Blomquist 2005; Blomquist and Bagnères 2010) The chemical profile is

49 altered by the widespread endoparasites of the genus *Xenos* in females of *Polistes*
50 *dominula* (Dapporto et al. 2007) and *Polistes ferrerii* (Torres et al. 2016) as well as by
51 a cestode in the ant *Temnothorax* (formerly *Leptothorax*) *nylanderi* (Trabalon et al.
52 2000) and by *Varroa* mites in the honeybee *Apis mellifera* (Cappa et al. 2016 b). Thus,
53 the cuticular mixture may signal the physiological condition of the *P. dominula* female
54 host, which is castrated by the *X. vesparum* parasite.

55 Even if the general knowledge of the epicuticular CHCs blend is fairly advanced,
56 to date the great informational content of these complex mixtures have been
57 investigated mostly in females (e.g., Lorenzi et al. 1997, 2011; Bruschini et al. 2010).
58 Males are largely neglected in chemical ecology as well as in neuroendocrine studies,
59 given the spectacular female social evolution (Boomsma et al. 2005; Beani et al. 2014;
60 Heinze 2016). Male CHC profiles have been analyzed only in a few *Polistes* species in
61 comparison to conspecific female cuticular blends (*P. annularis*, Espelie and Hermann
62 1990; *P. metricus*, Layton et al. 1994; *P. atrimandibularis* and *P. biglumis bimaculatus*,
63 Lorenzi et al. 1996), but no studies have investigated whether the cuticular chemical
64 profile differs by sex and parasitism status, or whether it supports the information
65 conveyed by male sexually selected traits in social wasps.

66 As regards male behavioural traits, lek-territoriality has been described in more
67 than twenty *Polistes* species (Beani 1996). Territorial males patrol, defend and mark
68 small contiguous territories on landmarks, whereas non-territorial males wander from
69 one perch to another without scent-marking and without exhibiting aggressive behavior
70 (*P. nimphus*, Turillazzi and Cervo 1982; *P. fuscatus*, Post and Jeanne 1983; *P.*
71 *dominula*, Beani and Turillazzi, 1988; *P. canadensis*, Polak, 1993; *P. semenowi*,
72 Lorenzi et al., 1994). Male mating success in *P. dominula* is affected by lek persistence,

73 territorial defense, scent marking, mate guarding and, as regards morphological traits,
74 large body size, well developed accessory glands (Beani and Zaccaroni 2015) and by
75 two yellow abdominal spots which act as visual signals. These condition-dependent
76 ornaments are sexually selected for their elliptical regular shape and small size (Izzo
77 and Tibbetts 2012, 2015).

78 Chemical communication could play a key role in sexual interactions with
79 potential mates and rivals, due to individual variation and plasticity of chemical signals
80 (Johansson and Jones 2007; Ingleby 2015). Territorial males rub their last gastral
81 sternites and hind leg on habitual perches; then they tap the substratum with their
82 antennae. Rubbing behaviour has been interpreted as a secretory release from
83 tegumental abdominal and leg glands (Beani and Calloni 1991a, b). Mating is more
84 frequent at these marked perches (Beani and Turillazzi 1988), after a prolonged
85 “antennal courtship” (Romani et al., 2005). When we pin *P. dominula* dead males (i.e.,
86 potential rivals) on routinely marked perches, the resident male first inspects through
87 antennation and then strikes the lures (de Souza et al. 2016). Following Cervo et al.
88 (1996), we considered this recurrent antennation behaviour as a ‘chemical cues
89 detection index’. Our analysis was focused on cuticular hydrocarbons used as potential
90 chemical signals in the mating context.

91 CHCs, due to their relatively high molecular weights, are non-volatile
92 compounds, perceived through antennation or at short distance without tactile
93 interaction (Brandstaetter et al. 2008, 2010). CHCs are exploited by *P. fuscatus* males
94 to assess mating status and relatedness with females (Ryan and Gamboa 1986), but their
95 hypothetical role in sex discrimination, and mate quality evaluation has not yet been
96 investigated in paper wasps. Assuming some involvement of CHCs in sexual

97 interactions of *P. dominula*, we here analyzed the qualitative and/or quantitative
98 differences of the cuticular blends: first, between males and females, according to CHC
99 sexual dimorphism in *Drosophila* (Foley et al. 2007) and other insects; second, between
100 wasps of both sexes parasitized or unparasitized by the strepsipteran *X. vesparum*, since
101 a slight effect on chemical signature has been described, but only in infected *P.*
102 *dominula* females (Dapporto et al. 2007); third, between territorial and non-territorial
103 males, in relation to male behaviour, body size and ornamental spots, if indeed CHCs
104 are involved in sexual selection, are condition-dependent and costly signals of quality
105 (Steiger and Stökl 2014). Significant qualitative and quantitative differences in CHCs
106 between sexes and between males of different quality would contribute to
107 understanding the role of chemical signals to assess sex and health status in a natural
108 environment, in synergy with male visual signals and territorial performance.

109 MATERIALS AND METHODS

110 Sample collection: territorial and non-territorial males

111 In August 2016, we carried out this study in a population of *P. dominula* in the
112 surroundings of Florence, Sesto Fiorentino, Italy (43°50'7"N, 11°11'46"E). The study
113 area consists of a flat landscape with scattered landmarks (trees, poles, fences, bushes).
114 We carried out behavioural observations between 10:00 h and 15:00 h, as this is the
115 time when males are more active at leks (Beani and Turillazzi 1988). We collected and
116 individually marked 81 non-parasitized *P. dominula* males with Testor's paint on
117 thorax; 35 males were collected from a set of 52 young *Platanus* spp. trees (spaced 5-
118 10 m from each other) and 46 were collected from the low shady branches of one big
119 linden tree (*Tilia* spp.), 20-30 m away from the set of *Platanus* trees. Observations were
120 carried out for 9 days after the capture-marking-release day (20 x 3-min periods per day

121 at both locations, i.e. *Platanus* and *Tilia* trees, 360 x 3-min periods altogether).

122 We identified as territorial each male that was re-sighted on the same *Platanus*
123 tree for at least 4 days (site fidelity) and which repeatedly marked peculiar perches,
124 Scent marking frequency was measured for a total of 9 min per male; observations were
125 non-consecutive, in case males went out of sight. In the last two days of field work, we
126 also measured aggressive behaviour of territorial males towards a lure male (see De
127 Souza et al. 2016). We did this by pinning, on a point of the trunk intensively rubbed
128 by the male, one lure consisting of a dead *P. dominula* male washed in pentane for 24
129 hr, to remove all chemical cues. In this way we were able to test the effect of rival visual
130 cues on territorial males) (Cappa et al. 2016 a). These lures (30 in all) had small
131 elliptical abdominal spots and their head-width ranged between 3.40-3.50 mm: thus,
132 simulated intruders had similar body size and abdominal spots. The observer was
133 located 2 m away from each lure and recorded inspections (without physical contact)
134 and strikes (with contact) for 3 min after the first interaction (frequency/3 min, 9 min
135 each male). At the end of the study period, we collected 20 territorial males out of 35
136 marked on the *Platanus* trees to analyze their CHCs.

137 During the same period, we described the behaviour of males collected and
138 individually marked on the low branches of the big linden. We labeled these males as
139 non- territorial when they were re-sighted on two different days but not on the same
140 site. On the last day of the observation period, we pinned a further 10 lures on the leaves
141 of *Tilia*, spaced 50-80 cm from each other, to test for the aggressive responses of non-
142 territorial individuals against intruders. At the end of the field study, we collected 24
143 non territorial males out of 46 marked on the *Tilia* tree to analyze their CHCs.

144 Sample collection: parasitized and non-parasitized wasps

145 At the end of August, we collected parasitized and non-parasitized wasps from a
146 flowering tall bush of *Campsis radicans* (Bignoniaceae), 80 m apart from *Tilia* tree,
147 without marking them or recording their behaviour. This bush, commonly known as
148 “trumpet creeper”, was intensively patrolled by *Polistes* and other hymenopterans, due
149 to its rich floral and extra-floral nectaries (Elias and Gelband, 1975). Trumpet creepers
150 were the preferred target of parasitized wasps in our study area (Beani et al., 2018).
151 Here we collected 16 non-parasitized *P. dominula* females, presumably gynes (wings
152 undamaged; at dissection, abundant fat bodies and ovaries with detectable oocytes); 9
153 parasitized females bearing one *X. vesparum* of either sexes, 2 parasitic males and 7
154 parasitic females (at dissection, female wasps had undeveloped ovaries due to parasitic
155 castration, see Beani et al. 2011) and 13 parasitized males infected by one *X. vesparum*
156 (6 parasitic males and 7 parasitic females). An additional 3 males, each parasitized by
157 a *X. vesparum* male, were collected from *Platanus* trees and from *Tilia* tree.

158

159 Male morphological measurements and abdominal spot categorization

160 Males were photographed using a digital camera (Canon A-620) under a
161 stereomicroscope. These pictures were used to measure male head width in pixels (a
162 reliable indicator of body size; Eickwort, 1969) and abdominal spots, including spot
163 Shape Index (SI; Izzo and Tibbetts 2012). SI compares the mean size of right and left
164 spots, measured in pixels, to the mean area of the idealized ellipses (length= L,
165 height=H) inserted into right and left spots. SI was calculated using the formula $SI =$
166 $A/(\pi \times 1/2 L \times 1/2 H)$. Wasps with regularly shaped spots have lower SI values, while
167 irregularly spotted wasps have higher SI values. Due to the difficulty of inserting
168 ellipses inside very irregular spots in our population, we also measured Spot mean area
169 as the average yellow area of right and left spots using ImageJ

170 (<http://rsbweb.nih.gov/ij/>). We calculated asymmetry in spot area as the difference
171 between right and left spot area. A subjective summary evaluation of right and left spot
172 shape and size, thereafter referred to as Spot category, was made by two observers blind
173 to predictions: 1= small, regular shape, round/elliptical; 2 = medium size, irregular
174 shape; 3 = large size, very irregular shape. These 3 typologies were the most common
175 in our samples, in which small irregular spots, medium regular or large regular ones
176 were not represented, according to the two blind observers.

177

178 Chemical analysis

179 We analyzed the CHCs of a total of 51 males (18 territorial males, 17 non-
180 territorial males, 16 parasitized males) and 25 females (9 parasitized females, 16 non-
181 parasitized females). We first checked whether males differed chemically from females;
182 second, we tested for the influence of the parasite on CHC profiles in both sexes; third,
183 in males we analyzed the association between CHCs and body size, ornamental spot
184 measures (SI, spot mean area, asymmetry in spot area, Spot category) and male
185 behaviour (territorial vs non-territorial males). Finally, in the subset of 18 territorial
186 males, we evaluated whether scent marking frequency and aggressiveness towards lures
187 (inspection and strike frequency) were related to morphological traits.

188 Immediately after collection, wasps were killed by freezing and weighed with a
189 precision balance (Precisa 125). We extracted cuticular compounds by dipping each
190 wasp in a glass vial with 1.5 ml pentane for 60 sec. Extracts were dried for 24 h at room
191 temperature and re-dissolved in 100 µl of heptane containing *n*-C20 as an internal
192 standard.

193 For quantification, we injected 2 µl of each extract (which included 800 ng of *n*-

194 C20) into a gas chromatograph with the flame ionization detection (GC-FID: Agilent
195 Technologies 6850 equipped with a J&W HP-1 nonpolar-capillary-column, 30 m L x
196 0.32 mm ID x 0.25 µm PT). For identification, we injected another 2 µl of the same
197 extracts into a gas chromatograph-mass spectrometer (Agilent Technologies 7000C
198 GC-MS Triple Quad equipped with a Zebron ZB-5HT capillary-column, 30 m L x 0.25
199 ID x 0.25 µm PT). The oven temperature for both GC-FID and GC-MS was
200 programmed as follows: from 70 °C to 150 °C at 30 °C/min and from 150 °C to 320 °C at
201 5 °C/min; 10 min at the final temperature of 320 °C. The carrier gas was helium at 1 bar
202 and 50 ml/min flux; the injector temperature was 250 °C. The output was registered with
203 the Agilent ChemStation program for GC-FID and Agilent Mass Hunter Workstation
204 program for GC-MS.

205 Identification and quantification of compounds

206 We identified the compounds using the GC-MS system and the diagnostic ions
207 and the M-15 of each peak, comparing the cuticular chemical profiles of *P. dominula*
208 previously described in Bonavita-Cougourdan et al. (1991) and Costanzi et al. (2013),
209 and we also identified new compounds in the extracts of males by the same technics.

210 For quantification, peak areas were integrated using the GC ChemStation
211 software, which allowed the calculation of the relative proportion of compounds.
212 Moreover, following Lorenzi et al. (2014) and Elia et al. (2017) we quantified the total
213 concentration of hydrocarbons on the wasps as the sum of all peak areas relative to the
214 area of the internal standard (*n*-C20), which corresponded to 800 ng, and we divided
215 this value by wasp weight (ng of hydrocarbons/mg of wasp).

216 Statistical analysis: morphological, behavioural and chemical data

217 We performed a first principal components Analysis (PCA) based on correlations
218 and varimax rotation on morphological data (head width, SI, spot mean area,
219 asymmetry in spot area and spot category) and a second PCA on the behavioural data
220 of territorial males (marking, inspecting, striking) to check for collinearity between
221 measures.

222 We processed the chemical data by computing the relative proportions of the
223 hydrocarbons, then transformed these compositional data using the log-ratio-
224 transformation (natural log of the proportion of each peak, divided by the geometric
225 mean of peak proportions) (Aitchison 1982). We then reduced the number of variables
226 (and checked for collinearity) by performing two PCAs (based on correlations, varimax
227 rotation), the first on the chemical profiles of males and females (n = 85 wasps, of which
228 60 males and 25 females), the second on males only.

229 In a preliminary analysis, we entered in a PCA both the chemical and the
230 morphological data; this resulted in morphological variables loading on PCs which
231 explained <5.5% variance. For this reason, we reverted into analyzing chemical and
232 morphological data separately. After summarizing the morphological, behavioural and
233 chemical traits by means of PCAs, we tested whether the resulting principal
234 components (morphological, behavioural and chemical PCs) were associated with sex
235 and parasitism or whether in territorial males, where behavioral data were available,
236 they predicted what behaviour the male displayed.

237 Using a Generalized Linear Model (GLM), we tested whether chemical PCs
238 varied as an effect of sex or parasitism. We also tested whether chemical and
239 morphological PCs predicted male territorial behaviour. To this aim we used a GLM
240 for binary data (males were either territorial or non-territorial). Finally, we checked

241 whether the morphology of the territorial males predicted their behaviour. To this aim
242 we performed another GLM on the single PC summarizing behavioural traits (as
243 response variable) and the two PCs on morphological data (as predictors).

244 Statistical analyses were conducted in R v.3.5.0 (“R Development Core Team”
245 2018) (MASS package, Bates et al., 2015), except for PCAs, which were performed in
246 IBM SPSS Statistics 22.0. For normally distributed data, we checked the statistical
247 models (GLMs) for homogeneity of variances and goodness of fit by plotting residuals
248 versus fitted values. Normality of the model residuals was assured by checking normal
249 probability plots. P-values were calculated by type II Wald chi-square tests. We report
250 regression slopes (β estimates) with standard errors as measures of effect sizes. The
251 initial models included all the biologically relevant terms and interactions. Then, we
252 removed the non-significant terms and re-ran the simplified models, unless this resulted
253 in increased AIC values. When not otherwise stated, descriptive statistics are given as
254 means \pm standard errors.

255

256 RESULTS

257 *Sex-specific hydrocarbon profiles and parasite effect*

258 *P. dominula* males and females had complex cuticular hydrocarbon blends that
259 included 70 peaks; these were homologous series of linear alkanes, methyl-branched
260 alkanes and linear alkenes between C₂₃ and C₃₇. Males and females had similar total
261 amounts of cuticular hydrocarbons (mean \pm sd: males 903.97 \pm 335.41 ng of
262 hydrocarbons/mg of wasp; females: 868.61 \pm 224.25) (estimates[male]: 35.36 \pm 67.72,
263 $\chi^2 = 0.273$, df = 1, P = 0.602). The infection by the parasite *X. vesparum* had no
264 significant effects on the total amount of CHCs (parasitism status was removed from a
265 preliminary model).

266 Males and females had quantitatively different profiles with a number of

267 hydrocarbons virtually present in one sex only (Fig. 1, Table 1). Indeed, the proportion
268 of hydrocarbons in the chemical profiles was sex-specific and was also affected by
269 *Xenos* parasites. The PCA on the chemical profiles of all the individuals (i. e. males and
270 females) produced two PCs explaining > 20% of the variance (PC1: 33.2%; PC2:
271 22.7%), where PC1 was significantly associated to sex (Fig. 2), and PC2 to parasite
272 presence (Fig. 3).

273 The difference between the chemical profiles of males and females was
274 determined by the hydrocarbons loading on PC1. Males had significantly higher PC1
275 values than females (estimates [male]: 2.016 ± 0.070 , $\chi^2 = 828.45$, $df = 1$, $P < 0.0001$)
276 (Fig. 2). In contrast, being parasitized by *X. vesparum* had no effect on the chemical
277 profile summarized by PC1 (estimate [being parasitized]: -0.089 ± 0.070 , $\chi^2 = 1.62$ df
278 $= 1$, $P = 0.203$) (the interaction sex*parasitism was non-significant and was removed
279 from a preliminary model). In the part of the chemical profile summarized by PC1,
280 males had higher amounts than females of 5-Methylhentriacontane (peak 34, PC1-
281 factor loading = 0.951), 2,x-Dimethyltriacontane (peak 30a, factor loading = 0.936), *n*-
282 Hentriacontene (peak 30, factor loading = 0.845), *n*-Tetracosane (peak P1b, factor
283 loading = 0.787), and *n*-Tricosane (peak P1, factor loading = 0.749), and lower amounts
284 of 5-Methylpentacosane (peak P2c), 7- Methylpentacosane (peak P2b) and 2-
285 Methylpentacosane + 3-Methylpentacosane (peak P2cd + P2d) (all these peaks had
286 factor loadings = -0.965); 13,17-+13,19-Dimethylhentriacontane (peak P35, factor
287 loading = -0.943), 9,13-+7,15-Dimethylhentriacontane (peak P36, factor loading = -
288 0.775) and of the blend of 9-+11-+13-+15-+17-Methyltriacontane (peak P45+P46,
289 factor loading = -0.717).

290 In contrast, the infection by the parasite determined the part of the chemical

291 profile summarised by PC2, and did it differently on males and females, as shown by
292 the significant interaction between sex and parasites (sex[male]*parasites[yes]:
293 estimates: -1.0529 ± 0.478 , $\chi^2 = 4.848$, $df = 1$, $P = 0.028$) (Fig. 3). Among non-
294 parasitized wasps, males had higher values of PC2 than females; among parasitized
295 wasps, females had higher PC2 values than males, suggesting that the effect of parasites
296 on CHC profiles of the females was stronger than that on males.

297 Parasitized wasps (and especially females) had higher amounts than non-
298 parasitized ones of the following compounds: a mixture of *n*-Docotriacontane + an
299 unidentified hydrocarbon (peak 39+40, factor loading = 0.939), 2-Methyloctacosane
300 (peak 14, factor loading = 0.857), 5-Methylnonacosane (peak 19, factor loading =
301 0.856), the co-eluting blend of 5,15--5,19-Dimethylhentriacontane (peak 37, factor
302 loading = 0.852), of 3-Methylnonacosane+7,11-Dimethylnonacosane (peak 21, factor
303 loading=0.803), 3,15,21-Trimethylnonacosane, *n*-Triacontane, 2,*x*-
304 Dimethylnonacosane, peaks 23+24+25, factor loading = 0.780), *n*-Tritriacontane + an
305 unidentified hydrocarbon (peak 44 , factor loading = 0.765), the blend of *n*-
306 Tetratriacontane +*x* (peak 50, factor loading = 0.740), *n*-Octacosane (peak 12 , factor
307 loading = 0.735), nonacosene (peak 14a, factor loading = 0.706).

308 *Territorial and non-territorial males: behaviour, hydrocarbons and ornaments*

309 Territorial males exhibited site fidelity, scent marking and attacks towards CHC-
310 deprived lure males, while such behaviours were not recorded for non territorial males.
311 Territorial males routinely landed and defended the same tree (sometimes two close
312 trees) from intruders for 4 days at least, rubbing the substrate with their abdomens and
313 legs on particular perches, e.g. a leaf, a twig, or a point of the trunk (scent marking,

314 mean frequency \pm SE: 3.13 ± 0.21). During trials with simulated CHC deprived
315 intruders, the resident males inspected and attacked the lures (inspections, mean
316 frequency \pm SE: 1.59 ± 0.12 ; s: 1.38 ± 0.12). Non territorial males flew from one branch
317 to another (no site fidelity) and never, or very rarely,, rubbed their gasters on the
318 substrate (no scent marking). These males were characterized by non aggressive
319 behavior towards lures as well as conspecifics and tolerated low inter-individual
320 distances: sometimes two males stopped and rested on the same leaf.

321 We then analysed whether chemical and/or morphological data reflected whether
322 males were territorial or not. The PCA on the chemical traits (only males) produced
323 eight PCs, of which three explained $> 10\%$ of the variance (PC1: 49.6%; PC2: 12.9%;
324 PC3: 11.9%). The PCA on the morphological traits resulted in two PCs (PC1: 41.1%;
325 PC2: 21.6%). Spot category and spot mean area (and asymmetry in spot area, although
326 at a lower extent) were summarized by PC1 (factor loadings for spot category: 0.877;
327 spot mean area: 0.798; asymmetry in spot area: 0.643) and PC2 (factor loadings for SI:
328 0.760; head width: -0.674).

329 Territorial males had peculiar morphological traits (Fig. 4): a significantly lower
330 spot category, a smaller spot mean area and, to a lesser extent, a lower asymmetry in
331 spot area than non-territorial males (PC1 on morphological data: estimates [territorial
332 males]: -1.308 ± 0.513 , $\chi^2 = 8.754$, $df = 1$, $P = 0.0031$). The head width (a proxy for
333 body size) and the SI did not differ significantly between territorial and non territorial
334 individuals. Similarly, no significant difference emerged in the chemical profiles of
335 these two groups (PC2 on chemical data: estimates [territorial males]: 0.580 ± 0.452 ,
336 $\chi^2 = 1.774$, $df = 1$, $P = 0.183$; non significant PC1 and PC3 covariates were removed
337 from preliminary models) (Fig. 4)

338 *Territorial males: behaviour, chemical profiles and morphological traits*

339 As regards territorial males (N=18), the PCA on the behavioural data produced
340 only one PC, which explained 80.7% of the variance and whose factor loadings were:
341 strike: 0.874; inspection: 0.833; marking: 0.714, thus this component efficiently
342 summarised the three behaviours. Territorial males with a lower Spot category and a
343 smaller Spot mean area performed significantly more markings, inspections and strikes
344 (Fig. 5), but had no peculiar chemical profiles, as shown by the highly significant
345 association between behaviour and morphological traits (PC1 on morphological data,
346 estimates: -0.957 ± 0.177 , $\chi^2 = 29.155$, $df = 1$, $P < 0.0001$), and by the lack of
347 significant association between behaviour and chemical profiles (PC2 on chemical data,
348 estimates: 0.169 ± 0.110 , $\chi^2 = 2.354$, $df = 1$, $P = 0.125$).

349 Finally, parasitized males did not differ significantly from non-parasitized males
350 in morphological traits, as summarized by the two PCs (PC1: estimates [parasitized
351 males]: -0.394 ± 0.292 , $\chi^2 = 1.821$, $df = 1$, $P = 0.177$; PC2: estimates [parasitized
352 males]: -0.147 ± 0.296 , $\chi^2 = 0.246$, $df = 1$, $P = 0.620$).

353

354 DISCUSSION

355 *Chemical discrimination of sex*

356 For the first time, our study compared chemical profiles of *P. dominula* between
357 sexes, parasitized and non-parasitized individuals, as well as in relation to male
358 mating strategies. The chemical profiles of *P. dominula* differed significantly between
359 sexes in a few sex-specific compounds and in the relative amount of others (Fig.1),
360 whereas the total CHC amount was similar. Sex discrimination via visual and
361 chemical cues has already been demonstrated in other social hymenoptera (Keeping

362 et al. 1986; Nonacs and Carling 1990; Passera and Aron 1996; Derstine et al. 2017;
363 Cappa et al. 2019) and in *P. dominula* in the social environment of the nest (Cappa et
364 al. 2016 a). Individual chemical profiles may play a relevant role also in the mating
365 context of leks, to assess the identity and the quality of a potential partner or rival:
366 This is suggested by the observations of successful or attempted copulations by
367 resident males with females landing inside their territory, whereas intruder males are
368 the target of aggressive interactions (Beani and Turillazzi, 1988; Beani and Zaccaroni
369 2015).

370 Laboratory experiments confirm a chemical-mediated attraction. In Y-tube
371 olfactometer bioassays, *P. dominula* females turned toward male odour (McKenzie et
372 al. 2008). Moreover, when tested in a wind tunnel, *P. exclamans* males exhibited
373 flights in response to airflow passed over females; they flew towards female-hexane
374 extracts of whole bodies and thoraces, whereas females were attracted towards male-
375 hexane extracts (Reed and Landolt 1990). Consistent with a close range pheromonal
376 effect in male-female contacts, extracts of the female mesosoma applied to female
377 models stimulate copulatory attempts in *P. exclamans* (Enquist et al. 2018).

378 In arena bioassays, *P. dominula* males rubbed their gasters when exposed to
379 chemical extracts of legs and gaster of both sexes (McKenzie et al. 2008), supporting
380 a double role for scent marking in attracting females and signaling territorial
381 ownership to males. In *P. exclamans* Vierick (Emquist et al. 2018), a species in which
382 males mark perches near female hibernacula or nests with gynes (Reed & Landolt
383 1990), male rubbing behaviour may be elicited by female CHC blends. In
384 Stenogastrinae, males of *Parischnogaster mellyi* rub the abdomen tip on the leaf
385 borders, leaving small dark spots (Beani and Landi, 2000; Beani et al. 2002) that
386 contain CHC blends similar to those on the cuticle, suggesting a possible role of these

387 marking spots in species and sex recognition, as females land on and copulate at these
388 hover-sites (Beani et al. 2002).

389

390 *Parasite influence on CHCs*

391 Regarding the effect of parasites on the wasp CHC profiles, many parasites of
392 social insects affect the CHCs of the host (Bagnères and Lorenzi 2010). Focusing on
393 *P. dominula*, the presence of a single *X. vesparum* parasite in the abdomen was enough
394 to alter the chemical signature of both males and female wasps, but the effect was
395 quantitative, not qualitative, as shown in the females of the ant *T. nylanderi* (Trabalon
396 et al. 2000), and significantly stronger in females than in males (Fig. 2 and 3).
397 Noticeably, host sex is a critical factor also for morphological and behavioural
398 changes driven by parasites: the primary target of infection are workers and gynes,
399 which are castrated by the parasite, whereas minor morphological effects (a subtle
400 change in accessory gland secretion) were found in parasitized males that were
401 sexually active in leks and not castrated (Cappa et al. 2014; Beani et al. 2017). Here,
402 we did not consider parasite load, because *P. dominula* wasps rarely harbour more
403 than one parasite (Hughes et al. 2004 b). A higher parasite load could more strongly
404 affect host hydrocarbon profiles, as described in *P. ferreri* (Torres et al. 2016).

405 The chemical alteration of the cuticular profile by parasites partially confirms
406 previous analyses in *P. dominula* and *P. ferreri* females (Dapporto et al. 2007; Torres
407 et al. 2016). The underlying mechanism leading to changes in epicuticular blend
408 probably occurs during the development of the endoparasite inside wasp larvae (Giusti
409 et al. 2008). These slight changes, a by product of the hosts altered development, may
410 be adaptively used by the parasite to manipulate the acceptance of infected individuals
411 by nestmates, e.g. in bees (Cappa et al. 2016 b; 2019). *P. dominula* workers, if

412 parasitized, desert the colony one week after emergence (Hughes et al. 2004), when
413 their chemical profile is still forming (Lorenzi et al. 2004) and the parasite is not yet
414 extruded; lacking clear chemical discrimination, parasitized workers are not the target
415 of aggressiveness by nest-mates (Hughes et al. 2004). The peculiar chemical profile
416 induced by the parasite could facilitate the aberrant summer aggregations mainly
417 formed by parasitized females (Hughes et al. 2004; Beani et al. 2018). According to
418 slight quantitative changes in the profile of parasitized wasps, winter aggregations
419 include both parasitized and non-parasitized females (Dapporto et al. 2007; Beani et
420 al. 2011). This weak chemical discrimination might explain why in preference trials,
421 male antennation and attempted copulations were directed towards both parasite-
422 castrated and non-parasitized gynes (Cappa et al. 2013). Noticeably, in these brief lab
423 trials full copulations were extremely rare. It is likely that a more accurate mate
424 assessment occurs in leks, where males simply ignore aggregations of parasitized
425 females (Beani, *pers. obs.*)

426

427 *Territorial behaviour of males with sexually selected ornaments*

428 Regarding mating strategies, there was no evidence of any significant chemical
429 difference between territorial and non-territorial males. Intriguingly, the relatively
430 flexible behaviour of *P. dominula* males, which sometimes switch from non-territorial
431 to territorial behaviour depending on the context (Beani and Turillazzi, 1988; Beani and
432 Zaccaroni, 2015), matches the weak differences in the hydrocarbon profiles that we
433 found in males adopting different mating strategies, as well as in body size. In contrast,
434 concerning ornamental traits (Fig. 4), territorial males had smaller elliptical abdominal
435 spots than non-territorial ones (as measured by means of spot area, spot category, spot
436 asymmetry). Moreover, in territorial males (Fig. 5) there was evidence of a significant

437 relationship between spots and behaviour: males with smaller and regular spots were
438 more active both in scent marking and in aggressive interactions with lures

439 Abdominal spots are highly variable and strongly influenced by larval
440 nourishment: they are elliptical and regularly shaped when males are reared with a rich
441 diet (Izzo and Tibbetts 2015). How color patterns are shaped during development is still
442 unknown in wasps, but large irregularly shaped spots and high asymmetry in s might
443 be due to stressors during the development, such as a restricted diet and low levels of
444 melanin, an honest indicator of male phenotypic quality (de Souza et al. 2016, 2018).
445 Visual cues, beside chemical cues, may play a critical role in sex discrimination (Cappa
446 et al. 2016) and mating success, at least in lab trials (Izzo & Tibbetts 2012). Intriguingly,
447 different measures of abdominal spots (including the non-parametric spot category,
448 easier to assess than shape index, and unrelated to both male behavioural and
449 morphological traits in our male population) may be adopted by females to distinguish
450 between males with different territorial strategies, highlighting how visual signals could
451 be also important in the communication system of social wasps (Tibbetts et al. 2009).

452 The yellow spots may be an example of constrained sexual signals which depict,
453 at the moment of emergence, the quality of the male, further described by the CHCs
454 (which conveyed information about parasite infection). In this respect, the CHCs might
455 inform the females about the current quality of the male, acting in concert with the
456 visual ornaments. Even if male- and female-attractants appear common in
457 hymenopterans, very few sex pheromones have been chemically identified, due to the
458 behavioural sophistication and the wide spectrum of communication modes in social
459 insects (reviewed by Ayasse et al. 2001). Lacking specific behavioural tests on *P.*
460 *dominula* in the field, we can advance the hypothesis that CHCs are potential cues to

461 assess sex and mate quality (e.g., parasite infection) at a short distance, after responding
462 to behavioural displays and visual stimuli, similarly to the dynamic multimodal
463 signaling in birds (Karubian et al. 2011).

464

465

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469

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- 633

634 FIGURE HEADINGS

635 Fig. 1. Average proportion of chemical compounds in the profiles of males and females
636 of *Polistes dominula*, parasitized or non-parasitized by *Xenos vesparum* (mean \pm se).

637 See Table 1 for the identification of the peaks.

638 Figure 2. Plot of PC1 versus PC2 of the chemical signatures of males and females
639 of *Polistes dominula*, parasitized or non-parasitized by *Xenos vesparum* ^[11]_[SEP]

640 Figure 3. The variation in the chemical profiles of males and females of *P. dominula*
641 (as summarized by PC2) parasitized or non-parasitized by *Xenos vesparum* parasites.

642 Figure 4. Plot of PC1 on morphological data versus PC2 on chemical data of Territorial
643 and Non-territorial males of *P. dominula*.

644 Figure 5. The association between morphological and behavioural data in *Polistes*
645 *dominula* Territorial males: males with smaller elliptical abdominal spots performed
646 significantly more markings, inspections and strikes.