



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

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Abstract

In the paper wasp *Polistes dominula*, cuticular hydrocarbons play a critical role to acquire information regarding conspecific individuals. However, the relationship between cuticular hydrocarbons, health status, and male sexually selected traits is poorly investigated. In this study, we characterized the cuticular hydrocarbon profile of adult male and female wasps, infected or not by the strepsipteran endoparasite *Xenos vesparum*, to assess whether the chemical signature provides information about sex and health status (parasite infection). Moreover, we tested whether the chemical profile reflects male quality as measured via morphological and behavioural (sexually selected) traits at leks. Our results showed that males and females had similar total amount of CHCs, quantitatively different profiles and, to a lesser extent, sex-specific chemical compounds. Cuticular profiles were influenced by the strepsipteran infection, and the effect was stronger in females (the primary host) than in males, according to the physiological castration of female but not of male hosts. Regarding territorial and non-territorial males, no significant difference emerged in their chemical profiles. Furthermore, sex-dimorphic visual signals (size, shape, and asymmetry of abdominal yellow spots) were related to the behavioural displays of territorial males. We hypothesize that cuticular hydrocarbons are potential multi-role cues to assess sex and health status in male and female wasps, in synergy with visual signals and territorial performance in signaling male quality.

Keywords Cuticular hydrocarbons · *Polistes dominula* · *Xenos vesparum* · Sexually selected traits · Sexual signals · Territorial behaviour · Sex differences

Introduction

Sex pheromones are chemical substances capable to elicit behaviours related to sex recognition, mate finding, and mate selection in insects, including social insects of both sexes (Landolt et al. 1998; Ayasse et al. 2001; Wen

et al. 2017). In primitively eusocial *Polistes* paper wasps (Hymenoptera, Vespidae), semiochemicals used in communication among conspecifics are typically multicomponent blends derived from numerous exocrine tissues, including the cuticle, which is “essentially a glandular layer” (Wheeler 1892, quoted in Jeanne 1996). Cuticular hydrocarbons (CHCs) were identified as “surface pheromones” involved in male–female interactions by Post and Jeanne (1984). CHCs are synthesized by the oenocytes and play a dual role, as a barrier against dehydration and as semiochemicals (Chung and Carroll 2015). Among the wide variety of semiochemicals (Keeling et al. 2004), CHCs allow the recognition of species, colony, physiological status, and age in *Polistes* (Singer et al. 1998; Lorenzi et al. 1996; Howard and Blomquist 2005; Blomquist and Bagnères 2010). The chemical profile is altered by the widespread endoparasites of the genus *Xenos* in females of *P. dominula* (Dapporto et al. 2007) and *P. ferreri* (Torres et al. 2016) as well as by a cestode in the ant *Temnothorax* (formerly *Leptothorax*) *nylanderi* (Trabalon et al.

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2000) and by *Varroa* mites in the honeybee *Apis mellifera* (Cappa et al. 2016b). Thus, the cuticular mixture may signal the physiological condition of the *P. dominula* female host, which is castrated by the *X. vesparum* parasite.

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

As regards male behavioural traits, lek territoriality has been described in more than 20 *Polistes* species (Beani 1996). Territorial males patrol, defend, and mark small contiguous territories on landmarks, whereas non-territorial males wander from one perch to another without scent marking and without exhibiting aggressive behaviour (*P. nimphus*, Turillazzi and Cervo 1982; *P. fuscatus*, Post and Jeanne 1983; *P. dominula*, Beani and Turillazzi, 1988; *P. canadensis*, Polak, 1993; *P. semenowi*, Lorenzi et al., 1994). Male mating success in *P. dominula* is affected by lek persistence, territorial defense, scent marking, mate guarding, and, as regards morphological traits, large body size, well-developed accessory glands (Beani and Zaccaroni 2015) and by two yellow abdominal spots which act as visual signals. These condition-dependent ornaments are sexually selected for their elliptical regular shape and small size (Izzo and Tibbetts 2012, 2015).

Chemical communication could play a key role in sexual interactions with potential mates and rivals, due to individual variation and plasticity of chemical signals (Johansson and Jones 2007; Ingleby 2015). Territorial males rub their last gastral sternites and hind leg on habitual perches; then, they tap the substratum with their antennae. Rubbing behaviour has been interpreted as a secretory release from tegumental abdominal and leg glands (Beani and Calloni 1991a, b). Mating is more frequent at these marked perches (Beani and Turillazzi 1988), after a prolonged “antennal courtship” (Romani et al. 2005). When we pin *P. dominula* dead males (i.e., potential rivals) on routinely marked perches, the resident male first inspects through antennation and then strikes the lures (de Souza et al. 2017). Following Cervo et al. (1996), we considered this recurrent antennation behaviour (Pardi 1948) as a ‘chemical cues detection index’.

Our analysis was focused on cuticular hydrocarbons used as potential chemical signals in the mating context.

CHCs, due to their relatively high molecular weights, are non-volatile compounds, perceived through antennation or at short distance without tactile interaction (Brandstaetter et al. 2008, 2010). CHCs are exploited by *P. fuscatus* males to assess mating status and relatedness with females (Ryan and Gamboa 1986), but their hypothetical role in sex discrimination, and mate quality valuation has not yet been investigated in paper wasps. Assuming some involvement of CHCs in sexual interactions of *P. dominula*, we here analyzed the qualitative and/or quantitative differences of the cuticular blends: first, between males and females, according to CHC sexual dimorphism in *Drosophila* (Foley et al. 2007) and other insects; second, between wasps of both sexes parasitized or unparasitized by the strepsipteran *X. vesparum*, since a slight effect on chemical signature has been described, but only in infected *P. dominula* females (Dapporto et al. 2007); third, between territorial and non-territorial males, in relation to male behaviour, body size, and ornamental spots, if indeed CHCs are involved in sexual selection, are condition-dependent and costly signals of quality (Steiger and Stöckl 2014). Significant qualitative and quantitative differences in CHCs between sexes and between males of different quality would contribute to understanding the role of chemical signals to assess sex and health status in a natural environment, in synergy with male visual signals and territorial performance.

Materials and methods

Sample collection: territorial and non-territorial males

In August 2016, we carried out this study in a population of *P. dominula* in the surroundings of Florence, Sesto Fiorentino, Italy (43°50'7"N, 11°11'46"E). The study area consists of a flat landscape with scattered landmarks (trees, poles, fences, and bushes). We carried out behavioural observations between 10:00 and 15:00 h, as this is the time when males are more active at leks (Beani and Turillazzi 1988). We collected and individually marked 81 non-parasitized *P. dominula* males with Testor's paint on thorax; 35 males were collected from a set of 52 young *Platanus* spp. trees (spaced 5–10 m from each other) and 46 were collected from the low shady branches of one big linden tree (*Tilia* spp.), 20–30 m away from the set of *Platanus* trees. Observations were carried out for 9 days after the capture–marking–release day (20×3-min periods per day at both locations, i.e., *Platanus* and *Tilia* trees, 360×3-min periods altogether).

We identified as territorial each male that was re-sighted on the same *Platanus* tree for at least 4 days (site fidelity) and which repeatedly marked peculiar perches. Scent marking frequency was measured for a total of 9 min per male; observations were non-consecutive, in case males went out of sight. In the last 2 days of field work, we also measured aggressive behaviour of territorial males towards a lure male (see De Souza et al. 2017). We did this by pinning, on a point of the trunk intensively rubbed by the male, one lure consisting of a dead *P. dominula* male washed in pentane for 24 h, to remove all chemical cues. In this way, we were able to test the effect of rival visual cues on territorial males (Cappa et al. 2016a). These lures (30 in all) had small elliptical abdominal spots and their head width ranged between 3.40 and 3.50 mm: thus, simulated intruders had similar body size and abdominal spots. The observer was located 2 m away from each lure and recorded inspections (without physical contact) and strikes (with contact) for 3 min after the first interaction (frequency/3 min, 9 min each male). At the end of the study period, we collected 20 territorial males out of 35 marked on the *Platanus* trees to analyze their CHCs.

During the same period, we described the behaviour of males collected and individually marked on the low branches of the big linden. We labeled these males as non-territorial when they were re-sighted on 2 different days but not on the same site. On the last day of the observation period, we pinned a further 10 lures on the leaves of *Tilia*, spaced 50–80 cm from each other, to test for the aggressive responses of non-territorial individuals against intruders. At the end of the field study, we collected 24 non-territorial males out of 46 marked on the *Tilia* tree to analyze their CHCs.

Sample collection: parasitized and non-parasitized wasps

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

vesparum (six parasitic males and seven parasitic females). An additional three males, each parasitized by a *X. vesparum* male, were collected from *Platanus* trees and from *Tilia* tree.

Male morphological measurements and abdominal spot categorization

Males were photographed using a digital camera (Canon A-620) under a stereomicroscope. These pictures were used to measure male head width in pixels (a reliable indicator of body size; Eickwort 1969) and abdominal spots, including spot Shape Index (SI; Izzo and Tibbetts 2012). SI compares the mean size of right and left spots, measured in pixels, to the mean area of the idealized ellipses (length = L , height = H) inserted into right and left spots. SI was calculated using the formula $SI = A / (\pi \times 1/2 L \times 1/2 H)$. Wasps with regularly shaped spots have lower SI values, while irregularly spotted wasps have higher SI values. Due to the difficulty of inserting ellipses inside very irregular spots in our population, we also measured Spot mean area as the average yellow area of right and left spots using ImageJ (<http://rsbweb.nih.gov/ij/>). We calculated Asymmetry in spot area as the difference between right and left spot area. A subjective summary evaluation of right and left spot shape and size, thereafter referred to as Spot category, was made by two observers blind to predictions: 1 = small, regular shape, round/elliptical; 2 = medium size, irregular shape; 3 = large size, very irregular shape. These three typologies were the most common in our samples, in which small irregular spots, medium regular, or large regular ones were not represented, according to the two blind observers.

Chemical analysis

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

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

For quantification, we injected 2 μl of each extract (which included 800 ng of *n*-C20) into a gas chromatograph with the flame ionization detection (GC-FID: Agilent Technologies 6850 equipped with a J&W HP-1 non-polar capillary column, 30 m L \times 0.32 mm ID \times 0.25 μm PT). For identification, we injected another 2 μl of the same extracts into a gas chromatograph–mass spectrometer (Agilent Technologies 7000C GC–MS Triple Quad equipped with a Zebron ZB-5HT capillary column, 30 mL \times 0.25 ID \times 0.25 μm PT). The oven temperature for both GC–FID and GC–MS was programmed as follows: from 70 to 150 $^{\circ}\text{C}$ at 30 $^{\circ}\text{C}/\text{min}$ and from 150 to 320 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C}/\text{min}$; 10 min at the final temperature of 320 $^{\circ}\text{C}$. The carrier gas was helium at 1 bar and 50 ml/min flux; the injector temperature was 250 $^{\circ}\text{C}$. The output was registered with the Agilent ChemStation program for GC–FID and Agilent Mass Hunter Workstation program for GC–MS.

Identification and quantification of compounds

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

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

Statistical analysis: morphological, behavioural, and chemical data

We performed a first principal component analysis (PCA) based on correlations and varimax rotation on morphological data (head width, SI, Spot mean area, Asymmetry in spot area and Spot category) and a second PCA on the behavioural data of territorial males (marking, inspecting, and striking) to check for collinearity between measures.

We processed the chemical data by computing the relative proportions of the hydrocarbons and then transformed these compositional data using the log-ratio-transformation (natural log of the proportion of each peak, divided by the geometric mean of peak proportions) (Aitchison 1982). We then reduced the number of variables (and checked for collinearity) by performing two PCAs (based on correlations and varimax rotation): the first on the chemical profiles of

males and females ($n = 85$ wasps, of which 60 males and 25 females) and the second on males only.

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

Using a generalized linear model (GLM), we tested whether chemical PCs varied as an effect of sex or parasitism. We also tested whether chemical and morphological PCs predicted male territorial behaviour. To this aim, we used a GLM for binary data (males were either territorial or non-territorial). Finally, we checked whether the morphology of the territorial males predicted their behaviour. To this aim, we performed another GLM on the single PC summarizing behavioural traits (as response variable) and the two PCs on morphological data (as predictors).

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

Results

Sex-specific hydrocarbon profiles and parasite effect

Polistes dominula males and females had complex cuticular hydrocarbon blends that included 70 peaks; these were homologous series of linear alkanes, methyl-branched alkanes, and linear alkenes between C_{23} and C_{37} . Males and females had similar total amounts of cuticular hydrocarbons (mean \pm SD: males 903.97 ± 335.41 ng of hydrocarbons/mg of wasp; females: 868.61 ± 224.25) (estimates [male]: 35.36 ± 67.72 , $\chi^2 = 0.273$, $df = 1$, $P = 0.602$). The infection

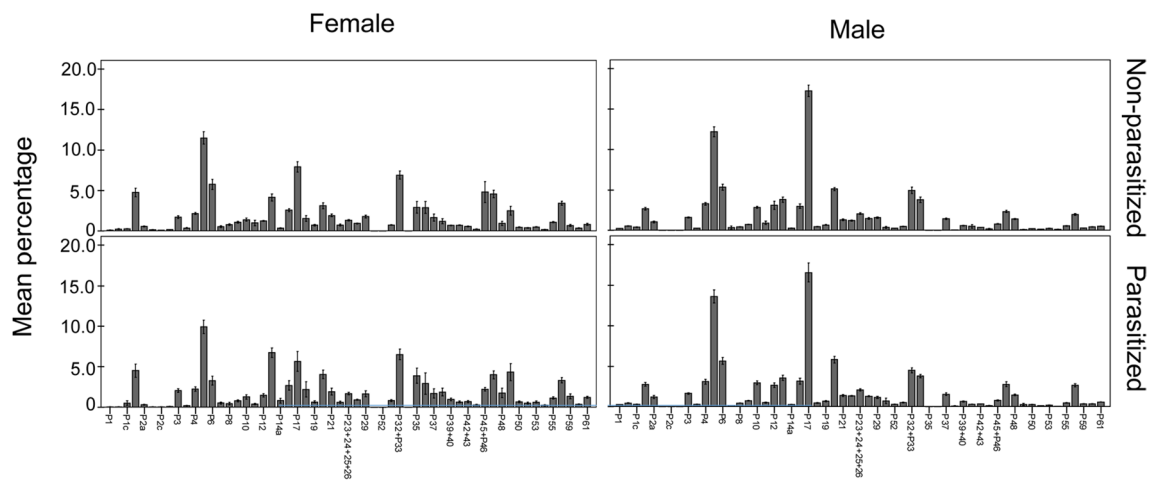


Fig. 1 Average proportion of chemical compounds in the profiles of males and females of *Polistes dominula*, parasitized or non-parasitized by *Xenos vesparum* (mean \pm se). See Table 1 for the identification of the peaks

by the parasite *X. vesparum* had no significant effects on the total amount of CHCs (parasitism status was removed from a preliminary model).

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

The difference between the chemical profiles of males and females was determined by the hydrocarbons loading on PC1. Males had significantly higher PC1 values than females (estimates [male]: 2.016 ± 0.070 , $\chi^2 = 828.45$, $df = 1$, $P < 0.0001$) (Fig. 2). In contrast, being parasitized by *X. vesparum* had no effect on the chemical profile summarized by PC1 (estimate [being parasitized]: -0.089 ± 0.070 , $\chi^2 = 1.62$, $df = 1$, $P = 0.203$) (the interaction sex \times parasitism was non-significant and was removed from a preliminary model). In the part of the chemical profile summarized by PC1, males had higher amounts than females of 5-methylhentriacontane (peak 34, PC1-factor loading = 0.951), 2,x-dimethyltriacontane (peak 30a, factor loading = 0.936), *n*-hentriacontene (peak 30, factor loading = 0.845), *n*-tetracosane (peak P1b, factor loading = 0.787), and *n*-tricosane (peak P1, factor loading = 0.749), and lower amounts of 5-methylpentacosane (peak P2c), 7-methylpentacosane (peak P2b) and 2-methylpentacosane + 3-methylpentacosane (peak P2 cd + P2d) (all these peaks had factor loadings = -0.965); 13,17- + 13,19-dimethylhentriacontane (peak P35, factor loading = -0.943), 9,13- + 7,15-Dimethylhentriacontane (peak P36, factor loading = -0.775) and of

the blend of 9- + 11- + 13- + 15- + 17-methyltriacontane (peak P45 + P46, factor loading = -0.717).

In contrast, the infection by the parasite determined the part of the chemical profile summarized by PC2, and did it differently on males and females, as shown by the significant interaction between sex and parasites (sex [male] \times parasites [yes]: estimates: -1.0529 ± -0.478 , $\chi^2 = 4.848$, $df = 1$, $P = 0.028$) (Fig. 3). Among non-parasitized wasps, males had higher values of PC2 than females; among parasitized wasps, females had higher PC2 values than males, suggesting that the effect of parasites on CHC profiles of the females was stronger than that on males.

Parasitized wasps (and especially females) had higher amounts than non-parasitized ones of the following compounds: a mixture of *n*-docotriacontane + an unidentified hydrocarbon (peak 39 + 40, factor loading = 0.939), 2-methyloctacosane (peak 14, factor loading = 0.857), 5-methylnonacosane (peak 19, factor loading = 0.856), the co-eluting blend of 5,15- + 5,19-dimethylhentriacontane (peak 37, factor loading = 0.852), of 3-methylnonacosane + 7,11-Dimethylnonacosane (peak 21, factor loading = 0.803), 3,15,21-trimethylnonacosane, *n*-triacontane, 2,x-dimethylnonacosane, peaks 23 + 24 + 25, factor loading = 0.780), *n*-Triacontane + an unidentified hydrocarbon (peak 44, factor loading = 0.765), the blend of *n*-Tetracontane + x (peak 50, factor loading = 0.740), *n*-octacosane (peak 12, factor loading = 0.735), and non-acosene (peak 14a, factor loading = 0.706).

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

Territorial males exhibited site fidelity, scent marking, and attacks towards CHC-deprived lure males, while such

Table 1 The list of cuticular hydrocarbons identified in females and males of the social wasp *Polistes dominula*. Peak number correspond to those in Fig. 1. Lines with a grey background highlight the main chemical differences between females and males of *P. dominula*

Peak number	females	males
peak 1	<i>n</i> -Tricosane	<i>n</i> -Tricosane
peak 1a	9-+11-Methyltricosane	
peak 1b	<i>n</i> -Tetracosane	<i>n</i> -Tetracosane
peak 1c	2-Methyltetracosane	2-Methyltetracosane
peak 2	<i>n</i> -Pentacosane	<i>n</i> -Pentacosane
peak 2a	9-+11-+13-Methylpentacosane	11-+13-Methylpentacosane
peak 2b	7-Methylpentacosane	
peak 2c	5-Methylpentacosane	5-Methylpentacosane
peak 2cd	2-Methylpentacosane	
peak 2d	3-Methylpentacosane	3-Methylpentacosane
peak 3	<i>n</i> -Hexacosane	<i>n</i> -Hexacosane
peak 3a	12-Methylhexacosane	12-Methylhexacosane
peak 4	2-Methylhexacosane	2-Methylhexacosane
peak 5	<i>n</i> -Heptacosane	<i>n</i> -Heptacosane
peak 6	9-+11-+13-Methylheptacosane	(9-+)11-+13-Methylheptacosane
peak 7	7-Methylheptacosane	7-Methylheptacosane
peak 8	5-Methylheptacosane	5-Methylheptacosane
peak 9	9,13-+11,15-Dimethylheptacosane	(9,13-+)11,15-Dimethylheptacosane
peak 9a	2-Methylheptacosane	
peak 10	3-Methylheptacosane	3-Methylheptacosane
peak 11	5,15-Dimethylheptacosane	5,15-Dimethylheptacosane
peak 12	<i>n</i> -Octacosane (+P)	<i>n</i> -Octacosane
peak 12a	(2,15-Dimethylheptacosane)	2,15-Dimethylheptacosane
peak 13a	8-+11-+12-+13-Methyloctacosane	8-+11-+12-+13-Methyloctacosane
peak 13b	(+P(squalene))	(+P(squalene))
peak 13b	6-Methyloctacosane	
peak 14	2-Methyloctacosane	2-Methyloctacosane
peak 14a		Nonacosane
peak 15		3-Methyloctacosane
peak 15b		2,12-+2,14-Dimethyloctacosane
peak 16	<i>n</i> -Nonacosane	<i>n</i> -Nonacosane
peak 17	9-+11-+13-+15-Methylnonacosane	(9-+)11-+13-+15-Methylnonacosane
peak 18	7-Methylnonacosane	7-Methylnonacosane
peak 19	5-Methylnonacosane	5-Methylnonacosane
peak 20	13,17-+9,15-Dimethylnonacosane	13,17-+9,15-Dimethylnonacosane
peak 21	3-Methylnonacosane+7,11-Dimethylnonacosane	3-Methylnonacosane
peak 22	5,15-Dimethylnonacosane	5,15-+5,17-Dimethylnonacosane
peak 23	3,15,21-Trimethylnonacosane	
peak 24 +	<i>n</i> -Triacontane	<i>n</i> -Triacontane
peak 25	2,x-Dimethylnonacosane	2,15-Dimethylnonacosane
peak 27+28	9-+10-+11-+12-+13-+14-Methyltriacontane	10-+11-+12-+13-+14-Methyltriacontane
peak 29	2-Methyltriacontane	2-Methyltriacontane
peak 30	Hentriacontene	Hentriacontene
peak 30a		2,x-Dimethyltriacontane
peak 31	<i>n</i> -Hentriacontane	<i>n</i> -Hentriacontane
peak 32	9-+11-+13-+15-Methylhentriacontane	(9-+11-+)13-+15-Methylhentriacontane
peak 33	(7-Methylhentriacontane)	7-Methylhentriacontane
peak 34		5-Methylhentriacontane
peak 35	13,17-+13,19-Dimethylhentriacontane	11,17-Dimethylhentriacontane
peak 36	9,13-+7,15-Dimethylhentriacontane	(9,13-Dimethylhentriacontane)
peak 37	5,15-+5,19-Dimethylhentriacontane	5,15-+5,19-Dimethylhentriacontane
peak 38	7,11,15-+9,13,21-Trimethylhentriacontane	
peak 39+40	(<i>n</i> -Docotriacontane)+x	(<i>n</i> -Docotriacontane)+x
peak 41	10-+12-+14-+16-Methydocotriacontane	
peak 42+43	Dimethyldocotriacontane+2-Methyldocotriacontane	
peak 44	<i>n</i> -Tritriacontane+x	
peak 45+46	9-+11-+13-+15-+17-Methyltritriacontane	(9-+11-+13-+15-+17-Methyltritriacontane)
peak 47	13,17-+11,15-+9,17-Dimethyltritriacontane	(13,17-+11,15-+9,17-Dimethyltritriacontane)
peak 48	7,17-Dimethyltritriacontane	
peak 49	7,11,15-+9,13,21-Trimethyltritriacontane	
peak 49b	5,17-Dimethyltritriacontane	5,15-Dimethyltritriacontane
peak 50	(<i>n</i> -Tetracontane)+x	
peak 51	(3,x-Dimethyltritriacontane)	
peak 52	14-+16-Methyltetracontane	
peak 53	2-Methyltetracontane+Dimethyltetracontane	
peak 54	<i>n</i> -pentatriacontane+x	
peak 55	11-+13-+15-+17-Methylpentatriacontane	
peak 57+58	9,15-+11,15-+13,17-Dimethylpentatriacontane	9,15-+11,15-+13,17-Dimethylpentatriacontane
peak 59	9,15,25-Trimethylpentatriacontane	
peak 60	17-+15-+13-+11-Methylheptacosane	
peak 61	Trimethylheptacosane	

x = unknown
() = traces

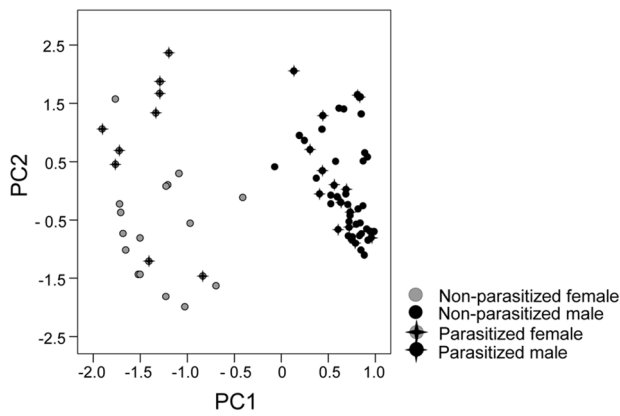


Fig. 2 Plot of PC1 versus PC2 of the chemical signatures of males and females of *Polistes dominula*, parasitized or non-parasitized by *Xenos vesparum*

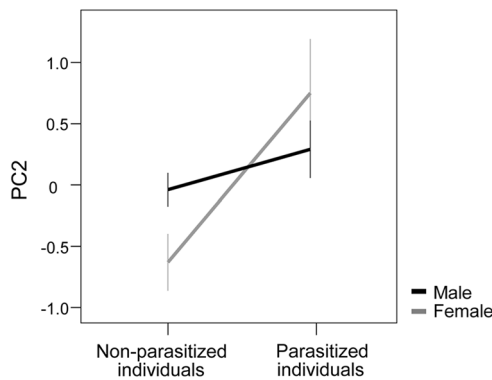


Fig. 3 The variation in the chemical profiles of males and females of *Polistes dominula* (as summarized by PC2) parasitized or non-parasitized by *Xenos vesparum* parasites

behaviours were not recorded for non-territorial males. Territorial males routinely landed and defended the same tree (sometimes two close trees) from intruders for 4 days at least, rubbing the substrate with their abdomens and legs on particular perches, e.g., a leaf, a twig, or a point of the trunk (scent marking, mean frequency \pm SE: 3.13 ± 0.21). During trials with simulated CHC-deprived intruders, the resident males inspected and attacked the lures (inspections, mean frequency \pm SE: 1.59 ± 0.12 ; s: 1.38 ± 0.12). Non-territorial males flew from one branch to another (no site fidelity) and never, or very rarely, rubbed their gasters on the substrate (no scent marking). These males were characterized by non-aggressive behaviour towards lures as well as conspecifics and tolerated low inter-individual distances: sometimes, two males stopped and rested on the same leaf.

We then analyzed whether chemical and/or morphological data reflected whether males were territorial or not. The PCA on the chemical traits (only males) produced eight PCs,

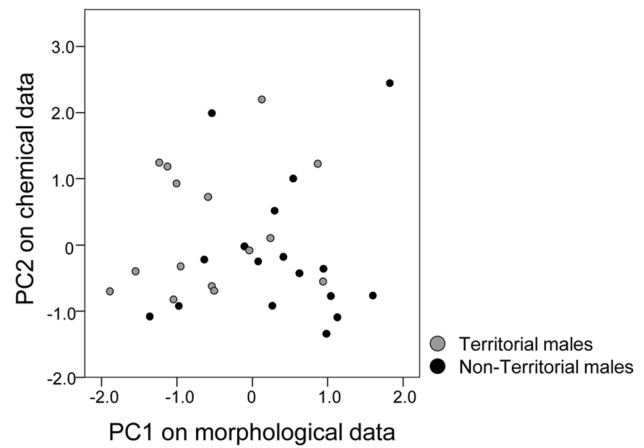


Fig. 4 Plot of PC1 on morphological data versus PC2 on chemical data of territorial and non-territorial males of *Polistes dominula*

of which three explained $> 10\%$ of the variance (PC1 49.6%; PC2 12.9%; PC3 11.9%). The PCA on the morphological traits resulted in two PCs (PC1 41.1%; PC2 21.6%). Spot category and Spot mean area (and Asymmetry in spot area, although at a lower extent) were summarized by PC1 (factor loadings for Spot category: 0.877; Spot mean area: 0.798; Asymmetry in spot area: 0.643) and PC2 (factor loadings for SI: 0.760; head width: -0.674).

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

Territorial males: behaviour, chemical profiles, and morphological traits

As regards territorial males ($N = 18$), the PCA on the behavioural data produced only one PC, which explained 80.7% of the variance and whose factor loadings were: strike: 0.874; inspection: 0.833; marking: 0.714, and thus, this component efficiently summarized the three behaviours. Territorial males with a lower Spot category and a smaller Spot mean area performed significantly more markings, inspections, and strikes (Fig. 5), but had no peculiar chemical profiles, as shown by the highly significant association between behaviour and morphological traits (PC1 on morphological data,

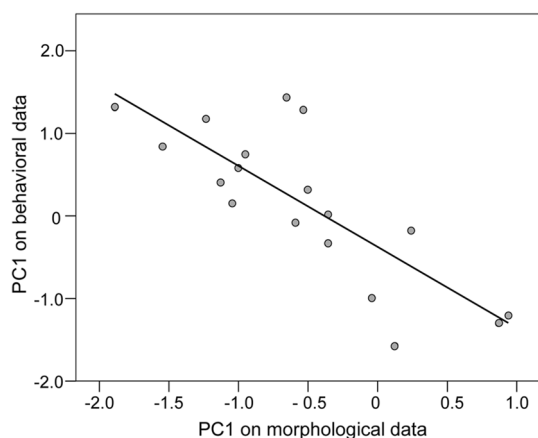


Fig. 5 The association between morphological and behavioural data in *Polistes dominula* Territorial males: males with smaller elliptical abdominal spots performed significantly more markings, inspections, and strikes

estimates: -0.957 ± 0.177 , $\chi^2 = 29.155$, $df = 1$, $P < 0.0001$), and by the lack of significant association between behaviour and chemical profiles (PC2 on chemical data, estimates: 0.169 ± 0.110 , $\chi^2 = 2.354$, $df = 1$, $P = 0.125$).

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

Discussion

Chemical discrimination of sex

For the first time, our study compared chemical profiles of *P. dominula* between sexes, parasitized, and non-parasitized individuals, as well as in relation to male mating strategies. The chemical profiles of *P. dominula* differed significantly between sexes in a few sex-specific compounds and in the relative amounts of others (Fig. 1), whereas the total CHC amount was similar. Sex discrimination via visual and chemical cues has already been demonstrated in other social Hymenoptera (Keeping et al. 1986; Nonacs and Carlin 1990; Passera and Aron 1996; Derstine et al. 2017; Cappa et al. 2019) and in *P. dominula* in the social environment of the nest (Cappa et al. 2016a). Individual chemical profiles may also play a relevant role in the mating context of leks, to assess the identity and the quality of a potential partner or rival: this is suggested by the observations of successful or attempted copulations by resident males with females landing inside their territory, whereas intruder males are the

target of aggressive interactions (Beani and Turillazzi 1988; Beani and Zaccaroni 2015).

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

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

Parasite influence on CHCs

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

The chemical alteration of the cuticular profile by parasites partially confirms previous analyses in *P. dominula* and *P. ferreri* females (Dapporto et al. 2007; Torres et al. 2016). The underlying mechanism leading to changes in epicuticular blend probably occurs during the development of the endoparasite inside wasp larvae (Giusti et al. 2007). These slight changes, a by product of the hosts' altered development, may be adaptively used by the parasite to manipulate the acceptance of infected individuals by nestmates, e.g., in bees (Cappa et al. 2016b, 2019). *P. dominula* workers, if parasitized, desert the colony 1 week after emergence (Hughes et al. 2004a), when their chemical profile is still forming (Lorenzi et al. 2004) and the parasite is not yet extruded; lacking clear chemical discrimination, parasitized workers are not the target of aggressiveness by nestmates (Hughes et al. 2004a). The peculiar chemical profile induced by the parasite could facilitate the aberrant summer aggregations mainly formed by parasitized females (Hughes et al. 2004a; Beani et al. 2018). According to slight quantitative changes in the profile of parasitized wasps, winter aggregations include both parasitized and non-parasitized females (Dapporto et al. 2007; Beani et al. 2011). This weak chemical discrimination might explain why in preference trials, male antennation and attempted copulations were directed towards both parasite-castrated and non-parasitized gynes (Cappa et al. 2013). Noticeably, in these brief lab trials, full copulations were extremely rare. It is likely that a more accurate mate assessment occurs in leks, where males simply ignore aggregations of parasitized females (Beani, *pers. obs.*).

Territorial behaviour of males with sexually selected ornaments

Regarding mating strategies, there was no evidence of any significant chemical difference between territorial and non-territorial males. Intriguingly, the relatively flexible behaviour of *P. dominula* males, which sometimes switch from non-territorial to territorial behaviour depending on the context (Beani and Turillazzi 1988; Beani and Zaccaroni 2015), matches the weak differences in the hydrocarbon profiles that we found in males adopting different mating strategies, as well as in body size. In contrast, concerning ornamental traits (Fig. 4), territorial males had smaller elliptical abdominal spots than non-territorial ones (as measured by means of Spot mean area, Spot category, and Asymmetry between spots). Moreover, in territorial males (Fig. 5), there was evidence of a significant relationship between spots and behaviour: males with smaller and regular spots were more active both in scent marking and in aggressive interactions with lures.

Abdominal spots are highly variable and strongly influenced by larval nourishment: they are elliptical and regularly

shaped when males are reared with a rich diet (Izzo and Tibbetts 2015). How color patterns are shaped during development is still unknown in wasps, but large irregularly shaped spots and high asymmetry in s might be due to stressors during the development, such as a restricted diet and low levels of melanin, an honest indicator of male phenotypic quality (de Souza et al. 2017, 2018). Visual cues, in addition to chemical cues, may play a critical role in sex discrimination (Cappa et al. 2016a) and mating success, at least in lab trials (Izzo and Tibbetts 2012). Intriguingly, different measures of abdominal spots (including the non-parametric Spot category, easier to assess than Shape Index, and unrelated to both male behavioural and morphological traits in our male population) may be adopted by females to distinguish between males with different territorial strategies, highlighting how visual signals could be also important in the communication system of social wasps (Tibbetts et al. 2009).

The yellow spots may be an example of constrained sexual signals which depict, at the moment of emergence, the quality of the male, further described by the CHCs (which conveyed information about parasite infection). In this respect, the CHCs might inform the females about the current quality of the male, acting in concert with the visual ornaments. Even if male and female attractants appear common in hymenopterans, very few sex pheromones have been chemically identified, due to the behavioural sophistication and the wide spectrum of communication modes in social insects (reviewed by Ayasse et al. 2001). Lacking specific behavioural tests on *P. dominula* in the field, we can advance the hypothesis that CHCs are potential cues to assess sex and mate quality (e.g., parasite infection) at a short distance, after responding to behavioural displays and visual stimuli, similarly to the dynamic multimodal signaling in birds (Karubian et al. 2011).

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