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Differentiating between gynes and workers in the invasive hornet *Vespa velutina* (Hymenoptera, Vespidae) in Europe

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Abstract

In theVespinae, morphological differences of castes are generally well-marked, except for some *Vespa* species, where it is difficult to distinguish between future queens and workers in autumn-winter colonies. Individual weights have widely been used as a distinguishing factor but recently cuticular hydrocarbon profiles seems to be the definitive tool, although much more expensive and time-consuming. Parameters such as size (mesoscutum width), wet and dry weight were analysed, throughout several colonies, to differentiate female castes (workers and gynes) in the hornet *Vespa velutina* in Europe. These parameters were compared to cuticular hydrocarbon profiles. The results showed that in late autumn, but not earlier, populations are divided into two size groups, which, based on their CHC profiles, can be hypothesized to correspond to workers and gynes. This differentiation mirrored a good separation by size that proves to be more accurate than weight (wet and dry). The size limit between workers and gynes is established at a mesoscutum width of 4.5 mm.

Keywords

caste differentiation, CHCs, chemical signature, size, weight, yellow-legged hornet

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Introduction

The Vespidae includes both solitary and eusocial groups with extensive variation among the social wasps (Cowan 1991). Caste polymorphism is one of the most widely studied point (Noll et al. 2004). Traditionally, it has been considered that Vespinae wasps (*Vespa, Provespa*, *Dolichovespula* and *Vespula*) present morphological differences between female castes, with queens being larger than workers (Felippotti et al. 2009, Jeanne and Suryanarayanan 2011). However, not all species present the same degree of caste differentiation. *Dolichovespula* shows the weakest caste differentiation (Greene 1991) and *Vespula*, the highest (Spradbery 1973). In the case of *Vespa* there are species, such as *Vespa mandarinia*, *V. affinis*, *V. crabro* or *V. simillima,* in which castes present clear size separation. By contrast, hornets like *V. tropica* and *V. analis*, show an overlap of caste sizes (Matsuura and Yamane 1990). So, in most vespine wasps, size variation among females is discontinuous, although without any clear external physical distinction between gynes and workers aside from size. It seems that *Vespa velutina* conforms to this pattern. Moreover, there are few studies of *V. velutina* on morphological differences between female castes and those use a complex wing morphometric procedure (Perrard et al. 2012).

The size difference between castes can be expressed in various ways. For example, mesoscutum width (MW) from tegula to tegula is one of the most-used parameters to distinguish castes in some Vespidae species (Noll et al. 1997, Felippotti et al. 2009; Felippotti et al. 2010). In contrast, in some other species it is hard to find morphological features to distinguish castes; for this reason, some authors have looked into other kinds of parameters. Strassmann et al. (1984) reported differences linked to the capability of gynes to overwinter. This explained why foundresses develop multistratified fat bodies whereas workers do not (Eickwort 1969, Toth et al. 2009). For that reason, many authors have used weight to distinguish between workers and gynes (Monceau et al. 2013, Rome et al. 2015).

Apart from size and weight, cuticular hydrocarbon profiles (CHCs) can be used to differentiate between castes in a colony (Liebig 2010, Darrouzet et al. 2014). CHCs are complex mixtures of long-chain aliphatic and methyl-branched alkanes and/or alkenes present on the epicuticle of these insects (Blomquist and Bagnères 2010). This layer of CHCs not only protects insects against desiccation (Gibbs and Rajpurohit 2010), but is also part of inter- and intraspecific communication (Howard and Blomquist 2005, Blomquist and Bagnères 2010). The pattern of cuticular chemical compounds is linked to several biological aspects such as, dominance, fertility (reproductives and non-reproductives) (Liebig 2010), workers' activity (Rahman et al. 2016), nesting sites (Steinmetz et al. 2003) or recognition between species, castes, nest mates (Howard and Blomquist 2005) and sexual mates (Spiewok et al. 2006).

In European populations of the yellow-legged hornet, *Vespa velutina*, CHC profiles differ between individuals, depending on caste and sex (Gévar et al. 2017), as they are in several other social insects (Liebig 2010), even though there is genetic homogeneity (Arca et al. 2015) and inbreeding (Darrouzet et al. 2015). These differences are based mainly on the relative quantities of the various compounds that make up the chemical signature.

The natural distribution of *Vespa velutina* ranges from Afghanistan to eastern China, Indo-China and Indonesia (Villemant et al. 2011). Nowadays, the *nigrithorax* form of this species is an invader in Europe, since about 2004 (Rortais et al. 2010) and in South Korea since 2003 (Kim et al. 2006). New colonies of *V. velutina* are established in the spring by mated queens, after the overwintering period. Colonies go through a period in which an increasingly large number of workers are produced in order to ensure colony growth, and then produce sexual individuals (males and gynes) in autumn (Monceau et al. 2013, Rome et al. 2015).

The aim of this study was (1) to study the dynamics of colony population and individual morphometric variations throughout the annual nesting cycle of *Vespa velutina* in Europe, measuring mesoscutum width, as an index of linear body size. As an alternative discriminator, (2) we tested the cuticular hydrocarbon (CHC) profiles of known autumn females. Finally (3), we compared the CHC profiles with size, wet weight, and dry weight with the goal of discovering rapid, simple and useful parameters for determining castes or groups.

Methods

Sample collection

In this study, 11 nests at different developmental stages were used. These nests were collected from June to December between 2011 and 2015 at different locations in the Basque Country (Spain) and Indre-et-Loire (France) (Table 1). In both countries the species was well established (Goldarazena et al. 2015; Rome et al. 2013). The collected nests were frozen, dissected and the individuals separated by sex. Only the females were used for this study. All of the individuals were kept frozen at -20°C until they were studied. Three types of data were analysed: size, weight, and CHC profile of individuals.

Table 1. Dates and locations of collected colonies.

Colony	Date	Location
1	02/12/2011	Civray de Touraine (Tours, France)
2	22/11/2013	Tours (Tours, France)
3	02/06/2014	Ibarrangelu (Biscay, Spain)
4	22/06/2014	Loiu (Biscay, Spain)
5	23/07/2014	Mungia (Biscay, Spain)
6	26/07/2014	Gatika (Biscay, Spain)
7	28/08/2014	Lasarte (Gipuzcoa, Spain)
8	30/08/2014	Astigarraga (Gipuzcoa, Spain)
9	01/10/2014	Mungia (Biscay, Spain)
10	26/10/2014	Maruri (Biscay, Spain)
11	13/11/2015	Civray de Touraine (Tours, France)

Size and weight analyses

Size of individuals: the mesoscutum width (MW) from tegula to tegula was measured in a stereomicroscope coupled to a camera system. The MW was used as an index of overall linear size (Noll and Zucchi 2002, Ohl and Thiele 2007). Size measurements are given in mm.

Weight of individuals: wet (WW) and dry weight (DW) were taken using a high precision balance (0.01mg). The wet weight was obtained after two hours of defrosting specimens to avoid moisture on the body surface. For dry weight, hornets were dried in an oven at 70°C for 24h (modified from Monceau et al. 2012). Weight measurements are given in g.

Chemical analyses

CHC profiles were analysed to determine the castes of individuals. CHCs were extracted by placing hornets in 1 ml of pentane and shaken for 2 minutes in a Wheaton™ V Vial™ glass. 500 µl of the extract was placed in another vial and stored at -20°C until the samples were analysed. Ten µl of standard n-eicosane (C20) (10^{-3} g/ml) was added to each sample and, immediately afterwards, 2 µl of sample was injected into a gas chromatograph (Agilent 7820A) coupled with a flame ionisation detector (FID). The analysis was carried out with a 413HP5 (30m × 320µm × 0.25µm) capillary column. The oven temperature programmed was from 50°C to 200°C (8°C/min), from 200°C to 315 (5°C/min) and 315°C for 5 min. The injection was in splitless mode and helium was used as a carrier gas (1.7 ml/min). All data were processed with ChemStation B.04.03 software. The relative proportions of each peak were calculated as described in Bagnères et al. (1990).

Statistical analysis

MW histograms were used to see how the sizes of individuals change throughout the season. All of the females in the eight Spanish colonies, including the queens, were used.

The XLSTAT 2014 add-on for Microsoft Excel® was used to perform the Gaussian mixture model (GMM), fitted using an EM algorithm, with the MW data of 350 individuals from the four late autumn colonies pooled together to detect potential size classes between reproductive and sterile castes. Using the same individuals, identical procedure was follow to verify whether potential weight (wet and dry) classes existed.

A Principal Components Analysis (PCA) of the individual CHC signatures of four autumn colonies was performed. The independent variables were the relative area of the most important peaks (≥ 0.1%) in the chromatogram. A Cluster Analysis (Pearson correlation index and k-nearest neighbour algorithm) was performed to define the chemical groups. After that, a Discriminate Analysis with cross-validation, over those

groups to test the fitness of categories separation, was performed. In order to test how the size or weight classes, got from GMMs, fit to PCA CHC profiles, distinct representations of the PCA plots were made. The analyses were carried out using IBM SPSS Statistics 23.

Results

The distribution of the morphometric MW variable in the different colonies from June to October is represented in Figure 1. The frequency distribution of mesoscutum width was unimodal throughout most of the colony cycle (from early June to mid October), with a single large individual (the queen) lying outside the mode. The distribution became bimodal late in the colony cycle with the appearance of new gynes.

Apart from the modality, individual numbers and body size also changed (Fig. 1). As the season went by, the number of individuals in each colony increased from N=20 in Colony 3 to N=249 in Colony 10. The same occurred with the sizes of individuals. In unimodal colonies, the MW of none of the hornets reached 4.5 mm, with the exception of the large individual which is outside the group. However, in late-season Colony 10, which was bimodal, the size of the MW varied from 3.79 mm to 4.49 mm for the population on the left, and from 4.61 mm to 4.87 mm for the one on the right. In most of the colonies represented in Fig. 1, the individual that is outside the unimodal distribution had a MW greater than 4.5 mm, except for Colony 6 where this was 4.48 mm. The MW of 4.5 mm was the threshold used to separate the two groups in the bimodal colony.

Figure 2 shows the Gaussian mixture model (GMM) of autumn colony data, performed to establish the threshold between the two populations according to size (MW) and weight (WW and DW).

The GMM analysis for MW split the distribution into two size classes, separated by a threshold or mid-point value of 4.5 mm (Fig. 2A). The 5% uncertainty level was set at 4.4 mm for workers and 4.58 mm for gynes. The same GMM analysis was performed for wet weight (WW) and dry weight (DW). In the case of WW (Fig. 2B) the model did not have the same bimodal distribution as MW. Even so, the threshold calculated was 0.618 g, with the 5% uncertainty level at 0.445 g for workers and 0.797 g for gynes. Unlike WW, the DW GMM did show a bimodal distribution (Fig. 2C), with a threshold value of 0.225 g separating the two groups. The 5% uncertainty value was 0.202 g for workers and 0.247 g for gynes.

For each of the three GMMs, the mid-point or threshold was compared to the highest values for the 5% uncertainty interval, in percentage terms, to check which of the three presented the smallest uncertainty interval. A higher percentage showed a lower uncertainty interval, resulting in a clearer separation between groups. These values were 98.25% for MW, 77.54% for WW, and 91.09% for DW.

The Cluster Analysis of the CHC profiles of the four late-season colony hornets, showed three clearly well-separated chemical groups, named as 1, 2 and 3. They are

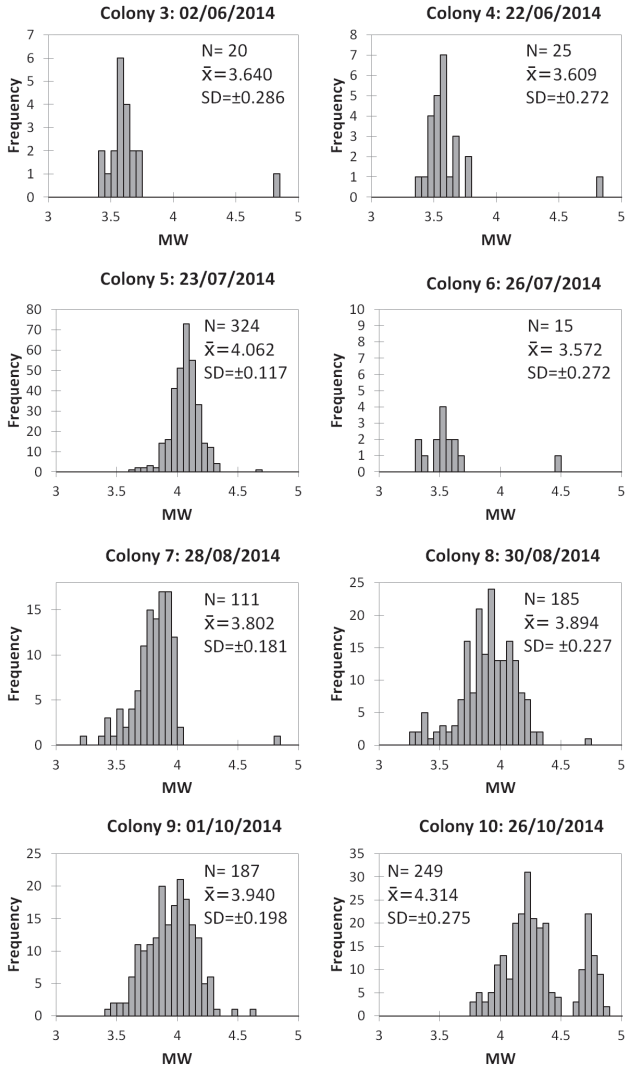


Figure 1. MW histograms. Histograms showing MW (mesoscutum width) from eight different colonies, sorted by collection date.

represented in the axes I and II of the ACP (Fig. 3). The Discriminant Analysis showed all the hornets were chemically well classified. The group 1 hornets showed to be chemically more similar to each other, since the dots cloud was more compact. The group 2 was more scattered, showing they were chemically more heterogeneous. The group 3 had very few individuals.

In the PCA of the figure 3, ordination plots were displayed according to the size or weight class of each hornet. In the size (MW) column (Figure 3), all individuals classified as "small" belonged to the same chemical group (group 1) and the "large" to the

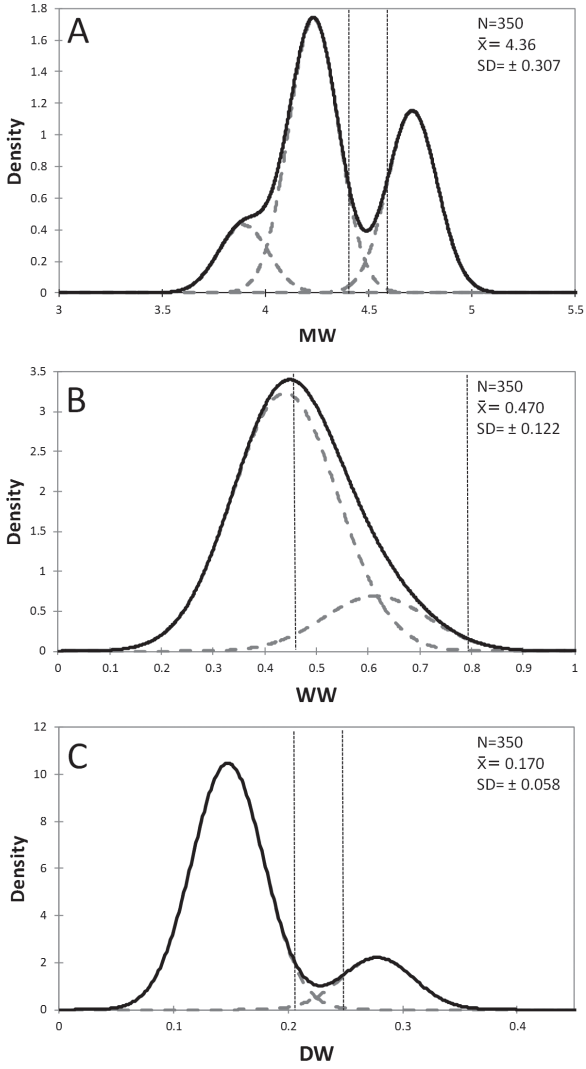


Figure 2. GMMs of hornet size, WW and DW. *Vespa velutina* size (**A**), wet weight (**B**), and dry weight (**C**) distribution using a Gaussian Mixture Model. Two-dimensional distribution is represented by continuous line **A** workers < 4.5 mm, gynes ≥4.5mm **B** workers < 0.618 g, gynes ≥ 0.618 g and **C** workers < 0.225 g, gynes ≥ 0.225 g. The dashed lines represent group densities. The 5% level of uncertainty is shown by dotted lines **A** 4.4 mm–4.58 mm **B** 0.445 g–0.797 g and **C** 0.202 g–0.247 g. 4 colonies: Colony 1, N= 30; Colony 2, N= 30; Colony 10, N= 240; Colony 11, N=50.

other two (groups 2 and 3). This showed a good agreement between both PCA chemical groups and size ones. There was an exception in Colony 1, where three individuals classified as "small" appeared in the group 2.

In the column showing the PCA for wet weight (Fig. 3), it can be observed that the three CHC groups did not match well to the two WW defined groups. In Colony

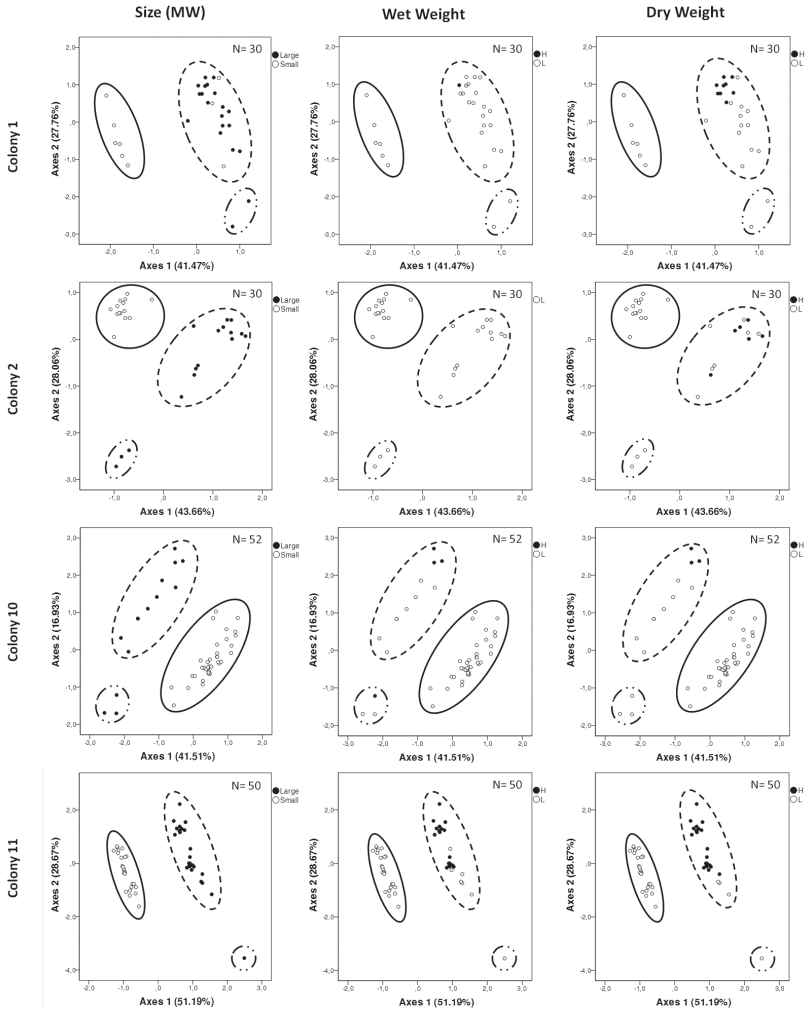


Figure 3. PCA of the three CHC profiles labelled by hornet size, WW and DW. Principal Component Analysis of CHC profiles in each of the four autumn colonies. Chemical groups are defined by continuous line: Group 1; dash line: Group 2 and dot-dash line: Group 3. PCA dots show representations according to GMMs size, wet weight and dry weight thresholds of hornets. Size, Black dots: Large females (MW ≥ 4.5 mm); White dots: Small females (MW < 4.5 mm). Wet weight, Black dots: Heavy fresh females (≥ 0.618 g); White dots: Light fresh females (< 0.618 g). Dry weight, Black dots: Heavy dry females (≥ 0.225 g); White dots: Light dry females (< 0.225 g).

1, all individuals, except one, were "light". In Colony 2 there were no hornets classified as "heavy". In Colony 10 there are four "heavy" individuals spread in the second and third CHC groups. In the case of Colony 11 all the "heavy" hornets were in the second chemical group, most of them in the top of the group.

Lastly, in the column showing the PCA for dry weight (Fig. 3), all colonies contained "heavy" individuals, which are located in the top part of the CHC group 2.

Discussion

Mesoscutun width (MW) seems to be one of the most common parameters used in morphometry, as it is relatively large and constant, thus minimising errors in measurement, and can be taken easily (Noll and Zucchi 2002, Ohl and Thiele 2007). As a result, this size parameter was chosen, among all the used measures, to study the dynamics of the *Vespa velutina* population as well as individual morphometric changes from June to October. The latter, had not been studied until now.

Early in the season, the number of individuals per colony was low and they were also smaller in size. However, close to the end of the colony life cycle, both individual numbers and sizes are larger and the individual size distribution changes from unimodal to bimodal. From June to early October, we observed that all of the unimodal colonies studied contained only one individual that was notably larger in size than the other females, being the queen of those colonies. Moreover, these females matched the size of individuals in the second population (MW > 4.5mm) in the autumn nests. In the other hand, females captured in early spring, which are overwintering survivor gynes, also presented MW > 4.5mm (Pérez-de-Heredia, personal observation). Therefore, it can be said that these larger autumn females will become the queens of the following year's colonies. This population dynamic is typical in aculeate colonies which are founded by a single queen. The first cohort is raised by the queen alone and comprises the smallest workers; the following cohorts increase in size until the largest workers appear. This happens together with, or is followed by, the production of gynes and males (Wilson 1971, Miyano 1981). At the same time as gynes are being produced, female size distribution starts turning from unimodal to bimodal. This bimodality corresponds to the differentiation between castes, workers and gynes (Spradbery 1973). This size increase in females, during the annual colony cycle, is associated with the trophic advantages of having more workers in the nest to feed larvae. Another explanation for this increase in individual size is the sizes of the cells where larvae are raised, which gradually increase as the nest grows larger (Spradbery 1972). Edwards (1980) showed that, in *Vespa crabro*, the size of individuals is conditioned by the size of the cells in which they are raised. There were two size classes among males, some of which were raised in worker cells and others in gyne cells.

The bimodality of the size parameter in late autumn colonies led us to consider size as a good caste differentiator. Nevertheless, hitherto, only the weight of individuals has been used to differentiate castes in *Vespa velutina*. For that reason, we also analysed WW and DW using the GMM procedure to establish the threshold for each of them and compare the results to MW, to determine the best caste predictor.

According to the three GMMs, the MW size presents less overlap bimodality between groups, making it more accurate and reliable than either of the weights. This can be explained because once an insect emerges as an adult; its body is enclosed in a solid, non-regenerative cuticle, making body plates invariable. Unless it is damaged, no morphological changes occur in any hardened (sclerotized) body part (O´Donnell 1998) regardless of insect age or physiological state.

The GMM for WW presents a greater overlap between groups, resulting in a unimodal distribution. This can be explained because there is great variability in the WW for individuals of the same size, influenced by differences in metabolic status, age of individuals (Hilligsøe and Holmstrup 2003) or by physiological variations, as occurs in collembolans (Verhoef 1981). By contrast, the GMM of DW presents a bimodal pattern, which means that the parameter is more constant for a given group of hornets and in consequence is more reliable.

Our study shows that the thresholds for separating the two classes or groups were 0.618 g for WW and 0.225 g for DW. These data differ a little from those observed by Rome et al. (2015), which considered that individuals with WW exceeding 0.593 g and DW exceeding 0.250 g were considered to be gynes, while those with lower weights were workers. These discrepancies in the DW may be due to differences in methodology, such as the temperature and drying time for the individuals. Even so, the variation in the DW rank linked to 5% uncertainty was very similar: 91.09% in our study and 87.72% in the data of Rome et al. (2015).

The three chemically-differentiated groups observed in the four autumn colonies, are explained as follows. Hornets of groups 2 and 3 presented sizes equal or bigger than 4.5 mm (except for three individuals in Colony 1). In addition, only hornets of the group 2 (classified as "large" hornets) presented high weights. So, following to Rome et al. 2015, it can be hypothesized that this group belongs to the gynes. The cuticular profiles discriminate by castes, workers being in chemical group 1 and gynes in group 2. Group 3, located apart from the other two, is an undefined chemical group, different from the other two.

The aforementioned three mismatched individuals in Colony 1 have the size of workers but they have the chemical signature of gynes. It is possible that, in some nests, this type of gyne could be raised in workers' cells, resulting in small gynes. This was also observed in *Vespula germanica* (Spradbery 1993), but further studies are needed to confirm that. In all cases large hornets always had gyne CHC profiles. This can be explained because, when gynes start emerging, the production of workers is interrupted (Matsuura and Yamane 1990, Monceau et al. 2013).

Group 2, consist of both high and low weights gynes. The gynes are the only members of the colony that will survive the winter (Monceau et al. 2014). Recently-emerged gynes spend some days inside the nest before leaving it to hibernate, as long as 13–14 days in the case of *Vespa affinis* (Martin 1993). During those days, they are fed by trophallaxis with substances regurgitated by workers and larvae. Most of this food is converted into fat reserves to last the winter (Matsuura and Yamane 1990). The workers, however, have no such energy reserve, and this makes them lighter than gynes (Martin 1993). For that reason we can assume that hornets with a large MW but low weights are young gynes which have had no time to feed enough to reach high weight. All these hornets have a similar chemical profile so, it can be concluded that PCA axis II discriminated the groups by age. Thus, the workers (group 1) are more homogeneous, because all of them have similar ages contrary to what hap-

pens in gynes (group 2) which have hornets with different ages. Finally, group 3 is comprised presumably by just emerged hornets, which have not had enough time to develop and get a defined chemical profile (Lorenzi et al. 2004). Thus, it can be hypothesized that they belong to the caste of the just emerged gynes. This is supported by the fact that there are no individuals of the chemical group 1 with a MW equal or bigger than 4.5mm.

According to the DW threshold of 0.250 g given by Rome et al. (2015), recently-emerged gynes which have no time to feed are classified in the group of light individuals, i.e. workers. The same happens with colonies collected at the end of autumn, when feeding conditions may not be ideal due to the lack of food or because there are not enough workers to feed larvae (Matsuura and Yamane 1990). Both workers and final instar larvae are feeders of recently-emerged hornets (Matsuura and Yamane 1990). So, the two castes tend to be lighter from November to December (Rome et al. 2015). The heaviest females in the chemical gynes group, which appeared close together, are probably the oldest ones. They have remained feeding in the nest for a longer time accounting for their greater amounts of reserves.

Since *Vespa velutina* was introduced into Europe, a number of scientific questions have been analysed regarding this invasive species. For some of them, it is crucial to discriminate between female castes to better understand some of the biological aspects, such as when the first gynes emerge and how many gynes are produced per nest. So, considering the data set out here, *V. velutina* seems to present distinctive morphological female castes depending on their MW. Moreover, the variable rank corresponding to the 5% uncertainty level in the GMM is lower in the MW than in the weight data, with less potential for error. This is confirmed by the results from the CHC profiles. Hornets with a MW of 4.5 mm or more are considered to be gynes, while those with a MW of less than 4.5 mm are considered to be workers. This MW size parameter is easier, faster and cheaper to measure than analysing CHC profiles. DW worked better than WW but neither of them is as accurate as MW at least with young or not well fed gynes.

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