

Analysis of the secondary nest of the yellow-legged hornet found in the Balearic Islands reveals its high adaptability to Mediterranean isolated ecosystems

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Abstract The yellow-legged hornet (*Vespa velutina*) was detected for the first time in the north of Spain in 2010, but was not detected in Majorca, Balearic Islands until 2015 and only one secondary nest, with 10 combs, was found in the northwest of the island. During 2016, nine more nests were found in the same region. To better understand the biology of *V. velutina* in isolated conditions, the following objectives were proposed: (I) describe the architecture and structure of nests; (II) analyse the shape of combs and develop a new method to confirm the circular pattern of breeding; (III) determine the colony size and (IV) determine the succession of workers and sexual individuals throughout the season. For these reasons, nests that were removed were frozen for at least 48 days until analysis. Our results show that this species has a high reproductive potential under isolated conditions. Results reveal that parameters such as weight, height, diameter, number of cells and total individual production are directly related. Moreover, each mature nest can produce up to 9,000 individuals and several hundred potential founder queens. All results inform formulation of an efficient control or eradication programme in the Balearic Islands, as we are in the early stages of invasion and intervention is essential to eradicate *V. velutina* on Majorca Island.

Keywords: architecture, breeding provision, caste differentiation, individual production, Latter's formula, Majorca, *Vespa velutina nigrithorax*

INTRODUCTION

The yellow-legged hornet (*Vespa velutina* Lepelletier 1836) is a social Hymenopteran of the family Vespidae. It is native to tropical and subtropical areas of Southeast Asia (Archer, 1994; Martin, 1995; Carpenter & Kojima, 1997). It was reported for the first time in south-west France in 2004 (Haxaire, et al., 2006; Rome, et al., 2009; Villemant, et al., 2011) and rapidly spread to nearby European countries: Spain (Castro & Pagola-Carte, 2010; López, et al., 2011), Portugal (Grosso-Silva & Maia, 2012), Italy (Demichelis, et al., 2014), Belgium (Bruneau, 2011; Rome, et al., 2013) and Germany (Witt, 2015). This species is also established in South Korea (Choi, et al., 2012; Choi, et al., 2013) and Japan (Ueno, 2014). The most recent incursion was in Great Britain in 2016, and Switzerland in 2017 (UK National Bee Unit, 2016; Budge, et al., 2017).

The introduction of *V. velutina* to Europe could lead to important economic and ecological impacts. The main impact of the yellow-legged hornet is the likely decrease in honeybee (*Apis mellifera*) populations (Tan, et al., 2007; Monceau, et al., 2013a; Monceau, et al., 2013b), as wasp larvae feed on the proteins of honeybees. Honeybees are considered one of the most important pollinators for agriculture, so the decrease of *A. mellifera* populations is anticipated to decrease the production of their crops resulting in economic losses for the farmers (Villemant, et al., 2011; Arca, et al., 2014). In addition, it is possible that the yellow-legged hornets attack humans when colonial nests are established in urban areas (Villemant, et al., 2006). In the particular case of Majorca, a yellow-legged hornet invasion could be devastating for the populations of honeybees, the fragility of the ecosystem (typical of the island ecosystems) and the impact on endemic insects.

The life cycle of *Vespa velutina* is annual. In optimal ambient conditions, when the temperature is high and the food resources are abundant, one founder queen will build an embryo nest (Edwards, 1980; Archer, 2010), after that the workers begin to emerge. In spring the workers build combs around the embryo nest; this is called the primary nest. The primary nest has an irregular structure with

the embryo nest in the centre (Spradbery, 1973). During summer, the colony increases and the primary nest is left and another nest is built in the same location, if the conditions are favourable (food resources, temperature, humidity, etc.). If the ambient conditions are unfavourable (cold conditions and limited food resources), they build the secondary nest in a different location, normally in large trees. This new nest is named the secondary nest and is larger than the primary nest, with the objective that the colony increases. The nests of this invasive species are classified as a calyptodomus type (concealed nest) (Fig. 1), having an external spherical structure, but the combs are of a conical structure. The upper combs have large diameters and the lower ones smaller diameters, with a slight narrowing in the last comb (Jeanne, 1975). When the reproductive caste emerges in autumn, the nest is

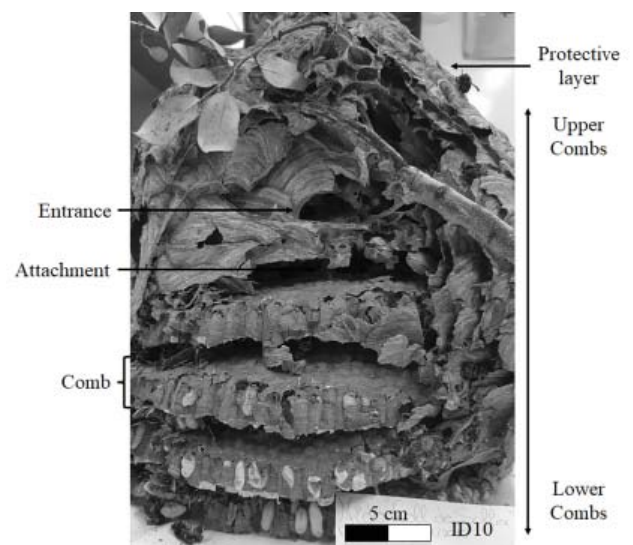


Fig. 1 Calyptodomus nest of *V. velutina*.

called mature, because it is possible to separate males from females by their morphology (Choi, et al., 2012; Rome, et al., 2015). The male hornets fertilise new founder queens, after which the colony dies during the winter. Only new founder queens survive the winter and build new nests the following season and start the annual life cycle (Edwards, 1980; Matsuura & Yamane, 1990).

The yellow-legged hornet is established in the northern regions of Spain (Navarra, Basque Country, Galicia and Cantabria) (Castro & Pagola-Cardé, 2010; López, et al., 2011), and in Catalonia (Pujade-Villar, et al., 2012). In 2015 it was reported in Soller (Majorca, Balearic Islands). The hornet was detected by a beekeeper and was identified by the laboratory of Zoology of the University of Balearic Islands. Together with the local authorities, an intensive survey was implemented to detect nests, as is described in Leza, et al. (2017). In 2015 only one nest of *V. velutina* was found in the north-west of the island. However, during 2016 nine more nests were found in the same region. At this moment, the invasion is in its early stages (Leza, et al., 2017), and is the first incursion on an island where eradication through locating and destroying nests can be used to control the spread; a scenario very different to mainland Europe. This immediate intervention plays an important role in the invasion or eradicating the species on the island.

Although the general structure and production of the nests of this species has been previously described in Asia (Spradbery & Kirk, 1978; Matsuura, 1991) and Europe (Rome, et al., 2011), it is important to study the nests in local conditions in order to find out if the adaptation of *V. velutina* is similar to other regions or if they would be unable to breed on the island. For this purpose, the detailed study of the nests found on Majorca (3,667 km², situated 176 km from the mainland) can be a useful tool to understand if this invasive species has the same biotic fitness or if they have some problems adapting in an island context. The results could help plan future surveys and possible dedicated control or eradication measures. Therefore, the study's goal was to better understand the biology of *V. velutina* in isolated conditions. For this reason, the following objectives were proposed: (I) describe the architecture and structure of nests; (II) analyse

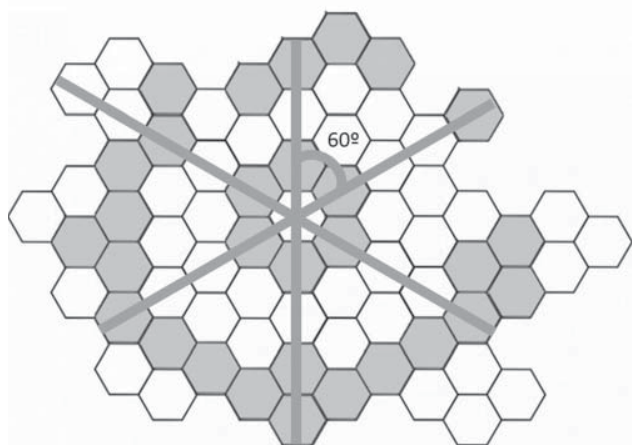


Fig. 2 Diagram of a nest comb. The grey cells represent operculated cells and the lines indicate the three diameters, which pass through the breeding centre and separated by 60°, where the stages of the individuals are determined.

the shape of combs and develop a new method to confirm the circular pattern of breeding; (III) determine the colony size and (IV) determine the succession of workers and sexual individuals throughout the season.

MATERIAL AND METHODS

Nest collection

Nine nests were located from August to November 2016, after an active search for nests using the triangulation method (Leza, et al., 2017). All nests were entirely removed and frozen for a minimum of 48 hours. Nests were kept frozen at -25°C until dissection.

All nests were located in the “Serra de Tramuntana”, in the north-west of Majorca (the exact location is shown in Table 1). This region has a meso-Mediterranean climate (Emberger classification), where there is more precipitation than in other parts of the island (mean of 1,400–1,600 mm per annum) and cooler temperatures.

Architecture and structure of nests

External morphology of nests was analysed and described. Weight, height and maximum diameter of each nest was measured and the number of combs was recorded. Total weight was the result of the weight of the structure and its individuals and the total height corresponds to the height of the whole nest with the external envelope.

Shape of combs

In order to check the circular organisation of the combs described in other species of wasps (Spradbery & Kirk, 1978; Matsuura, 1991), a new method was proposed. It follows a similar methodology of comparison between two sequences of DNA (Brudno, et al., 2004). In our study the sequences were the diameters of combs and the nucleotides were the different brood stages, as follows: every developmental stage (empty cell, egg, larvae, prepupae, pupae and teneral adult) in cells across three diameters in each comb was analysed and compared with each other (the first with the second, the second with the third and the first with the third) (Figs. 2 & 3).

Each diameter comparison received an arbitrary categorisation: “2” was assigned if the stage was the same in cells at the same distance from the breeding centre, “1” if one of the stages was before or after the other stage (for example: in one diameter it is a larva and the other a

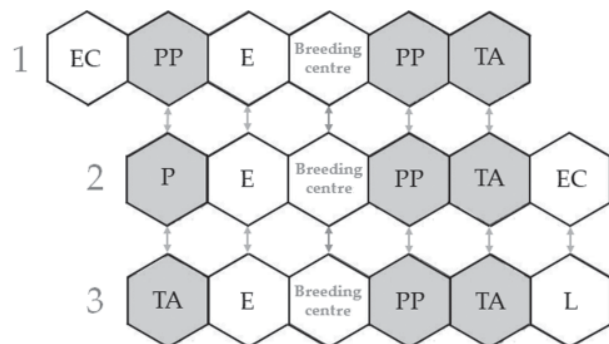


Fig. 3 Example of the three diameters, aligned in the breeding centre, and comparing the development stages at the same distance from the centre. EC = Empty cell, E = Egg, L = Larva, PP = Prepupa, P = Pupa and TA = Teneral adult.

Table 1 Characteristics of the nine nests. L = number obtained by Latter's formula. GP = individual production.

	ID1	ID2	ID3	ID4	ID5	ID6	ID7	ID8	ID9
UTM	39°45'26.33"N 2°42'37.04»E	39°46'56.30"N 2°45'40.72»E	39°46'0.80"N 2°41'4.30"E	39°46'5.80"N 2°44'25.70"E	39°44'17.29"N 2°38'7.82"E	39°45'36.7"N 2°39'14.3»E	39°47'36.5"N 2°46'02.6»E	39°47'28.0"N 2°46'03.5"E	39°44'14.3"N 2°41'29.5"E
Date removed	23/08/2016	31/08/2016	8/09/2016	13/09/2016	14/09/2016	21/09/2016	17/10/2016	2/11/2016	24/11/2016
No. of combs	6	5	5	5	2	6	7	5	9
Weight (g)	5,090.0	2,884.12	3,882.6	2,073.7	136.3	3,313.0	2,893.4	1,448.9	3,583.3
Height (cm)	49.3	30.6	41.5	14.4	7.0	18.3	32.6	18.6	47.6
Max diameter (cm)	33.0	25.5	24.6	24.6	12.0	36.0	23.0	21.2	38.0
Workers	1338	789	285	615	127	684	610	351	151
No. of females	4	0	1	0	0	2	40	15	316
Error interval	0	0	1	0	0	0	0	0	180
Founders	0	0	1	0	0	0	0	0	0
Total	1350	810	293	624	127	701	653	367	656
Males	0	0	0	0	0	436	0	134	429
No. of adults	1,350	810	293	624	127	1,137	653	501	1,085
L. cells	6,197.75	2,712.0	3,077.25	2,778.75	200.0	7,008.25	2,916.25	2,412.75	9,355.0
L. eggs	131.25	196.5	16.5	203.0	1.25	155.5	1.25	74.25	40.25
L. larvae	1,055.0	774.25	947.25	1,066.75	176.25	563.75	809.0	36.2	440.5
L. meconium	2,074.0	796.25	1,202.0	1,186.75	0.0	3,370.75	1,239.25	1,629.75	7,750.25
GP	4,610.25	2,577.0	2,458.75	3,080.5	304.5	5,227.0	2,702.5	2,241.2	9,316.0
GP/cell	0.74	0.95	0.80	1.11	1.52	0.75	0.93	0.93	1.0

prepupa) and "0" if it does not coincide with any of the previous cases, as long as both cells have some stage or are empty (for example: in one diameter it is an egg and the other a pupa) If one diameter had more cells than the other, those cells in a diameter that did not have their partner in the other would not receive any value. For each diameter comparison the sum of each arbitrary punctuation was divided by the number of cells multiplied by "2", the maximum arbitrary punctuation, obtaining a coincidence percentage with the circular organisation.

Colony size

The number of cells was estimated with Latter's formula (Latter, 1935): $N = (3n/2 + 1) \cdot n/2$, where N is the total number of cells in one comb and n is the number of cells counted across its maximum diameter. This formula was extrapolated to estimate the number of eggs, immature stages (larvae and pupae) and meconium pellets (meconium is the gut content eliminated immediately by an individual when moulting from larval to pupal instars and was recorded only as presence or absence, indicating that at least one individual had bred). The number of adults was counted manually.

The estimated total individual production of a nest was defined as the sum of the estimated number of eggs, immature stages and meconium pellets, estimated with Latter's formula, and adults.

Pearson's and Spearman's rank correlations were made between the estimated individual production and the following variables: number of combs, weight, height, diameter, cells, eggs, immature stages or meconium pellets.

Sexual and caste differentiation

Females and males were distinguished by morphological differences (apex of last sternite bilobate in male but sharp in female). For females, founders and workers were distinguished based on their wet weight. Below 593.09 mg individuals can be considered as workers and individuals weighing over 593.09 mg can be considered as potential future queens. The 5% level of uncertainty was reached beyond 525.44 mg for workers and below 664.84 mg for founders. Dry and wet weights are strongly correlated ($\rho = 0.88$, $p < 2.2 \cdot 10^{-16}$) with the following linear regression formula: $y (W_{wet}) = 2.05 \cdot (W_{dry}) + 80.59$ and dry and wet weights and proved to be useful to discriminate workers and queens (Rome, et al., 2015). Every female was weighed with a precision balance (ADAM NBL 423i: 420 g capacity and precision of 0.001 g).

Statistical analysis

RStudio 3.3.2 software (R core team 2016) was used for analysis. It evaluated the correlation factor and its significance differences between the nest characteristics

(Kruskal - Wallis and its Dunn *post-hoc*).

RESULTS

Architecture and structure of nests

Table 1 presents the characteristics of the nine *V. velutina* nests collected from August to November 2016 in Majorca. All the nests found in Majorca analysed in this work were secondary nests (no embryo nests were found inside), presented a calyptodomus typology and had ovoid morphology.

The number of combs within nests analysed ranged from five to nine (the last one, ID9, found in November), except for one nest (ID5, found in September) that had only 2 combs. The weight varied from 136 g (ID5) to 5,090 g (2811.7 ± 482.4), the height from 7 cm (ID5) to 49.3 cm (28.9 ± 5.1) and the maximum diameter from 12 cm (ID5) to 38 cm (26.4 ± 2.7) (Table 1).

Shape of combs

The lower combs had a high coincidence percentage (88.6%) with a circular organisation. However, the coincidence percentage drops in the upper combs to 62.1%. A t-test was applied to observe if the mean of coincidence percentage with a circular organisation of the two upper combs was different from the two lower combs, which produced a $p = 0.0065$, so the circular organisation is lost ascending in the combs because the percentage in upper combs was lower than lower combs (Table 2).

Also, in upper combs there is reduced individual production, in the number of eggs and larvae; lower than in the first lower comb. In lower combs there are more immature stages so the individual production moves to the lower combs. There were significant differences relating to the number of cells between combs ($p = 0.0006$), and also in individual production ($p < 0.0001$). In both cases, cells and individual production, the significant differences were for the first and second lower combs.

Colony size

The evaluation of the total number of cells in the 50 combs of the nine mature nests, using Latter's formula, revealed that the number of cells ranged from 200 (ID5) to 9,355 ($4,073.1 \pm 947.5$) and the general production (which is the sum of the estimated number of eggs, immature stages, meconium pellets (estimated with Latter's formula) and adults) varied between 304.5 (ID5) and 9,316 individuals ($3,613.08 \pm 853.67$) (Table 1).

Spearman's rank correlation test showed that diameter and the general production are highly correlated ($\rho = 0.895$, $p = 0.001$) indicating that diameter is a good parameter for estimating the colony size. The general production follows the exponential function: $y = 0.1778x^{2.8995}$, where y is the

Table 2 Values obtained after applying the arbitrary categorisation. Total = sum of coincidence values between two diameters. Max = sum of the number of paired cells multiplied by the arbitrary categorisation "2", it corresponds to a 100% coincidence percentage with a circular organisation. Total / Max = index of coincidence to a circular arrangement of breeding.

Combs	1			2			3			4			5		
Total	15	19	12	26	27	32	32	30	22	27	36	25	27	24	32
Maximum	16	22	14	40	40	44	52	46	46	50	58	52	40	42	52
Total/ Maximum	0.938	0.864	0.857	0.650	0.675	0.727	0.615	0.652	0.478	0.540	0.621	0.481	0.675	0.571	0.615
Mean	0.886			0.684			0.582			0.547			0.621		

general production of the nest and x is the largest diameter of the nest.

Sexual and caste differentiation

Males started to appear during autumn (ID6, ID8 and ID9), except for ID7, which was removed in October and had no males (Table 1). Season starts at 21 of September in north hemisphere and males of these three nests represented 57.9% of the adults found (999 males; 1,724 females in nests ID6, ID8 and ID9). All other nests (ID1-ID5) were found and removed before the first fortnight in September, and had not produced males.

Caste differentiation was determined by weighing all individuals and a weight increase was observed over time. Individuals in the last nests collected, weighed 63 mg more than those in the first nests removed in summer. During the dissection of the nine nests, a total of 5,581 females were weighed. The 97.4% of females found in the first eight nests (ID1-ID8) were workers and only the 1.3% of the females were in the uncertainty interval. However, in the last nest found (ID9) the percentage of females in the uncertainty interval was 48.2%.

DISCUSSION

All the nests found in Majorca during 2016 had a calyptodomus typology, and the number of combs within nests analysed ranged from 5 to 9. In comparison, the general production in mature nests found in France (4,797.75±606.40) revealed that the analysed nest, under isolated conditions, had similar production. So, the nests found in Majorca presented the same morphology as those nests found in other regions of Europe (Rome, et al., 2015).

Here, we suggest that the diameter of the nest is a good parameter to estimate the colony size, and the general production follows the exponential function: $y = 0.1778x^{2.8995}$ where y is the general production of the nest and x is the largest diameter of the nest. This is interesting in order to analyse the fitness of the species, and provide an easy way to analyse it as, by taking only one measurement (the largest diameter), the potential of each nest can be estimated.

Regarding the shape of combs, we provide a new method to check the circular organisation. The lower combs had a high coincidence percentage with a circular organisation and the coincidence percentage drops in the upper combs. The loss of the circular organisation when ascending in the combs and the higher number of immature individuals (such as eggs and larvae), in the lower combs, and pupae and meconium pellets in upper combs, is due to the fact that this species of genus *Vespa* does not clean the cells after adult emergence (Janet, 1903), limiting each cell to produce between one and four individuals (Archer, 2008). This pattern is similar to the nest structure of *V. crabro* (Nadolski, 2012). Other species of *Vespa* have a higher number of meconium pellets per cell (Yamane & Makino, 1977; Yamane, 1992; Archer, 1993; Makino & Yamane, 1997), with four as the maximum (Archer, 2011) before the queen stops laying eggs inside the combs. Moreover, some authors suggest that un-cleaned combs are the reason the offspring are found in lower combs, which are cleaner than upper combs (Janet 1895). Moreover, the nest analysed presented the lower combs with more immature stages, so the individual production moves to the lower combs, which corresponded with Martin (1991, 1992).

Regarding the sexual and caste differentiation, it is important to note that the method of caste differentiation

used in this work, based on the wet weight, was not very useful in our analysis as many individuals were in the error interval, and the increase in weight of 63 mg in workers was observed in autumn. Other authors proposed alternative methods for caste differentiation, such as Perrard, et al. (2012), who used size and nerve structure of the wings to distinguish individuals. Other possible methods are by genitalia differentiation or molecular methods used for other species of Hymenoptera (Barchuk, et al., 2007). So, for future research we will use these other methodologies. The presence of males during autumn is important information for a management plan on the island as it indicates the possibility that new founder queens can mate and create new nests the following season, signifying the beginning of the expansion of the invasive species.

In conclusion, the analysis of the secondary nests of the yellow-legged hornet found in the Balearic Islands reveals the high adaptability of this species to Mediterranean isolated ecosystems, which has important implications for the development of an effective eradication plan.

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