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Delayed sexual maturity in males of *Vespa velutina*Juliette Poidatz¹, Christophe Bressac², Olivier Bonnard¹ and Denis Thiéry¹¹INRA, UMR1065 Santé et Agroécologie du Vignoble, ISVV, Bordeaux Sciences Agro, Villenave d'Ornon, France and ²IRBI, Université François Rabelais, UMR CNRS 7261, Tours, France

Abstract *Vespa velutina* var *nigrithorax* (Lepelletier, 1835) is an invasive predator of bees accidentally introduced in France in 2004, and it is having a serious impact on apiculture and ecosystems. Studying the reproduction of an invasive species is key to assess its population dynamic. This study explores the sexual maturation of *V. velutina* males and the evolution of their fertility. The main studied parameters were physiologic (spermiogenesis, spermatogenesis) and anatomic (testes size and structure, head width). Two populations of males were described based on their emergence period: early males in early summer or classic males in autumn. Each testis has an average of 108 testicular follicles. Spermatogenesis is synchronous, with only 1 sperm production wave, and completed, on average, at 10.3 d after emergence with the degeneration of the testes. The sperm counts in seminal vesicles of mature males are 3×10^6 in October/November and 0.8×10^6 in June. In comparison, females store 0.1×10^6 sperm in their spermathecae. The early males emerged from colonies made by fertilized queens. The reproductive potential of these early males seemed limited, and their function in the colony is discussed. The sperm stock evolution in autumn males suggests the occurrence of a reproductive pattern of male competition for the access to females and a single copulation per male. The synchronicity of male and foundress emergences and sexual maturation is of primary importance for the mating success and the future colony development.

Key words male fertility; seminal vesicles; spermiogenesis; sperm stock; testis; Vespidae; yellow-legged hornet

Introduction

Mating success is key to understand and predict population dynamic patterns in animals. Due to their rapid expansion, invasive species are particularly susceptible to the mating potential of both females and males. In most studies, the reproductive potential is considered as how females can mate and produce the next generation. Even if, usually, males are considered a nonlimited and disposable resource in terms of mating potential (Page, 1986; Fjerdingstad & Boomsma, 1998; Crozier & Fjerdingstad, 2001), numerous studies on different insect orders still assess the importance of sperm production in their

population dynamic (Diptera *et al.*, 1985; Yamagishi *et al.*, 1992; Mack *et al.*, 2003; Hymenoptera, Allen *et al.*, 1994; Stein & Fell, 1994; Stein *et al.*, 1996; Chevrier & Bressac, 2002; Baer & Boomsma, 2004; Araujo *et al.*, 2010; Beani *et al.*, 2014; Baer 2014).

Male sperm donation is an essential factor for the reproductive success in all sexual animals, especially in social insects (Boomsma *et al.*, 2009; Baer, 2014). In Hymenoptera, diploid females result from the fertilization of 1 oocyte by a spermatozoon, while haploid males are the result of nonfertilized oocytes (Hartl, 1971; Cook & Crozier, 1995). Certain situations of poor male fertility result in constrained females that produce male-biased sex ratios (Lacoume *et al.*, 2009; Nguyen *et al.*, 2013; Chirault *et al.*, 2015). Such outcome was evidenced in nonsocial Hymenoptera; however, it could be of major importance in social species as well because the colony success

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depends on the workers, that is, diploid females (Plowright & Palet, 1979). Then, the sex-ratio of the queen's offspring will depend to some extent on the sperm stock in the spermatheca. Males are classically produced by unfertilized eggs laid either by queens or workers with functional ovaries (see Spradbery, 1973 for hornets). In hornets, it only occurs when the foundress is missing (Takahashi et al., 2004; Spiework et al. 2006).

Spermatogenesis is a useful indicator of male sexual maturation. Sperm production has been described in several social hymenoptera such as Formicidae (Wheeler & Krutzsch, 1992), Apidae (Cruz-Landim, 2001; Araujo et al., 2005; Mônica et al., 2005), and some Vespidae (Bushrow et al., 2006). In such cases, the spermatogenesis is generally synchronic, that is, a single wave of sperm production occurs, and the testis produces a determined quantity of sperm that is transferred to the seminal vesicles until copulation (Roosen-Runge, 1977); then, the testis degenerates in the adult male. In some parasitoid wasps, the spermatogenesis is continuous (Damiens et al., 2003; Bressac et al., 2008; Nguyen et al., 2013); that is, it occurs along the entire or almost the entire life of males. The reproductive system varies in male Hymenoptera and shows differences in its structure, in the time of testis degeneration, in its size, in its morphology, and in both number and quality of the spermatozoa (Dirks & Sternburg, 1972; Watson & Martin, 1974; Simmons & Siva-Jothy, 1998; Morrow & Gage, 2000; Damiens et al., 2002; Damiens & Boivin, 2005).

The yellow legged hornet, *Vespa velutina var nigrithorax* (Lepelletier, 1835), is an invasive predator of arthropods and especially of honeybees, which was accidentally introduced in France around 2004 (for a review, see Monceau et al., 2014; Arca et al., 2015). This specie is now present in half of the French territory (Monceau et al., 2014 for a review), in Italy (Porporato et al., 2014), Portugal (Grosso-Silva & Maia, 2012; Bessa et al., 2016), Spain (López et al., 2011), and more recently England and Belgium (2016). In Asia, *V. velutina* has also recently colonized Korea (Kim et al., 2006; Choi et al., 2012) and Japan (Ueno, 2015).

After 6 months of worker production, hornet queens of temperate climatic regions produce a large amount of future gynes and males (Du Buysson, 1904 [1905]; Spradbery, 1973; Edwards, 1980; Matsuura & Yamane, 1990). Newly emerged males stay in the nest around 8 d in *V. simillima* (Martin, 1991) and 8–11 d in *V. affinis* (Martin, 1993), profit from food, and increase their weight until their nuptial flights (West-Eberhard, 1969; Kasuya, 1983). This phenomenon has already been observed in *V. velutina* nests in captivity (e.g., Monceau et al., 2013b).

The occurrence of female multiple mating is low in hornets (Foster et al., 1999, Foster & Ratnieks, 2000; Strassman, 2001), hence, *V. velutina* foundress has been shown to mate more than once (2.4 mating on average; 8 max; Arca et al., 2012). If the spermatozoa production allows to fill up the spermatheca, then the observed multiple mating would promote the hypothesis that foundresses mate with several partners to either increase the colony size and genetic diversity (Loope et al., 2014), or limit the risks of colony infection by diversification of the daughters immune system (Baer & Schmid-Hempel, 1999, 2001; Cremer et al., 2007). Jaffe et al. (2012) showed that in such cases of multi-mating, paternities are strongly biased toward 1 or very few males. Studying the reproduction of an invasive species is a key to assess its population dynamic, and acquire information on both male mating potential and the sperm need of queens is important to enlarge our knowledge on the reproductive biology of this invasive predator but also to plan new strategies to contrast/monitor its expansion.

The aim of present experimental study was to analyze the sexual maturation of *V. velutina* wild males in different periods of their life cycle. Several questions are raised in the present paper: (i) Does the production of sperm increase in time? (ii) Is sperm produced during their entire life? (iii) When are the males most fertile? (iv) What are the sexual anatomic changes during a male's life and how long does the spermatogenesis last? (v) Are there differences in those parameters between October/November and June males? To answer these questions, we dissected 98 wild *V. velutina* males of different ages, at 2 different periods and from 7 different field collected nests. We also investigated their testis morphology and their sperm availability.

Materials and methods

Maintenance and origin of the hornets

In autumn, 4 colonies of *V. velutina* were collected in the Bordeaux area (France) ($n = 72$ individuals); nests 1, 2, and 4 were collected in Bordeaux on 14/10/2014 (44°49'12.712–0°34'39.128), on 23/10/2014 (44°51'18.094–0°34'48.615), and on 10/11/2014 (44°49'26.977–0°33'4.568), respectively; nest 3 was collected on 6/11/2014 in Latresnes (44°47'5.287–0°30'22.226). The nests were “mature,” as the largest combs were approximately 40–60 cm wide. During late spring, we collected 3 nests from different places in Bordeaux - le Haillan on 25/06/2015 (44°52'23.401–0°40'40.49) and observed the emergence

of 26 males during this period. The nests were young, with a comb diameter between 50 and 110 mm.

The nests combs of the collected nest in the 2 seasons were maintained in aerated plastic boxes in the dark in a climatic chamber at 23 ± 1 °C. Two sizes of boxes were used according to the comb size: 130 mm × 130 mm × 205 mm boxes and 265 mm × 215 mm × 360 mm boxes. We examined the adult emergences twice a day; resulting in an uncertainty less than 12 h in the emergence date. The newly emerged hornets of both seasons were kept in similar conditions in a climatic chamber at 23 ± 1 °C, 12 h light : 12 h dark, to homogenize their development speed. For each individual, both nest origin and emergence date were recorded. The different emerged adults were grouped in meshed lid boxes by sex and nest, with water, honey and a shelter. Males were raised in such conditions until they were dissected. The raising boxes had an adapted size of the sampled group (5 hornets in 110 mm × 110 mm × 160 mm boxes, and 10 hornets in 130 mm × 130 mm × 205 mm boxes). In total, 98 virgin males of different ages, from nonmelanized pupae (the youngest stage observed here—2 individuals) to 62-d-old adults, were dissected.

Males dissection

The head width of the hornets was measured using a digital caliper (Linear Tools 2001, 0–150 mm) that was placed on the larger length of the face, from one eye to another. The dissection was performed in a Petri dish (70 mm diameter) with Ringer's solution (Hayes, 1953). After killing the hornet by deep freezing, the abdomen was separated from the thorax by cutting the waist, and it was dissected by opening between the 2nd tergite and the abdomen apex using precision forceps (Dumont, Montignez, Switzerland 5Ti and 5I). The entire reproductive

tract could thus be extracted (Fig. 1). Close to the last tergite, the aedigium is connected to the seminal glands (Fig. 1C) and the seminal vesicles (Fig. 1B), which are connected to the testis (Fig. 1A), near to the first tergite, via the deferent canal.

Testes size

Once the testes and the pair of seminal glands and vesicles were separated from the rest of the organs with ultra-precise forceps (Dumont 55 I), for each individual a picture was stored (Binocular magnification 63). Then, the testes area and length were measured using ImageJ.1[®] software analysis. These measures of testes surfaces (TS) were used to describe the dynamic of their size, while the testes diameters (length) were used to compare males between the seasons, with a correction of the testes length by the head width.

Testes structure

The testes of 15 October/November males were flattened to assess their structure. In hymenopterans, the testes are made of testicular tubules also named follicles. Follicles are assembled by groups as petals of a flower (Fig. 2). First, the average testicular tubules numbers in each group was counted, then the total number of testicular tubules in each testis and individual.

Spermatogenesis

As in all insects, spermatogenesis occurs in cysts where the future sperm cells are grouped throughout their multiplication and differentiation (Dallai, 2014). To characterize each follicle maturity, the differentiation stages of the

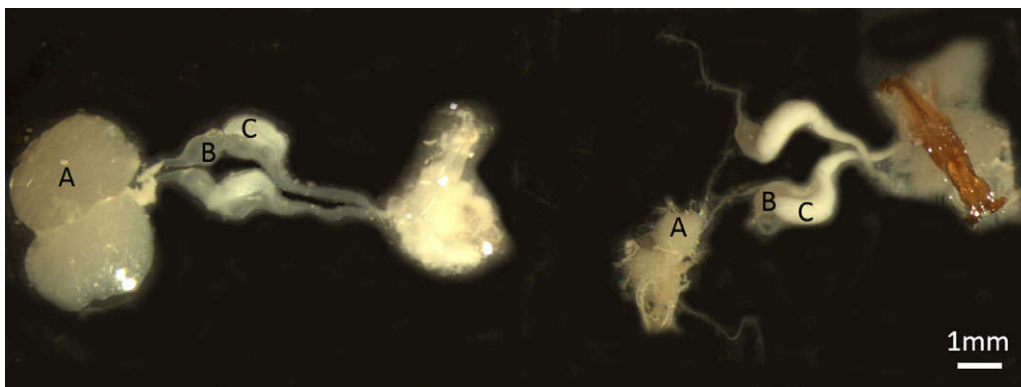


Fig. 1 Tractus of a black pupae (left) and a 13-d-old *Vespa velutina* male (right). (A) Testes, (B) seminal vesicles, (C) seminal glands (binocular picture, ×0.63).

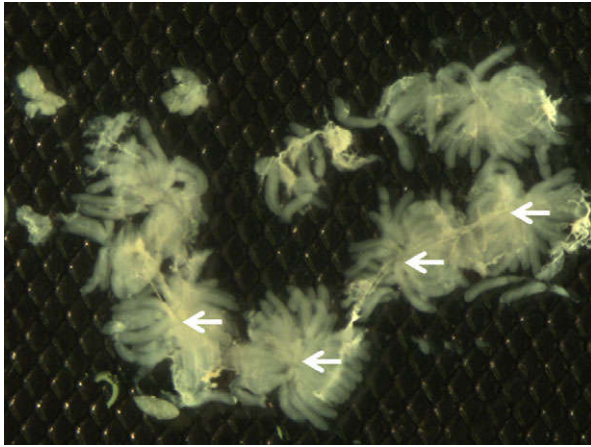


Fig. 2 Photograph of a *Vespa velutina* male testis spread on a microscope slide. The white arrows indicate the centers of testicular follicles groups.

cysts were assessed using DAPI coloration in 25 males at different ages from 0 to 15 d.

Histological section

Freshly dissected male tracts were fixed by incubation for 48 h in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) with 0.1 mol/L sucrose. They were washed in 0.1 mol/L cacodylate buffer (10 min) and water (3×10 min), dehydrated in a graded series of ethanol solutions (50% 2×10 min, 70% 3×15 min, 90% 3×20 min, 100% 3×20 min) and propylene oxide (100%, 3×20 min), and embedded in Epon resin (Sigma-Aldrich, USA), which was allowed to polymerize (24 h for 37 °C, 48 h at 60 °C).

Semifine sections (500 nm thick) were cut with a “Leica Ultracut UCT” ultramicrotome, stained with toluidine blue for 30 s at 60 °C, washed with distilled water for 5 s, ethanol 100% for 10 s, and distilled water again for 20 s, dried at 60 °C and embedded in Epon resin, which was allowed to polymerize for 48 h at 60 °C.

Sperm production

The seminal glands were removed from the seminal vesicles, which contain mature sperm, using a sharp needle and directly transferred in a drop of saline, on a microscope slide. The seminal vesicle was pierced and a homogeneous solution of sperm was obtained by gently rotating the forceps in the sperm mix until its coloration shifted from white to transparent. The vesicle wall

fragments were removed after pressing it to ensure to collect all of the spermatozoa. After ethanol fixation and DAPI staining method for nuclei (Bressac & Chevrier, 1998), slides were observed under an epifluorescence microscope (magnification $\times 40$). From the total 72 autumn males, we counted the sperm in both seminal vesicles in 69 of them, and in 1 seminal vesicle in the 3 others. Note that, 14 out of 26 males dissected in June were 10–20 d old. Due to this undetermined male age, the corresponding data was only used for a comparison of sperm production with $n = 59$, 10- to 20-d-old October/November males.

Fertility of the June queens

In order to check if the presence of males in June was due to a lack of sperm in the queens’ spermatheca, the 3 queens of the 3 different nests collected in June (from which the June males emerged) were dissected. After killing the queens by freezing, their spermatheca was extracted, the content of it was spread in a Ringer solution drop (description above) on a microscope slide and, as we did earlier with the males seminal vesicles, a homogeneous sperm mix that was fixed with ethanol and colored with a DAPI solution was made.

Sperm counting methodology

From the microscope slides of males and females, 10 microscope fields randomly chosen were counted (5 fields in each seminal vesicle preparation for males, e.g., 10 fields par male, and 10 fields for the female’s spermatheca). The methodology of sperm preparation with the Ringer’s solution drops was sufficiently homogeneous; thus, the counting variability was considered acceptable. Then, the cumulative surfaces of the fields were extrapolated to the entire surface of the preparations, which was measured using an ellipsoid formula ($a \times b \times \pi$, where a and b are the maximum dimensions of the quasi circular preparation). To estimate the total number of spermatozoa produced by 1 male (n), we added the number of spermatozoa from both seminal vesicles; for the 3 males where only 1 seminal vesicle was mounted, the count was multiplied by 2. Such method is equivalent to a dilution but without risk of sperm loss or destruction in the course of successive manipulations.

Statistics

Data is given as mean \pm standard error. All of the statistical tests were performed using R3.1.2. © software. For the

comparison of the testes diameter between autumn males and summer males, we eliminated a potential bias linked to the male size by dividing the testes length by the head width. To test the differences between the summer and autumn populations, we assessed the normality of the data using a Shapiro test and, then, a Student's test was used if the data was normally distributed or a Kruskal–Wallis rank test otherwise. To test the correlation between the different morphological elements, we used a Spearman's correlation test if at least 1 argument had a nonnormal distribution.

The modeling of the evolution of testes' area and sperm amount through time was performed with Matlab[®].

The colony 3 was overrepresented in the sampled males ($n = 57$ individuals/72 October/November males), therefore, because of statistical rigor, no comparisons between colonies were made.

Results

Testes size and structure

The testes surface (TS) decreased with age regardless of the male emergence time, that is, autumn or summer (Fig. 3). We estimated the sample equation (exponential function) using a fitting function and the analysis of the curve variation allowed us to assess the stabilization period when the decrease was inferior to 0.05%. Likewise, a stabilization point was found at 10.3 d (9.58–11.18) with 95% confidence. After this period, the testes area was established and stabilized at $1.10 \pm 0.39 \text{ mm}^2$, which corresponds to a diameter of $1.33 \pm 0.30 \text{ mm}$. This degeneration of the testes is easily visible in the histological sections when

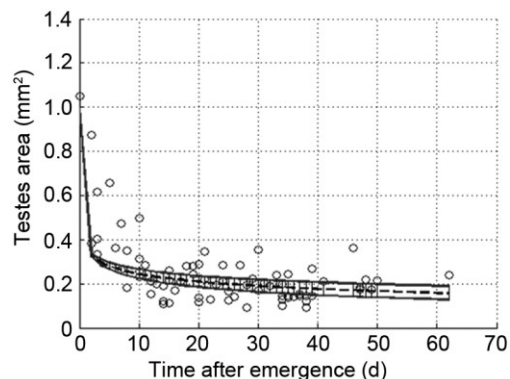


Fig. 3 Evolution of the average testes surface of *Vespa velutina* males with their age.

comparing of Fig. 4(A–C) (young males) to Fig. 4(D, E) (old males).

The testes were made of 9.14 ± 1.95 groups of testicular tubules. Each group contained an average of 11.33 ± 2.55 testicular tubules. Each testis had an average of 108.34 ± 27.18 testicular tubules, and each individual possessed an average of 201.21 ± 72.07 testicular tubules.

Spermatozoa differentiation stages in the testes

The testes were more or less active as a function of the male age and produced different stages of spermatids. We observed characteristic spermiogenesis stages, which differed in both nucleus shape and number per cyst at different ages. At first in white pupae, we observed not fully elongated nuclei spermatids (Fig. 4F), second, early elongated spermatids that stay connected by both extremities (Fig. 4G). Third, fully elongated spermatids that are connected by 1 extremity (Fig. 4H). By the end of the process, the mature spermatozoa were free (Fig. 4I) and were transferred to the seminal vesicles. From the emergence to 10 d, cysts of immature spermatid stages were found, and all of the spermatozoa matured after the 10th day.

October/November versus June males

The number of spermatozoa in the seminal vesicles of October/November and June males varied as a function of their age (Fig. 5). Before 10 d, mature spermatozoa were anecdotic in the seminal vesicles. All of the individuals from the 2 samples with ages between 10 and 26 d were considered (June: $n = 21$; October/November: $n = 26$), and tested for different parameters.

First, we examined the sperm production (Fig. 6), which was significantly lower in June males than in October/November ones (average $7.62 \times 10^5 \pm 6.49 \times 10^5$ and $5.56 \times 10^6 \pm 4.18 \times 10^6$ sperm, respectively; KW test, $k = 13.21$, $df = 1$, $P < 0.01$). The sperm amount variability was higher in October/November males. June males had smaller testes diameters than the October/November males ($1.12 \pm 0.36 \text{ mm}$ and $1.27 \pm 0.27 \text{ mm}$, respectively), even after correcting for head width ($0.21 \pm 0.07 \text{ mm}$ and $0.24 \pm 0.05 \text{ mm}$, respectively) (KW test, $k = 4.37$, $df = 1$, $P = 0.036$). The head width of the June males was significantly smaller than the October/November one (5.24 ± 0.18 and $5.45 \pm 0.15 \text{ mm}$, respectively) (Student's *t*-test, $P < 0.01$, $n = 47$). The higher longevity at 23 °C for a male was 62 d; thus, a survival up to 2 months in laboratory conditions is expected.

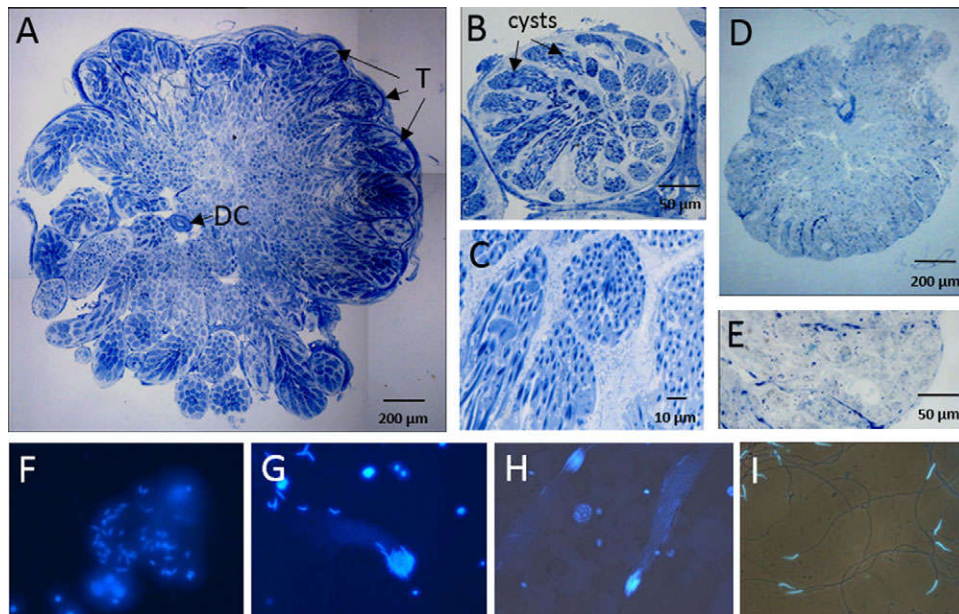


Fig. 4 (A) Histological section of a 0-d-old *Vespa velutina* (Vv) male testis, formed by tubules (T) and a deferent canal (DC) in the center. (B) Zoom on a tubule section of a 0-d-old Vv male showing developing cysts. (C) Transversal sections of cysts showing spermatid nuclei in dark blue and cytoplasm in clear blue. (D) Histological section of a 10-d-old Vv male degenerated testis. (E) Zoom on a histological section of a tubule of a 10 d old Vv male, no cysts are visible in the empty tubules. Spermatids of a Vv white pupae (F, unelongated nuclei), 0-d-old Vv (G, grouped short nuclei, elongating flagellum), 5-d-old Vv (H, grouped elongated nuclei, elongated flagellum), and 10-d-old Vv (I, free sperm from seminal vesicles).

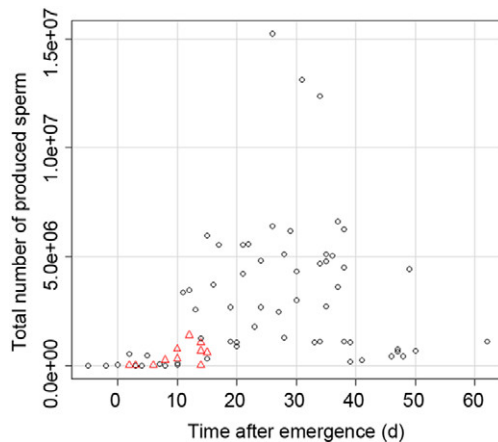


Fig. 5 Evolution of the number of spermatozoa in the seminal vesicles of *Vespa velutina* males with their age (triangles: June males, circles: October/November males).

There was no correlation between the male head width and the number of spermatozoa (Spearman's test, $\rho = -0.19$, $P = 0.24$) and its testis length (Spearman's test, $\rho = -0.09$, $P = 0.51$), nor between the testis surface and the number of sperm produced (Spearman's test, $\rho = -0.14$, $P = 0.36$).

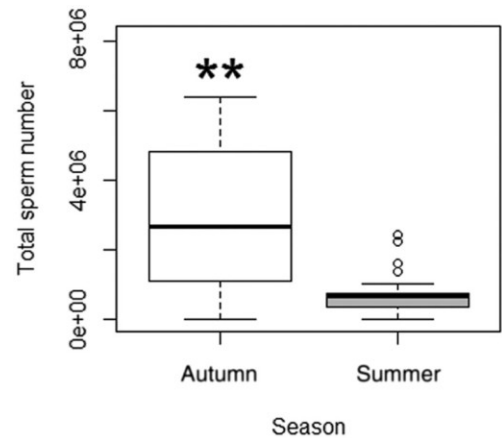


Fig. 6 Sperm production, depending on the emergence period, in 10- to 25-d-old males, KW rank test.

Sperm stock in June queens

Half of the emerging individuals collected from the nests between the 28/06/2015 and the 15/07/2015 were males. The 3 queens of these June nests weighed 521.4, 454.8, and 509.6 mg, and the sperm contents in their

spermatheca were 102.4×10^3 , 111.6×10^3 , and 136.1×10^3 spermatozoa, respectively.

Discussion

Male sexual maturation

The general structure of the *V. velutina* male tract is similar to that of other hymenopterans, and it is known that the testicular follicles number per testis varies significantly among species. The basal Apidae, for example, can have 3 (Mellitinae and Apidae *s. stricto*; Roig-Alsina *et al.*, 1993) to 28 tubules (*H. foveolatum*; Genissel *et al.*, 2009), while *Apis mellifera* can have 250 tubules (Chapman, 1998). In 3 parasitoid wasps of the Chalcidoidea family, Fiorillo *et al.* (2008) described only a single testicular tubule. Instead, the number of tubules varies from 1 to 25 in Formicidae (Wheeler & Krutzsch, 1992). With an average of 201 tubules, *V. velutina* seems to invest strongly in sperm production, at the same level as male honeybees, which is an extreme among hymenopterans.

In *V. velutina* males, the spermatogenesis begins at the pupal stage and ends in the adult stage, at approximately 10 d. Compared with other hymenopterans, this maturation period is considerably long; in general, the male maturation is close to emergence (Araujo *et al.*, 2005; Boomsma *et al.*, 2005; Fiorillo *et al.*, 2008). At 25 °C, we observed a pupal development period of average 15 d (our unpublished data, $n = 3$, by marking a new sealed brood cell and checking daily its opening). These results were obtained in controlled conditions, and maturation dates may differ to some extent in natural conditions. Still these results seem coherent with previous observations made on other *Vespa* species: the sexual maturation period could be related to the period when males stay in their nest for feeding (around 8 d for *V. simillima*; Martin, 1991 and 8–11 d in *V. affinis*; Martin, 1993), and the sealed brood developmental period of *V. simillima* was assessed at 15 ± 0.6 d by Martin's (1991) model on Matsuura's (1984) data. As expected for Vespidae, the spermatogenesis of this species is a synchronic phenomenon. All cysts of developing spermatid were at the same stage in the testis. We found only 1 wave of synchronous sperm release, and then the testes size significantly decreased. Only matured sperm stored in the seminal vesicle can be transferred during mating to the female. Even if our experiments took place in laboratory with regulated conditions, it is probable that during the first 10 d of the adult stage, males are not able to mate, at least with sperm transfer. We showed a declining dynamic in the sperm availability in the seminal vesicles after 40 d. However, the majority of the males

were from nests 1 and 2 after this date; thus, we cannot assert whether this phenomenon is either related to the age of the hornets, the nest identity, or a physiological issue. Likewise, the survival of males of more than 40 d might be a laboratory artifact. In the wild, males are probably unable to live as long.

In the autumn, an important variation in the spermatozoa production was observed among males emerged from the same nest. All of the imago males were kept under the same controlled climatic conditions after emerging; therefore, the sperm production could be linked to the quantity and type of food received by the males when they were larvae. In summer, the variations of sperm production among the nests were not significant, probably because the nests were smaller and the food was more equally distributed. Such food dependence on both male abundance and their sperm supply was already observed in *Bombus terrestris* under lab conditions (Genissel *et al.*, 2002).

The sperm production after 10 d was in the range of 1.5×10^5 to 1.5×10^7 per male, with an average at 3×10^6 , which is in large excess compared to what was found in the queens spermathecae in early summer (26 times more, average $116.7 \times 10^3 \pm 17.41 \times 10^3$). For comparison, a normally sized drone has an average 11.6×10^6 sperm in its seminal vesicle (Schlüns *et al.*, 2003). Giving the offspring size of *V. velutina* foundresses, 1000 to 10 000 individuals, we can estimate that the sperm found in the spermatheca is ca. 10 times fold larger than the offspring production, and a male has 26 times more than needed for the whole paternity of workers and females of the complete progeny. If all of the natural populations are similar to the dissected foundresses, instances of sperm-limited females would be rare in nature. Schlüns *et al.* (2003) observed a connection between drone's size and fertility, as did Beani and Zaccarini (2015) and Beani *et al.* (2014) on *Polistes*, but for *V. velutina* males, the sperm quantity in the seminal vesicles could not be related to the body size of the hornet males in the same season. Still we observed that the June males were statistically smaller and less fertile than the October/November ones.

Considering the sperm allocation in males, only multiple mating experiments could provide insight into the ability of males to invest their sperm in successive females. However, neither the male tract observation—devoid of partitioned seminal vesicles or ejaculatory bulb—nor the sex ratio observed in nature (1/3 foundress vs. 2/3 males in autumn; Rome *et al.*, 2015) are in favor of the ejaculate parsimony or strategic ejaculation of males (Wedell *et al.*, 2002). Moreover, the present results show that *V. velutina* males are unable to rebuild their sperm stock after exhaustion. In the future, it could be interesting to study the role

of seminal fluid in ejaculate competition in *V. velutina*, as did den Boer *et al.* (2010), to have more clues about a putative sperm stock constrain.

In *V. crabro*, males actively seek females using specific odors (Spiework *et al.*, 2006). Likewise, macrogglomeruli, which are implicated in sexual pheromone detection, have been described in *V. velutina* males (Couto *et al.*, 2016). The fertility pattern is in accordance with such physiological traits and implies a reproduction strategy of male competition for the access to females and male investment in only 1 copulation.

June males

June males are smaller than October/November males, what is logical and well known in vespids because the nests cell's size are smaller in this season, but moreover, our results show that those males have a poor reproductive capacity. This production bears a cost for the colony, especially when food is limited, and food sharing would be more advantageous for worker larvae. An early male production was already observed in *V. velutina* in summer nests (Arca, 2012, Monceau *et al.*, 2013b), and also demonstrated by male captures in July (Monceau *et al.*, 2013a). The queens of the June nests, from which these June males came from, had a full sperm stock when dissected, and early diploid males were described in this species (Arca, 2012, Darrouzet *et al.*, 2015): we can then hypothesize that a big part of June males analyzed in our work was diploid, and that it could affect their fertility the same way it did on *Bombus terrestris* (Duchateau & Mariën, 1995).

In 2004, Cowan and Stahlhut studied the vespid wasp *Euodynerus foraminatus*, which produces fertile haploid and diploid males and did not find differences in their offspring viability or fertility. Thus, it could be relevant to conduct tests to assess if *V. velutina* queens are able to discriminate males based on their genetic makeup (haploid / diploid) or their sperm content.

Conclusions

Males of *Vespa velutina* present a delayed sexual maturation. Under lab conditions, they can transfer sperm only 10 d after emergence. They produce sperm in excess compared to the need of a single mating. *V. velutina* males have a high amount of testicular follicles, close to the number found in *Apis mellifera*. We found a large variability in sperm quantity among males even from the same nest, which could be attributed to differences others than genetic diversity.

The reproductive strategy of hornets is based on the synchronicity between sexually mature males and foundress emergence. Such parameters are of great importance for the success of mating and the future colony development. If males are able to copulate only once with a maximum sperm stock, and they are in excess compared to females (male-biased sex ratio), then the occurrence of virgins or females with little sperm stock would be reduced. Such reproductive process could be linked with the invasive success of *V. velutina*. Studies on male sperm potential should be included when considering the entire reproductive strategy of this invasive species to control its expansion.

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Disclosure

The authors have no conflict of interest to declare.

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