AXE 1 : Etude de la biologie de la reproduction chez *Vespa velutina*.

Comme présenté en introduction, un moyen pour limiter le nombre de colonies d'un insecte social est de perturber sa reproduction ou l'initiation de sa colonie. Peu de choses sont connues chez les reproducteurs de *V. velutina*, *i. e.* les mâles et les femelles reproductrices : c'est un verrou de connaissances qui a reçu très peu de travaux et que nous nous attacherons à explorer dans ce premier axe. Ce volet portera sur la biologie, la physiologie et le comportement des reproducteurs de *V. velutina*. Nous traiterons (1) des mâles et de leur maturation sexuelle, (2) de la biologie des fondatrices suivant l'espèce (*V. velutina* vs *V. crabro*) et suivant le lieu de capture, et de (3) l'accouplement chez *V. velutina.*

Mâle de *V. velutina* émergeant de sa cellule (Photo J. Poidatz)

A.1. Physiologie des mâles de *Vespa velutina*

A.1.1 Généralités sur les mâles d'hyménoptères.

Comparé à la quantité énorme d'études se rapportant aux femelles des hyménoptères dans la littérature, peu de travaux ont été faits sur la physiologie des mâles. Les principaux mâles ayant été étudiés sont ceux des fourmis (Wheeler & Krutzsch 1992), et des abeilles (Cruz-Landim *et al.* 1980, Cruz-Landim 2001, Moors *et al.* 2005, (*Apis mellifera*), Araujo *et al.* 2005, Mônica *et al.* 2005, Velthuis *et al.* 2005 (abeilles mélipones), Gracielle *et al.* 2009 (Megalichilinae)). La physiologie des mâles de quelques guêpes a également été étudiée : chez *Ancistrocerus antilope* (Eumeninae) (Bushrow *et al.* 2006), chez des polistes (Wenzel 1987, Beani *et al.* 2014), ainsi que chez *Pegoscapus* (Chalcidoidea) (Fiorillo *et al.* 2008) et *Fopius arisanus* (Braconidae) (Quimio & Walter 2000). Mais, la physiologie reproductrice d'aucun mâle d'une espèce de frelons n'avait jusqu'à présent été étudiée.

Comme nous l'avons vu en introduction, la très grande majorité des mâles de *V. velutina* sont produits en automne, en même temps que les gynes, mais en fin de printemps 2015, nous avons trouvé de nombreux mâles dans les nids collectés *in natura* (>50% de la population). Des mâles précoces ont déjà été observés chez cette espèce (Arca *et al.* 2012, Monceau *et al.* 2013a, Darrouzet *et al.* 2015), et au-delà de la raison de la présence de mâles précoces (point qui sera traité en **A.2.3**), nous avons voulu comparer ici la maturation sexuelle et les capacités reproductrices de ces deux types de mâles.

Chez les *Vespidae* la production de mâles a parfois été considérée par certains auteurs comme une production parasite (Montagner 1964, Spradbery 1973). Ils étaient décrits comme étant incapables de participer aux tâches majeures de la colonie (collecte et distribution des ressources ou construction ou défense du nid). De plus, pour faire leurs réserves glucidiques avant le vol nuptial, les mâles réquisitionnent de la nourriture auprès des larves (exsudats) et des ouvrières de la colonie : par exemple l'augmentation de poids des mâles observée chez *V. affinis* avant de quitter leur nid est de 38%, Martin 1993. Puis les mâles sont dans la plupart des espèces de vespidés chassés de la colonie par les ouvrières. Ainsi des comportements agonistiques de la part des ouvrières de *V. velutina* envers les mâles ont déjà été observés en captivité (Monceau *et al.* 2013a). Mais le rôle des mâles dans les colonies de vespidés est de plus en plus discuté et controversé, et semble être plus complexe. Ainsi

chez *Polistes chinensis*, un rôle social des mâles après l'émergence a été mis en évidence (Kasuya 1983). Chez les mâles de *V. velutina pruthii,* présent naturellement au Pakistan (Perrard *et al.* 2014), Perveen & Shah décrivirent en 2013 leur capacité à défendre la colonie. De plus, Couto *et al.* 2016 ont mis en évidence une morphologie du cerveau particulière chez les mâles de *V. velutina var nigrithorax*, assez proche de celui des ouvrières de cette espèce. L'étude de la physiologie de reproduction des mâles de *V. velutina* apportera des éléments supplémentaires dans la compréhension de la complexité de cette caste.

A.1.2 Organisation du tractus reproducteur mâle.

La période de maturité des mâles pour la reproduction ainsi que la quantité de spermatozoïdes produits sont des paramètres essentiels pour comprendre leurs capacités reproductrices. Le tractus des mâles de *V. velutina* est organisé comme suit (Figure 10). Les vésicules séminales contiennent les spermatozoïdes prêts à être transférés à la femelle lors d'un accouplement.

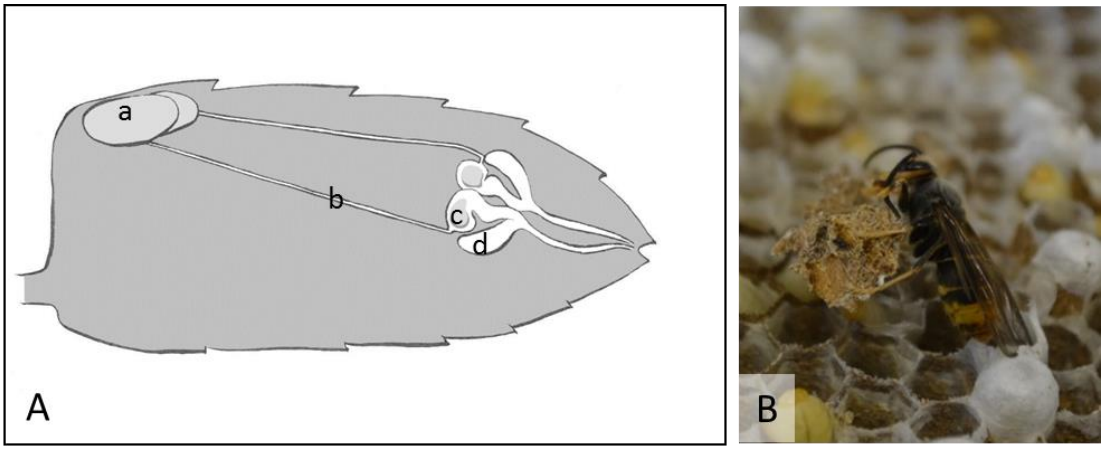

Figure 10 : A : coupe longitudinale de l'abdomen et schéma du tractus reproductif d'un mâle de *V. velutina*, B : émergence d'un mâle de cette même espèce. a : testicules, b : vase déférent, c : vésicules séminales, d : glandes séminales (Schéma et photo J. Poidatz).

Lors de l'étude de la production spermatique d'un mâle, les vésicules séminales sont isolées (Figure 11), détachées de leur glandes séminales associées, et les spermatozoïdes contenus dans les vésicules peuvent ainsi être mis en solution.

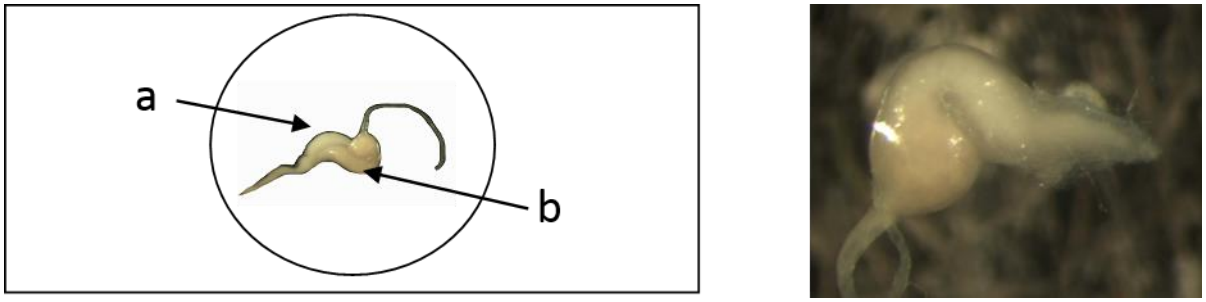

Figure 11: A : schéma d'une lame de microscope où est déposée la glande séminale (b) attachée à la vésicule séminale (a). B : vésicule séminale détachée de la glande séminale. On remarque la « pelote » de spermatozoïdes, beige argentée, dans la partie ronde de la vésicule. (Photos-shéma, J. Poidatz)

A.1.3 Structure des testicules et spermatogenèse.

Les testicules des hyménoptères (Figure 12.A) sont structurés en groupes de follicules séminaux (Figures 12.B, C, D), où sont produits les spermatozoïdes (la **spermatogenèse**) (Fiorillo *et al.* 2008). Le processus de différentiation des cellules souches, les spermatides (Figure 12.E), en spermatozoïdes, est appelé **spermiogénèse**.

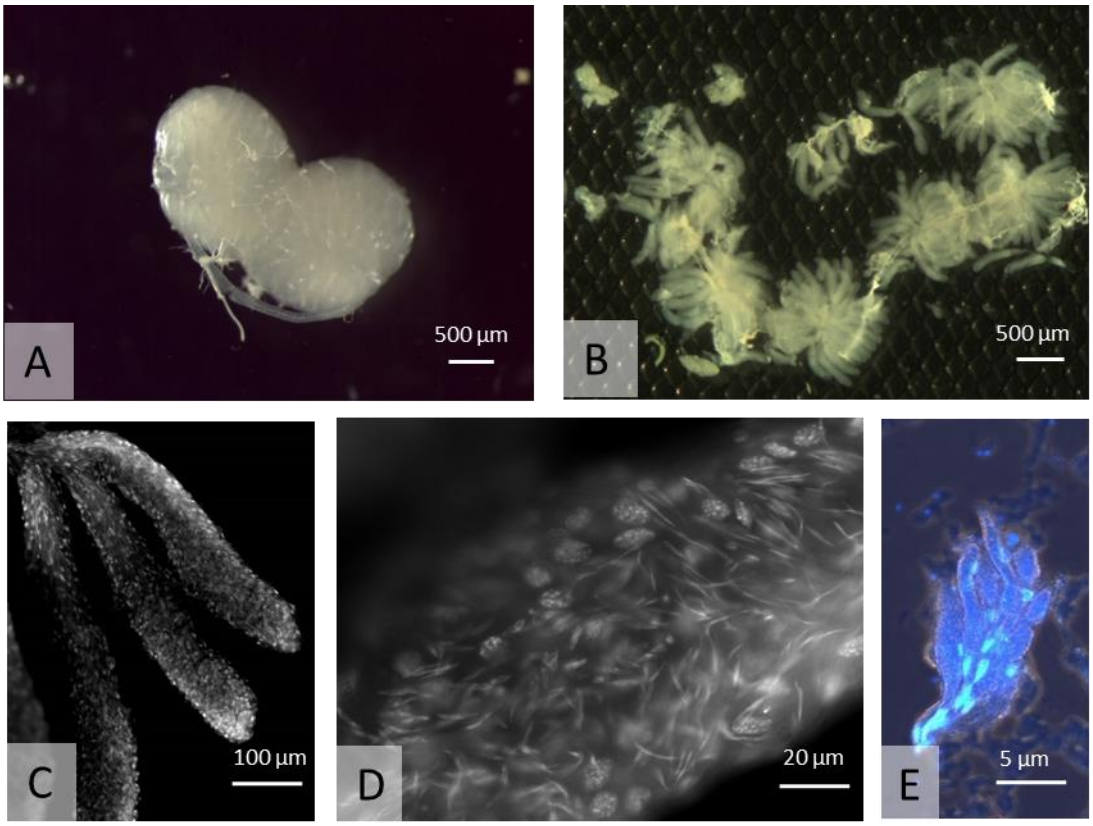

Figure 12 : Focus sur la spermatogénèse chez *V. velutina*. A. Testicules de frelons (X5). B. Un testicule de frelon étalé, mettant en évidence une organisation typique en 'régimes de bananes' des follicules séminaux. C. Follicules séminaux d'un jeune mâle (coloration DAPI, X45). D. Vue de spermatides en cours de différenciation à l'interieur d'un follicule séminal (Coloration DAPI, X100). E. Exemple de spermatides en cours de différenciation où les noyaux et flagelles s'allongent. (Photos A,B par J. Poidatz et C, D, E par C. Bressac)

La spermiogenèse observée chez *V. velutina* est classique. Elle passe, tout d'abord, par la division des cellules initiales, rondes, les spermatocytes 1 (Bowen 1920). Une fois que les cellules se sont suffisemment multipliées (256 noyaux), les noyaux se condensent, s'allongent, et les flagelles commencent à grandir. Les spermatides ainsi formés vont pouvoir se séparer d'abord en gardant un point d'attache à l'extremité du noyau, puis seront libérés totalement, finiront leur maturation avant de pouvoir enfin être transférés dans la vésicule séminale (Figure 13).

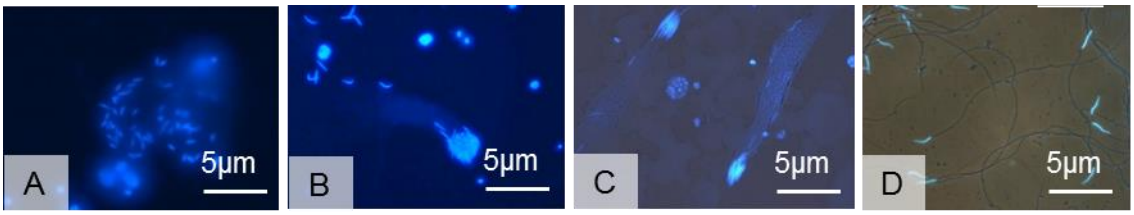

Figure 13 : Spermiogenèse chez *V. velutina* : A : spermatides d'une pupe non mélanisée (les noyaux sont rond) ; B : spermatides d'un mâle émergent (les noyaux des spermatides sont courts et soudés, les flagelles s'allongent) ; C : spermatides d'un mâle de 5 jours (les noyaux des spermatides sont encore groupés mais bien allongés, les flagelles sont également bien allongés) ; D : spermatozoïdes d'un mâle de 10 jours (issus des vésicules séminales) (Photos J. Poidatz).

Chez la plupart des hyménoptères, la spermatogenèse se déroule avant ou peut après l'émergence de l'imago (**spermatogenèse synchrone**), entrainant par la suite une dégénerescence des testicules : c'est le cas chez *V. velutina* (voir Article 1 et la différence entre la Figure 14.A et 14.B après 10 jours). Chez d'autres hyménoptères ce phénomène est continu (**spermatogenèse continue**), *i.e.* au lieu d'une vague unique de production de spermatozoides, ils sont produits par les testicules tout au long de la vie du mâle (Damiens *et al.* 2003, Bressac *et al.* 2008, Nguyen *et al.* 2013). Dand le cas d'une spermatogenèse synchrone, on observe au niveau des testicules une disparition des spermatides en division dans les follicules après leur vague de production (Figure 14). Après cela, la taille des testicules diminue suite à leur dégénèrescence.

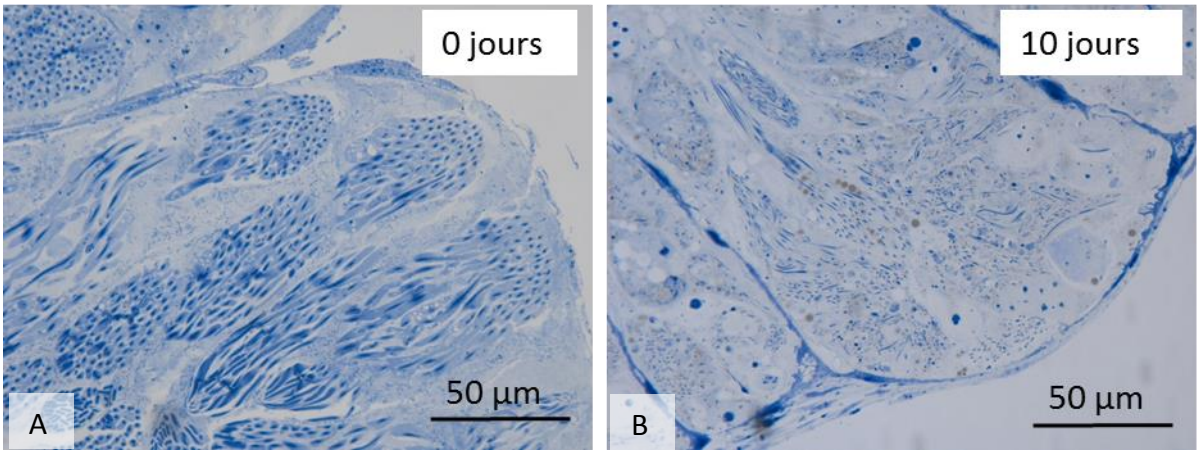

Figure 14 : Coupes d'un testicule de mâle de *Vespa velutina* : A : testicule d'un jeune mâle juste après émergence, de nombreux spermatides remplissent les follicules distendus, B : testicule d'un mâle de 10 jours, les follicules sont alors vidés et rétrécis suite à la dégénérescence testiculaire. (Photos C. Bressac)

A.1.4 Production de mâles précoces chez *V. velutina.*

La production de mâles précoces chez *V. velutina* avait été décrite pour la première fois par Arca (2012) lors de sa thèse, qui posa l'hypothèse de la présence de mâles diploïdes dans la population. Depuis, des mâles précoces ont également été observés par Monceau *et al.* 2013a, puis Darrouzet *et al.* 2015 ont observé des mâles dans certaines colonies dès le mois d'Avril, et ont montré que la majorité des mâles produits tout au long de l'année étaient **diploïdes**. Rappelons que les hyménoptères sont des insectes haplodiploïdes, et donc que les femelles sont issues d'œufs fécondés, et les mâles d'œufs non fécondés. Pour exprimer le sexe femelle, deux versions d'un même gène sexuel doivent être lues. Dans le cas des mâles diploïdes, on a affaire à un œuf fécondé, où une mutation sur le locus de ce gène empêche sa double lecture. Ainsi l'œuf fécondé donnera naissance à un individu exprimant le sexe mâle au lieu du sexe femelle, mais avec une double information génétique. Les mâles diploïdes apparaissent dans des populations consanguines où cette mutation est conservée.

Le coût de la diploïdie varie chez les hyménoptères, et la fréquence de mâles diploïdes est souvent considérée comme un indicateur de déclin des populations (Zayed *et al.* 2004). En effet, la production

de mâles diploïdes augmente le taux de mortalité des colonies en diminuant leur vitesse de développement, ces males précoces ne pouvant pas œuvrer pour la colonie comme les ouvrières (collecte de ressource, défense, exploration, soin au couvain) (Plowright & Palet 1979, Ross & Fletcher, 1986). De plus, les mâles diploïdes sont décrits comme quasiment stériles et incapables de se reproduire chez beaucoup d'espèces, ou encore ils peuvent être moins choisis par les gynes pour s'accoupler (El Agoze *et al.* 1994). Leur descendance potentielle, triploïde, est stérile et avec une viabilité réduite (Thiel & Weeda 2014).

Bien souvent des **mécanismes** permettent **d'éviter** la consanguinité qui augmenterait les risques d'accouplements entre porteurs de la mutation, résultant en la production de mâles diploïdes. Chez les polistes invasifs *Polistes dominulus* par exemple, les males diploïdes sont moins choisis par les reines pour les accouplements (Liebert *et al.* 2010). Chez *V. velutina*, Monceau *et al.* 2013a décrivent que sur des nids en captivité les jeunes mâles sont chassés hors de leur colonie par les ouvrières, ce qui pourrait aider à limiter les accouplements intra-nidaux.

D'autres mécanismes pouvant **compenser** la diploïdie peuvent se mettre en place chez certaines espèces : par exemple en 2004, Cowan & Stahlhut, n'ont observé aucune différence entre la viabilité ou la fertilité des descendants produit des mâles diploïdes chez la guêpe *Euodynerus foraminatus*: cela mettrait en cause des phénomènes de **compensation physiologiques** qui rendraient les spermatozoïdes haploïdes chez les mâles diploïdes. Pour les espèces capables de compenser la diploïdie, la production de mâles tout au long de l'année qu'elle permet est une force qui contribue à maintenir un taux de reproduction élevé avec des individus proches génétiquement. Chez certains hyménoptères se reproduire dans son nid natal avant de se disperser est chose commune (Pamilo 1985). La fréquence de femelles triploïdes dans les populations d'hyménoptères sociaux comme les guêpes et les fourmis semblerait même être grandement sous-estimée (Krieger *et al.* 1999). Des études supplémentaires comportementales seraient de ce fait très intéressantes à faire chez *V. velutina* pour estimer le « coût » réel de cette diploïdie chez cette espèce.

Axe 1

L’ article présenté ci-après a pour objectif à la fois de décrire la dynamique de maturation sexuelle des mâles de *V. velutina*, mais également de comparer la fertilité de mâles suivant leur période d’émergence, précoces ou automnaux.

ARTICLE 1: Delayed sexual maturity of males in Vespa velutina.

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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 × 106 in October/November and 0.8 × 106 in June. In comparison, females store 0.1 × 106 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.

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, Letsinger et Gromko, 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; Araújo *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.*, 2005; Baer, 2014). In Hymenoptera, diploid females result from the fertilization of 1 oocyte by a spermatozoon, while haploid males are the result of non-fecundated 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 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; Araújo *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.*, 2014a; Arca *et al.*, 2015). This specie is now present in half of the French territory (Monceau *et al.*, 2014a 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, 1903, 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.*, 2013a).

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). Jaffé *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.

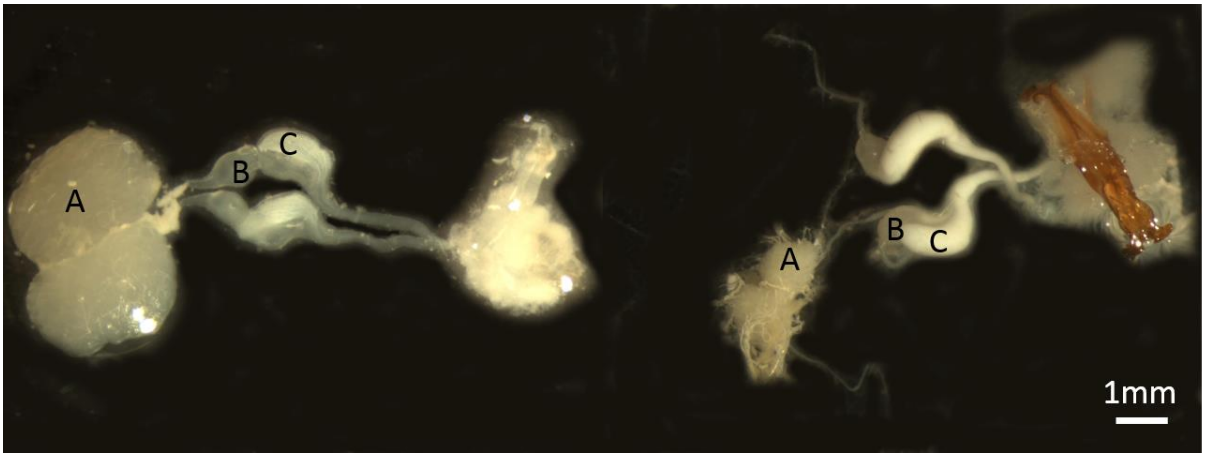

Figure 1. Tractus of a black pupae (left) and a 13-d-old *Vespa velutina* male (right). (A) Testes, (B) seminal vesicles, (C) seminal glands (binocular picture, ×0.63).

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.

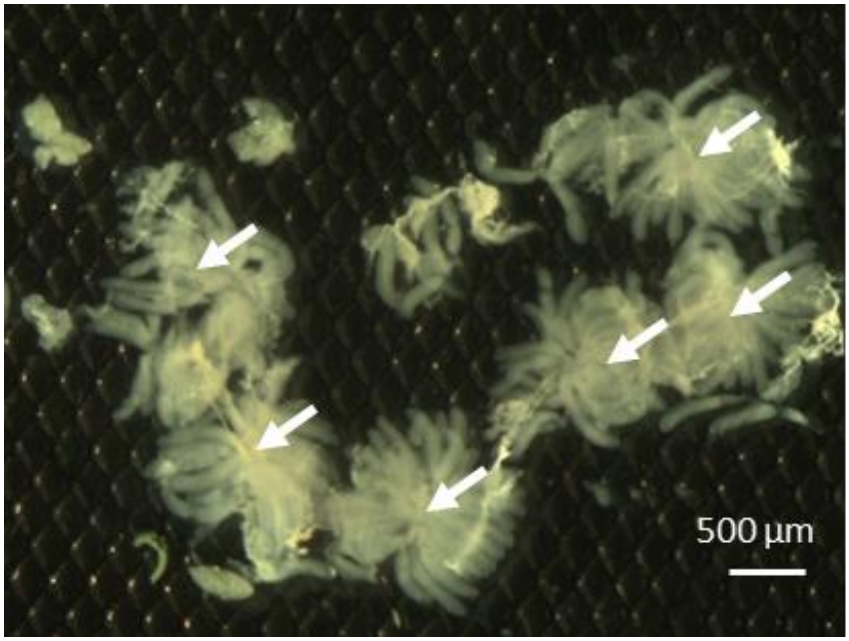

Figure 2. Photograph of a *Vespa velutina* male testis spread on a microscope slide. The white arrows indicate the centers of testicular follicles groups.

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 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 ×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 ± 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 non-normal 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 ± 0.39 mm2, which corresponds to a diameter of 1.33 ± 0.30 mm. This degeneration of the testes is easily visible in the histological sections when comparing of Fig. 4(A–C) (young males) to Fig. 4(D, E) (old males).

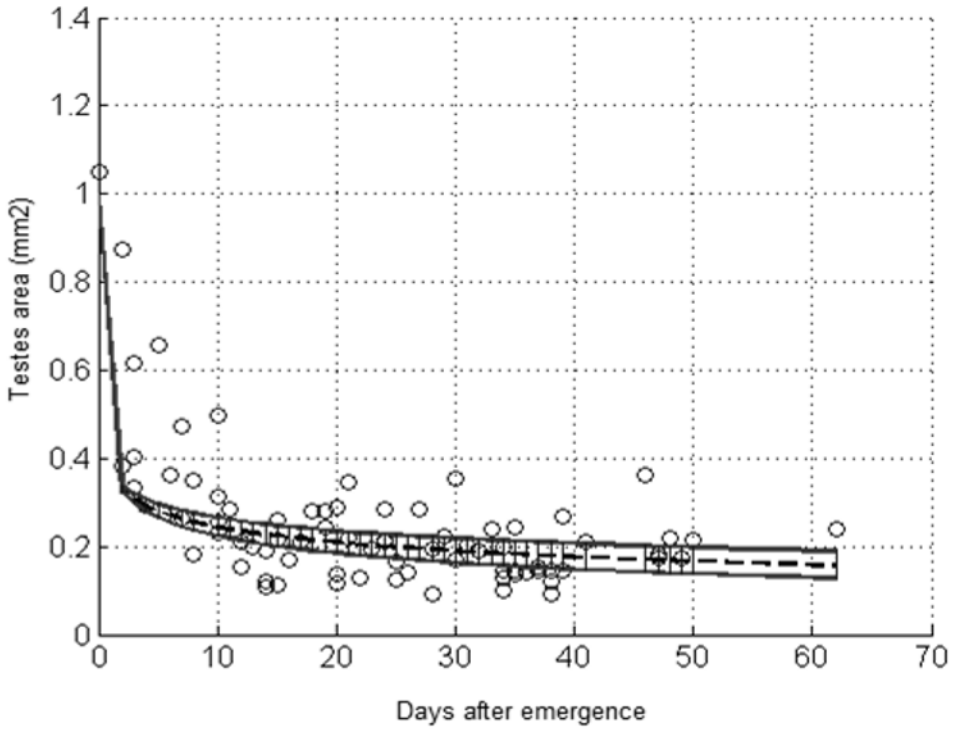

Figure 3. Evolution of the average testes surface of *Vespa velutina* males with their age.

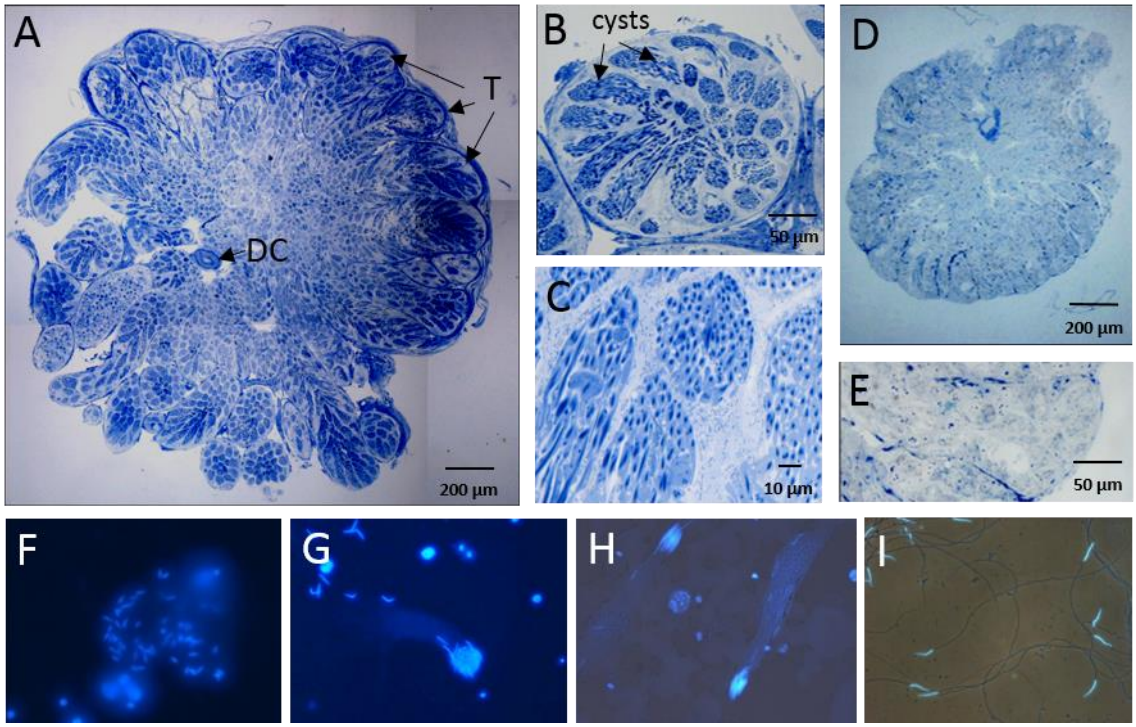

Figure 4. (A) Histological section of a 0-d-old *Vespa velutina* (*Vv*) male testis, formed by tubules (T) and a deferent canal (DC) in the center. (B) Zoom on a tubule section of a 0-d-old *Vv* male showing

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).

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.

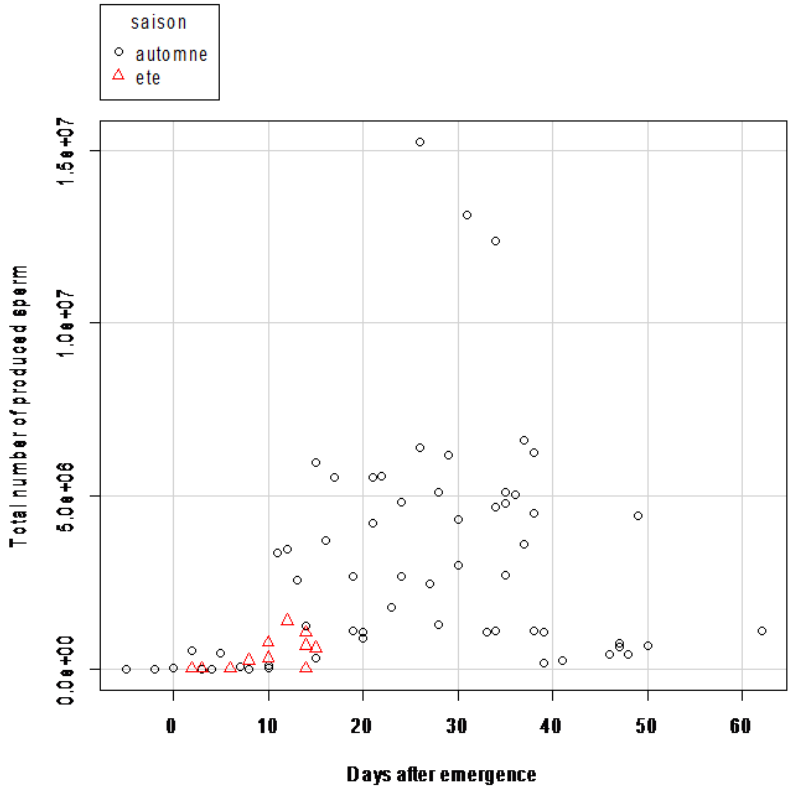

Figure 5. Evolution of the number of spermatozoa in the seminal vesicles of *Vespa velutina* males with their age (triangles: June males, circles: October/November males).

First, we examined the sperm production (Fig. 6), which was significantly lower in June males than in October/November ones (average 7.62 × 105 ± 6.49 × 105 and 5.56 × 106 ± 4.18 × 106 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 ± 0.36 mm and 1.27 ± 0.27 mm, respectively), even after correcting for head width (0.21 ± 0.07 mm and 0.24 ± 0.05 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 ± 0.15 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.

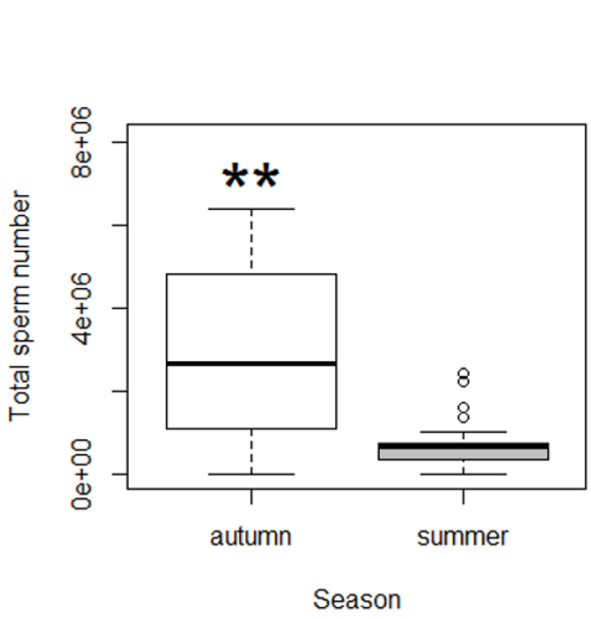

Figure 6. Sperm production, depending on the emergence period, in 10- to 25-d-old males, KW rank test.

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).

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 × 103, 111.6 × 103, and 136.1 × 103 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 parasitoids 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 extend 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 × 105 to 1.5 × 107 per male, with an average at 3 × 106, which is in large excess compared to what was found in the queen's spermathecae in early summer (26 times more, average 116.7 × 103 ± 17.41 × 103). For comparison, a normally sized drone has an average 11.6 × 106 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, macroglomeruli, 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.*, 2013a), 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.

Acknowledgments

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A.2 Physiologie des fondatrices de *V. velutina.*

Chez les insectes sociaux, la fertilité des fondatrices est l'un des paramètres les plus importants dans le succès reproducteur des colonies *(i.e.* la quantité de nouvelles reines et mâles produits en fin d'année). La quantité d'œufs que la reine va pondre et la quantité de spermatozoïdes en stock nécessaire pour féconder ces œufs auront des effets directs sur la taille de la colonie, sa vitesse de croissance, étant dépendante du nombre d'ouvrières (haplodiploidie, voir **A.1**), mais aussi de leurs performances.

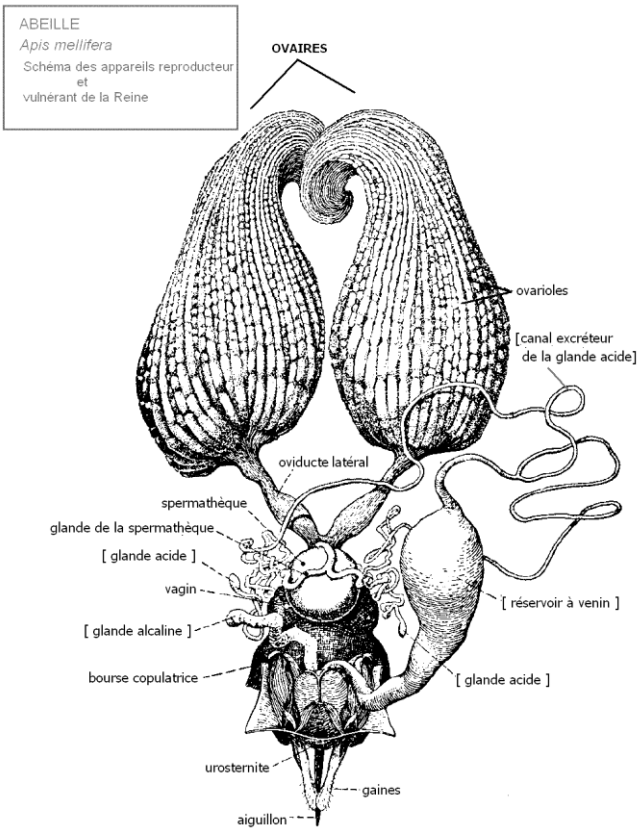
Dans cette partie nous nous attacherons à décrire et à comparer des caractères liés à la dispersion, la survie et la fertilité des fondatrices de frelons entre différentes espèces (*V. crabro, V velutina*), mais également entre des *V. velutina* provenant de sites envahis depuis plus ou moins longtemps.

A.2.1 Organisation du tractus reproducteur femelle.

A.2.1.1 Ovaires et production d'œufs.

L'organisation du tractus des femelles reproductrices chez *V. velutina* est classique comparée à celle d'autres hyménoptères (voir Figure 15), avec deux ovaires constitué d'ovarioles dans lesquels les œufs sont produits. Ces ovaires sont reliés par un oviducte par lequel les œufs matures passeront, pour être au passage fécondés ou non par des spermatozoïdes stockés dans la spermathèque attenante, sous régulation de la reine (démontré chez *A. mellifera* par Ratnieks & Keller 1998).

Figure 15 : exemple d'organisation de l'appareil reproducteur chez l'abeille : schéma basé sur Snodgrass 1956. L'organisation de celui de la reine de frelon est assez proche, bien que les ovaires n'atteignent pas cette taille en proportion (4-5 étages de production max).



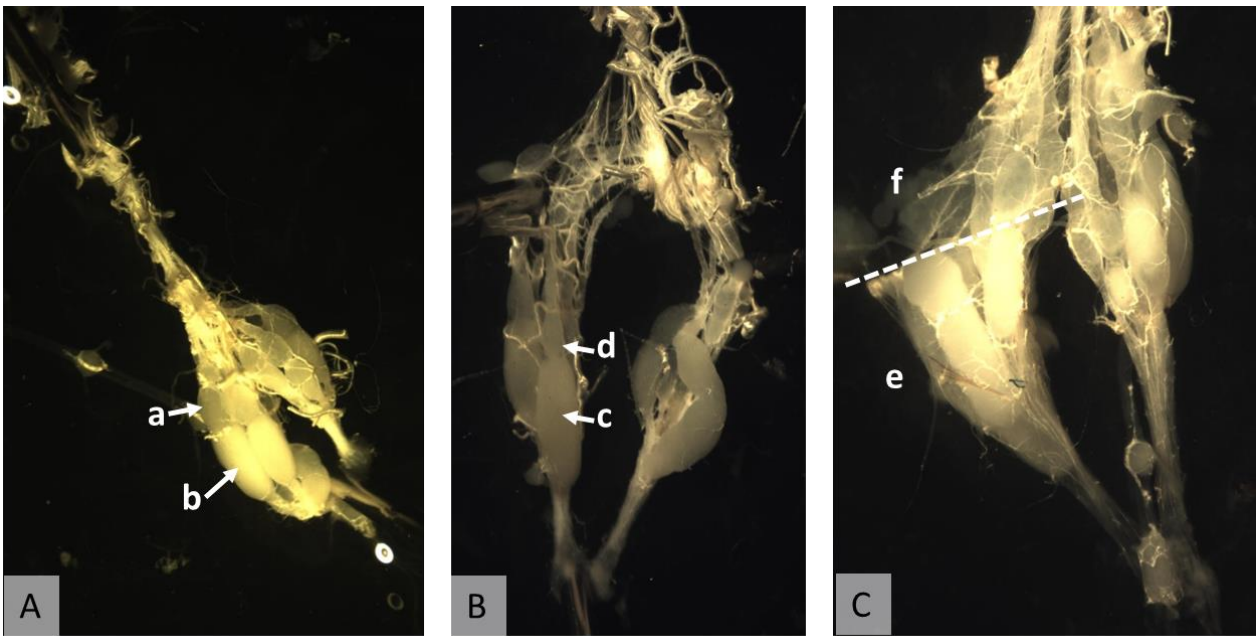


Figure 16 : Ovaires de *V. velutina* à différents stades de développement (X5). A : ovaires en début de maturation : les cellules nourrices (ou accompagnatrices) (a) sont presque de la même taille que les œufs en préparation (b). B: ovaire presque matures : les œufs (c) sont déjà 2 fois plus grands que leurs cellules accompagnatrices (d). C : ovaires matures : la production d'œufs commence à se faire sur plusieurs étages (e,f), les œufs atteignent leur taille de ponte et s'opacifient. (Photos J. Poidatz)

Chez *V. velutina*, les ovaires sont constitués de 8 ovarioles, dans lesquelles les œufs seront produits (Figure 16). Les œufs en cours de préparation sont couplés à leur cellule accompagnatrice – nourrice (Figure 16 A, D). Les ovaires des fondatrices commencent leur maturation seulement en sortie d'hibernation, au printemps. La production d'œufs peut se faire sur plusieurs étages une fois la colonie dans sa phase d'expansion.

Les reines constituent des réserves pour leur hibernation sous la forme de **corps gras.** Ces réserves sont pour la grande majorité constituées au nid avant le départ des reines pour leur vol nuptial et leur hibernation (Martin 1993). Les corps gras remplissent littéralement l'abdomen des reines avant leur départ : ils entourent les organes, en particulier les ovaires, mais sont également présents sur les tergites et sternites[1]. Chez *V. mandarinia*, le poids des reines est réduit de 40% suite à la consommation de ces réserves durant l'hibernation (Matsuura 1966). Ce qui reste de ces corps gras sert également de réserve d'énergie pour produire des œufs. La quantité de corps gras avant et après hibernation est indéterminé chez *V. velutina* actuellement, mais nous explorerons leur présence en début de printemps dans le Manuscrit 2.

A.2.1.2 La spermathèque

La spermathèque est l'organe dans lequel la reine stocke les spermatozoïdes après s'être accouplée, afin de les utiliser pour la fertilisation de ses œufs tout au long de sa vie. Les spermatozoïdes sont compressés dans une capsule rigide pour une meilleure conservation. La spermathèque est enveloppée par une enveloppe externe appelée réservoir, reliée à des glandes accessoire de taille et de forme extrêmement variable suivant les espèces (Figure 17). Les femelles de *Vespa velutina*, qu'elles soient ouvrières ou reines possèdent toutes une spermathèque, tout comme chez *Apis mellifera* : aucune différence n'a d'ailleurs put être démontrée entre la taille des spermathèque entre ces deux castes chez cette dernière espèce (Gotoh *et al.* 2008).

[1] Tergites et sternites = plaques cuticulaires dorsales et ventrales respectivement de chaque segments de l'abdomen d'un insecte

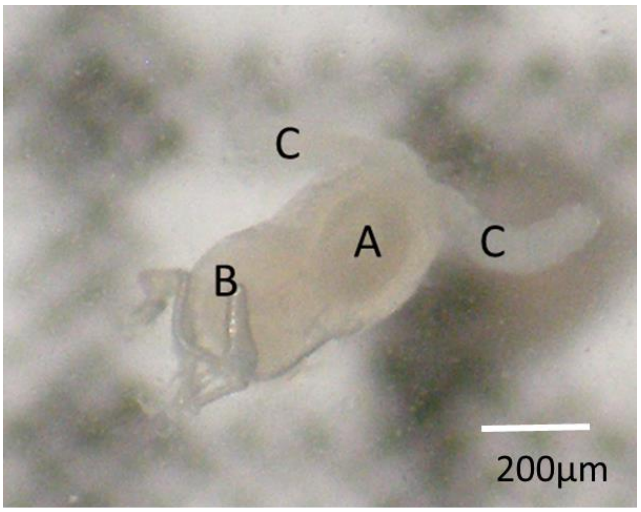


Figure 17 : Spermathèque de *V. velutina* (A), avec son réservoir (B) et ses glandes accessoires effilées (C).

Manuscrit 2: Comparison of reproductive traits of foundresses in a native and an invasive hornet in Europe.

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*Soumis à **Journal of Insect Physiology** (Septembre 2017)*

Abstract

The population dynamics of annual social hymenoptera such as vespids depend largely on the fertility of the foundresses, which, in turn, is a key factor in the context of biological invasions. The native European hornet *Vespa crabro* (*Vc*) and the invasive Asian hornet *Vespa velutina* (*Vv*) have generally similar ecological traits, e.g. nesting and feeding habits, although they differ in colony size, which is higher in *Vv*. Furthermore, in contrast to *Vc*, *Vv* is more specialized in its predatory habits, intensively hunting honey bees at the hive. Comparing the morphological and reproductive traits of two closely related species occupying the same ecological niche, one of which is a native species and the other an alien, can help us to gain an understanding of the invasion process. To this end, we here compare reproductive (ovarian size and maturation, fat level, spermatheca size and sperm stock, fecundity) and morphological (head size, weight) traits of the foundresses of these two hornet species. We observed that ovarian maturation began approximately one month earlier in *Vv* than in *Vc*, and that the fat level in the former was lower. We found twice the number of sperm in the mated foundresses of *Vv* than in those of *Vc* (more than 100×10^3 and less than 50×10^3 sperm, respectively), in a 16% smaller spermatheca in *Vc*. Furthermore, the sperm of *Vv* was found to be 65% shorter than that of *Vc*. The precocity and higher potential fecundity of *Vv* queens may have favoured this species over *Vc* in terms of predatory behaviour, and thereby contributed to its invasiveness.

Keywords: *Vespa velutina*, *Vespa crabro*, spermatheca, sperm stock, ovarian maturation, fat reserves, species competition

Introduction

The proliferation of an alien insect generally has detrimental effects on native species in the invaded area, particularly in terms of competitive exclusion when they have similar ecological niches (Mooney and Cleland 2001). Reproductive potential is recognized as key factor in understanding population dynamics and the potential for invasiveness (see, Moller 1996; Sakai *et al.* 2001 for a review). We might expect alien species to possess promoting traits linked to invasiveness (such as dispersal, fecundity *etc*) more developed than non-invasive ones, that will enhance population growth, including dispersal, establishment, and proliferation (Hudina *et al.* 2014, Chapple *et al.* 2012; Holway and Suarez 1999; Blackburn *et al.* 2009; Weis 2010, Monceau *et al.* 2015a). These life traits allow invasive species to outcompete the local species, thereby facilitating more successful invasion. Given the adaptability conferred by their sociality, social insects are good candidates for biological invasions (Moller 1996; Suarez *et al.* 1999; Cervo *et al.* 2000; Beggs *et al.* 2011). The European hornet *Vespa crabro* (Lin. 1758) is the only hornet originally distributed in Continental Europe (Archer 1994). This species is protected in some European countries for its ecological value (for example in Germany), and was considered an endangered species even before the arrival of *Vespa velutina* in Europe (Erlandson, 1988). The Yellow-legged Asian hornet, *Vespa velutina* var. *nigrithorax* (Lepelletier 1835), is native to East Asia, and was accidentally introduced to South France from China around 2004 (Monceau *et al.* 2014a, Arca, 2015). The introduced *V. velutina* (*Vv*) subsequently spread to neighbouring European countries, including Spain, Italy, Portugal, Belgium, and Germany, and has more recently been recorded in England and Scotland (Monceau and Thiéry 2017). The ecological niche of *Vv* is very similar to that of *V. crabro* (*Vc*): they both hunt arthropods, are scavengers, and consume ripe fruit. However, due to its larger colonies and outbreaks in the invaded areas, *Vv* has an enhanced impact on the local biodiversity. Furthermore, although reports of predation on domestic honeybees by *Vc* are largely anecdotal (Baracchi *et al.* 2010), *Vv* predates them in huge amounts, including both imagoes and larvae (Matsuura and Yamane 1990, Monceau *et al.* 2013c). The mass hunting of bees by *Vv* is therefore an additional threat to the beekeeping industry, which is already in crisis owing to multifactorial causes (Oldroyd 2007; Flynn 2008; Brown & Paxton 2009; Kluser *et al.* 2010, Le Conte

et al. 2010, Tan *et al.* 2007). Several species of Asian hornets are known to attack honeybee colonies, using collective hunting strategies of varying degrees of sophistication (Matsuura *et al.* 1990), even if hives do represent a risky resource to exploit, since honeybees are defensive and can sometime kill the hunters (Tan *et al.* 2012, Arca *et al.* 2014). One hypothesis for the paucity of bees predation by *Vc* is that a *Vc* nest produces low numbers of workers, which is not conducive to risky attacks on hives. The capacity to produce large number of workers could thus be a key factor in optimal exploitation of such a resource. Monceau *et al.* 2015a showed that the seasonal phenologies in *Vv* and *Vc* overlaps to some extent. Even if they do not compete directly for food sources, either direct or indirect interspecific competition between these two species at the initiation stage is likely. *Vc* prefers cryptic sites for nest construction (Langowska *et al.* 2010**)**, whereas mature *Vv* nests are mostly found in open sites, typically in tree canopies (Monceau *et al.* 2013a, 2014a). Nevertheless, numerous *Vv* colonies are initiated in roofs and underground, and two month later, some of those colonies relocate to more open sites (Matsuura and Yamane 1990). During this critical period of nest initiation, it seems probable that interspecific competition would occur. Monceau *et al.* (2015b) compared several behaviours of *Vc* and *Vv* foundresses, including aggressiveness and exploration, and showed that *Vv* outperforms *Vc* in such traits, which can be advantageous for invasion and competition with *Vc*. In the present study, we compared different fertility traits of the two hornet species that are prerequisites for larger colonies, and could thus increase or reduce the impact of the invasive *Vv*, not only on *Vc*, but also on honeybees.

The fertility of social Hymenoptera with an annual cycle, such as *Vespa* species, depends on different criteria, notably the number of eggs produced (Fletcher and Ross 1985; Reeve 1991; Reeve and Nonacs 1992, Foster *et al.* 2004), physiological investment in reproduction through ovarian development (Cini *et al.* 2013, Makino 2016), and precocity in establishing and developing a colony. In *Vespa* species, the eggs are laid by a single queen, whereas hormonal castration of the workers prevents them from mating and laying eggs (Foster *et al.* 2000). If the queen disappears for any reason, some workers can undergo ovarian maturation but will lay only unfertilized eggs (Matsuura and Yamane 1990). All females develop from fertilized eggs (diploid), whereas the males develop from unfertilized eggs (haploid). The queen's fertility therefore influences the size and the structure of

the colony (Takahashi *et al.* 2002), and sperm stored by queens after copulation are an essential resource for the production of workers and colony growth. In this regard, sperm morphology should also be taken into consideration because longer sperm occupies more space, and thus an equal spermathecal volume would contain less long sperm than short sperm.

Hornet gynes remain in their nest for a short period after emergence to increase their fat reserves, which will serve for both hibernation and egg production (Matsuura 1984, Matsuura and Yamane 1990, Martin 1993). After mating, the gynes conserve the sperm compacted in their spermatheca for the rest of their lives. The size and shape of the female spermatheca are species-specific (Gotoh *et al.* 2008), and in *Vespidae* comprises a single epithelial layer, subdivided into three main regions: a globular reservoir, a spermathecal duct, and a Y-shaped spermathecal gland (Martins *et al.* 2005). After hibernating, and a short period of food collection, ovarian maturation occurs for the duration of reproductive life (Matsuura 1984, Matsuura and Yamane 1990, Makino 2016). The queen (foundress) subsequently establishes a colony, and gradually uses spermatozoa to fertilize (or not) the eggs she is laying.

The objective of this study was to answer the following questions related to the fertility of queens of two European hornet species, the native *V. crabro* and the invasive *V. velutina*. (i) What is the proportion of foundresses that are fertilized in spring in each species? (ii) Are there differences between *V. velutina* and *V. crabro* in the spermatheca and the characteristics of its contents? (iii) Are there differences in the timing of ovarian maturation in the two species? Obtaining insights on such aspects is not only very important for gaining an understanding of the invasiveness of *Vv* but is also of general interest, as it addresses the problem of biological invasions, one of the current major threats to native wildlife. Moreover, to date, the reproductive biology of hornets in Europe has been poorly investigated. This study is based on an examination of 237 hornet queens (184 *Vv* and 53 *Vc*) in which we measured size and weight, described their sexual maturation, measured their spermatheca diameter, and assessed the amount and morphology of spermatozoa contained in their spermathecae.

Material and methods

Insects

Foundresses of the species *Vespa velutina* (VV) and *V. crabro* (VC) were collected from 9th March 2015 to 21st May 2015 (VV = 182, VC = 22), and from 18th March 2016 to 11th May 2016 (VV = 107, VC = 31). All the foundresses were captured using bottle food traps (lager containing 5% red fruit syrup) at different locations in the vicinity of Bordeaux (France) (for more details, see Table 1). Before being dissected, the insects were maintained for 1 to 48 h in plastic boxes (10 × 20 × 15 cm) within a climatic chamber at 23 ± 1°C, LD 12/12, and were provided with *ad libitum* water and honey.

Dissection and measurements

The hornets were killed by cooling in a freezer for 5 min, without degrading the sperm, so that they could be weighed using an electronic balance (AS 220/C/2; Radwag 2011, Poland) immediately prior to dissection.

- Head width

The head width of the hornets was measured using an electronic calliper (0–150 mm, Stainless hardened, e = 0.01 mm) at the largest distance between the eyes.

- Fat level

Under a stereomicroscope (OLYMPUS SZ61), the abdomen of the insect was separated from the remainder of the body, and was then immobilized with dorsal face uppermost on a dissection surface. The sternites were then removed, thereby enabling the fat level to be assessed, as described for *Polistes* in Beani *et al.* (2011). The amount of fat was classified based on a scale from 0 to 4, with 0 indicating the absence of extra fat and 4 representing an abdomen filled with fat. All the measurements were performed by a single experimenter (JP) to limit observer bias.

- Ovary development

After removing the superficial abdominal fat with clamps, the number of ovarioles that constitute each ovary was assessed. Ovarian development was assessed by evaluating the stage of the ovarioles, as described in Beani *et al.* (2011). This evaluation is illustrated in Figure 1. To assess if the queen had already laid eggs, we looked for the remains of yellow bodies in the lower portion of the flat ovarioles (S), as evidence of previous egg laying. The number of eggs in each ovariole ready to be laid was also counted. To determine if an egg was ready (O), we initially examined its colour: eggs at this stage change from a cream colour to a characteristic pearly colour. We also compared the size of the egg with the average size of the eggs that could already be observed in the tractus (stage OV).

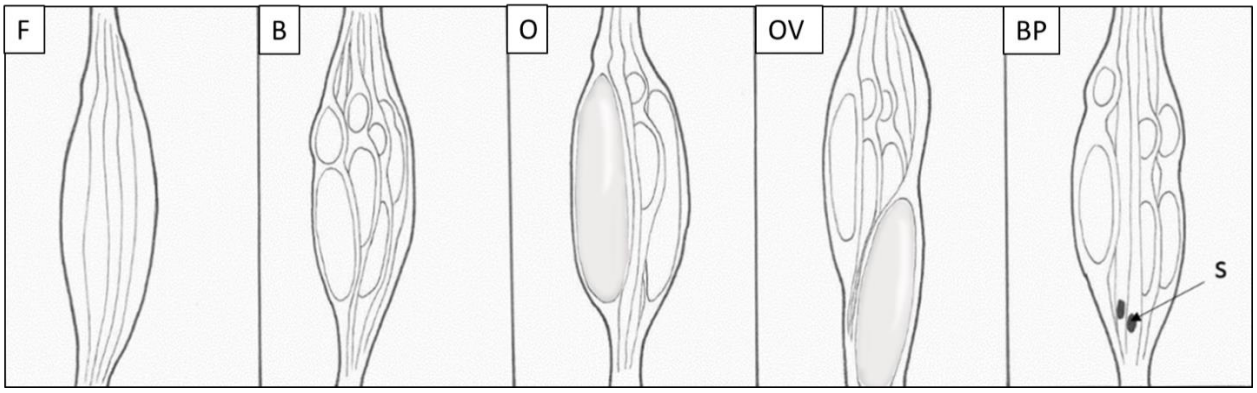

Fig 1. Ovariole development stages: F: flat ovarioles (one side), B: oocytes in formation, O: eggs, OV: eggs already in the oviduct, BP: no egg ready, but egg(s) already laid, S: spots of yellow body residuals from previously laid eggs. (Schema J. Poidatz).

- Spermatheca size

The spermatheca was extracted from the abdomen using precision clamps (Dumont, 55I). The spermatheca was then placed in a drop of Ringer's solution on a microscope slide. Photographs were taken using a camera (CAMEDIA C-7070) at magnification ×6 (Figure 2). The diameter of the spermatheca reservoir (Fig. 2AB a) was measured using the ImageJ1® image processing Software. In

order to avoid a year effect, when comparing the weight and the spermatheca diameter of the two species, we applied a correction factor, obtained by dividing the spermatheca diameter by the head width of the queen. For six specimens of *V. crabro*, we were unable to take good enough photographs of the spermatheca, and thus only 48 queens of this species were used for this part of the analysis.

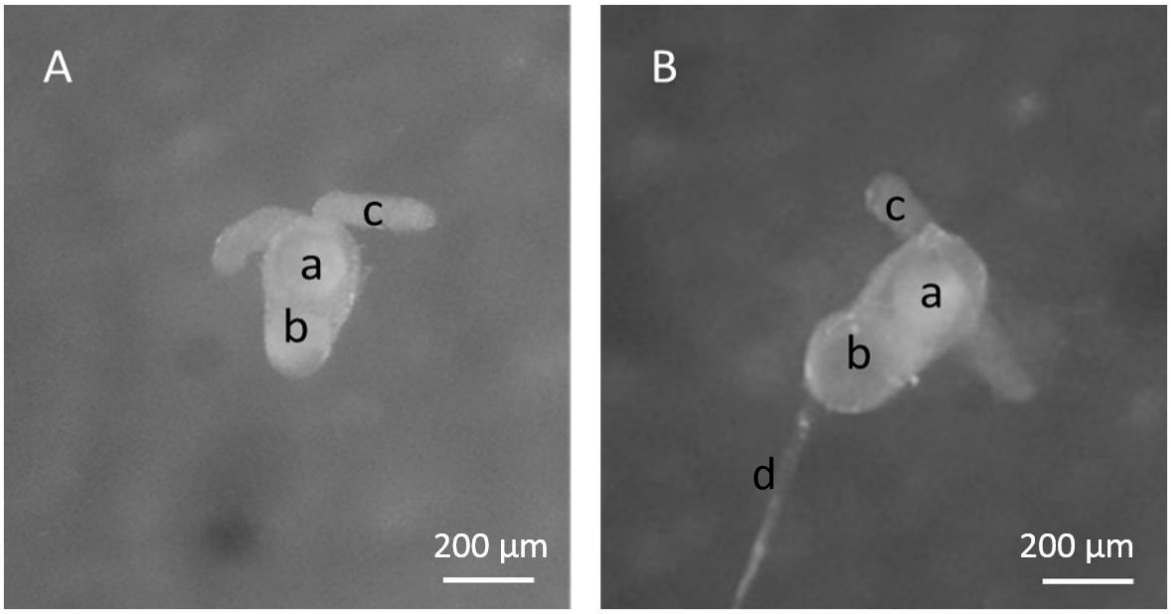

Fig. 2. Photographs of the spermatheca of *Vespa velutina* (A) and *V. crabro* (B) containing a condensed mass of sperm (a) in the reservoir (b), and the spermathecal accessory gland (c). The spermathecal duct is visible in *V. crabro* (d).

- Sperm count

After removing the external spermatheca envelope, the spermatheca content was spread in a drop of Ringer's solution by pressing it with clamps. The sperm fixation method described for parasitoids by Bressac and Chevrier, (1998) was applied. This method consists of a homogenization of the mix by clamp rotation, ethanol fixation, and DNA staining using 4′-6-diamidino-2-phenylindole (DAPI). This method has previously been used for counting sperm in seminal vesicles and for a description of spermiogenesis in male *V. velutina* (Poidatz *et al.* 2017). Having initially counted sperm five times on two slides, in 5, 10, and 15 microscope fields, we decided to count sperm in 10 microscope fields (Average spread = 25%). We counted all the visible sperm nuclei, except those in which half of the

nuclei was missing. Although this led to reduced numbers, it prevented sperm loss or destruction during successive manipulations. For four urban *V. velutina* queens, we were unable to perform a sperm count. Sperm length was assessed in 15 randomly selected individuals in each species. In total, the sperm and nuclei lengths of 130 spermatozoa of *V. velutina* and 150 spermatozoa of *V. crabro* were measured from photographs (magnification ×100) using ImageJ1® software.

Statistics

Results are presented as the means ± SD. Analyses were performed using R 3.2.2 statistical software. A Shapiro test was used to assess the normality of the data. To assess the strength of the explanatory variables, we performed an ANOVA for the continuous variables or a GLM for discrete variables, with the fixed effects being 2015 or 2016, the species (*V. crabro* or *V. velutina*), and the sampling site category for *Vv* (urban or rural). To compare sperm length, number, and morphological characteristics, a Student's t-test was used when the data were normally distributed; otherwise, a Kruskal–Wallis test was used. To examine the correlation between different parameters, we used a Pearson correlation test if the arguments had a normal repartition, and a Spearman correlation test if at least one argument did not. For the comparison of fat level between species, which was based on a visual scale, we used a Wilcoxon rank sum test.

In the comparison between species, a correction factor obtained by dividing by the head width was applied for the weight and the spermatheca diameter to avoid a year effect.

Results

Interspecific comparisons (means ± SD)

The **head width** was significantly smaller in *Vv* than that in *Vc* (respectively mean ±SD: 5.83 ± 0.09 mm and 6.66 ± 0.27 mm; t-test, $p < 0.0001$, N = 237). For both species, individuals heads were smaller in 2016 (*Vv*: 5.82 ± 0.09 mm and *Vc*: 6.53 ± 0.22 mm) than in 2015 (*Vv*: 5.84 ± 0.09 mm and

Vc: 6.90 ± 0.16 mm) [t-test p = 0.01 for *Vv* (N = 184) and p < 0.001f or *Vc* (N = 53)]. The queens of *Vv* and *Vc* **weighed** 540.38 ± 77.48 mg and 985.12 ± 150.71 mg, respectively (significant difference: t-test, p < 0.001, N = 237). See Fig 3.a for an illustration.

The **spermatheca diameter** corrected by the head width of *Vv* queens (0.09 ± 6.10^{-3} mm, N = 184) was significantly lower than in *Vc* (0.10 ± 5 × 10^{-3}mm, N = 48), (t-test, p < 0.001).

The **amount of sperm** in *Vv* spermathecae (111.56 ± 29.65 × 10^3 sperm) was significantly higher than that in *Vc (*48.26 ± 19.19 × 10^3 sperm), (Kruskal–Wallis test: k = 123.74, p < 0.001, N = 237) (Figure 3.b).

The average length of *Vv* spermatozoa (122.17 ± 19.99 µm) was smaller than that of *Vc* (201.68 ± 26.86 µm) (Kruskal–Wallis test: k = 206.26, p < 0.001, N = 280). Sperm nuclei length was, respectively, 12.31 ± 1.30 µm and 14.61 ± 1.25 µm [t-test: p < 0.001, 95% CI (1.992; 2.594), N = 280].

The **fat level (category)** of *Vv* queens (2.78 ± 0.67) was significantly lower than for *Vc* queens (3.43 ± 0.61) (Wilcoxon rank sum test, p < 0.001, N = 204). A strong correlation was found between foundress fresh weight and fat level (Spearman test, p < 0.001). The fat level did not change with time in *Vv* queens during the sampling period (R=0.072, least square, t=0.07, p=1, n=185). Moreover, when we compared the fat level of *Vv* queens trapped in the first month in 2015 with that of *Vc*, we again observed a lower fat level in *Vv*, with less variability (Wilcoxon rank sum test with continuity correction: W = 2176.5, p < 0.001, N*Vv* = 53, N*Vc* = 48).

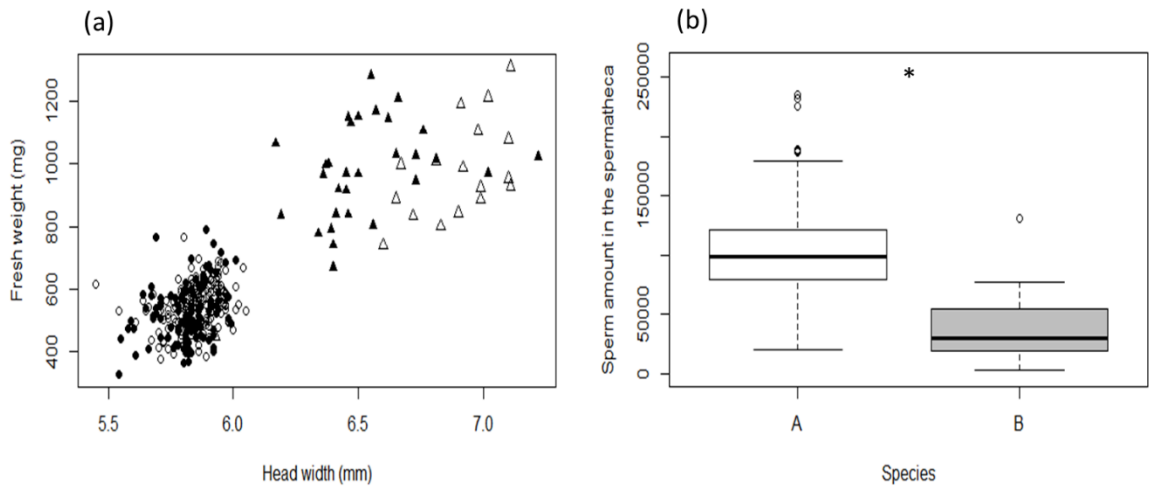

Fig. 3. (a) Head size as a function of fresh weight in the foundresses of *Vespa velutina* (circles) and *V. crabro* (triangles) in 2015 (white) and 2016 (black). **(b)** Total amount of sperm in spermathecae of the foundresses of *Vespa velutina* (A) and *V. crabro* (B) Kruskal–Wallis test P<0.05.

Among the ovaries dissected from 53 Vc foundresses, we observed 50 ovaries with 16 ovarioles and three ovaries with 14 ovarioles. We observed 16 ovarioles in all the ovaries dissected from 184 Vv foundresses. Ovarian development began approximately 15 days earlier in *Vv* than in *Vc*. Furthermore, *Vv* queens carrying three eggs ready to be laid in their ovarioles were found one month earlier than *Vc* queens. At the end of the sampling period, we observed a maximum of seven eggs in *Vv* ovarioles, but a maximum of only three in *Vc*. The maximum egg production in the ovarioles of *Vv.* queens was observed in the second half of April in spring 2015, and at this time, yellow bodies were visible in the ovarioles of some *Vv* queens, indicating that they had begun to lay eggs.

Correlations between the different parameters

The data obtained from correlation matrices for the two species are summarized in Table 2. In *Vv*, we observed significant correlations between head width, sperm contained in the spermatheca and spermatheca diameter. In *Vc*, a significant correlation was observed between weight and spermatheca

diameter. In both species, we observed a correlation between weight and head width (tendency for *Vv*), and a significant correlation between weight and sperm content in the spermatheca.

Table 2. Correlation matrix of the different parameters observed in foundresses of the hornet species *Vespa velutina* and *V. crabro*. ●: p > 0.05, ▪: p > 0.01. Abbreviations: Head = head width, Weight = fresh weight, spt diam = spermatheca diameter, sperm = amount of sperm in the spermatheca. p= Pearson correlation test, s = Spearman correlation test.

	***V. velutina* (N = 289)**				***V. crabro* (N = 53)**			
	Head	Weight	spt diam	sperm	Head	Weight	spt diam	sperm
Head	-	▪0.013 p	**●0.659** p	**●0.095** s	-	**●0.931** p	0.003 p	0.9×10^{-5} s
Weight	▪0.013 p	-	0.006 p	**●0.380** s	**●0.931** p	-	**●0.61**p	**●0.185** s
spt diam	**●0.659** p	0.006 p	-	0.0005 s	0.003 p	**●0.616** p	-	0.005 s
sperm	**●0.095** s	**●0.380** s	0.0005 s	-	0.9×10^{-5} s	**●0.185** s	0.005 s	-

Discussion

The *Vv* queens were 79% lighter than *Vc* queens and their head width was on average 18% smaller, which is consistent with the species descriptions by Linnaeus (for *Vc*) and Lepelletier (for *Vv*). For both species, all the queens captured during spring were found to be mated. The spermathecae of the two hornet species observed here had the same approximately spherical shape, and differed only in size, being 16% larger in *V. crabro*, which is a very similar proportion compared with this insect's total size. This is consistent with the known morphology of spermathecae in Vespids (Martins *et al.* 2005). The sperm morphology was very similar in the two *Vespa* species, being thin, elongated, and slightly curved, which is consistent with the observations of Mancini *et al.* 2009. Compared with the sperm of *Vc*, those of *Vv* were 65% shorter with an 18% shorter nucleus, but were 43% more

numerous. Initiation of egg production and oviposition were one month earlier in *Vv* than in *Vc*. *Vespa crabro* had higher fat reserves with a smaller amount variation than *Vv*, in correlation with its total weight.

In addition to outperforming *Vc* in behaviours potentially related with nest initiation (Monceau *et al.* 2015b), our study demonstrates that *Vv* also outperforms *Vc* in egg production timing. However, it should be noted that we measured egg production at the beginning of the cycle during the sampling period (for illustration, a detailed description of the development cycle of *Vespa affinis* is presented by Martin 1991), and thus the foundresses had not attained their maximal egg production rate. Accordingly, we are unable to predict the final egg production based on the measurements made in the present study. Furthermore, the yellow bodies observed in the ovarioles of *Vv* queens are evidence of oviposition, but cannot be used to determine the number of egg laid, as demonstrated by Cini *et al.* 2013. The fate of egg maturation should be linked to nest foundation. As mentioned in the introduction, a significant proportion of the mature *Vv* nests are found in relatively cryptic sites (Rome *et al.* 2011), and are thus potentially suitable for *Vc*, and a large proportion of the *Vv* nests are initiated in such sites before relocation. Competition during this critical period would appear to be plausible. As the number of produced gynes is considerably higher in *Vv* compared with *Vc*, (spring trapping, Monceau *et al.* 2012), this would enhance the potential efficacy of such occupation. The time differential in the reproductive cycle of *Vv* and its high fecundity could therefore be advantageous to this species in terms of founding and defending nest sites. Indeed, the timing of worker production is linked to the timing of colony foundation: 'first come first served'. The workers will defend the nest, explore, and locate resources with very limited competition during this initial period, which is a key factor for potential colony expansion. The early production of *Vv* workers could thus be advantageous in terms of colony defence against the larger *Vc* queens at the beginning of the colony cycle, the latter of which could attempt to usurp nests already founded by the congeneric *Vv*. Although interspecific usurpation of colonies between *V. velutina* and *V. crabro* has yet to be reported, it is not so rare in vespid species (Spradbery 1973, Edward 1980, Matsuura and Yamane 1990, Cervo *et al.* 2004).

In addition to local exclusion for nest initiation where the two species are present, which may involve a substantial proportion of the *Vc* foundresses, there may also be temporal exclusion. Accordingly, to counter the aggressiveness and strength of *Vc*, *Vv* seemed to have develop a strategy based on rapid reproduction of large numbers of individuals. The high precocity of *Vv* in its ovarian function and the larger stock of sperm allow this species to produce more workers earlier, which can compete with other vespids for resource collection. Numerous cases of aggressive interaction between *Vc* and *Vv* workers have been observed at feeding sites (D. Thiéry, pers. Obs.). This precocity of *Vv* may also be advantageous with respect to feeding on bee colonies. For example, when hunting as a group, *Vv* workers could benefit from rapidly reducing beehive defences, thereby ensuring a longer and safer hunting period. The high quality of such resources would undoubtedly contribute favourably to the number and quality of the next generation of *Vv* reproducers, as has also been hypothesized by Matsuura (1988).

Our finding of a lower variation in the levels of fat reserves in *Vc* compared to *Vv* could be biased by the lower number of sampled queens in this species (N = 54 *Vc* vs. N *Vv* = 183). However, we can assume that during initiation period, the fat level is a critical parameter amongst the life traits of the foundresses. Low fat level could have several repercussions, including a lower number or size of eggs, a higher requirement for sugar at this period, and less resistance to climatic variation (Strohm, 2000; Toth 2005; Weissel *et al.* 2012). A paucity of fat in *Vv* could be compensated by the production of a higher number of gynes compared with *Vc*. The fact that the fat level did not vary with time in the captured *Vv* queens could suggest that the queens with fat reserves lower than level 2 in our analysis did not survive through winter, possibly explaining why we found only 2.65% of the dissected *Vv* foundresses in this case. Alternatively, it could indicate that in this environment, the queens of *Vv* are able to maintain or enhance their fat level above the level 2 in our scale (*i.e.* there is sufficient rich resources available).

Recently, Kovacs *et al.* (2012) suggested that 'mating should not adversely affect female viability in social insects'. We can thus assume that a large majority of the queens in these species are fertilized in the autumn, or that unfertilized gynes do not overwinter. To date, *Vv* matings *in natura*

have all been observed at the end of autumn, on the ground adjacent to nests (J. Poidatz, O. Bonnard pers obs) or on the ground under or adjacent to nectariferous plants (J. Poidatz, pers obs.; K. Monceau pers com.).

The observation that there was two times more sperm in the spermathecae of *Vv* than in those of *Vc* is consistent with the following observations. (1) **Population size**: colonies of *Vv* are considerably larger than those of *Vc*, with 15000–30000 individuals annually produced in *Vv* colonies and 700–1400 individuals produced in *Vc* colonies (Nadolski 2012; Rome *et al.* 2015). (2) **Mating number**: multiple mating occurs in *Vv* as demonstrated by Arca (2012). There are on average 2.4 matings per gyne in *Vv*, (a maximum of 8 in France), and the first queen introduced into Europe was mated in China by at least 5 males prior to its introduction (Arca 2015). In contrast, only approximately 1.1 matings per gyne have been described for *Vc* (Foster *et al.* 1999, Spiewok *et al.* 2006). (3) The **spermatozoa** of *Vv* are 0. 65 times shorter than those in *Vc* and are more compacted within the reservoir. Compared to spermatozoa stored by females, *Vv* males had sperm in large excess (Poidatz *et al.* 2017). Sperm could exhibit morphological variations as a consequence of selective constraints on male paternity by sperm competition (Wedell *et al.* 2002; Snook 2005) due to frequency of female multiple matings. The shorter sperm in *Vv* is consistent with selection of high concentrations in ejaculates, whereas the longer *Vc* sperm would be less constrained by numbers because females typically mate only once. The multiple mating observed in *Vv* foundresses is a particular characteristic compared to other *Vespa* species (Strassman 2001, Cole 1983), and is a very useful life trait for an invasive species, as illustrated in the case of the single queen introduced into France (Arca 2012). Multiple mating in *Vv* could be a strategy to ensure both high potential fecundity and general brood genetic diversity, as has been observed for other social hymenopterans (Page and Metcalf 1982; Cole 1983; Crozier and Page 1985; Ross 1985; Ratnieks and Visscher 1989; Keller and Reeve 1994; Boomsma and Ratnieks 1996; Schmid-Hempel and Crozier 1999). The correlation observed in between the weight and the spermathecal content in both *Vespa* species is interesting, because it could suggest that heavier gynes could in some way optimize their mating(s), which has been observed in

fruit flies (Blay and Yuval 2014), or they may prove more attractive to potential mates. This last hypothesis has been demonstrated in moths (Xu and Wang 2009).

The problem of allelic diversity conservation has, nevertheless, to be raised here. In hymenopterans, multiple mating in populations with low diversity can cause consanguinity issues, and, for example, an increase in the proportion of diploid males in a population, which could lead to its extinction (Zayed *et al.* 2004). Diploid males are homozygous at the complementary sex determiner (*csd*) locus, and their cost to the colony growth is important, since, unlike workers, they do not forage or partake in brood care. Moreover, they often have low reproductive capacities (Harpur *et al.* 2013). In France, diploid males have already been detected in the *V. velutina* populations (Arca 2012, Darrouzet *et al.* 2015). However, in some cases, invasive social insects can overcome the genetic load at the *csd* locus via balancing selection, as demonstrated for the invasive bee *Apis cerana* in Australia by Gloag *et al.* 2016. This invasive population did experience a handicap due to diploid males, which was enhanced by the founder effect; however, rapid selection at the *csd* locus favoured equal allele frequencies. With regards to *V. velutina*, its multiple mating tendency could favour a similar balancing effect by enhancing the initial allele diversity in the inoculum and the selection of rare alleles at the *csd* locus, thereby diminishing the consanguinity effect.

The findings of this study bring to light several common traits but also differences in the fertility potential and fecundity of the queens of the endangered European hornet *V. crabro* and those of the invasive Asian hornet *V. velutina*. *Vv* queens are earlier in preparing eggs than *Vc* queens, and also have shorter but twice the number of sperm contained in a smaller spermatheca compared with *Vc*. The precocity and high fertility of *Vv* queens probably favours it over *Vc*, not only in terms of potential local exclusion during nest initiation, but also via temporal exclusion for territory and resource defence. The number, physiology, and precocity of *Vv* foundresses may have helped this species to compensate for its smaller size compared with the congeneric *Vc*. All these differences could explain the high brood production in *Vv*, and thus the high number of workers able to predate on bee hives. They also have important implications for the production of gynes in subsequent

generations. Collectively, these observations might explain the observed supremacy of *Vv* over *Vc*, and hence the rapid colonization of this invasive species across European countries.

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A.2.2 Sélection spatiale ou « Spatial sorting hypothesis »

Lors d'une invasion biologique, les organismes invasifs sont soumis à différentes pressions de sélection au cours de leur installation, entrainant une évolution rapide de leur phénotype (Sax *et al.* 2005, Weiss-Lehman *et al.* 2017). Après leur arrivée dans un nouvel environnement, les traits de dispersion des organismes invasifs sont bien souvent sujets à des évolutions extrêmes : c'est l'hypothèse du « Spatial sorting », *i.e.* d'une évolution spatiale de certaines caractéristiques avec l'invasion (Travis & Dytham 2002). En particulier, il est supposé que sur les fronts d'invasion les capacités de dispersion et d'exploration les plus optimales sont favorisées (Phillips *et al.* 2008). Cette hypothèse génère des différences dans les capacités de dispersion à travers le temps et l'espace (Alford *et al.* 2009). Ce phénomène a par exemple été observé très peu de temps (moins de 10 ans) après l'invasion du crapaud buffle *Buffo marinus* en Australie (taille des pattes plus grandes et comportement de dispersion favorisé sur les fronts d'invasion (Phillips *et al.* 2007, Phillips *et al.* 2008, Alford *et al.* 2009)), ou encore chez la coccinelle asiatique *Harmonia axyridis* après son arrivée en Europe (ailes plus grandes sur les fronts d'invasion (Lombaert *et al.* 2014)), et très récemment sur le coléoptère Ca*llosobruchus maculatus* (Ochocki & Miller 2017). Chez les insectes sociaux, la dispersion se fait via les fondatrices, qui vont fonder leurs colonies plus ou moins loin de leur nid d'origine. C'est pourquoi nous testerons cette hypothèse de sélection spatiale sur des fondatrices de *V. velutina* en Europe. La relocalisation d'une colonie peut avoir lieu dans la première phase de développement de la colonie (voir **Introduction** et **Axe 2**) mais reste dans un environnement proche ; le transport passif d'une colonie entière est peu probable, mais les fondatrices seules peuvent aisément être transportées passivement, surtout pendant leur phase hivernante, le plus souvent dans du bois.

Si les paramètres de dispersion et de reproduction des frelons asiatiques suivent l'hypothèse évolutive du Spatial sorting, des variations entre les *V. velutina* issus de diverses régions envahies il y a plus ou moins longtemps devraient pouvoir être isolées. Le protocole et les premiers résultats des expérimentations sur le sujet sont présentés en **Annexe 1.1 et 1.2.**

A.2.3 Une sélection spatiale existe-t-elle chez *Vespa velutina* ?

Les protocoles et résultats préliminaires des expérimentations menées ou prévues pour étudier ce phénomène évolutif chez *V. velutina* en Europe sont regroupés en Annexes 1.1 et 1.2. Nous présentons néanmoins ci-après un résumé de leur principe et des principaux résultats.

Résumé

L'aire de répartition du frelon asiatique invasif *V. velutina* augmente de manière particulièrement rapide en Europe, interrogeant quant aux possibles facteurs favorisant sa dispersion. Ces facteurs peuvent jouer de manière directe sur l'insecte (capacités intrinsèques, paysage, ressources, climat) mais également indirecte, par transport passif par l'homme. L'hypothèse évolutive du « **Spatial sorting** » suppose l'existence d'un phénomène de sélection très rapide à travers le temps et l'espace de traits dispersifs chez des organismes invasifs. **Cette hypothèse s'applique-t-elle à *V. velutina*** ? Chez cette espèce comme chez tous les vespidés, la dispersion se fait par les fondatrices pendant leur **phase solitaire**, *i.e.* à l'Automne après leur accouplement lors de la recherche de site d'hibernation, et au Printemps en sortie d'hibernation lors de la recherche de site d'initiation de leur colonie. Les frelons sont des **insectes volants**, et une grande part de leurs capacités dispersives reposent sur leur taille, la taille de leurs ailes, et la quantité de muscles alaires (dans leur thorax).

Dans une première étude préliminaire, une comparaison triple de traits de fertilité et de dispersion de fondatrices de *V. velutina* entre trois zones envahies depuis plus ou moins longtemps (Aquitaine, France >11ans ; Bretagne, France ~5-6 ans et Ligurie, Italie ~1an) a été réalisée au printemps 2016. Nous avons posé l'hypothèse dans cette étude qu'une évolution spatiale puisse être détectée chez les reines de *V. velutina*, qui favoriserait des meilleurs traits dispersifs dans les sites les plus récemment envahis, et des meilleurs traits reproductifs dans les sites les plus anciennement envahis. Dans cette première étude, nous avons ainsi capturé 388 fondatrices au total dans ces trois zones, et avons comparé des traits liés à la fertilité (leur maturation ovarienne, leur stock de spermatozoïde dans leur spermathèque, ainsi que leur quantité de corps gras), et des traits liés à leur dispersion (taille, poids, envergure, taille/poids du thorax en proportion avec le reste du corps). De nombreuses différences sur

plusieurs indicateurs pouvant avoir des conséquences importantes sur les dynamiques invasives de *V. velutina* dans les trois régions envahies plus ou moins tardivement ont ici été mises en évidence. Les fondatrices italiennes sont plus petites et légères, avec un thorax plus lourd proportionnellement et des ailes plus courtes que chez les françaises. Les reines Italiennes stockent également plus de gras et de spermatozoïdes, et semblent donc contrairement à notre hypothèse, avoir des capacités reproductives au moins aussi performantes que les Françaises. Aucune similitude n'a pu être observée entre la Ligurie et la Bretagne, pourtant les plus récemment envahies, la Bretagne s'avérant être plus proche de l'Aquitaine pour tous les traits observés. Mais l'identification des causes sous-jacentes ayant entrainé ces différences reste ici difficile à établir, tout comme leur interprétation et les implications dispersives engendrées. De plus l'évolution spatiale de certains traits pourrait ici avoir été modifiée par des effets environnementaux (climat, ressources) ou liés au transport passif qui pourrait court-circuiter une potentielle évolution.

Suite à cette première étude nous confirmant que des différences morphologiques existent bien chez V. velutina au moins dans trois sites à travers l'Europe, nous avons cherché à répondre de manière plus précise à la question de l'existence d'une sélection spatiale de traits dispersifs chez *V. velutina* et de faire la part des facteurs climatiques et de transport passif dans leur expression. Ainsi nous avons proposé dans une **deuxième étude** de travailler sur des échantillons bien plus importants **répartis de manière homogène dans toute l'Europe,** et recouvrant différents climats. Pour cela, nous avons lancé une **campagne internationale de collecte de reines de frelons au printemps 2017** grâce à un projet de science participatif nommé **« Eurofrelon ».** Un site internet multilingue (https://sites.google.com/site/eurofrelon/home) et une diffusion des directives à l'échelle nationale et internationale ont permis de récolter environ 6 000 fondatrices provenant de 250 sites à travers l'Europe. Nous allons associer des mesures morphologiques et génétiques pour ces échantillons, afin de voir apparaître des potentiels patterns évolutifs et la présence de pression de sélection dans les populations de front d'invasion, et de faire la part de la dispersion liée à une évolution intrinsèque des frelons, du climat, et du transport passif par l'homme. Ce projet, lancé cette année en partenariat avec le CNRS de Toulouse (col M. Liohreau et A. Wystrach), devrait pourvoir commencer à fournir des premiers résultats l'année prochaine.

A.3 Description du comportement de reproduction de *V. velutina*

A.3.1 La reproduction chez les frelons

La constitution d'un stock de spermatozoïdes disponibles pour la fertilisation des œufs est une étape clé de la reproduction pour les espèces comme les frelons, qui ne s'accouplent qu'à un seul moment de leur vie (Page & Metcalf 1982). L'étude du comportement de reproduction chez un insecte social passe par la description de plusieurs paramètres, tels que le choix du site de reproduction, le choix du partenaire, la fréquence de reproduction par mâle et par femelle pendant la période reproductive, les processus d'attraction (parades), les conditions environnementales (température, humidité) et physiologiques (âge, hormones) optimales *etc*... (Cole 1983). Il existe différentes étapes dans ce comportement : la recherche ou attraction du partenaire (odeurs, couleurs, présents), l'acceptation de l'accouplement, l'accouplement (transfert de gamètes), la fin de l'accouplement. Une fois l'enchainement de ces comportements et les paramètres jouant dessus établis, une modélisation précise de l'impact de modification de différents paramètres pourra être réalisée. Peu d'études existent sur ce sujet concernant les vespidés, à part *Mischocyttarus drewseni* (Jeanne & Bermùdez 1980) et un travail assez général sur abeilles et guêpes (Alcock *et al.* 1978).

Chez *V. crabro* ainsi que chez *V. mandarinia*, il a pu être démontré que les reines produisent des phéromones attractives pour les mâles (Batra 1980). La présence de macro glomérules dans les lobes antennaires du cerveau des mâles de *V. velutina* laisse supposer que de telles phéromones existent et qu'elles auraient une fonction importante chez cette espèce (Couto *et al.* 2016).

Les reproductions multiples chez les frelons sont rares, avec par exemple des nombres d'accouplement moyens de 1.1 par reine chez *V. crabro*, et de 1.3 chez *V. mandarinia* (Foster *et al.* 1999). Les reines de *V. velutina* s'accouplent pourtant en moyenne 2.4 fois, avec un maximum de 8 observé (Arca 2012) : cet investissement dans la reproduction semble donc particulièrement élevé chez cette espèce de frelon, et pourrait expliquer en partie les très fortes populations observées (> 15000 individus par

colonie). Bien souvent de très fortes capacités de reproduction vont de pair avec de fortes capacités d'invasion (Moller *et al.* 1996).

Chez les hyménoptères, les sites où se déroulent les accouplements sont très diversifiés selon les espèces. Ils peuvent se dérouler (1) **près des nids** : par exemple chez *V. mandarinia*, les mâles attendent la sortie des gynes pour s'accoupler (Matsuura 1984), ou encore par exemple chez les abeilles, où les faux-bourdons effectuent une congrégation autour de la reine à sa sortie de la ruche lors d'un vol nuptial (Heidinger *et al.* 2014). L'accouplement peut également se situer (2) **sur des sites stratégiques** : de nourriture, comme le font les *V. crabro*, ou de passage, par exemple les mâles de bourdons patrouillent de feuilles en feuilles en attendant le passage d'une jeune reine. Pour *V. velutina*, le site exact des accouplements est encore mal connu, des témoignages ayant été rapportés d'observations sur des sites de nourriture (arbres mellifères, pers. com. AAAFA), mais également à proximité de nids (obs. pers. J. Poidatz). Contrairement à l'accouplement chez *Apis mellifera* qui a lieu en vol, les frelons s'accouplent au sol, avec une position caractéristique, dite « en S ». (Figure 18).

Figure 18: Accouplement de plusieurs espèces de frelons, avec la position en « S » caractéristique. A : *Vespa crabro* le frelon européen (Khruner ©), B : *V. mandarinia*, le frelon mandarin (Japan, Alastair Macewen ©), C : *V. velutina*, le frelon asiatique à pattes jaunes (Source Choi *et al.* 2012).

Le choix du partenaire peut se faire sur différents critères pouvant être physiques (taille, taille relative de certains organes, couleur), physiologiques (odeurs (phéromones), âge), mais également génétiques (appartenance à la même fratrie) (Crozier & Page 1985)... L'âge de reproduction dépend du développement de l'individu, de sa stratégie de recherche de partenaire, et de l'acceptation par ce dernier. Chez certaines espèces, les accouplements sont des phénomènes sociaux qui impliquent de

nombreux partenaires : par exemple chez les abeilles, la jeune reine va effectuer des vols nuptiaux durant une semaine et s'accoupler en vol avec une congrégation de mâles (~20 accouplements) (Cole 1983, Palmer & Oldroy 2000, Tarpy *et al*. 2004).

Tous ces éléments et leurs parts respectives dans la reproduction sont encore inconnus chez *V. velutina*, et nous avons tenté d'explorer leur caractérisation dans **l'Annexe 1.3** présentée ci-après.

A.3.2 Reproduction chez *Vespa velutina*

Nous avons tenté de répondre à plusieurs questions concernant la reproduction *V. velutina* grâce à des études comportementales en condition contrôlées. Nous avons voulu à la fois estimer la fréquence de reproduction des mâles et des femelles chez *V. velutina*, et évaluer les critères de choix du partenaire chez cette espèce (âge, proximité génétique, fertilité). Les expérimentations réalisées sont présentées en Annexe 1.3, dont voici un résumé.

Résumé

Afin de mesurer l'implication de certains paramètres liés aux choix du partenaire dans les accouplements chez *V. velutina*, nous avons effectué trois expérimentations. Dans la première, nous avons mesuré **l'évolution du comportement de couples suivant leur âge et leur origine** (intra ou extra-colonial), par analyse vidéo des 33 couples mis en présence 15 minutes dans des petites enceintes. Nous avons ainsi pu observer que l'agressivité des gynes envers les mâles augmentait fortement avec l'âge des gynes. Les deux sexes étaient caractérisés par des comportements très différents, les femelles étant plus agressives et plus enclines au toilettage et à l'immobilité, quand les mâles passaient le plus clair de leur temps à se déplacer et rechercher le contact antennaire avec les femelles. Seulement deux tentatives d'accouplement ont été observées dans cette configuration. Dans une deuxième expérimentation, nous avons mesuré **la quantité d'accouplements tentés par des mâles lorsqu'ils sont mis en présence en groupe avec une femelle immobilisée** dans une enceinte

assez grande. Nous avons pu observer plus d'une trentaine de tentatives d'accouplements dans cette configuration, 30 sur 33 reines ayant été sujettes à au moins une tentative d'accouplement. Certains mâles ont montré un comportement de gardiennage envers la reine. Deux mâles ont semblés devenir attractifs pour d'autres après avoir tenté de s'accoupler avec la reine (une tentative d'accouplement entre deux mâles a pu être observée). Enfin, les mâles ont tenté en général très rapidement de s'accoupler aux femelles, en moyenne 10 secondes après mise en présence de la femelle dans l'enceinte. Dans un troisième temps, **une femelle a été mise en présence avec plusieurs mâles cette fois-ci sans entraves, de manière à ce qu'un comportement plus « naturel » puisse être observé**. Le nombre de tentative d'accouplement observé a été bien inférieur à celui avec les femelles immobilisées, malgré tout quelques tentatives ont été observées. Contrairement aux observations effectuées dans la première expérimentation où les femelles étaient très agressives envers le mâle, les femelles étaient ici plus enclines à la fuite qu'à l'agressivité. Les couples ayant tenté de s'accoupler ont à chaque fois été disséqués, sans jamais parvenir à détecter de spermatozoïdes dans les tractus des femelles : nous n'avons pas su pour l'instant observer de réel accouplement chez *V. velutina*. **Des observations *in natura* devront être mise en place pour avancer dans la description et la compréhension du comportement reproductif chez *V. velutina.***