Competition between the native and the introduced hornets *Vespa crabro* and *Vespa velutina*: a comparison of potentially relevant life-history traits

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**Abstract**

1. Invasive alien species are a major threat to biodiversity. In addition to predation and parasitism, native species might suffer from competition when invasive alien species occupy a similar ecological niche.

2. We focused on the potential interspecific interaction between two hornets: the Asian yellow-legged hornet, *Vespa velutina*, a high-concern invasive alien species recently arrived in Europe, and the native European hornet, *Vespa crabro*. The two species share a similar ecological niche and *V. velutina* is rapidly expanding across Europe, which suggests that *V. crabro* might suffer from competition.

3. We investigated, under laboratory controlled conditions, two life history traits that might cause the two species to compete: i) the ability of workers to find food sources and their flexibility in exploiting them (through individual food item choice tests and exploration assays), and ii) the worker resistance to pathogens (through immune challenge tests).

4. We show that trophic preference of both species highly overlaps, with a marked dietary preference for honeybees compared to other insect prey and non-prey protein items. No differences have been observed in the exploratory behaviour of both species. Finally, constitutive antibacterial activity was greater in workers of the native species than in workers of the invasive hornet.

5. Our laboratory study provides a first assessment under controlled conditions of the factors affecting competition between workers of two hornet species and proposes a framework to assess, in wild contexts, the magnitude of the competition and the impact of the introduced *V. velutina* on the native *V. crabro*.

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**Introduction**

In the "era of globalization", increased trades have resulted in and still produces a legacy of biological invasions (Meyerson & Mooney 2007; Hulme 2009), which causes severe ecological and economic impacts across the globe. Invasive alien species (IAS) are indeed one of the leading threats to native wildlife, human health and food safety/production (Clavero & Garcìa-Berthou 2005; Crowl et al. 2008; Pejchar & Mooney 2009; Butchart et al. 2010; Vilà et al. 2010; 2011), with an associated economic impact estimated in hundreds of US$ billion each year (Pimentel et al., 2005; Pysek & Richardson, 2010). The arrival and spread of IAS are in particular considered among the main drivers of worldwide biodiversity loss (Clavero & Garcìa-Berthou 2005). Part of this impact can be explained by direct effects of IAS presence, as in the case of introduction of IAS which act as predators, parasites or pathogens of native species (Mooney & Cleland 2001; Tompkins et al. 2003; Gurevitch & Padilla 2004; Clavero & Garcìa-Berthou 2005; Salo et al. 2007). Introduction of alien predators and parasites/pathogens outside their natural geographical range can create novel ecological contexts in which the adaptive responses of native prey and hosts may not be successful (Tompkins et al. 2003; Strauss et al. 2006; Salo et al. 2007). Indeed, alien predators and parasites appear to have a relevant effect on native species (Kats & Ferrer 2003; Gurevitch & Padilla 2004; Salo et al. 2007; Roy et al. 2012; Nazzi & Le Conte 2016).

A large and generally more difficult to predict and assess effect is related to competition, either mediated by second order ecological interactions (e.g. indirect dispersal and transmission of pathogens or parasites) or by competition for space and other resources between the IAS and native species (exploitation competition, Reitz & Trumble 2002; Duyck et al. 2004). The last scenario often occurs when the introduced IAS occupy an ecological niche very similar to the one filled by a native species (competitive exclusion principle,

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Hardin 1960*)*. Examples of native species ecological displacement and decline due to exploitation competition are more and more common in both plant and animal species, either vertebrates or invertebrates (Holway 1999; Brown et al. 2002; Duyck et al. 2004; Gherardi & Cioni 2004; Bevins 2008; Strubbe & Matthysen 2009; Vilà et al. 2011). Exploitation competition due to invasive species is indeed considered to be a major determinant of invertebrate species spatial displacement (Reitz & Trumble 2002).

Understanding the potential life history traits that cause native and introduced species to compete is thus important to know, evaluate and prevent/reduce competition, and therefore, in turn, the loss of native biodiversity.

Here, we investigated the potential life history traits affecting the competition between two hornets species: the native European hornet, *Vespa crabro* Linnaeus, 1758 (Hymenoptera: Vespidae), and the recently introduced alien invasive *Vespa velutina* Lepetier, 1836 (Hymenoptera: Vespidae), also known as yellow-legged hornet.

*Vespa velutina* is an invasive hornet species native of South East Asia (Monceau et al. 2014a). Its presence was first recorded in South of France, in 2004 (Haxaire et al. 2006); since then the species rapidly spread across France and Europe (Rome et al. 2009; Villemant et al. 2006; Villemant et al. 2011a; Robinet et al. 2016). At present, *Vespa velutina* is found in different European countries (e.g. Spain, Portugal, Italy, Belgium and United Kingdom, Monceau et al. 2014a, Robinet et al. 2016), also in areas disjointed from the front of invasion (e.g. Balearic Islands, Mar Leza Salord pes.comm. and Veneto region, Italy, Bortolotti and Cervo 2016). Based on climatic suitability models the potential invasion risk of the species (Villemant *et al*. 2011a) has been estimated to concern most of the European territory.

The main threat posed by *Vespa velutina* is on beekeeping activities, as the yellow-legged hornet is a specialized predator of honeybees (Monceau et al. 2014a). Such predation can be intense during summer and autumn, and represents a further threat to honeybee

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populations, which are already suffering a noteworthy decline all over Europe because of several factors (Goulson et al. 2015). In addition to the economic impact on apiculture, the invasive hornet has also a potentially significant ecological impact, due to its predation on a vast array of insect species (Spradbery 1973; Matsuura & Yamane 1990), some of which (honeybees included) provide valuable ecosystem services, such as pollination, as well as a potential impact on human health (Monceau et al. 2014a), since envenomation of Vespa velutina can induce severe allergic or toxic reactions, resulting in organ failure and death (Liu et al. 2015).

Due to competition for a similar ecological niche, *Vespa velutina* may also be a threat for the native hornet species. The European hornet, *Vespa crabro,* represents one of the two hornet species native of Europe along with the Oriental hornet, *Vespa orientalis*. The species is found throughout Europe, differently from *Vespa orientalis*, which occurs only in the southern countries. European hornets show a very similar lifecycle compared to the yellow-legged hornet (Matsuura & Yamane 1990; Matsuura 1991; Takahashi et al. 2004; Monceau et al. 2014a) (see Material and Methods).

Similarities in life history traits create several dimensions of potential competition between the invasive and the European species (Monceau et al. 2015a). Competition over shared resources can occur in two main phases of the hornet lifecycle: colony foundation (Spring, Matsuura 1991) and colony growth (late Summer/Autumn; Matsuura 1991). During colony foundation, foundresses of the two species might compete for nesting sites, (Edwards 1980; Matsuura & Yamane 1990; Matsuura 1991), but due to the different nesting habits (see Material and Methods), competition for nesting sites might become relevant only under very high *Vespa velutina* population density.

In the phase of colony growth, workers of the two species might compete over two main challenges they face: finding food and resisting disease transmission.

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The nutritional requirements differ between adult hornets and their larvae, with adults mainly feeding on carbohydrates and larvae on proteins (Raveret Richter 2000). Foragers collect protein sources mainly through summer and autumn to feed the developing brood (Spradbery 1973; Edwards 1980); the gathering of proteins increase during the rearing of sexuals, especially gynes, as they require more proteinaceous food to build up their fat storage (Spradbery 1973; Edwards 1980; Monceau et al. 2015a), as it occur for other social wasps, where the quality and quantity of fat bodies reflect nutritional status, and particularly protein uptake, during larval development (Hunt 2007; Daugherty et al,.2011). Both *Vespa crabro* and *Vespa velutina* prey on a wide range of arthropods (Spradbery 1973; Matsuura & Yamane 1990), with a preference for honeybees in apiaries (Matsuura & Yamane 1990; Matsuura 1991; Baracchi et al. 2010; Monceau et al. 2013a, 2014a, 2015a). Their similar feeding habits and partially overlapping phenologies suggest that the two species may likely compete for food (Monceau et al. 2014a; 2015a). It is however largely unknown how much the feeding spectrum of the two species overlap, and whether they are similarly placed along the specialist/generalist continuum. Indeed, *Vespa velutina* is reported to specialize on honeybee prey (Tan et al. 2007, 2012), but probably both the yellow-legged hornet and the European hornet should be classified as semi-generalists (Matsuura 1991; Monceau et al. 2013b). Under this perspective, any trait that facilitates or enhances the efficiency in food finding, processing and uptaking might give an advantage to a species over the other. For example, behavioural traits such as boldness and explorative tendency, which are thought to play a relevant role in colony founding, differs between foundresses of the two species, favouring *Vespa velutina* invasion and potentially enabling it to outcompete *Vespa crabro* (Monceau et al. 2015b).

A second main challenge to colony survival and species diffusion is represented by pathogen pressure. Social insect colonies represent a preferential target for parasitic and pathogen infections, since they usually consist of large numbers of closely related individuals

that frequently interact favouring the spread of parasites and pathogens among colony members (Cremer et al. 2007). Moreover, the costant internal enviroment mantained within a nest of a social insect species to favour brood development creates optimal condition to pathogens and parasite growth (Cremer et al. 2007). Ability to resist pathogen infections is therefore a crucial trait for the ecological success of a species and to predict its invasive potential (Traniello et al. 2002; Lee & Klasing 2004; Prenter et al. 2004; Nadolski 2013).

The role of pathogen pressure in shaping biological invasions is still debated, as IAS might either benefit from the absence of specialized pathogens, the so-called "enemy release hypothesis" (Colautti et al. 2004; Liu & Stiling 2006), or suffer from the presence of pathogens with which they did not coevolve (Prenter et al. 2004). The "evolution of increased competitive ability" hypothesis predicts that invasive species are subjected to less predation and parasitization than sympatric native species, and thus can allocate resources from defence and immunity to growth and fecundity, thereby achieving higher fitness (Lee & Klasing 2004; Liu & Stiling 2006; Manfredini et al. 2013). A higher individual antibacterial activity could be advantageous for the colony not only in the case of reproductive individuals, but also for sterile workers. Foragers are exposed to pathogens at foraging hotspots (Durrer & Schimd-Hempel 1994) and they may represent routes for bringing new infections into the colony (Cremer et al. 2007); therefore, a stronger immune system in workers could enhance colony efficiency in foraging activities allowing the invasive hornets to outcompete the native species.

Here, we evaluated the potential competition of *Vespa velutina* and *Vespa crabro* over these two contexts. First, we investigated if workers of the two species differ, at the individual level, in two traits that could affect resource finding and exploitation: (i) boldness and exploratory tendencies, which are likely to be correlated to the species ability to rapidly find and exploit food sources; and (ii) preference for different food items, which could provide

valuable information on the dietary flexibility of the two species to understand where to place them in the specialist/generalist continuum and to evaluate their likelihood to compete for food. Then we compared the immune ability of *Vespa velutina* and *Vespa crabro* workers through an immune challenge by using as a proxy the individual antibacterial activity in the two species.

By focusing on individual life history traits under controlled laboratory conditions, where confounding variables such as colony size and brood abundance can be controlled for, this paper provides a first insight into the possible factors affecting competition between these two hornet species and propose a framework that future studies could use to assess, in wild contexts, the magnitude of the competition and the impact of the introduced yellow-legged hornets on native hornet species.

**Material and methods**

*Species biology*

*Vespa velutina* and *Vespa crabro* show a very similar lifecycle (Matsuura & Yamane 1990; Matsuura 1991; Takahashi et al. 2004; Monceau et al. 2015a). Single queens start their colonies in spring after a wintering diapause, the colony grows in size throughout summer, with the production of thousands of workers, and new generations of sexuals (i.e. males and gynes) are produced in late summer/early autumn. Mating occurs during the fall, and a new generation of mated queens enter hibernation (Matsuura & Yamane 1990; Matsuura 1991; Takahashi et al. 2004; Monceau et al. 2014a). The main differences between *Vespa velutina* and *Vespa crabro* regard the length of the annual lifecycle, which is longer in the invasive species, going from February/March till November and the size of the colony, with the yellow-legged hornet building bigger nests which contain a consistently higher number of

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individuals (Monceau et al., 2015a). Moreover, while *Vespa crabro* usually builds its nest in confined spaces such as tree cavities (Edwards 1980; Matsuura & Yamane 1990), *Vespa velutina* nests both in confined and exposed sites, apparently with a preference for the latter (Monceau et al. 2014a).

*Sample collection and rearing*

*Vespa velutina* and *Vespa crabro* workers were emerged in the laboratory from combs collected in the field. *Vespa velutina* combs with sealed brood were collected during the months of October and November 2015 in the surroundings of Ventimiglia (Imperia, Liguria, Italy), from five nests that were gathered by local beekeepers. *Vespa crabro* combs were collected during the same months in the surroundings of Florence (Tuscany, Italy), from four nests. Combs from different nests were maintained at 26±2 °C in separated glass cages (50 × 50 × 50 cm). Workers were collected at emergence, individually marked with a spot on the thorax with Uni Posca® paint markers using different colors according to day of emergence and nest of origin and transferred in groups of 10-15 individuals to 15 × 15 × 15 cm glass cages with a mesh wire side, at room temperature, with *ad libitum* water and sugar as food, until behavioural or immune challenge assays were performed. At the end of the assays all workers were dissected in order to confirm their worker phenotype, by checking the fat storage in their abdomen; in *Vespa velutina*, the size of workers and gynes may largely overlap, but, as in other vespid species which go through a winter diapause, reproductive gynes present well-developed fat bodies for overwintering (Hanson & Olley 1963; Spradberry 1973; Perrard et al. 2012) clearly visible on the internal surface of their tergites and sternites, while workers have very scant or null fat deposit on their abdominal segments (Beani et al. 2011; Cappa et al. 2013). At the time of behavioural experiments or immunochallenge assays, all used workers of both species were on average eight days old (*Vespa crabro*: 8.620+-5.454;

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range:5-36; *Vespa velutina*: 8.434+-3.146; range:5-20) and no significant differences exists in age between the two species (t-test: t=0.630, p=0.528, n=221 vs 252).

*Behavioural assays: explorative tendency and feeding preference*

1) *Boldness and exploration assays*

To assess the explorative behaviour of *Vespa velutina* and *Vespa crabro* workers, we measured two behavioral traits (boldness and exploration) that have already been investigated in queens of the two species (Monceau et al. 2015b). The two traits were measured at the same time using an open-field apparatus modified from that used by Monceau and co-workers (2015b). The apparatus was represented by an experimental arena consisting of a square opaque acclimatization box ($15 \times 15 \times 15$ cm) connected via a trapdoor (Ø 3 cm) to one side of a rectangular transparent test box ($32 \times 24 \times 16$ cm) virtually divided in 24 equivalent sections ($8 \times 8 \times 8$ cm). Each part of the apparatus was carefully washed with 96% ethanol between trials. Each worker (*Vespa velutina*, N=22; *Vespa crabro*, N=21) was kept in the opaque box for 5 min of acclimatization before the trial; the trapdoor was then opened to allow the hornet to explore freely the test box for 10 minutes or to return to the opaque box as a refuge.

Following Monceau et al. (2015b), we directly (real-time) measured two behaviours: (i) the latency to the first exit from the acclimatization box after trapdoor opening, which was used as a measure of *boldness* (i.e., the lower is the score, the bolder is the individual); (ii) the number of different sections visited, which was used as a measure of *exploration* (maximal score = 24). Trials were performed in the central hours of the day, from 11:00h to 15:00h, when workers are most active (pers. obs.). One worker of each species was tested at the same time in one of two identical open-field apparatus.

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*2) Feeding preference assays*

In order to assess the food preference and diet flexibility of *Vespa velutina* and *Vespa crabro* workers for different food sources, food choice trials were performed. Individual workers (*Vespa velutina*, N=123.; *Vespa crabro*, N=118) were kept without food for 1 hour before the trials; each worker was then transferred to a plastic transparent cage (20 × 15 × 14 cm) and left for 10 minutes for acclimatation. At the end of the acclimatation period, different food sources were introduced inside the cage through a slide tray (9 × 3 cm). Food sources were presented in small circular plastic cups (Ø 2.5 cm × 1 cm) on the tray at one end of the cage. Each food source was separated by 0.5 cm from the other/s. Workers were then observed for 10 minutes and the time spent feeding/manipulating on each food item was directly recorded. Four trials were performed, 3 with protein baits and 1 with sugar baits. Each worker was used only once. In a first choice trial we assessed the workers' preference for one specific prey item, *Apis mellifera* honeybee foragers (presented as dead individuals, killed by freezing), with respect to generic protein sources: minced meat versus fish (canned tuna). We then assessed the preference of the workers for the two protein non-prey items (meat versus fish), and for honeybee foragers compared to another potential prey item, the paper wasp *Polistes dominula* (presented as dead individuals, killed by freezing), whose nests are plundered by *Vespa crabro* in late summer/early autumn (pers. obs.) and which belongs to a genus which is part of the diet of another hornet, *Vespa tropica* (Matsuura 1991). Both A. *mellifera* and *P. dominula* where collected in the field (surroundings of Florence) while foraging. Finally, we evaluated the workers' preference for different carbohydrate sources: honey, honeybee sugar candy (sucrose and corn syrup, 3:1) and grape. We chose grape as a potential carbohydrate source since European hornets along other social wasps are often seen foraging on grapes, and they indeed appear to play a relevant role in the ecology of yeast strains involved in the production of fermented beverages (Stefanini et al. 2012; 2016). The four trials were

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performed during central hours of the day, from 11:00h to 15:00h, in a random order. Sample size were the following: Meat, fish, honeybee: *Vespa velutina* N=31; *Vespa crabro* N=31; Meat, fish: *Vespa velutina* N=30; *Vespa crabro* N=30; Honeybee, *Polistes* sp.: *Vespa velutina* N=30; *Vespa crabro* N=33; Honey, honeybee sugar candy, Grape: *Vespa velutina* N=30; *Vespa crabro* N=28).

*Antibacterial activity assays*

To compare the ability of hornet workers of the two species to remove bacterial cells from their haemolymph (i.e. bacterial clearance), workers belonging to each species were injected with the Gram-negative bacteria *Escherichia coli*, an immune elicitor commonly used to test antibacterial activity in insects (Yang and Cox-Foster 2005; Manfredini et al. 2010; Gätschenberger et al. 2013; Manfredini et al. 2013; Cappa et al. 2015; Polykretis et al. 2016). We chose to measure bacterial clearance as a good proxy of workers immunity since different parameters linked to antimicrobial immune response (e.g. number of haemocytes, phagocytosis, nodule formation, PO activity, encapsulation response) appear correlated in insects' immunity (Gillespie et al. 1997; Cotter and Wilson 2002; Lambrechts et al. 2004; Schimd-Hempel 2005) and injection of live bacteria provide an integrative view of the activation of the an organism's immune system (Charles & Killian 2015). *E. coli* is not naturally found in *Vespa velutina* and *Vespa crabro*, thus, we could exclude its presence in our hornet workers prior to artificial infection. Pathogens, such as *E. coli*, that do not infect wild insect populations are often used in laboratory bioassays to elicit the immune response and induce the production of antimicrobial peptides (Gillespie et al. 1997; Siva-Jothy et al. 2005).

In order to select the infectious bacterial cells and minimize the competing effect by other possible microorganisms, we used the *E. coli* tetracycline-resistant strain XL1 Blue

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(Stratagene, La Jolla, CA, USA). Bacterial cultures were grown aerobically in Luria-Bertani (LB) complex medium (Miller, 1972) containing tetracycline at a concentration of 10 μg/mL overnight at 37 °C in a shaking incubator. After centrifugation, bacteria were washed twice in phosphate-buffered saline (PBS), re-suspended and diluted to the desired concentration with PBS (~1,5 x $10^8$ cells/ml). The approximate amount of bacterial cells in the solution was determined using a haemocytometer (Neubar) and confirmed by plating the bacterial solution on LB agar (dilutions $10^{-6}$, $10^{-7}$) and counting the colony forming units (CFUs) that grew overnight at 37 °C. Each hornet (*Vespa velutina*, N=69; *Vespa crabro*, N=52) was infected by injecting 1 μL of inoculum, containing ~1,5 x $10^5$ cells, with a Hamilton™ micro syringe between the 2nd and the 3rd tergite (Yang & Cox-Foster, 2005). Before injection, workers were cooled down in a refrigerator (T 4°C) to facilitate their manipulation. After infection, hornets were introduced in groups of about 10, separated for species and colony of origin into 15 × 15 × 15 cm glass cages with a mesh wire side previously rinsed with 96% ethanol, provided with *ad libitum* sugar cubes as food. Twenty-four hours later, during which the hornets were maintained under controlled conditions, (20 ± 2 °C; 55% RH), each worker was inserted in a sterile plastic bag with 10 mL of PBS after removing the sting and the venom sac, in order to avoid a possible reduction of the bacterial count due to the presence of antimicrobial peptides in the bee venom (Baracchi et al., 2011). We chose the timeframe of twenty-four hours from the bacterial challenge as it is a widely used procedure in insect immunity studies since it provides a view of the organism rapid response to the microbial infection (Gillespie et al. 1997; Siva-Jothy et al. 2005; Charles and Killian 2015).  Each sample was then processed with a Stomacher® 400 Circulator at 230 rpm for 10 min in order to homogenize the hornet body and extract haemolymph and content of the internal organs in the PBS. Afterwards, 0.1 mL of serially diluted PBS suspensions (dilutions $10^{-1}$, $10^{-2}$) of each sample were plated on LB solid medium added with tetracycline (10 μg/mL) and incubated

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overnight at 37°C. The following day, the colonies grown on the plate surface were counted and the viable bacterial count was expressed as Colony Forming Units (CFUs) per worker. At least 3 control hornets per colony for each species (*Vespa velutina N = 12; Vespa crabro, N = 12*) were injected with 1 µL of PBS, homogenized and plated following the same procedure of *E. coli-infected* workers, to ensure absence of other bacterial strains capable of growing on our LB agar plates added with tetracycline (10 µg/mL).

A total of 121 hornets were infected with *E. coli* and plated: 69 *Vespa velutina* workers, 52 *Vespa crabro* workers. Workers age range was 3-14 days post-emergence, at least 10 workers were infected from each of 8 nests (4 for each species).

*Statistical analysis*

In order to account for the non-independence of data (i.e. workers belonging to the same colony), we used a generalized estimating equations (GEE) approach, which extends the generalized linear model to allow for analysis of correlated observations such as clustered data (Burton et al 1998), and it is robust against misspecification of the error structure model and more relaxed on distributional assumptions (Hubbard et al., 2010, Overall & Tonidandel 2004). For all GEEs, model selection was performed on the basis of the "quasilikelihood under the independence model" criterion QIC, by choosing the model parameters that resulted in the smallest QIC (Pan 2001).

We assessed differences in boldness and exploratory activity between the two species using the following model parameters: boldness or exploration activity as dependent variables; tweedie probability distribution; log link fuction; independent working correlation matrix; fixed effect: species; subject effect: colony of origin. Feeding preferences were assessed, for all experiments, both using the time spent feeding on a bait item and the latency to first item as dependent variables. In the first case we used the following model parameters: tweedie

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probability distribution, log link fuction, independent working correlation, species, bait and their interaction as fixed effects, colony of origin and individual as subject effects. In the case of latency as dependent variable all the parameters were the same except for the probability distribution, which was a negative binomial one for the honeybee vs wasps experiment, and a gamma one for all the other experiments. The influence of species on anti-bacterial response was assessed using log-transformed CFU count as a dependent variable, species as a fixed effect and colony origin as a subject effect, and the following parameters: independent working correlation matrix and gamma-log link distribution. All analyses used a model-based estimater and a type III analysis. Stastistical Analyses were performed in SPSS 20.0 (SPSS, 2011).

**Results**

*1. Boldness and exploratory activity*

Neither boldness nor exploratory tendencies differed between *Vespa velutina* and *Vespa crabro* workers. There was no difference either in the time of latency to the first exit from the acclimatization box after trapdoor opening or in the number of visited sections for workers of the two species (Boldness: Wald chi-square=1.713, df=1, p=0.191; Exploration: Wald chi-square=0.396, df=1, p=0.529; Fig. 1).

*2. Feeding preference*

*Proteins sources*

*Meat, fish, honeybee*

The total time spent feeding on any protein item differed between species (Wald chi-square=9.108, df=1, p=0.003) with *Vespa velutina* spending more time on protein items than

*Vespa crabro* (Fig 2, top left). The time spent feeding on each item differed among items for workers of both species (Wald chi-square=337.895, df=1, p<0.001), with both species spending more time in feeding on honeybees than on fish or meat (pairwise comparisons, honeybee vs meat: p>0.001, honeybee vs fish: p>0.001, meat vs fish: p=0.181). However, the interaction between species and bait was significant (Wald chi-square=13.906, df=1, p=0.001), which suggest that, while both species spent more time on honeybees, *V. velutina* tended to spend more time on fish than meat, while for *V. crabro* the opposite trend was observed (even if this was not significant after multiple comparison correction, Fig 2, top left).

The latency time to reach each food item confirmed the pattern shown by time of feeding, with honeybee being the food item reached most promptly by both species (Wald chi-square=356.477, df=1, p<0.001, pairwise comparisons between honeybee and meat or fish, for both species, all p<0.001). However, there was a significant effect of the species-bait interaction (Wald chi-square=38.287, df=1, p<0.001), with *Vespa crabro* showing smaller latency time for meat than fish, and *Vespa velutina* showing the opposite pattern (even if none of the pairwise comparison was significant, p>0.100). No differences between species were found in the general latency time toward any protein item (Wald chi-square=0.184, df=1, p=0.668).

A strong feeding preference for honeybee was also corroborated by the analysis of the number of switches from one food item to another. When the honeybee was found as the first food item, both *Vespa crabro* and *Vespa velutina* switched to other food items less often than when the first found item was meat or fish (*Vespa crabro*: proportion of individuals switching from honeybee to meat or fish=0.217, from meat or fish to honeybee=1, $\chi^2$ =9.28, df=1, p=0.002; *Vespa velutina*: proportion of individuals switching from honeybee to meat or fish=0.412, from meat or fish to honeybee=1, $\chi^2$=6.99, df=1, p=0.008).

*Honeybee versus Polistes sp*

The time spent on each prey item differed between items for both *Vespa velutina* and *Vespa crabro*, with workers of both species spending more time on *A. mellifera* honeybee baits than on *Polistes dominula* (Wald chi-square=19.195, df=1, p<0.001, Fig. 2, bottom left). The total time spent on any prey item did not differ between species (Wald chi-square=0.575, df=1, p=0.448, Fig 2, bottom left). Interaction between species and bait was not significant (Wald chi-square=1.107, df=1, p=0.293).

The latency time to reach each food item confirmed the pattern shown by time of manipulation, with honeybees being the reached most promptly than wasps by both species (Wald chi-square=73.624, df=1, p<0.001). General latency time was significantly smaller in *Vespa velutina* than in *Vespa crabro* (Wald chi-square=5.170, df=1, p=0.023). Interaction between species and bait was not significant (Wald chi-square=1.711, df=1, p=0.191).

The preference for the honeybee over the wasp was confirmed for both species also when comparing the number of switches from one food item to another, but in this case the difference was significant only for *Vespa velutina* (proportion of individuals switching from honeybee to wasp=0.263, from wasp to honeybee=0.889, $\chi^2$=7.26, df=1, p=0.007) but not for *Vespa crabro* (proportion of individuals switching from honeybee to wasp=0.550, from wasp to honeybee=0.846, $\chi^2$=1.92, df=1, p=0.192).

*Meat versus fish*

In meat versus fish trials, species had a significant effect on the time spent feeding on items (Wald chi-square=271.327, df=1, p<0.001), with *Vespa velutina* spending more time on baits than *Vespa crabro* (Fig.2, top right). Bait had only a slightly significant effect (Wald chi-

square=4.124, df=1, p=0.042), with more time spent feeding on fish than meat in both species (Fig 2, top right). Interaction between specie and bait was not significant (Wald chi-square=0.024, df=1, p=0.876). Latency time analyses overall confirmed the results: species had a significant effect (Wald chi-square=4.423, df=1, p=0.035), with *Vespa velutina* being faster than *Vespa crabro* in starting to feed. Nor bait nor the interaction between bait and species had a significant effect (Wald chi-square=0.031, df=1, p=0.859; Wald chi-square=0.189, df=1, p=0.664). This pattern was also confirmed by comparing the number of switches from one food item to another. The proportion of cases in which an individual switched from one item to another was not different, in any species, whether the first chosen item was meat or fish (*Vespa crabro*: proportion of individuals switching from meat to fish=0.600, from fish to meat=0.444, $\chi^2$=0.05, df=1, p=0.823; *Vespa velutina*: proportion of individuals switching from meat to fish=0.455, from fish to meat=0.714, $\chi^2$=0.820, df=1, p=0.365).


### *Carbohydrate sources*

*Honey, honeybee sugar candy, grape*

Workers of the two species spent a different amount of time feeding on any carbohydrate item (Wald chi-square=8.525, df=1, p=0.004), with *Vespa Crabro* spending more time on carbohydrate baits than *Vespa velutina* (Fig.2, bottom right). Bait type had a significant effect (Wald chi-square=13,666, df=1, p=0.001), with more time spent manipulating honey and grape than candy. However, the interaction between species and bait was significant (Wald chi-square=7,053, df=1, p=0.029). showing that *Vespa crabro* was spending a similar amount of time on all carbohydrate sources (all pairwise comparison >0.05) while *Vespa velutina* was spending more time on honey and grape than on candy (all pairwise comparisons p<0.005). The analysis of latency time showed that the two species did not differ in the overall latency

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time to reach carbohydrate items (Wald chi-square=0.592, df=1, p=0.441), nor the kind of bait influenced the latency time (Wald chi-square=1.402, df=1, p=0.496)- Finally, no significant interaction between species and bait was found (Wald chi-square=1.964, df=1, p=0.375)

*3. Immune ability*

Workers of the two species exhibited significantly different response to *E. coli* infection (Fig. 3). *Vespa crabro* workers had a significantly higher anti-bacterial response (or bacterial clearance) than *Vespa velutina* workers (Wald chi-square=6.165, df=1, p=0.013). The bacterial loads found in the homogenate of native hornets were significantly lower than those found in invasive yellow-legged hornets (Fig. 3). No bacteria were detected in the plates of PBS-injected samples of both species. There was no correlation between worker age and individual bacterial clearance (Spearman rho=0.154, N=121, p=0.091).

**Discussion**

Our results show a similar pattern of exploratory behaviour and a marked overlapping of feeding preferences in workers of the two hornet species, suggesting that invasive *Vespa velutina* might represent a potential competitor for the European hornet, at least in terms of foraging and food source consumption or exploitation. As regards the exploratory behaviour, differently from what previously found for queens of the two species (Monceau et al. 2015b), with *Vespa velutina* queens bolder and more prone to exploration compared to *Vespa crabro*, workers were found similar in terms of both boldness and exploratory activity. The comparable exploratory tendencies of hornet workers of the two species could be due to the fact that workers, as their duties mainly consist of providing building material and food (thus usually spatially closer to the colony), might be less under the selective pressure for dispersal that likely acts on the foundress phenotype. Taking together the results about the foundress

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and worker phenotypes, we suggest that competition between the two species might be a two step process: *Vespa velutina* might outcompete *Vespa crabro* during colony foundation because of higher explorative tendencies of queens, and it might later outcompete *Vespa crabro* for resource exploitation during summer and autumn because of its foraging strategy, with a high number of foragers patrolling and defending food sources (Tan et al. 2007; Monceau et al. 2014b), rather than individual differences in boldness and exploratory behaviour.

Behavioural assays investigating the dietary preferences clearly highlighted a strong preference for honeybee prey in workers of both the invasive and native hornet compared to other generic protein sources potentially available. Such preference is quite interesting since it underlines a rather evident specialization in feeding behaviour of both species towards *A. mellifera* honeybee prey, although both species are considered semi-specialists (Matsuura 1991). Previous work indicated that honeybees might represent one-third to two-thirds of dietary protein of *Vespa velutina* (Villemant et al. 2011b), but the proportion was suggested to depend on the nest location and surrounding environment (Villemant et al. 2011b; Monceau et al. 2014a). Thus, it is likely that, in case of beehive availability, hornets of both species would concentrate their foraging effort on the preferred prey, therefore increasing the chances of competition. Honeybee colonies represent an excellent protein source for a growing nest of hornets (Ono et al. 1995); the high concentration of potential prey and the lack of effective defensive strategies (Tan et al. 2013; Tan et al. 2012; Arca et al. 2014) could explain the dietary preferences showed by workers in our trials.

The results of our laboratory assays are mirrored by the intense predation observed on beehives in the field for both species but, especially, for yellow-legged hornets (Baracchi et al. 2010; Tan et al. 2007; 2012; Monceau et al. 2013a; b; 2014b). The similar pattern of exploratory activities and the overlapping preference for honeybee prey observed for both

species in our laboratory trials further corroborate the hypothesis of a possible competition for the preferred food source (i.e. honeybees) between native and invasive hornets in the field. Moreover, considering a) the different predation strategies adopted by foragers of the two species in the field (Baracchi et al. 2010; Tan et al. 2007; Monceau et al. 2013b; 2014b), b) that *A. mellifera* is able to defend, at least partially, from *Vespa crabro* attacks (Baracchi et al., 2010) while is not able to counteract *Vespa velutina* attacks (Arca et al. 2014) and c) that *Vespa velutina* drastically outnumber *Vespa crabro* both in colony density and colony size (Monceau et al. 2014b; Monceau & Thiery 2016), it is predictable that the native European hornet may be easily displaced by the invasive one apiaries.

Indeed, while *Vespa crabro* has a relatively mild predation impact on honeybees, with only few hornets patrolling beehives in order to catch bees (Baracchi et al. 2010), *Vespa velutina* specializes in hawking honeybee foragers returning to their nest (Tan et al. 2007), imposing a much higher predation pressure on beehives, with tens of hornet foragers patrolling the hive entrances (Tan et al. 2007; Monceau et al. 2013b, 2014a).

Feeding preference towards honeybees was confirmed for both species also when the preferred prey was presented with alternative Hymenoptera prey items (*Polistes* wasps). The higher attraction of workers of both species towards honeybee prey could be explained by the fact that the relatively small colonies of paper wasps and the scant number of *Polistes* foragers encountered in the field may be a less valuable source of protein for hornets, especially when honeybee prey are available

The feeding preference towards honeybees and, to a lesser extent, wasps is unlikley to be due to the presence of prey's haemolymph, which might be considered as a sugar-reward for hornets, as if hornets were attracted by bees (or wasps) only or mainly for the sugar content of hemolymph, we would not expect to observe hornets manipulating preys as they

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usually do when foraging, by removing heads and legs and carrying the thorax, a behavioral pattern that indeed was very clear.

In the absence of the preferred prey item, *Vespa velutina* workers showed a significantly higher consumption of both the meat and fish baits compared to *Vespa crabro*. The higher feeding rate towards general protein sources in the yellow-legged hornet is a trait found in other opportunistic predatory species, favouring their invasion success (Rehage et al. 2005; Eloranta et al. 2011; Almeida et al. 2012), and might explain the aggregated distribution of yellow-legged hornet nests, observed at a local scale, in anthropic areas at the seafront in the proximity of fishery activities (Monceau & Thiery 2016). Invasive Vespids are often opportunistic foragers and are attracted to seafood products, which can be used as bait in food traps (Spradbery 1973; Edwards 1980; Matsuura & Yamane 1990; Pereira et al. 2013; Monceau et al. 2014, 2015a; Unelius et al. 2014). The attraction of *Vespa velutina* towards characteristic seafood odors, such as p-xylene (Couto et al. 2014), which is a component of fish odors (Piveteau et al. 2000; Grigorakis et al. 2003; Varlet et al. 2006), might explain the higher feeding rate towards general protein sources *Vespa velutina* workers in our trials. However, the dramatic preference of *Vespa velutina* for honeybee bait over meat and fish baits clearly suggests that meat or fish baited traps might be of little efficacy when used within or near apiaries.

Interestingly, *Vespa crabro* workers showed a higher feeding rate when it comes to carbohydrate sources compared to *Vespa velutina* workers. A possible explanation refer to potential differences in the physiology and morphology of the two species, and in primis in the size difference between them with *Vespa crabro* being bigger that *Vespa velutina* (Monceau et al., 2014). Whatever the explanation, this specular difference, with workers of the invasive species spending more time on protein sources, while native hornet workers consuming more carbohydrates, seems to further highlight the ability of the invasive species

to outperform the native one in foraging. In fact, while protein items are collected and manipulated by workers to feed the developing brood in the nest, carbohydrates are used by adults to supply their energetic needs.

Overall, our laboratory assays on feeding behaviour suggest that *Vespa velutina* workers should be more prone to exploit and collect protein items with lower energetic demands than *Vespa crabro* workers, both traits should underline a higher foraging efficiency in workers of the yellow-legged hornet.

When it comes to individual immunity, the results of our immune challenge showed that workers of the native species were significantly more immunocompetent than *Vespa velutina* workers in terms of ability to remove bacteria from their haemolymph. The reduced immunocompetence in *V. velutina* workers might be linked to a higher degree of inbreeding in the invasive species with respect to the native one due to invasion bottleneck (Darrouzet et al. 2015), although this is not observed in inbred populations of honey bees which present a similar immune response when compared with outbred populations (Lee et al. 2013). Our results, however, seem to support the "evolution of increased competitive ability" hypothesis (Lee & Klasing 2004; Liu & Stiling 2006). If yellow-legged hornet workers left behind their natural enemies, they should afford to invest their resources in other activities rather than immunity. A less costly immune system could reduce the individual energetic demands and explain the lower consumption of highly energetic carbohydrate sources compared to *Vespa crabro* workers observed in our trials on trophic habits.

Overall, our laboratory study highlights a number of potentially relevant life-history traits that could allow workers of the invasive Asian hornet to outperform workers of the native species in the likely case of competition during the phase of colony growth when workers unrelentingly forage outside the nest to provide for food and nest-building material.

Although workers of the two species are similar in terms of exploratory behaviour, under standardized laboratory conditions *Vespa velutina* workers showed a higher ability in exploiting protein sources, crucial for colony provision, with apparently lower energetic needs than *Vespa crabro* workers. If we take into account also the aforementioned differences in predatory strategies of the two species in the field, it appears plausible that the native hornet species might be easily outcompeted and displaced by the invasive one at foraging hotspots.

The results of our work give new insights in the biology of the invasive yellow-legged hornet and provide a basis for evaluating its impact on native potential competitors in the field. Indeed, two complementary approaches can be adopted for the study of competition: a top-down approach, which collect evidence of competition and try to figure out potential influencing factors, and a bottom-up approach, which infers potential competition from the comparison of life history traits. While the top-down approaches might have the advantage of showing the order of magnitude and the direction of competition based on field-rooted studies, the bottom up approach adopted in our study has the potential to reveal possible competition even before clear/evident effects are recognized, allowing researchers to act in advance before competition occurs. This is particularly valuable in the case of recently arrived and fast spreading IAS, such as *Vespa velutina* in Europe.

**Author Contribution Statement**

AC, FC, IP and RC conceived and designed research. AC, FC, IP and IP collected the data. AC analyzed the data. AC and FC wrote the manuscript. LB and RC provided material, facilities and reagents. All authors read and approved the manuscript.

**Compliance with Ethical Standards**

Authors declare that they have no conflict of interest.

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**Figure captions**

**Fig.1** Boldness (the latency to the first exit from the acclimatization box) and Exploration (the number of different sections visited) of *Vespa velutina* (Vv) and *Vespa crabro* (Vc) workers. For each sample, boxes, horizontal lines inside the boxes and short horizontal lines ("whiskers") respectively represent the 25-75 percent quartiles, the median and the minimal and maximal values, ns= non significant comparison, see text for details.

**Fig.2** Comparison of feeding preferences (time spent on each item) of *Vespa velutina* (Vv) and *Vespa crabro* (Vc) workers. For each sample, boxes, horizontal lines inside the boxes and short horizontal lines ("whiskers") respectively represent the 25-75 percent quartiles, the median and the minimal and maximal values. See result section for significant comparisons.

**Fig.3** Comparison of antibacterial activity (viable bacterial count expressed as Colony Forming Units, CFUs, per worker) of *Vespa velutina* (Vv) and *Vespa crabro* (Vc). For each sample, boxes, horizontal lines inside the boxes and short horizontal lines ("whiskers") respectively represent the 25-75 percent quartiles, the median and the minimal and maximal values.