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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. This study 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. Under laboratory-controlled conditions, two life-history traits that might cause the two species to compete were investigated: (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. The results show that trophic preference of both species highly overlaps, with a marked dietary preference for honeybees compared with other insect prey and non-prey protein items. No differences were 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. This 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*.

Key words. biodiversity loss, immunity, interspecific competition, invasive alien species, Vespidae, yellow-legged hornet.

Introduction

In the 'era of globalisation', increased trades have resulted in and still produce 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 billions of dollars (US) each year (Pimentel *et al.*, 2005; Pyšek & Richardson, 2010). The arrival and spread of IAS, in particular, are 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 that act as predators, parasites or pathogens

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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 effect that is generally more difficult to predict and assess is related to competition, 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; 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 if we are to understand, evaluate and prevent/reduce competition and, in turn, the loss of native biodiversity.

Here, we investigated the potential life-history traits affecting the competition between two hornet 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 the yellow-legged hornet.

Vespa velutina is an invasive hornet species native of Southeast Asia (Monceau *et al.*, 2014a). Its presence was first recorded in the south of France in 2004 (Haxaire *et al.*, 2006); since then, the species spread rapidly across France and Europe (Villemant *et al.*, 2006, 2011a; Rome *et al.*, 2009; Robinet *et al.*, 2016). At present, *V. velutina* is found in different European countries (e.g. Spain, Portugal, Italy, Belgium and the UK; Monceau *et al.*, 2014a, Robinet *et al.*, 2016), and also in areas that are not contiguous with the invasion front [e.g. the Balearic Islands (S. Mar Leza, pers. comm.) and the Veneto region of Italy (Bortolotti & 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 *V. velutina* is on beekeeping activities, as the yellow-legged hornet is a specialised predator of honeybees (Monceau *et al.*, 2014a). Such predation can be intense during summer and autumn, and represents a further threat to honeybee populations, which are already suffering a noteworthy decline throughout Europe because of several factors (Goulson *et al.*, 2015). In addition to the economic impact on apiculture, the invasive hornet also has 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), as envenomation of *V. 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, *V. velutina* may also be a threat to the native hornet species. The European hornet, *V. crabro*, represents one of the two hornet species native to Europe, along with the Oriental hornet, *V. orientalis*. The species is found throughout Europe, unlike *V. orientalis*, which occurs only in the southern countries. European hornets have a very similar life cycle to the yellow-legged hornet (Matsuura & Yamane, 1990; Matsuura, 1991; Takahashi *et al.*, 2004; Monceau *et al.*, 2014a) (see Materials and Methods).

Similarities in life-history traits create several dimensions of potential competition between the invasive and European species (Monceau *et al.*, 2015a). Competition over shared resources can occur in two main phases of the hornet life cycle: 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 *V. 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.

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 increases during the rearing of sexuals, especially gynes, because they require more proteinaceous food to build up their fat storage (Spradbery, 1973; Edwards, 1980; Monceau *et al.*, 2015a), as is the case 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 *V. crabro* and *V. 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 probably compete for food (Monceau *et al.*, 2014a, 2015a). It is, however, largely unknown how much the feeding spectra of the two species overlap, and whether they are similarly placed along the specialist/generalist continuum. Indeed, *V. velutina* is reported to specialise on honeybee prey (Tan *et al.*, 2007, 2012), but both the yellow-legged hornet and the European hornet should probably 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 one 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, differ between foundresses of the two species, favouring *V. velutina* invasion and potentially enabling it to outcompete *V. 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, as 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 constant internal environment maintained within a nest of a social insect species to favour brood development creates optimal conditions for pathogen and parasite growth (Cremer *et al.*, 2007). The 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 specialised 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 parasitisation 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 in the case of sterile workers. Foragers are exposed to pathogens at foraging hotspots (Durrer & Schmid-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 *V. velutina* and *V. 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 find and exploit food sources rapidly; 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 of competing for food. We then compared the immune ability of *V. velutina* and *V. 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 proposes 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.

Materials and methods

Species biology

Vespa velutina and *V. crabro* have very similar life cycles (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 autumn, 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 *V. velutina* and *V. crabro* are to do with the length of the annual life cycle, which is longer in the invasive species, going from February/March to November, and the size of the colony, with the yellow-legged hornet building bigger nests which contain a consistently higher number of individuals (Monceau *et al.*, 2015a). Moreover, while *V. crabro* usually builds its nest in confined spaces, such as tree cavities (Edwards, 1980; Matsuura & Yamane, 1990), *V. velutina* nests in both confined and exposed sites, apparently with a preference for the latter (Monceau *et al.*, 2014a).

Sample collection and rearing

Vespa velutina and *V. crabro* workers 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 from the area around Florence (Tuscany, Italy), from four nests. Combs from different nests were maintained at 26 ± 2 °C in separated glass cages ($50 \times 50 \times 50$ cm). Workers were collected at emergence, individually marked with a spot on the thorax with Uni Posca® (Milano, Italy) paint markers using different colours according to day of emergence and nest of origin, and transferred in groups of 10–15 individuals to $15 \times 15 \times 15$ 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 *V. velutina*, the size of workers and gynes may largely overlap, but, as in other Vespid species that go through a winter diapause, reproductive gynes present well-developed fat bodies for overwintering (Hanson & Olley, 1963; Spradbery, 1973; Perrard *et al.*, 2012) clearly visible on the internal surface of their tergites and sternites, while workers have very scant or null fat deposits on their abdominal segments (Beani *et al.*, 2011; Cappa *et al.*, 2013). At the time of behavioural

experiments or immunochallenge assays, all workers of both species were, on average, 8 days old (*V. crabro*: 8.620 ± 5.454, range 5–36; *V. velutina*: 8.434 ± 3.146, range 5–20) and there were no significant differences 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

Boldness and exploration assays. To assess the explorative behaviour of *V. velutina* and *V. crabro* workers, we measured two behavioural traits (boldness and exploration) that had 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 et al. (2015b). The apparatus was represented by an experimental arena consisting of a square opaque acclimatisation box (15 × 15 × 15 cm) connected via a trapdoor (diameter 3 cm) to one side of a rectangular transparent test box (32 × 24 × 16 cm) virtually divided in 24 equivalent sections (8 × 8 × 8 cm). Each part of the apparatus was carefully washed with 96% ethanol between trials. Each worker (*V. velutina*, $N = 22$; *V. crabro*, $N = 21$) was kept in the opaque box for 5 min of acclimatisation before the trial; the trapdoor was then opened to allow the hornet to explore freely the test box for 10 min 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 acclimatisation box after trapdoor opening, which was used as a measure of boldness (i.e. the lower the score, the bolder the individual); and (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 11.00 to 15.00 hours, when workers are most active (A. Cini & F. Cappa, pers. obs.). One worker of each species was tested at the same time in one of two identical open-field apparatuses.

Feeding preference assays. In order to assess the food preference and diet flexibility of *V. velutina* and *V. crabro* workers for different food sources, food choice trials were performed. Individual workers (*V. velutina*, $N = 123$; *V. 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 min for acclimatisation. At the end of the acclimatisation period, different food sources were introduced into the cage through a slide tray (9 × 3 cm). Food sources were presented in small cylindrical plastic cups (diameter 2.5 cm, height 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 min and the time spent feeding/manipulating each food item was recorded directly. Four trials were performed, three with protein baits and one 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 with another potential prey item, the paper wasp *Polistes dominula* (presented as dead individuals, killed by freezing) – the nests of *P. dominula* are plundered by *V. crabro* in late summer/early autumn (R. Cervo, pers. obs.) and it belongs to a genus that is part of the diet of another hornet, *Vespa tropica* (Matsuura, 1991). Both *A. mellifera* and *P. dominula* were 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 because European hornets and other social wasps are often seen foraging on grapes, and they do 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 performed between 11.00 and 15.00 hours in a random order. Sample sizes were as follows (for *V. velutina* and *V. crabro*, respectively): meat, fish, honeybee: $N = 31$ and $N = 31$; meat, fish: *V. velutina*, $N = 30$ and $N = 30$; honeybee, *Polistes* sp.: $N = 30$ and $N = 33$; honey, honeybee sugar candy, grape: $N = 30$ and $N = 28$.

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 & Cox-Foster, 2005; Manfredini *et al.*, 2010, 2013; Gätschenberger *et al.*, 2013; Cappa *et al.*, 2015; Polykretis *et al.*, 2016).

We chose to measure bacterial clearance as a good proxy of workers' immunity as different parameters linked to antimicrobial immune response (e.g. number of haemocytes, phagocytosis, nodule formation, phenoloxidase activity, encapsulation response) appear correlated in insects' immunity (Gillespie *et al.*, 1997; Cotter & Wilson, 2002; Lambrechts *et al.*, 2004; Schmid-Hempel, 2005) and injection of live bacteria provides an integrative view of the activation of an organism's immune system (Charles & Killian, 2015). *Escherichia coli* is not naturally found in *V. velutina* and *V. crabro*, and we could therefore 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 minimise the competing effect by other possible microorganisms, we used the *E. coli* tetracycline-resistant strain XL1 Blue (Stratagene, La Jolla, California). Bacterial cultures were grown overnight aerobically in Luria-Bertani (LB) complex medium (Miller, 1972) containing tetracycline at a concentration of 10 μg ml^{-1} at 37 °C in a shaking incubator. After centrifugation, bacteria were washed twice in phosphate-buffered saline (PBS), resuspended and diluted to the desired concentration with PBS

($\sim 1{,}5 \times 10^8$ cells ml^{-1}). The approximate amount of bacterial cells in the solution was determined using a haemocytometer (Neubauer, Padova, Italy) 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 (*V. velutina*, $N = 69$; *V. crabro*, $N = 52$) was infected by injecting 1 μl of inoculum, containing $\sim 1{,}5 \times 10^5$ cells, with a Hamilton™ (Bonaduz, Switzerland) micro syringe between the second and third tergites (Yang & Cox-Foster, 2005). Before injection, workers were cooled down in a refrigerator (temperature 4 °C) to facilitate their manipulation. After infection, hornets were introduced in groups of about 10, separated for species and colony of origin into $15 \times 15 \times 15$ 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 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 time frame of 20 h from the bacterial challenge because it is a widely used procedure in insect immunity studies as it provides a view of the organism's rapid response to microbial infection (Gillespie *et al.*, 1997; Siva-Jothy *et al.*, 2005; Charles & Killian, 2015). Each sample was then processed with a Stomacher® (Worthing, West Sussex, U.K.) 400 Circulator at 230 rpm for 10 min in order to homogenise 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}) from each sample were plated onto LB solid medium with added tetracycline (10 μg ml^{-1}) and incubated overnight at 37 °C. The following day, the colonies grown on the plate surface were counted and the viable bacterial count was expressed as CFUs per worker. At least three control hornets per colony for each species (*V. velutina*, $N = 12$; *V. crabro*, $N = 12$) were injected with 1 μl of PBS, homogenised 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 with added tetracycline (10 μg ml^{-1}).

A total of 121 hornets were infected with *E. coli* and plated: 69 *V. velutina* workers and 52 *V. crabro* workers. The workers' age range was 3–14 days post-emergence, and at least 10 workers were infected from each of eight nests (four 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 generalised estimating equations (GEE) approach, which extends the generalised 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 (Overall & Tonidandel, 2004; Hubbard *et al.*, 2010). For all GEEs, model selection was performed on the basis of the 'quasi-likelihood 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 function; independent working correlation matrix; fixed effect: species; subject effect: colony of origin. Feeding preferences were assessed, for all experiments, using both 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 probability distribution, log link function, 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 distribution for the honeybee versus wasps experiment, and a gamma distribution 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 estimator and a type III analysis. Statistical analyses were performed in SPSS 20.0 (SPSS, 2011) and PAST (Hammer *et al.*, 2001).

Results

Boldness and exploratory activity

Neither boldness nor exploratory tendencies differed between *V. velutina* and *V. crabro* workers. There was no difference either in the time of latency to the first exit from the acclimatisation box after trapdoor opening or in the number of visited sections for workers of the two species (boldness: Wald $\chi^2 = 1.713$, d.f. = 1, $P = 0.191$; exploration: Wald $\chi^2 = 0.396$, d.f. = 1, $P = 0.529$; Fig. 1).

Feeding preference

Protein sources.

Meat, fish, honeybee. The total time spent feeding on any protein item differed between species (Wald $\chi^2 = 9.108$, d.f. = 1, $P = 0.003$) with *V. velutina* spending more time on protein items than *V. crabro* (Fig. 2, top left). The time spent feeding on each item differed among items for workers of both species (Wald $\chi^2 = 337.895$, d.f. = 1, $P < 0.001$), with both species spending more time feeding on honeybees than on fish or meat (pairwise comparisons: honeybee versus meat, $P > 0.001$; honeybee versus fish, $P > 0.001$; meat versus fish, $P = 0.181$). However, the interaction between species and bait was significant (Wald $\chi^2 = 13.906$, d.f. = 1, $P = 0.001$), which suggests 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 $\chi^2 = 356.477$,

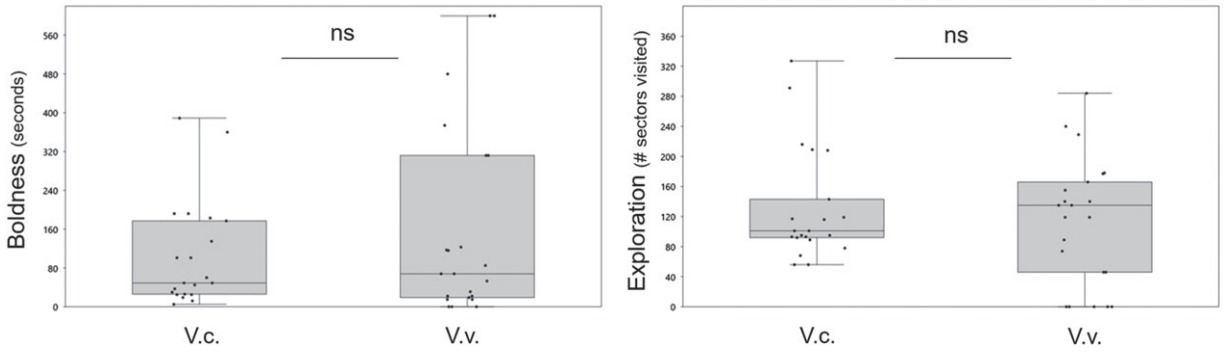

Fig. 1. Boldness (the latency to the first exit from the acclimatisation box) and exploration (the number of different sections visited) of *Vespa velutina* (V.v.) and *Vespa crabro* (V.c.) workers. For each sample, boxes, horizontal lines inside the boxes and short horizontal lines ('whiskers') represent the 25–75% quartiles, the median and the minimal and maximal values, respectively. ns, nonsignificant comparison. See the text for details.

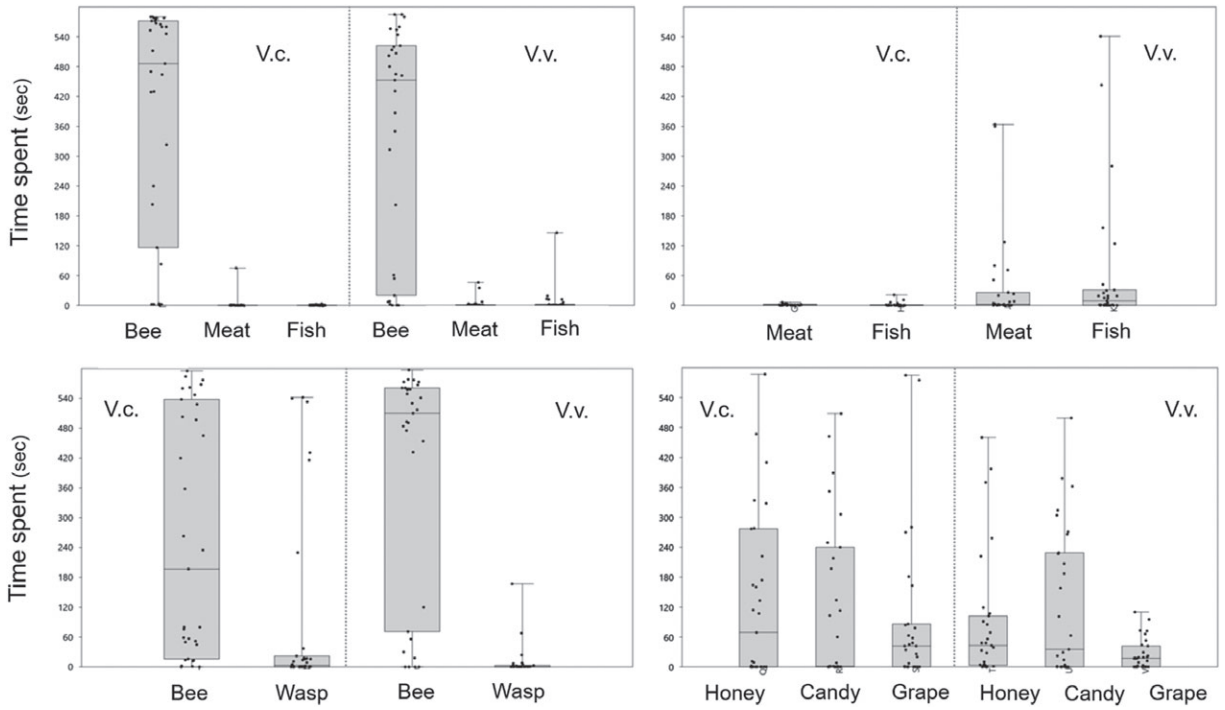

Fig. 2. Comparison of feeding preferences (time spent on each item) of *Vespa velutina* (V.v.) and *Vespa crabro* (V.c.) workers. For each sample, boxes, horizontal lines inside the boxes, and short horizontal lines ('whiskers') represent the 25–75% quartiles, the median, and the minimal and maximal values, respectively. See the Results section for significant comparisons.

d.f. = 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 $\chi^2 = 38.287$, d.f. = 1, $P < 0.001$), with *V. crabro* showing smaller latency time for meat than for fish, and *V. 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 $\chi^2 = 0.184$, d.f. = 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 *V. crabro* and *V. velutina* switched to other food items less often than when the first found item was meat or fish (*V. crabro*: proportion of individuals switching from honeybee to meat or fish = 0.217, from meat or fish to honeybee = 1, $\chi^2 = 9.28$, d.f. = 1, $P = 0.002$; *V. velutina*: proportion of individuals switching from honeybee to meat or fish = 0.412, from meat or fish to honeybee = 1, $\chi^2 = 6.99$, d.f. = 1, $P = 0.008$).

Honeybee versus* Polistes *sp. The time spent on each prey item differed between items for both *V. velutina* and *V. crabro*, with workers of both species spending more time on *A. mellifera* honeybee baits than on *P. dominula* (Wald $\chi^2 = 19.195$, d.f. = 1,

$P < 0.001$; Fig. 2, bottom left). The total time spent on any prey item did not differ between species (Wald $\chi^2 = 0.575$, d.f. = 1, $P = 0.448$; Fig. 2, bottom left). Interaction between species and bait was not significant (Wald $\chi^2 = 1.107$, d.f. = 1, $P = 0.293$).

The latency time to reach each food item confirmed the pattern shown by time of manipulation, with honeybees being the item reached most promptly (versus wasps) by both species (Wald $\chi^2 = 73.624$, d.f. = 1, $P < 0.001$). General latency time was significantly smaller in *V. velutina* than in *V. crabro* (Wald $\chi^2 = 5.170$, d.f. = 1, $P = 0.023$). Interaction between species and bait was not significant (Wald $\chi^2 = 1.711$, d.f. = 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 *V. velutina* (proportion of individuals switching from honeybee to wasp = 0.263, and from wasp to honeybee = 0.889, $\chi^2 = 7.26$, d.f. = 1, $P = 0.007$) but not for *V. crabro* (proportion of individuals switching from honeybee to wasp = 0.550, and from wasp to honeybee = 0.846, $\chi^2 = 1.92$, d.f. = 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 $\chi^2 = 271.327$, d.f. = 1, $P < 0.001$), with *V. velutina* spending more time on baits than *V. crabro* (Fig. 2, top right). Bait had only a slightly significant effect (Wald $\chi^2 = 4.124$, d.f. = 1, $P = 0.042$), with more time spent feeding on fish than meat in both species (Fig. 2, top right). Interaction between species and bait was not significant (Wald $\chi^2 = 0.024$, d.f. = 1, $P = 0.876$). Latency time analyses overall confirmed the results: species had a significant effect (Wald $\chi^2 = 4.423$, d.f. = 1, $P = 0.035$), with *V. velutina* being faster than *V. crabro* in starting to feed. Neither bait nor the interaction between bait and species had a significant effect (Wald $\chi^2 = 0.031$, d.f. = 1, $P = 0.859$; Wald $\chi^2 = 0.189$, d.f. = 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 (*V. crabro*: proportion of individuals switching from meat to fish = 0.600, and from fish to meat = 0.444, $\chi^2 = 0.05$, d.f. = 1, $P = 0.823$; *V. velutina*: proportion of individuals switching from meat to fish = 0.455, and from fish to meat = 0.714, $\chi^2 = 0.820$, d.f. = 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 $\chi^2 = 8.525$, d.f. = 1, $P = 0.004$), with *V. crabro* spending more time on carbohydrate baits than *V. velutina* (Fig. 2, bottom right). Bait type had a significant effect (Wald $\chi^2 = 13{,}666$, d.f. = 1, $P = 0.001$), with more time spent manipulating honey and grape than candy. However, the interaction between species and bait was significant (Wald $\chi^2 = 7{,}053$, d.f. = 1, $P = 0.029$), showing that *V. crabro* was spending a similar amount of time on all carbohydrate sources (all pairwise comparisons > 0.05), while *V. 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 time to reach carbohydrate items (Wald $\chi^2 = 0.592$, d.f. = 1, $P = 0.441$), nor did the kind of bait influence the latency time (Wald $\chi^2 = 1.402$, d.f. = 1, $P = 0.496$)- Finally, no significant interaction between species and bait was found (Wald $\chi^2 = 1.964$, d.f. = 1, $P = 0.375$).

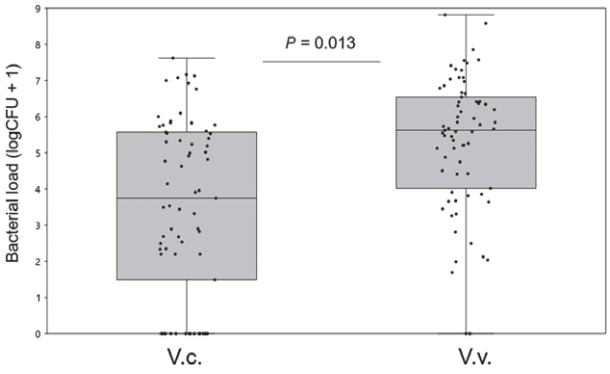

Fig. 3. Comparison of antibacterial activity [viable bacterial count expressed as colony forming units (CFU) per worker] of *Vespa velutina* (V.v.) and *Vespa crabro* (V.c.). For each sample, boxes, horizontal lines inside the boxes, and short horizontal lines ('whiskers') represent the 25–75% quartiles, the median value, and the minimal and maximal values, respectively.

Immune ability

Workers of the two species exhibited significantly different responses to *E. coli* infection (Fig. 3). *Vespa crabro* workers had a significantly higher anti-bacterial response (or bacterial clearance) than did *V. velutina* workers (Wald $\chi^2 = 6.165$, d.f. = 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 *V. 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, in contrast to what was previously found for queens of the two species (Monceau *et al.*, 2015b), with *V. velutina* queens bolder and more prone to exploration than *V. crabro* queens, workers were found to be 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 (and they are thus usually spatially closer to the colony), are under less selective pressure for dispersal than is likely to be the case for the foundress phenotype. Taking together the results regarding the foundress and worker phenotypes, we suggest that competition between the two species might be a two-step process: *V. velutina* might outcompete *V. crabro* during colony foundation because of higher explorative tendencies of queens, and it might later outcompete *V. 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 hornets compared with other generic protein sources potentially available. Such a preference is quite interesting as it underlines a rather evident specialisation 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 the dietary protein of *V. 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.*, 2012, 2013; Arca *et al.*, 2014) could explain the dietary preferences shown 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 (Tan *et al.*, 2007, 2012; Baracchi *et al.*, 2010; Monceau *et al.*, 2013a, 2013b, 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 the different predation strategies adopted by foragers of the two species in the field (Tan *et al.*, 2007; Baracchi *et al.*, 2010; Monceau *et al.*, 2013b, 2014b), the fact that *A. mellifera* is able to defend itself, at least to some extent, from *V. crabro* attacks (Baracchi *et al.*, 2010), while is not able to counteract *V. velutina* attacks (Arca *et al.*, 2014), and the fact that *V. velutina* drastically outnumbers *V. crabro* in both colony density and colony size (Monceau *et al.*, 2014b; Monceau & Thiéry, 2016), it is predictable that the native European hornet may be easily displaced by the invasive species.

Indeed, while *V. crabro* has a relatively mild predation impact on honeybees, with only a few hornets patrolling beehives in order to catch bees (Baracchi *et al.*, 2010), *V. velutina* specialises 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 also confirmed for both species 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 unlikely to be due to the presence of the prey's haemolymph, which might be considered a sugar-reward for hornets, because, if hornets were attracted by bees (or wasps) only (or mainly) for the sugar content of their haemolymph, we would not expect to observe hornets manipulating prey as they usually do when foraging, by removing heads and legs and carrying the thorax, a behavioural pattern that was very clear.

In the absence of the preferred prey item, *V. velutina* workers showed a significantly higher consumption of both the meat and fish baits compared with *V. 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 & Thiéry, 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.*, 2014a, 2015a; Unelius *et al.*, 2014). The attraction of *V. velutina* to characteristic seafood odours, such as p-xylene (Couto *et al.*, 2014), a component of fish odours (Piveteau *et al.*, 2000; Grigorakis *et al.*, 2003; Varlet *et al.*, 2006), might explain the higher feeding rate towards general protein sources of *V. velutina* workers in our trials. However, the dramatic preference of *V. 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, *V. crabro* workers showed a higher feeding rate when it comes to carbohydrate sources than did *V. velutina* workers. A possible explanation concerns potential differences in the physiology and morphology of the two species, and in the size difference between them, with *V. crabro* being bigger that *V. velutina* (Monceau *et al.*, 2014a). Whatever the explanation, this difference, with workers of the invasive species spending more time on protein sources, and 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 energy needs.

Overall, our laboratory assays on feeding behaviour suggest that *V. velutina* workers should be more prone to exploit and collect protein items with lower energy demands than *V. crabro*

workers, and both traits should underline a higher foraging efficiency in yellow-legged hornet workers.

When it comes to individual immunity, the results of our immune challenge showed that workers of the native species were significantly more immunocompetent than *V. velutina* workers in terms of the 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 honeybees 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 could afford to invest their resources in other activities rather than immunity. A less costly immune system could reduce the individual energy demands and explain the lower consumption of high-energy carbohydrate sources compared with *V. 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 standardised laboratory conditions, *V. velutina* workers showed a higher ability in exploiting protein sources, crucial for colony provision, with apparently lower energy needs than *V. crabro* workers. If we also take into account 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 this study provide new insights into the biology of the invasive yellow-legged hornet and provide a basis for evaluating its impact on potential native competitors in the field. Indeed, two complementary approaches can be adopted for the study of competition: a top-down approach, which collects evidence of competition and tries to determine the potential influencing factors; and a bottom-up approach, which infers potential competition from the comparison of life-history traits. While the top-down approach 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 evident effects are recognised, allowing researchers to act before competition occurs. This is particularly valuable in the case of recently arrived and fast-spreading IAS, such as *V. velutina* in Europe.

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AC, FC, IP and RC conceived and designed the research. AC, FC, IP and IP collected the data. AC analysed the data. AC and FC wrote the manuscript. LB and RC provided material, facilities and reagents. All authors read and approved the manuscript. The authors declare that they have no conflict of interest.

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