



Immune competence of the invasive hornet *Vespa velutina* and its native counterpart *Vespa crabro*: a comparison across caste and sex

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With 2 figures and 1 table

Abstract: Understanding the major mechanisms that allow an alien species to become invasive is crucial for limiting the impact of such species, and individual immunity seems to be a crucial trait for their large range colonization when introduced to a new area. Indeed, a high resistance to pathogens and parasites could favour the establishment and proliferation of an alien species in a newly colonized region. A striking example of recent successful invasion is represented by the Asian hornet, *Vespa velutina nigrithorax*, which has invaded Western Europe threatening bees and beekeeping activities. Investigating the biology of this species is fundamental to understand how *V. velutina nigrithorax* has become invasive in Europe and this knowledge could be crucial for limiting its impact. Here, by performing bacterial challenge bioassays, we compare the immune competence of different castes and sex of a population of the invasive Asian hornet, *V. velutina nigrithorax*, and of the native European hornet, *V. crabro*. Our results demonstrate that the two species differ in their immunocompetence with respect to caste: a higher level of immunocompetence was found in the reproductive females of the Asian hornet which were resistant to bacterial challenge when compared to workers and males of both species and to reproductive females of the native European hornet. The high pathogen resistance in reproductive females of the Asian hornet, might therefore represent a key factor contributing to the ecological success and spread of this invader.

Keywords: invasive alien species, insect immunity, Asian hornet, European hornet, antibacterial response

1 Introduction

A fundamental question to be addressed when dealing with alien species is why some become widespread and abundant (*i.e.* invasive alien species, IAS), while others do not become established after introduction into a new area (Sax et al. 2007). Resistance to parasites and pathogens is one key feature for the ecological success of a species (Hatcher & Dunn 2011) and likely plays a central role in the potential invasiveness of some species with respect to others (White & Perkins 2012). IAS can encounter novel parasites and pathogens in their new range, and we might expect that a higher degree of resistance or immunocompetence could be a key feature of successful IAS, enabling them to become established after introduction in a new region (Prenter et al. 2004; Dunn et al. 2012; White & Perkins 2012). On the other hand, transmis-

sion of new parasites and pathogens from invading to native species may help the invasion process by eliminating native competitors (Reynolds 2013; Vilcinskas 2015); whilst the ‘release’ of invaders from their natural enemies (*i.e.* parasites, pathogens, predators and competitors) should also facilitate invasions (Prenter et al. 2004; Dunn et al. 2012).

The enemy release hypothesis (ERH) postulates that an IAS introduced into a new area should experience a rapid spread due to lack of pressure by natural enemies from its native range and could direct their resources towards reproduction and growth, while reducing their investment in immunity (Liu & Stiling 2006). Indeed, individuals have limited amount of resources, and trade-offs in energetic investment among reproduction, growth, dispersion abilities and immune system have been demonstrated in different taxa (Vogelweith et al. 2014; Schwenke et al. 2016). ERH

has been widely investigated with regards to invasive plant species (for a review see Liu and Stiling 2006). Less numerous are instead the studies comparing the immune ability of invasive *versus* native species in animals (Rigaud & Moret 2003; Wilson-Rich & Starks 2010; Vilcinskas et al. 2013).

A striking example of successful invader is represented by the yellow-legged hornet, *Vespa velutina nigrithorax* (Monceau et al. 2014). One single queen (Arca et al. 2015) of this hornet was accidentally introduced from China to south-western France in the early 2000s and the species has since then spread in neighbouring countries (López et al. 2011; Monceau et al. 2014; Keeling et al. 2017; Granato et al. 2019). The yellow-legged hornet is a serious pest in its invasive range because it preys heavily upon domestic honeybees, *Apis mellifera*, (Monceau et al. 2013) and might interact and compete with the native European hornet, *Vespa crabro*, since the two species mostly share similar life cycles and ecological niches (Matsuura & Yamane 1990; Cini et al. 2018; Kwon & Choi 2020). In both species, a single mated foundress initiates a colony in spring after a wintering diapause and performs all tasks necessary for colony maintenance and brood rearing until the first workers emerge, then the colony rapidly grows in size throughout summer. In autumn, sexuals (*i.e.* males and gynes) are produced, mating takes place, workers and males die before winter, while a new generation of mated females enters hibernation until the following spring, when the colony cycle starts anew (Matsuura & Yamane 1990; Monceau et al. 2014). The two species differ in the length of the annual life cycle, which is longer in the invasive species (Monceau et al. 2014); in the colony size, with the yellow-legged hornet building larger colonies both in the invasive and native range (Starr 1992; Choi et al. 2012; Rome et al. 2015); and in the number of mating events, with queens of the invasive *V. velutina* showing a higher degree of polyandry with a mean mating frequency of 3.6 mates (Arca et al. 2015) against 1.11–1.13 for *V. crabro* queens (Foster et al. 1999; Takahashi et al. 2004).

Hornets of the genus *Vespa* are social species with a caste system and a very different life history among individuals belonging to different castes and these differences may reflect on individual immunocompetence (Laughton et al. 2011; Koch et al. 2013; Cappa et al. 2015). At present, several studies have shown that both species of hornets can be infected by parasites and pathogens, such as viruses (especially those derived from their bee prey, Yañez et al. 2012; Forzan et al. 2017; Garigliany et al. 2017; Mazzei et al. 2018, 2019; Dalmon et al. 2019), entomopathogenic fungi (Poidatz et al. 2018; 2019) and parasitic nematodes (Villemant et al. 2015). However, comparative studies on the immune ability of the two species or between the invasive and native populations of *V. velutina* are still lacking. Thus, the aim of the present study is to investigate, for the first time, the antibacterial response of individual hornets towards an unspecific pathogen with respect to caste and sex. To avoid biases linked to a possible coevolution between the differ-

ent species of hornets and specific pathogens, and to exclude pathogens' presence prior to artificial infection, we subjected individual hornets to immune challenge with the Gram-negative bacteria *Escherichia coli*, an immune elicitor used to test antibacterial activity in insects (Gätschenberger et al. 2013; Cappa et al. 2020; Cini et al. 2020a). We compared the immunocompetence of workers and sexuals (males and reproductive females) of the invasive *V. velutina* and native *V. crabro* to assess if immune ability changes according to caste or sex and between the invasive and native species. Moreover, we compared the immune response of reproductive females, responsible for the founding of new colonies and therefore essential for the spread of the invasive species. We chose to assess the immunocompetence of reproductive females in two delicate phases of the colony cycle: during spring, when foundresses start a new colony and become queens, and in autumn, when virgin gynes (*i.e.* reproductive females which will become future foundresses the following season if they survive) emerge from the nests before mating and entering the winter diapause. The comparison of the immune response between the native and invasive species may provide useful information to understand the main drivers favouring the invasion of *V. velutina* in Europe and, at the same time, to develop selective and sustainable control strategy for the invader with a low impact on native species.

We predict that, if the ecological success of the yellow-legged Asian hornet is somehow linked to a high degree of resistance to parasite and pathogens (Rigaud & Moret 2003; White & Perkins 2012), *V. velutina* hornets should outperform the native *V. crabro* when tested in immune challenges, especially when it comes to reproductive females, responsible for the species spread. On the other hand, if the release of their natural enemies plays a relevant role in the invasion process of *V. velutina*, we could expect individuals of the invasive yellow-legged hornet to be less immunocompetent than those of the native species regardless of caste and sex. The results of this study will represent a first caste assessment with respect to individual immunity in social vespids, since comparative studies carried out so far on the immune ability of different castes of social Hymenoptera have essentially focused on honeybees and ants (Baer et al. 2005; Laughton et al. 2011; Koch et al. 2013).

2 Material and methods

2.1 Sample collection and rearing

Vespa velutina and *V. crabro* workers and sexuals (*i.e.* males and reproductive gynes) emerged in the laboratory from combs collected in the field during the months of October and November 2016 in Liguria and Tuscany (Italy), from nine nests for *V. velutina* and four nests for *V. crabro*. All *V. velutina* specimens were collected in Liguria. Three out of four nests of *V. crabro* were instead collected in Tuscany since, after the arrival of *V. velutina* in Liguria, it is quite

difficult to find colonies of the European hornet, probably due to competition with the Asian invader (Cini et al. 2018; Kwon & Choi 2020). All newly-emerged individuals tested in bioassays were reared and pupated in the field. We chose to use newly-emerged individuals to (i) have specimens of known and comparable age (Moret & Schmid-Hempel 2009; Armitage & Boomsma 2010); (ii) avoid contaminations with environmental microorganisms after eclosion; and (iii) control for the effects of activities such as foraging (workers) and mating (sexuals) on individual immunity (Doums & Schmid-Hempel 2000; Baer et al. 2006; Alaux et al. 2010). Thus, by choosing to compare lab-eclosed workers, males and gynes we reduced the effect of potential confounding variables when assessing the immune response of the different castes and sex in the two species. Combs were maintained at 26 ± 2 °C in separated glass cages ($50 \times 50 \times 50$ cm). Hornets were collected at emergence, individually marked with a spot on the thorax with UniPosca® paint markers using different colours for day of emergence and nest of origin. Hornets were separated per colony and sex in groups of 10–15 individuals, into $15 \times 15 \times 15$ cm glass cages with a mesh wire side, with *ad libitum* water and sugar as food, at 23 ± 1 °C under natural daylight, until immune challenge assays were performed (within 2 weeks from emergence).

The following spring, between April and May, foundresses of the two species were collected from the same areas using modified bottle traps containing beer added with blackcurrant syrup as generic attractant (Monceau et al. 2012). Once more, all *V. velutina* foundresses were collected in Liguria, while those of the native *V. crabro* were collected from traps located in both regions. A steel mesh or polystyrene square was suspended over the liquid to prevent

foundresses from drowning. Traps were checked twice a day to collect the captured foundresses. Foundresses of both species were marked with a spot on the thorax using different colours and housed as the lab-emerged hornets, with the difference that they were isolated to prevent fights. Field-collection was the only way to have spring foundresses after overwintering under natural conditions, as a consequence, it was not possible to know their colony of origin. Foundresses collected in the field were maintained in the laboratory for at least two or three days to check their behaviour. Only foundresses that appeared in good conditions (i.e. showing inconspicuous behaviour and no sign of reduced coordination) were used in immune bioassays.

2.2 Immune challenge

To evaluate the hornets' ability to remove bacterial cells from their haemolymph (i.e. bacterial clearance), workers, sexuals and spring foundresses of both species were challenged with the mutant strain *E. coli* XL1 Blue (Stratagene, La Jolla, California), not naturally found in *V. velutina* and *V. crabro*, so to exclude its presence in our hornets prior to artificial infection (Cini et al. 2018; 2020b) (Fig. 1).

Bacterial cultures of *E. coli* tetracycline-resistant XL1 Blue were grown overnight aerobically in Luria-Bertani (LB) complex medium added with 10 µg/mL tetracycline at 37 °C in a shaking incubator. After centrifugation, bacterial pellets 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, measured as optical density with an Eppendorf BioPhotometer® 6131). Hornets of different caste (for simplicity we use the term caste for both females foundresses, gynes, workers and for males) were infected by

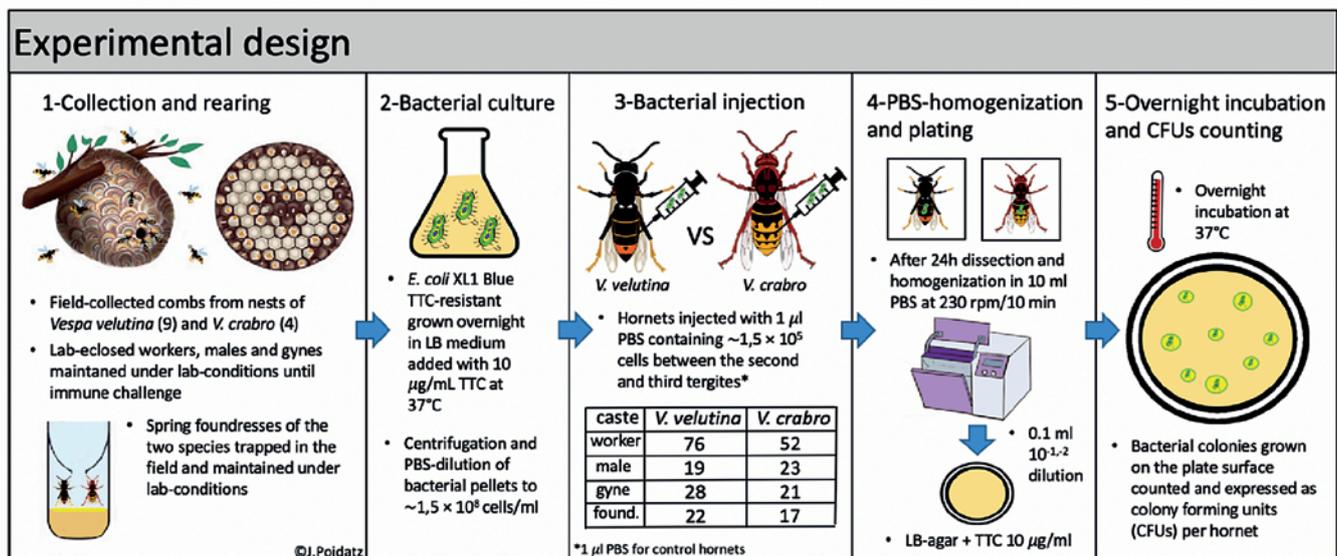


Fig. 1. Experimental design used in this study. Hornets of the two species were injected with the mutant strain of *E. coli* XL1-Blue TTC-resistant, 24h post-infection individuals were homogenized and plated on LB-agar added with TTC. After overnight incubation at 37°C the bacterial colonies grown on the plate surface were counted and expressed as colony forming units (CFUs) per hornet.

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 (Cini et al. 2018). Before injection, hornets were cooled down in a refrigerator (temperature 4 °C) for 15 minutes to facilitate manipulation. After infection, individuals were introduced in groups of about 5–10, separated for species, sex and colony into $15 \times 15 \times 15$ cm glass cages provided with *ad libitum* sugar cubes and water. Spring foundresses of the two species were isolated after bacterial infection under the same conditions. Twenty-four hours later, during which all hornets were maintained under controlled conditions (20 ± 2 °C, 55% RH), each hornet was inserted in a sterile plastic bag with 10 ml PBS after removing the sting apparatus of females, to avoid a possible reduction of bacterial counts due to the antimicrobial peptides contained in the venom (Baracchi et al. 2011). Dissection of lab-eclosed female hornets of both species also allowed to confirm their caste, by checking the fat storage in their abdomen. In *V. crabro*, workers and gynes are easily recognizable due to a rather conspicuous difference in body size with the former smaller than the latter (Perrard et al. 2012). As regards *V. velutina*, instead, the size of workers and gynes can largely overlap, but, as in other Vespidae species that go through a winter diapause, reproductive gynes present well-developed fat bodies for overwintering (Spradbery 1973; Perrard et al. 2012; Cappa et al. 2013, Poidatz et al. 2018) clearly visible on the internal surface of their tergites and sternites, while workers have very scant or null fat deposits on their abdominal segments (Cappa et al. 2019a, b). Each sample was then processed with a Stomacher® (Worthing, West Sussex, U.K.) 400 Circulator at 230 rpm for 10 min to homogenise the hornet body and extract haemolymph 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 added with tetracycline ($10 \mu\text{g ml}^{-1}$) and incubated overnight at 37 °C. The following day, colonies grown on the plate surface were counted and the viable bacterial count was expressed as colony forming units (CFUs) per hornet. A total of 144 *V. velutina* and 112 *V. crabro* hornets were infected with *E. coli* and plated (workers: *V. velutina*, $N = 76$; *V. crabro*, $N = 52$; males: *V. velutina*, $N = 19$; *V. crabro*, $N = 22$; gynes: *V. velutina*, $N = 28$; *V. crabro*, $N = 21$; foundresses: *V. velutina*, $N = 21$; *V. crabro*, $N = 17$) (Table S1). The lab-eclosed hornets' age range was 3–14 days post-emergence. For the field-collected spring foundresses it was not possible to assess the nest of origin and the exact individual age, although they were surely at least four or five months old, having survived through the overwintering diapause, till the spring founding phase.

For each species, control hornets of different caste and colony (*V. velutina*, $N = 30$: 9 workers, 9 males, 9 gynes, 3 foundresses; *V. crabro*, $N = 27$, 9 workers, 9 males, 6 gynes, 3 foundresses) were injected with 1 μl of PBS, homogenised and plated. To ensure absence of other bacterial strains capa-

ble of growing on our LB agar plates, the media was supplemented with tetracycline ($10 \mu\text{g ml}^{-1}$).

2.3 Statistical analyses

To evaluate the difference in bacterial clearance (log-transformed number of CFUs) among different groups of hornets of the two species we used a generalized mixed model with a Gamma probability distribution, log-link function and Satterthwaite approximation. The model included nest of origin as a random factor to take into account possible pseudoreplicates and intrinsic difference among colonies in immune ability, caste, species and age as fixed factors and included also the interaction between caste and species. Significance of post-hoc pairwise comparisons was adjusted using sequential Sidak correction for multiple comparisons. All the statistical analyses were performed using IBM SPSS Statistics 16.0 (SPSS Inc., Chicago, IL).

3 Results

The model revealed an effect of species, caste and their interaction on the hornet ability to clear bacteria from the hemolymph, while bacterial clearance was not influenced by individual age (Table 1).

The effect of the species and caste on the immune ability is demonstrated by the fact that the invasive *V. velutina* appeared more able to clear bacteria than the native *V. crabro*; but, this was true only for females of the two species belonging to the reproductive caste. Indeed, considering the response of the different groups of hornets, workers and males had a comparable rate of bacterial clearance among them both intra- and across species (Fig. 2, Table S2). Reproductive females presented instead different pattern between species: at the foundation stage (Spring), foundresses of both species had a higher ability to clear bacteria from the hemolymph than workers and males (Fig. 2, Table S2). Conversely, only gynes (Autumn sample) of the invasive *V. velutina* showed a pattern of immune response consistent with conspecific spring foundresses, while gynes of the native *V. crabro* were not only less able to clear bacteria than the foundresses of the same species, but also the least immunocompetent group of hornets among those tested in the two species (Fig. 2, Table S2). As regards intraspecific comparisons, in the native *V. crabro*, spring foundresses had the highest antibacterial response with respect to all the other groups, workers and males displayed a comparable rate of bacterial clearance, while reproductive gynes were the lowest immunocompetent group (Fig. 2, Table S2); in the invasive *V. velutina* gynes and foundresses had a similar rate of bacterial clearance and were consistently more able to clear bacteria from their hemolymph than both conspecifics workers and males, which were instead similar in their immune response.

Table 1. Bacterial clearance depends on caste and species.

Source of variance	F	df1	df2	P
corrected model	29.291	15	117	<0.0001
caste	27.301	2	209	<0.0001
species	86.692	1	17	<0.0001
caste*species	68.869	3	96	<0.0001
age	1.873	8	216	0.066
Covariance random factor	Z	Estimate	St.error	P
colony of origin	1.520	0.10	0.007	0.129

Results from the GLZ show that the hornet ability to clear bacteria from their hemolymph depends on the individual caste (i.e. workers, males, gynes and foundresses), the species and the interaction between these two factors, while not on the individual age.

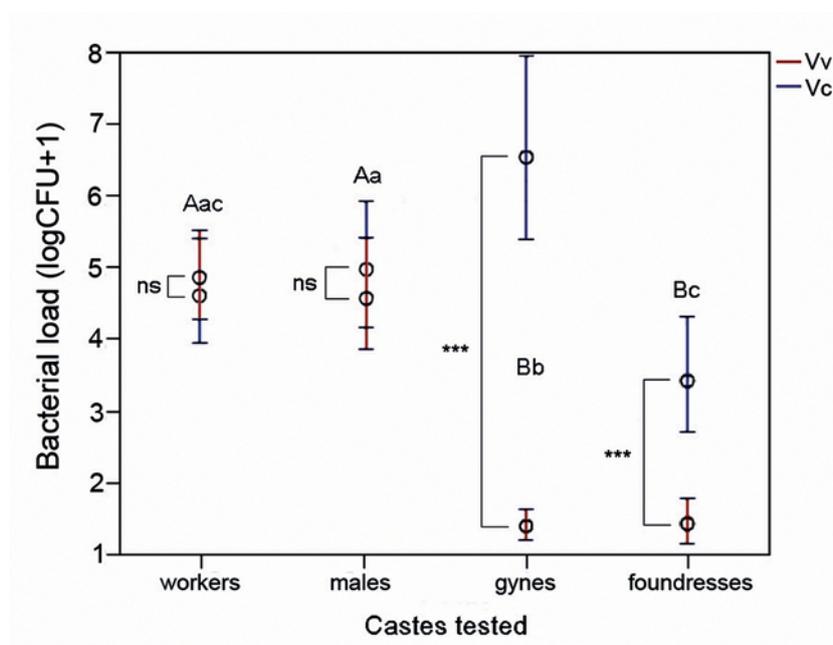


Fig. 2. Comparison of antibacterial activity [viable bacterial count expressed as the log transformation of colony forming units (CFU) per hornet] of different caste and sex (i.e. workers, males, gynes and foundresses) of *Vespa velutina* (Vv) and *Vespa crabro* (Vc). Pairwise contrasts: capital letters refer to within species comparisons among *V. velutina* castes (A vs B, $P < 0.0001$); lowercase letters refer to within species comparisons among *V. crabro* castes (a vs b, $P < 0.05$; a vs c, $P < 0.05$; b vs c, $P < 0.01$); * refer to between species comparisons ($***P < 0.0001$); ns: not significant.

The age difference between our lab-closed hornets and the spring foundresses was not a bias due to our sampling procedure, but an intrinsic characteristic of our experimental design. Indeed, it is not possible to compare the immunocompetence of foundresses, workers and males of comparable age, since workers and males die at the end of the colony cycle, and do not survive until the following spring. It would be possible to argue that the effects of age on the immune system of hornets may take a longer time span to manifest. Our results, however, did not show an effect of individual age on the hornet antibacterial response over the two weeks age

span that we considered, and, in addition, gynes and foundresses of *V. velutina* showed a comparable rate of bacterial clearance despite their considerable age difference (Table 1).

4 Discussion

Our results revealed different immune response patterns with regards to caste and sex in the two species of hornets. Overall, workers and males did not show noticeable differences in their immune response either among them or

between the two species. Conversely, reproductive females were more able to clear bacterial cells from their hemolymph in the invasive *V. velutina* than in the native *V. crabro*. The pattern of immune response was similar for foundresses, more immunocompetent than both workers and males, but differed with respect to gynes in the two species. In fact, gynes exhibited the lowest ability to clear bacterial cells in the native species, while the invasive ones had a rate of bacterial clearance comparable to that of conspecific foundresses, well above the immune response showed by all the other hornet groups of both species.

The difference observed between gynes of the two species is rather puzzling, and it may be linked to differences in the biology of the two species and to peculiar, albeit not sufficiently described yet, characteristics of their colony cycle. Despite these gaps and based on the available knowledge on the invasive yellow-legged hornet and the native European hornet, it is possible to provide several potential explanations. The lower immunity of *V. crabro* gynes with respect to conspecific spring foundresses might suggest that during the winter diapause there might be a selection of the more immunocompetent females, which will found the new colonies the following spring. In fact, overwintering sites might represent a hotspot for parasite and pathogen transmission and the longer hibernation phase of *V. crabro* could increase the risk of exposure to pathogens and parasites in hibernacula (Sayama et al. 2013). Early emergence of *V. velutina* foundresses (Monceau et al. 2015), might instead allow resource collection that could boost up individual immunity; although our results demonstrated that, differently from *V. crabro*, gynes and foundresses of the yellow-legged hornets have a comparable high level of antibacterial response both before and after hibernation. Furthermore, it has been showed that once introduced in Europe, the yellow-legged hornet has experienced a strong bottleneck and this might have selected only the most immunocompetent females (Prenter et al. 2004; Dunn et al. 2012; White & Perkins 2012; Arca et al. 2015). Similarly, also the invasion dynamics and polyandry documented in foundresses of *V. velutina* in the invasive range could play a role in the higher immunity observed in reproductive females of the yellow-legged hornet (Moller et al. 1999; Arca et al. 2015; Simone-Finstorm et al. 2016). In fact, it has been inferred that the invasion of Europe by *V. velutina* started with a single queen that had mated with multiple males (around 3–4 according to Arca et al. 2015) and subsequent studies confirmed this single introduction for the European population (Budge et al. 2017; Granato et al. 2019); it is therefore likely that the immunocompetence of the resulting European population might be impacted by the genetic background and strongly depends on the immune abilities of the first invasive foundress and its mates (Moller et al. 1999; Arca et al. 2015; Simone-Finstorm et al. 2016). The difference between gynes and foundresses of the two species is quite intriguing and further research, for example by evaluating the individual immunocompetence in the

native range of the yellow-legged hornet or assessing the effects of mating on immunity (Baer et al. 2006), is required to unveil the mechanisms responsible for the change in individual immunocompetence from gynes to foundresses of *V. crabro* and for the strong resistance to bacterial pathogens in reproductive females of *V. velutina* and the weak antibacterial response of *V. crabro* gynes.

The higher immune ability of foundresses in both species with respect to males and workers may be linked to the fact that they have to survive to hibernation, mostly in the soil (D. Thiéry, *pers. obs.*), and found a new colony the following spring (Matsuura and Yamane 1990; Monceau et al. 2014). Workers and males, instead, as in other temperate species of social Hymenoptera, complete their tasks before the end of the annual colony cycle and do not overwinter as the mated reproductive females (Matsuura and Yamane 1990); thus, a higher investment in immunity might be unnecessary for these individuals with their shorter lifespan and produced in large number by each colony to fulfill their tasks. As expected from this, a lower immunocompetence of males and workers with respect to reproductive females has been documented also in other species of social Hymenoptera (Haddad et al. 2007; Laughton et al. 2011; Meriggi et al. 2019).

The similar immune response between workers of the two species may appear in contrast with the results previously reported by Cini et al. (2018), which compared the same worker samples and found a slightly higher immunocompetence in the workers of the native *V. crabro*. In this case, however, the two groups of workers were compared in our model with the other six groups of males, gynes and foundresses and it is likely that the multiple pairwise contrast comparisons faded away the difference already on the verge of significance between the two groups of workers.

Overall, the results of our immune characterization of the native and invasive species of hornets seem to corroborate the hypothesis that a higher immunocompetence of reproductive females of *V. velutina*, alongside other behavioral adaptations (Monceau et al. 2015), could help the invader in outperforming the native species and favour the invasion process. A decreased immunocompetence as consequence of the release of natural enemies after invasion is not supported instead by our findings (Prenter et al. 2004; Dunn et al. 2012) despite the high reproductive investment of *V. velutina* foundresses which produce thousands of offspring during the colony cycle. In this case, it appears that the reproductive potential and long-term fitness of foundresses is not traded off with individual immunocompetence, differently to what happens in other social insect species (Baer et al. 2006).

It is important to point out once more that in this pioneering study we assessed the hornets' immune response to an unspecific immune elicitor, which is not naturally encountered by hornets in their environment, and it is possible that the different hornet castes and species might show different degree of resistance depending on the species of pathogens encountered or enact other immune strategies to cope

with infection (Schmid-Hempel 2005). Our use of a mutant *E. coli* strain in the immune challenge allowed us to measure the immune response of hornets to a generic pathogen, bypassing the potential effects of coevolution that we could expect between the different species of hornets and their natural pathogens either in the native or invasive range. It will be worthwhile to investigate if the considerable difference in the innate immune system of reproductive females of *V. velutina* compared to workers and males is reflected also by other proxies of individual immunity such as hemocyte number, phenol oxidase activity or encapsulation response (Schmid-Hempel 2005). However, the methods used to evaluate these immune parameters are often intrusive and it is not always simple to carry out multiple measures on the same individuals and with a limited number of specimens. Thus, future studies should explore the intriguing caste and species immune differences demonstrated in the present work, using different pathogens from both the native and invasive range and evaluating survival and other immunity-linked parameters after infection.

Our findings could also provide a starting point for future applicative research aimed at containing the spread of the invasive *V. velutina*. At present, effective strategies to control the species are still lacking, since nest detection and destruction using chemical insecticides are often difficult, costly and ecologically impactful (Monceau et al. 2014; Turchi & Derijard 2018). A desirable strategy when dealing with IAS should contemplate the use of specific biocontrol agents targeting the invader that should be sufficiently safe for the native community and the environment. However, the lower resistance, at least to our specific bacterial pathogen, of reproductive females of the native *V. crabro*, both at the gyne and foundress stage with respect to *V. velutina*, should be taken into account if a biocontrol strategy using microbial agents is to be used in the field to target *V. velutina* and contain its spread in Europe. The use of non-selective agents of control against the yellow-legged hornet could in fact have more harmful impact on the less immunocompetent reproductive females of the native species rather than contain the invader.

Author contribution statement: FC, AC and RC conceived the study and designed the experiments; FC, AC and JP collected samples; FC, NM and JP performed the experiments. AC and NM analysed the data. RC and DT provided material and facilities. JP designed the methodology figure. FC wrote the original draft and all authors reviewed draft of the manuscript. All authors read and approved the final manuscript.

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Animal rights statement: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Human rights statement: This article does not contain any studies with human participants performed by any of the authors.

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Appendix

Table S1. Lab-enclosed hornets of each caste and sex collected from different nests used in the immune challenge (both bacteria-injected individuals and PBS-injected controls are considered in the total).

Nest	species	worker	male	gyne
1	<i>V. velutina</i>	16	–	–
2	<i>V. velutina</i>	32	–	–
3	<i>V. crabro</i>	10	–	–
4	<i>V. crabro</i>	18	13	13
5	<i>V. crabro</i>	14	11	–
6	<i>V. crabro</i>	10	8	14
7	<i>V. velutina</i>	15	12	–
8	<i>V. velutina</i>	13	10	–
9	<i>V. velutina</i>	–	6	–
10	<i>V. velutina</i>	1	–	12
11	<i>V. velutina</i>	4	–	11
12	<i>V. velutina</i>	4	–	8
13	<i>V. velutina</i>	–	–	6

Table S2. Pairwise contrasts:

within species			
species	caste	contrast estimate	P
<i>Vespa crabro</i>	workers – males	–0.382	0.306
	workers – gynes	–1.929	0.003
	workers – foundresses	1.194	0.087
	males – gynes	–1.547	0.039
	males – foundresses	1.576	0.039
	gynes – foundresses	3.122	0.002
<i>Vespa velutina</i>	workers – males	0.261	0.773
	workers – gynes	3.462	<0.0001
	workers – foundresses	3.434	<0.0001
	males – gynes	3.200	<0.0001
	males – foundresses	3.172	<0.0001
	gynes – foundresses	–0.028	0.895
between species			
caste	species	contrast estimate	P
workers	Vc – Vv	–0.246	0.556
males	Vc – Vv	0.397	0.494
gynes	Vc – Vv	5.145	<0.0001
foundresses	Vc – Vv	1.994	<0.0001