Universitat de les Illes Balears

DOCTORAL THESIS

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GENETIC CHARACTERIZATION, HABITAT MODELLING AND AUTOMATED DETECTION OF THE YELLOW-LEGGED HORNET *VESPA VELUTINA* (HYMENOPTERA: VESPIDAE): TOOLS FOR THE MANAGEMENT OF THIS INVASIVE ALIEN SPECIES IN EUROPE

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Doctor by the Universitat de les Illes Balears

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Dr. Maria del Mar Leza Salord, Dr. José Antonio Jurado Rivera and Dr. Joana Cursach Seguí, professors of the Universitat de les Illes Balears,

DECLARE:

That the thesis titled GENETIC CHARACTERIZATION, HABITAT MODELLING AND AUTOMATED DETECTION OF THE YELLOW-LEGGED HORNET *VESPA VELUTINA* (HYMENOPTERA: VESPIDAE): TOOLS FOR THE MANAGEMENT OF THIS INVASIVE ALIEN SPECIES IN EUROPE, presented by Cayetano Herrera López to obtain a doctoral degree, has been completed under our supervision and meets the requirements to opt for an European Doctoral degree mention.

For all intents and purposes, we hereby sign this document.

Palma, 18 of July 2023

Dr. Maria del Mar Leza Salord	Dr. José Antonio Jurado Rivera	Dr. Joana Cursach Seguí

"*Mientras no se reconozca que la Naturaleza no tiene fronteras políticas, que los animales migran cruzando amplios territorios, que los ríos pasan por muchos países, que los océanos bañan innumerables costas y que el aire lo respiramos todos, el deterioro ecológico de nuestro planeta continuará de manera inevitable*"

Gerald Durrell - Lee Durrell

Acknowledgments:

Con esta memoria llegamos a la meta de un viaje que se inició en noviembre de 2018, y en este apartado quiero dedicar un agradecimiento a todas esas personas que me han acompañado en algún momento del camino. En esta aventura ha habido buenos y malos momentos, pero siempre acompañado de personas que han significado mucho para mí. Por ello, espero haber reconocido a todos y todas que han participado en este proyecto, y pedir disculpas si no he mencionado a alguien que se lo merecía.

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En 2021, tuve la oportunidad de hacer una estancia breve de un mes en el Departamento de Ciencias Ambientales de la Universidad de Girona. Allí tuve la gran suerte de conocer a **Nuria Roura**, mi supervisora durante ese periodo. Recuerdo que el primer día me recibió en su casa como si de un amigo de toda la vida se tratase y me brindó toda su ayuda desde aquel día. En el mismo grupo conocí a **Josep Maria**, en las salidas de campo a **Emili** y **Kilian** y fuera del ámbito laboral a **Fran**, **David**, **Lucas** y **Jordi**. Todos ellos hicieron que la experiencia fuera genial!

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List of publications

This thesis is in the form of a "Compendium of scientific publications". Five of the hereby presented articles have been peer-reviewed and published in prestigious scientific journals. The last two presented articles are respectively submitted to a journal of an equivalent level to the previous five.

Leza, M., **Herrera, C.**, Picó, G., Morro, T., & Colomar, V. (2021). Six years of controlling the invasive species *Vespa velutina* in a Mediterranean island: The promising results of an eradication plan. *Pest Management Science*, 77(5), 2375-2384.

<https://onlinelibrary.wiley.com/doi/10.1002/ps.6264>

Quality index:

"Pest Management Science" is a scientific journal published by Wiley. Its main area is Entomology, and its first publication was in 1970. The last calculated impact factor is from 2022 and its JCR (Journal Citation Report) value is 4.463. It classifies in position 8/100 in the Entomology category, making it a first quartile (Q1) and first decile (D1) journal. Its average JIF (Journal Impact factor) percentile is 92.50 and its article influence score is 0.810.

Herrera, C., Jurado-Rivera, J.A. & Leza, M. (2023). Ensemble of small models as a tool for alien invasive species management planning: evaluation of *Vespa velutina* (Hymenoptera: Vespidae) under Mediterranean island conditions. *Journal of Pest Science* 96, 359–371.

<https://link.springer.com/article/10.1007/s10340-022-01491-7>

Quality index:

"Journal of Pest Science" is a scientific journal published by Springer, publishing papers about Entomology. The journal was launched in 1925 and classifies in position 3/100 in the Entomology category, making it a Q1 and D1 journal. The latest year for which an impact index is 2022, with a value of 5.742 according to JCR, placing it with the highest journal impact factor in Entomology. Its average JIF percentile is 97.50 and its article influence score that year was equal to 0.996.

Herrera, C., Williams, M., Encarnação, J., Roura-Pascual, N., Faulhaber, B., Jurado-Rivera, J. A., & Leza, M. (2022). Automated detection of the yellow-legged hornet (*Vespa velutina*) using an optical sensor with machine learning. *Pest Management Science* 79(3), 1225-1233.

<https://onlinelibrary.wiley.com/doi/10.1002/ps.7296>

Quality index:

"Pest Management Science" is a scientific journal published by Wiley. Its main area is Entomology, and its first publication was in 1970. The last calculated impact factor is from 2022 and its JCR (Journal Citation Report) value is 4.463. It classifies in position 8/100 in the Entomology category, making it a Q1 and D1 journal. Its average JIF (Journal Impact factor) percentile is 92.50 and its article influence score is 0.810.

Leza, M., **Herrera, C.**, Marques, A., Roca, P., Sastre-Serra, J., & Pons, D. G. (2019). The impact of the invasive species *Vespa velutina* on honeybees: A new approach based on oxidative stress. *Science of The Total Environment*, 689, 709-715.

<https://www.sciencedirect.com/science/article/abs/pii/S0048969719330839>

Quality index:

“Science of The Total Environment” is a scientific journal by Elsevier, and its main topic is Environmental Sciences. The journal was launched in 1972 and its impact factor in 2022 resulted to be 10.754. Its position within the Environmental Sciences category is 26/279, making it a Q1 and D1 journal. It has an article influence score of 1.397 and a JIF percentile of 90.86.

Herrera, C., Leza, M., & Martínez-López, E. (2020). Diversity of compounds in *Vespa* spp. venom and the epidemiology of its sting: A global appraisal. *Archives of Toxicology*, 94(11), 3609-3627.

<https://link.springer.com/article/10.1007/s00204-020-02859-3 >

Quality index:

"Archives of Toxicology” is a scientific journal published by Springer, publishing papers about Toxicology. The journal was launched in 1930 and classifies in position 12/94 in the Toxicology category, making it a Q1 journal. The latest year for which an impact index is 2022, with a value of 6.168 according to JCR, its average JIF percentile is 87.77 and its article influence score that year was equal to 1.037.

Herrera, C., Ferragut, J.F., Leza, M., & Jurado-Rivera, J.A. Invasion genetics of the yellow-legged hornet *Vespa velutina* in the Westernmost Mediterranean archipelago. *Paper submitted*.

Herrera, C. Pinto, M.A., Leza, M., & Jurado-Rivera, J.A. Niche modelling and landscape genetics of the yellow-legged hornet (*Vespa velutina*): an integrative approach for evaluating central–marginal population dynamics in Europe. *Paper submitted*.

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SUMMARY

Global change constitutes one of the main threats to the terrestrial biosphere and its biodiversity, with important implications for human society. Among the main drivers stand out invasive alien species: species introduced by humans either accidentally or intentionally in areas outside their native range, usually with high dispersal capacity and producing negative impacts on native biota. The introduction of exotic species has increased due to the increase in maritime traffic and globalization, where the geographical and ecological barriers that keep species in their native ranges are fading away. In fact, invasive alien species are the second cause of biodiversity loss globally, and the first one in insular ecosystems. The yellow-legged hornet *Vespa velutina nigrithorax* is the first invasive hornet accidentally introduced into Europe. It was first detected in southern France in 2004, from where it has spread to Spain, Portugal, Belgium, Germany, the Netherlands, Italy, Switzerland, the United Kingdom, and Ireland. It is a general predator of insects with a special impact on honeybees, thus representing a serious threat to wild biodiversity and beekeeping. Currently, this species is included in the *Catálogo Español de Especies Exóticas Invasoras* and catalogued by the European Union as an invasive alien species of Union concern. The objective of this PhD thesis is to investigate the invasion and expansion patterns of the yellow-legged hornet *Vespa velutina nigrithorax* Buysson 1905 in Europe, to propose and evaluate an eradication plan applied in Mallorca, as well as developing a new automated detection method as a basis for the generation of management tools. To achieve these goals, methodologies from different disciplines such as population genetics, ecological niche models and the automated classification of Hymenoptera species based on their flight characteristics have been implemented. This invasive species was first reported from Mallorca island in 2015 where its early detection and management made its eradication possible, becoming the first European territory to eradicate it. Later in 2021, a new nest was detected and removed on the island, and no more nests or adults have been detected to date. The individuals and nests genotyped on the island, as well as samples and data obtained from different European and Asian areas, allowed us to determine that there were two independent entries in Mallorca, from Italy and Catalonia in 2015 and 2021, respectively, and with signs of bottleneck and effect founder. The ecological niche models calibrated on the island of Mallorca obtained good evaluation parameters and indicated that there are suitable areas for this invasive species to expand and establish on the main Mediterranean islands. Moreover, we detected a central-peripheral pattern of the European *V. velutina* populations, with lower allelic richness as we move away from the introduction focus to low environmental suitability areas. Finally, we demonstrate that flight features can be used with a machine learning model to differentiate *V. velutina* from six other Hymenopteran species under laboratory conditions. The derived results are very useful to improve detection and study the spatiotemporal patterns of this species, propose biosecurity measures in ports to prevent its arrival in new areas, analyse suitable areas for its establishment and define management measures and eradication plans.

RESUMEN

El cambio global constituye una de las principales amenazas de la a biosfera terrestre y su biodiversidad, con importantes implicaciones para la sociedad actual. Entre los principales impulsores destacan las especies exóticas invasoras: especies introducidas por los humanos (accidental o intencionadamente) en áreas fuera de su distribución natural, normalmente con una alta capacidad de dispersión y produciendo impactos negativos en la biota nativa. La introducción de especies exóticas se ha visto incrementada debido al aumento del tráfico marítimo y la globalización, donde las barreras geográficas y ecológicas que mantienen a las especies en sus áreas de distribución nativas se están desvaneciendo. De hecho, las especies exóticas invasoras suponen la segunda causa de pérdida de biodiversidad a nivel global, y la primera en ecosistemas insulares. El avispón asiático *Vespa velutina nigrithorax* es el primer avispón invasor introducido accidentalmente en Europa. Se detectó por primera vez en el sur de Francia en 2004, desde donde se ha expandido a España, Portugal, Bélgica, Alemania, Países Bajos, Italia, Suiza, Reino Unido e Irlanda. Es un depredador generalista de insectos que tiene un especial impacto sobre las abejas de la miel, por lo que representa una grave amenaza para la biodiversidad silvestre y la apicultura. Actualmente, esta especie está incluida en el Catálogo Español de Especies Exóticas Invasoras y catalogada por la Unión Europea como especie exótica invasora de preocupación para la Unión. El objetivo de esta tesis doctoral es investigar los patrones de invasión y expansión del avispón asiático *Vespa velutina nigrithorax* Buysson 1905 en Europa, proponer y evaluar un plan de erradicación aplicado en Mallorca, así como desarrollar un nuevo método de detección automatizado como base para el desarrollo de herramientas para su gestión. Para lograr estos objetivos se han implementado metodologías de diferentes disciplinas como la genética de poblaciones, modelos de nicho ecológico y la clasificación automatizada de especies de himenópteros en función de sus características de vuelo. Se detectó esta especie en la isla de Mallorca en 2015 donde su detección temprana y gestión hicieron posible su erradicación, convirtiéndose en el primer territorio europeo en erradicarla. Posteriormente en 2021, se detectó y retiró un nuevo nido en la isla, y hasta la fecha no se han detectado más nidos ni adultos. Los individuos y nidos genotipados en la isla, así como de muestras y datos obtenidos de diferentes zonas de Europa y Asia, permitieron determinar que hubo dos entradas independientes en Mallorca, de Italia y Cataluña en 2015 en 2021, respectivamente, y con signos de cuello de botella y efecto fundador. Los modelos de nicho ecológico calibrados en la isla de Mallorca obtuvieron buenos parámetros de evaluación e indicaron que existen áreas idóneas para que esta especie invasora se expanda y establezca en las principales islas del Mediterráneo. Además, detectamos un patrón central-periférico en las poblaciones europeas de *V. velutina*, con menor riqueza alélica a medida que nos alejamos del foco de introducción hacia áreas de baja idoneidad ambiental. Finalmente, demostramos que las características de vuelo se pueden usar con un modelo de aprendizaje automatizado para diferenciar *V. velutina* de otras seis especies de himenópteros en condiciones de laboratorio. Los resultados obtenidos son de gran utilidad para mejorar la detección y estudiar los patrones espaciotemporales de esta especie, proponer medidas de bioseguridad en puertos para evitar su llegada a nuevas zonas, analizar áreas idóneas para su establecimiento y definir medidas de gestión y planes de erradicación.

RESUM

El canvi global constitueix una de les principals amenaces de la biosfera terrestre i la seva biodiversitat, amb importants implicacions per a la societat actual. Entre els principals impulsors destaquen les espècies exòtiques invasores: espècies introduïdes pels humans (accidentalment o intencionadament) en àrees fora de la seva distribució natural, normalment amb una alta capacitat de dispersió i produint impactes negatius sobre la biota nativa. La introducció d'espècies exòtiques s'ha vist incrementada a causa de l'augment del trànsit marítim i la globalització, on les barreres geogràfiques i ecològiques que mantenen les espècies als seus rangs nadius s'estan esvaint. De fet, les espècies exòtiques invasores suposen la segona causa de pèrdua de biodiversitat a nivell global i la primera en ecosistemes insulars. La vespa asiàtica *Vespa velutina nigrithorax* és la primera vespa invasora introduïda accidentalment a Europa. Es va detectar per primera vegada al sud de França el 2004, des d'on s'ha expandit a Espanya, Portugal, Bèlgica, Alemanya, els Països Baixos, Itàlia, Suïssa, el Regne Unit i Irlanda. És un depredador generalista d'insectes amb un impacte especial sobre les abelles de la mel, per la qual cosa representa una greu amenaça per a la biodiversitat silvestre i l'apicultura. Actualment, aquesta espècie està inclosa al *Catálogo Español de Especies Exóticas Invasoras* i catalogada per la Unió Europea com a espècie exòtica invasora de preocupació per a la Unió. L'objectiu d'aquesta tesi doctoral és investigar els patrons d'invasió i expansió de la vespa asiàtica *Vespa velutina nigrithorax* Buysson 1905 a Europa, proposar i avaluar un pla d'eradicació aplicat a Mallorca, així com desenvolupar un nou mètode de detecció automatitzat com a base per al desenvolupament d'eines per a la seva gestió. Per assolir aquests objectius s'han implementat metodologies de diferents disciplines com la genètica de poblacions, models de nínxol ecològic i la classificació automatitzada d'espècies d'himenòpters en funció de les característiques de vol. Es va detectar aquesta espècie a l'illa de Mallorca el 2015 on la seva detecció primerenca i gestió van fer possible la seva erradicació, convertint-se en el primer territori europeu en erradicar-la. Posteriorment el 2021, es va detectar i retirar un nou niu a l'illa, i fins ara no s'han detectat més nius ni adults. Els individus i nius genotipats a l'illa, així com de mostres i dades obtingudes de diferents zones d'Europa i Àsia, van permetre determinar que hi va haver dues entrades independents a Mallorca, des d'Itàlia i Catalunya el 2015 el 2021, respectivament, i amb signes de coll d'ampolla i efecte fundador. Els models de nínxol ecològic calibrats a l'illa de Mallorca van obtenir bons paràmetres d'avaluació i van indicar que hi ha àrees idònies perquè aquesta espècie invasora s'expandeixi i s'estableixi a les principals illes del Mediterrani. A més, vàrem detectar un patró central-perifèric a les poblacions europees de *V. velutina*, amb menor riquesa al·lèlica a mesura que ens allunyem del focus d'introducció cap a àrees de baixa idoneïtat ambiental. Finalment, demostrem que les característiques de vol es poden fer servir amb un model d'aprenentatge automatitzat per diferenciar *V. velutina* d'altres sis espècies d'himenòpters en condicions de laboratori. Els resultats obtinguts son de gran utilitat per millorar la detecció i estudiar els patrons espaitemporals d'aquesta espècie, proposar mesures de bioseguretat a ports per evitar la seva arribada a noves zones, analitzar àrees idònies per establir-les i definir mesures de gestió i plans d'erradicació.

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GENERAL INTRODUCTION

Global change and loss of biodiversity

Since the industrial revolution (c. XVIII), a series of social, politic, and economic changes have improved the quality of human well-being and life spans, a better access to education, health care and information (Sage, 2019). Nonetheless, all these changes began to affect the correct biogeochemical system functioning at global scale, giving rise to a new geological era: the Anthropocene (Lewis & Maslin, 2015). This new geological era is characterized by a rapidly technological development, an explosive growth of the human population (rising 8 billion people nowadays) and the increase of production and consumption (Lewis & Maslin, 2015). The interaction between these factors has led to a growing exploitation of natural, mineral and fossil resources, and the expansion of agriculture areas and human-made infrastructures (Lewis & Maslin, 2015). Surprisingly, in 2020 the anthropogenic mass (which include all human-made mass) exceeded overall biomass in Earth (Elhacham et al., 2020), and is expected to exceed almost triple of the world dry biomass by 2040. This human activity has changed the composition of atmosphere, land, and oceans, affecting to the correct biosphere function. This set of planetary-scale changes produced by human activities is known as global change (Steffen et al., 2005), and has diverse impacts on Earth including the increase of temperatures and sea level, severe weather events, ocean acidification and loss of biodiversity. Paradoxically, human well-being depends directly on the correct ecological services provided by the biosphere, such as climate and water regulation, soil formation, pollination, food production or nutrient cycling, among other natural resources (Costanza et al., 2017).

The loss of biodiversity generally refers to the decrease of species diversity and abundance in the different ecosystems around the planet, but it also include loss of ecological interactions (i.e. pollination or predation) or genetic diversity (i.e. species populations), leading directly to the complexity of the ecosystem and its resilience to new disturbances (Pauls et al., 2013; Sage, 2019; Valiente-Banuet et al., 2015). This biodiversity crisis has serious drawbacks for ecosystems and human well-being since it can reduce productivity or enhance new diseases (Vanbergen et al., 2018). In the context of global change, biodiversity loss follows an exponential growth, with extinction rates between 100 – 1000 times pre-human background extinction levels nowadays, and 10000 times greater in future scenarios (De Vos et al., 2015). The biodiversity intactness index, based on models of the effects of land use on species abundance, concludes that nearly 15% of global species have been reduced to date (Johnson et al., 2017; Newbold et al., 2016). In other words, assuming that there exist 5 to 9 million animals species the loss will affect 11.000 to 58.000 species annually (Dirzo et al., 2014). Indeed, 60% of largest terrestrial herbivores, 40% of amphibians, 20% of all mammals and 14% of birds are considered endangered (Johnson et al., 2017; Ripple et al., 2015) and populations of several insect groups have declined (Dirzo et al., 2014). In this regard, most ecosystems (tropical rainforest, woodlands, grasslands, savannas and aquatic environments) are in several degraded state (Parr et al., 2014; Ramankutty et al., 2008; Sage, 2019; Steffen et al., 2011), and well preserved areas are increasingly sparse and isolated, being more vulnerable to new disturbances (Brook et al., 2008; Haddad et al., 2015).

Global change drivers

According to Sage (2019), main global change drivers include: CO_2 emissions and enrichment in atmosphere and oceans, climate warming and altered precipitation, stratospheric ozone reduction and tropospheric ozone enrichment, land transformation, freshwater appropriation and

degradation, overuse of natural populations, invasive alien species, ecosystem eutrophication and extensive use of pesticides. Among the drivers mentioned, other authors emphasize the problem of CO_2 emissions, nitrogen deposition, climate change, land use change and invasive alien species (Sala et al., 2000).

CO_2 emissions directly affects to biosphere ecophysiology (including plants, fungi and animals), though natural process such as photosynthesis, respiration and carbon storage, and lead to cascading effects through ecosystems (Sala et al., 2000; Ziska, 2008). Human activity promotes the increase of CO_2 emissions though use of fossil fuels or deforestation (Steffen et al., 2005). Free atmospheric CO_2 have raised 415 ppm in 2019, 135 ppm higher than in 1750, and it is continuing to increase approximately 3 ppm per year (Sage, 2019). The current CO_2 atmospheric enrichment may be faster than most species can adapt, leading to a maladaptation of the current flora in a future scenario (Bell & Collins, 2008).

Nitrogen deposition represents the most significant eutrophication driver, because of farm fertilizers, industrial pollutants and urban garbage (Fowler et al., 2013). Nitrogen increase not represents a toxic threat to plants, but those plants well adapted to eutrophic environments will be benefit and will grow in higher rates than other plants (Sage, 2019). This situation transforms the landscape and simplify the food webs used by other consumers, such as animals (Stevens et al., 2018).

Global temperature has increased 1ºC since 1800s, and future scenarios consider the possibility of increase another 1 – 4.8ºC by 2100 (Tollefson, 2020). Despite Earth's temperature has variated through geological eras, the rate at which it is growing during the Anthropocene is faster than previous geological records (Masson-Delmotte et al., 2013). Past temperature variations have allowed species adapt to new conditions through natural selection, evolution and demographic processes such as migration (Bell & Collins, 2008; Davis & Shaw, 2001). Nonetheless, within this temperature increase many species will not be able to respond as faster as temperature increase and clime changes (Sage, 2019).

Land exploitation provides food, residencies and transportation networks to human activities (Foley et al., 2005). Nonetheless, land cover is dynamically interacting with atmosphere, oceans, and global biogeochemistry, producing habitat fragmentation and disrupting important ecological processes like pollination or herbivory (Ellis et al., 2010). A recent study published in 2021 concludes that nearly 32% of Earth' surface has been affected by land use changes (~ 43 million km^2), four times greater than previously thought (Winkler et al., 2021). De Baan et al. (2013) conclude that land use changes reduce species richness globally, highlining the necessity of ecosystem regeneration to avoid loss of biodiversity.

Finally, global shipping network has produced a world without geographical and ecological barriers for species. In this regard, alien species incursion have increased worldwide and future models expect a higher increase of annual invasions (Sardain et al., 2019). Normally, alien species remain at low population densities being "harmless" to ecosystem, nonetheless, a percentage of species proliferate, spread and persist to natural control (Mack et al., 2000). When this happens, invasive alien species can disrupt natural communities by displacing natural species, modifying ecological networks, altering fire regime, or enhancing animal and human diseases, among others (Mack et al., 2000; Sage, 2019).

Invasive alien species

The arrival of new species to an area is a natural process. Species present many strategies to expand its natural distribution through active (i.e., migration) or passive dispersal (i.e., wind-driven), searching for new feed sources or refuges (Croteau, 2010). What it is not a natural process is the actual rate of dispersal of species though human-mediated transport.

Invasive alien species are introduced species by humans either accidentally or intentionally in areas outside of their native ranges, with high spread capacities, and producing negative impacts on native biota (Simberloff, 2004). Moreover, bioinvasions represent one of the main drivers of loss of biodiversity and ecosystem instability, in addition to causing large economic costs for society and a threat to global health (Bacher et al., 2018; Carpenter et al., 2018; Mazza et al., 2014). To get an idea, invasive alien species represents the second most important driver of loss of biodiversity at global scale (Chapin III et al., 2000), being the first most important driver in insular ecosystems (Brooke et al., 2007). Unfortunately, this problem is increasing (Sardain et al., 2019), and constitutes a priority research area in international global change programs (Essl et al., 2020; Latombe et al., 2022; Roura-Pascual et al., 2021).

Invasive species normally expand spite of human intervention to control them, hence, they start to disassemble the ecological community, so hugely that biological invasions are considered one of the pillars of biodiversity extinction (together with habitat degradation, overexploitation, and extinction cascades) (Sage, 2019). Moreover, the negative impacts of invasive species also include the modification of ecosystem processes (such as nutrient recycling and fire regimes), reduction of agricultural production and threat to animal and human health (Mack et al., 2000; Sakai et al., 2001). All these negative impacts represent an economic cost to countries and continents. For example, costs of bioinvasions in Europe were estimated in 116.61 billion € between 1960 and 2020, being UK (with 17.60 billion €) and Spain (with 16.19 billion €) the countries that reported the largest invasion costs (Haubrock et al., 2021). In this regard, the most impacted sectors were agriculture (29.94 billion €, ~26%), forestry (20.86 billion €, ~18%), authorities and stakeholders (17.83 billion €, ~15%), public and social welfare (7.59 billion €, ~7%) and health (4.97 billion €, ~4%) (Haubrock et al., 2021).

Not all alien species are able to proliferate, spread and persist into the new range, since they need to surpass a series of natural barriers in each stage of biological invasion to acquire an invasive behaviour (Figure 1). The first stage requires a propagule of a species (eggs, seeds, larvae, vegetative material, mature individuals, etc.) is sampled and human-mediated transported beyond the boundaries of its native range (Lieurance et al., 2022; Novak, 2007). The most probably human-mediated transport is by shipping, due to 80% of world trade is done by sea transport (UNCTAD, 2021). Once a species has surpassed its native boundaries helped by global trade, the following stage is its introduction in the new range (accidentally or intentionally). During this process, probably some immigrants dead due to biotic and abiotic interactions, and a small fraction will be able to establish in the new range. Success establishment will be possible when the species could survive and maintain enough individuals in wild populations (Blackburn et al., 2011). Finally, different life history traits will allow the alien species to spread through the new territory, producing with the pass of time negative impacts on environment, economy and health (Lieurance et al., 2022).

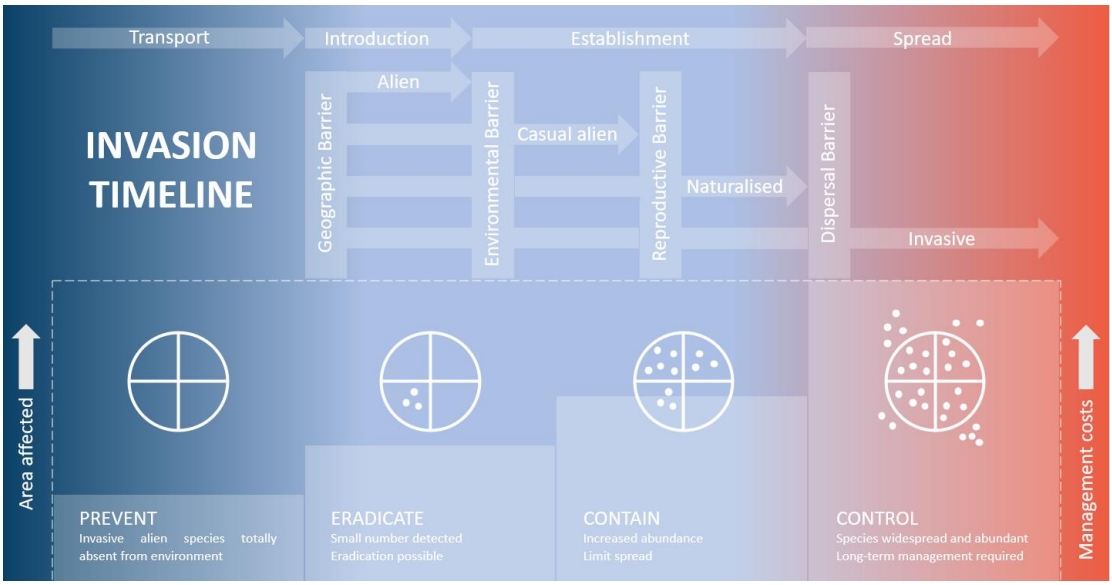

Figure 1.- Schematic representation of invasion timeline, with stages of biological invasions (adapted from Blackburn et al., 2011), major barriers limiting the spread of alien species (according to Richardson et al., 2000) and the management actions required at each stage (edited from Invasive Species Council, Australia).

The number of species that have spread to new ranges has increased substantially as a result of expansion of shipping transport (Sardain et al., 2019). For instance, there are approximately 14000 alien taxa currently in Europe (Katsanevakis et al., 2015), including terrestrial and aquatic free living and parasitic organisms. About 10% of them are considered as invasive alien species, based on Williamson & Fitter (1996), and 88 species are strictly regulated and included in the list of invasive alien species of Union concern: 41 plants (between them 15 aquatic plants, 6 trees, 5 grasses, 6 climbing plants, and 9 others) and 47 animals (between them 13 mammals, 6 birds, 4 reptiles and amphibians, 10 fishes, and 14 invertebrates).

According to Roques et al. (2009), more than 90% of the alien terrestrial invertebrates in Europe are insects. Literature cites that insects have been responsible for the spread of infectious diseases that have affected human and animal health (Crowl et al., 2008; Mellor et al., 2000), devastated crops (Oerke, 2006), damaged forests (Aukema et al., 2010), destroyed infrastructure (Su, 2002), altered ecosystem functions (Kenis et al., 2009) and weakened ecosystem resilience to other disturbances (Charles & Dukes, 2007). Moreover, there are the main animal group which have produced the most economic losses to human society (Bradshaw et al., 2016). In Europe, invasive insects have represented an annual cost of 3.4 billion € in goods and services and 9.3 million € in human health (Bradshaw et al., 2016).

Management and prevention of invasive alien species

The management of invasive alien species aims to reduce both their environmental and socioeconomic impacts and constitutes a great societal challenge for this century (Robertson et al., 2020). The topic is addressed in the United Nations Sustainable Development Goals (UN, 2015) and the associated targets of the Convention on Biological Diversity (CBD, 2010), that provides prevention measures regarding the introduction of invasive alien species on how to

reduce their associated negative impacts and a list of priority species. According to EU regulation, management is defined as "lethal or non-lethal action aimed at the eradication, population control or containment of a population of an invasive alien species, while also minimising the impact on non-targeted species and their habitats" (European Parliament and the Council of the European Union, 2014). The management of invasive alien species may include different strategies, depending on the stage of invasion (Figure 1): early detection and remove of a small population, containment of stablished species or biological, chemical, and physical control or species already spread. On the other hand, prevention is generally more environmentally desirable and cost-effective than management (Figure 1). Therefore, priority should be given to the listing of invasive alien species that are not yet present in an area or are at an early stage of invasion and of invasive alien species that are likely to have the most significant adverse impact (European Parliament and the Council of the European Union, 2014). The prevention of invasive alien species may include also different strategies: legal regulation of alien species trade or more and accurate inspections of vessels and cargo in harbours. Likewise, environmental education and public awareness about the impacts of invasive alien species is often an important part of bioinvasions prevention and essential for other phases of their management (Shackleton, Adriaens, et al., 2019), since opinions and attitudes of citizens can potentially affect the introduction and correct management of biological invasions (Sosa et al., 2021). The knowledge of citizens about the invasive status and negative impacts of invasive species seems to increase public support during management efforts (Novoa et al., 2017; Shackleton, et al., 2019). Moreover, United Nations Educational, Scientific and Cultural Organization recent report about issues and trends in education for sustainable development includes invasive alien species in its learning objectives on threats to biodiversity, along with habitat loss, deforestation, fragmentation and overexploitation (Leicht et al., 2018). Finally, there is a need to fully understand current and emerging pressures of invasive alien species acting on both species and ecosystems (Navarro et al., 2017), to assess long-term changes in biodiversity and provide an evidence base for environmental policy and decision-making (Navarro et al., 2017; Proença et al., 2017; Sakata et al., 2021).

The yellow-legged hornet (*Vespa velutina nigrithorax*)

Over the last two decades *Vespa velutina nigrithorax* Buysson 1905 has become one of the most challenging insects invading several regions of the globe (Choi et al., 2012; Laurino et al., 2020; Takahashi et al., 2018). This hornet is native from Asia were the species dwells from middle east to Indonesia and coexists with other species of hornets (Smith-Pardo et al., 2020). It is now also present in Europe, South Korea and Japan (Choi et al., 2012; Monceau et al., 2014b; Takahashi et al., 2018). The yellow-legged hornet represents the first case of an invasive alien Vespidae predator in Europe (Monceau et al., 2014b), where it was first reported from the French department of Lot-et-Garonne in 2004 (Haxaire et al., 2006). Since then, it has successfully colonised France and neighbouring countries: Spain, Portugal, Belgium, Germany, Holland, Italy, Switzerland, the United Kingdom and Ireland (Dillane et al., 2022; Keeling et al., 2017).

As other social wasps, the yellow-legged hornet is an opportunistic forager and generalist predator upon insects, preying mainly on flying Diptera and social Hymenoptera (Perrard et al., 2009; Verdasca et al., 2021), with a notable preference for honeybees (*Apis mellifera*) (Cini et al., 2018). Carbohydrates are the main source of energy for adults, while the diet of the offspring is mainly based on proteins (Raveret Richter, 2000). Such proteins are collected by the queen during the

embryo nest stage and then by workers the rest of the year (Monceau et al., 2014b). Rome et al. (2021) examined the diet of *V. velutina* based on morphological analysis, revealing that a single colony could prey upon at least 159 different species of insects (11 orders, 43 families) and consume about 11 kg of insect biomass in one season. Regarding the impact on honeybee populations, the yellow-legged hornets pray in front of the hives, disrupting their foraging activity (Requier et al., 2019) and inducing oxidative stress on the individuals (Leza et al., 2019). In addition, it may represent a threat to human health due to its sting, when nests are established in urban areas or when people get to close to the colonies in nature (de Haro et al., 2010).

In Europe there is a native hornet species, the European hornet *Vespa crabro* Linnaeus 1758. Nonetheless, both species are easily differentiated from each other by colour and size (Figure 2) (Monceau et al., 2014b). The general appearance of *V. crabro* is brown and brownish yellow, while *V. velutina* is black and yellow (Figure 2). Sex attributes are similar in both species: females present a sting while males not. Moreover, the antennae are shorter and thinner in females (Monceau et al., 2014b).

Figure 2.- Females of the yellow-legged hornet (*Vespa velutina*) (left) and the European hornet (*V. crabro*) (right).

Like other eusocial insects, *V. velutina* has an annual and haplodiploidy life cycle (females come from fertilized eggs, diploid, and males from unfertilized eggs, haploid). The life cycle starts when a single queen founds an embryo nest (4 – 20 cm of diameter) in early spring (Archer, 2010). This stage is critical due to its high vulnerability to predators and parasites, hence the queen must produce effective workers as soon as possible. When the first generation of workers emerge, the queen will focus exclusively on laying eggs. The colony grows and develops exponentially through the summer, producing up to several thousand of sterile workers. When the location of the embryo nest allows increasing the population of the colony, workers build a higher nest enveloping the embryo nest called primary nest. If the location does not allow increasing the colony, they move and workers builds a new nest called the secondary nest (40 – 100 cm), normally located in more open spaces. The reproductive caste (next year founder queens and males) emerges by the end of autumn, and the previous colony dies. Only next year founder queens will survive the winter and start a new cycle the next spring (Monceau et al., 2014b).

The yellow-legged hornet is included in the *Catálogo Español de Especies Exóticas Invasoras* (RD 630/2013) and listed by the European Union as Invasive Alien Species of Union Concern (European Commision 2016). According to these regulations, measures are urgently needed to prevent and early detect new introductions, as well as to identify and control established populations. Moreover, a recent literature review conducted by Lima et al. (2022) revealed that

life history traits, morphology and the sting venom properties are some of the most studied topics, but there are still large unknowns regarding the real impacts caused by this species and the most efficient measures to manage the invasion.

Control and management plans

Since the species is currently established in Europe, management efforts have to focus mainly on the containment and control of its populations to reduce its expansion and its potential impacts (Turchi & Derijard, 2018). Major control and management plants are based on the localization and removal of nests, queen trapping during the spring season, poisoned baits, and mechanical passive traps (Turchi & Derijard, 2018). However, there is no clear coordination among countries and there are no uniform criteria to stablish eradication measures.

Invasion genetics

While there is a wealth of studies on different aspects of the biology, ecology, and management of *V. velutina* in Europe, only a few of them have focused on the genetics of the invasion (Arca et al., 2015; Budge et al., 2017; Quaresma et al., 2022). Genetic analyses conducted with individuals from France, Italy or Portugal concluded that the European invasion by yellow-legged hornets represented a genetic bottleneck event that explains their low levels of genetic diversity, probably as a result of a single introduction of a multi-mated queen (Arca et al., 2015). Nonetheless, this has not meant any limitation for the species to expand throughout the European continent.

Invasion risk areas

Anticipating the possible distribution of an invasive species is essential for prevention, early detection, and control (Broennimann et al., 2007), and indispensable for a conservation plan (Peterson & Robins, 2003). Ecological niche models have been used to predict the invasion extents from a great diversity of invasive organisms (Guisan & Thuiller, 2005). Continental-scale models have been developed for this invasive species (Villemant et al., 2011) and have coincided with the dispersal pattern of this species recorded over the years in Europe (Laurino et al., 2020).

Genetic diversity

Genetic diversity plays a key role in alien species persistence and resilience in a new environment (Hoffmann & Willi, 2008). Propagule pressure during the introduction stage, selection pressure during establishment, factors related to demographic history or ecological conditions may affect the genetic diversity of an invasive populations and its spread through the territory (Bacon et al., 2017; Lockwood et al., 2005).

Monitoring

Tracking *V. velutina* populations can aid the management of the invasion and increase understanding of its spatio-temporal patterns species (Monceau et al. 2014a). Current methods for assessing its distribution and dynamics, such as visual observations, trapping, and nest location, are labour intensive and can harm non-target species (Turchi & Derijard, 2018). Hopefully, various technology-based methods are being tested, such as harmonic radar (Milanesio et al. 2017), radio-telemetry tracking (Kennedy et al. 2018), thermal imaging (Reynaud & Guerin-Lassous 2016), and citizen science programs (Carvalho et al., 2020).

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OBJECTIVES

The main objective of this PhD thesis is to investigate the invasion and expansion patterns of the yellow-legged hornet *Vespa velutina nigrithorax* Buysson 1905 in Europe, to propose and evaluate an eradication plan applied in Mallorca, as well as developing a new automated detection method as a basis for the development of tools for its management. To this end, the following specific objectives were established, according to the different chapters:

1. To contribute the first field study of the strategy of eradication of the pest *V. velutina* that has been established in Mallorca (the Balearic Islands).
2. To investigate both the origin and the genetic structure of the invading populations in Mallorca island.
3. To expand the current knowledge on the invasion genetics of *V. velutina* in Europe based on mitochondrial (cytochrome oxidase subunit I gene) and nuclear (microsatellites) DNA analyses of the invading populations in Spain.
4. To identify the ecological factors determining the presence of *V. velutina* and to predict potentially suitable areas of invasion under Mediterranean island conditions.
5. To test the central-marginal hypothesis in invasive *V. velutina* populations from France, Italy, Spain, and Portugal.
6. To contribute to the development of an automated system to monitor populations of *V. velutina* in the field based on features derived from the wingbeat recordings of a flying insect sensor.

Chapter 1

Six years of controlling the invasive species *Vespa velutina* in a Mediterranean island: The promising results of an eradication plan

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Abstract

BACKGROUND

The yellow-legged hornet, *Vespa velutina nigrithorax*, is an invasive alien species (IAS) which was accidentally introduced in Europe from Asia. This social insect preys primarily on honeybees but also on other pollinators and insects. Consequently, the establishment of this IAS has a negative impact on biodiversity, pollination, and economy. There is no clear coordination and uniformed methods for eradication measures between countries. Here we present the first field study of the strategy of eradication of the IAS species *V. velutina* that has been conducted in the westernmost Mediterranean archipelago.

RESULTS

We investigated the combination of different eradication methods, such as trapping; the use of the citizen science data for detection of presence, the active search of nests and the removal of nests using mechanical methods. The progression of the number of secondary nests found was 1 (2015), 9 (2016) and 20 (2017), with zero during 2018, 2019 and 2020, and just one embryo nest in 2018. More than half of the nests (58%) were detected thanks to citizen science data. The people sent us adult detections, and we started the triangulation method to find the nests. The last hornet found in the traps was in June 2018.

CONCLUSION

Early detections of the IAS are crucial to minimise their effects, and citizen science may offer an important source of information to determine the presence and distribution of *V. velutina*. The findings we present here indicate successful management for this globally significant IAS and could contribute to advance the 'science of eradication'.

Keywords: alien species; Asian hornet; eradication; island; Majorca; yellow-legged hornet

Introduction

The yellow-legged hornet (*Vespa velutina nigrithorax* Buysson 1905) is an invasive alien species (IAS) which was accidentally introduced in Europe from Asia. It is native to tropical and subtropical areas of South-East Asia. It was reported for the first time in south-west France in 2004 (Haxaire et al., 2006) and rapidly spread to nearby European countries. (Laurino et al., 2020). In the case of Spain, the yellow-legged hornet is established in the northern regions (Navarra, Basque Country, Galicia and Cantabria) (López et al., 2011) and in Catalonia (Pujade-Villar et al., 2013). Biological invasions around the world have experienced an unprecedented increase during recent decades as a result of global change, worldwide trade (García-Díaz et al., 2015) and human mobility, causing severe environmental and socioeconomic impacts (Paini et al., 2016).

In the case of *V. velutina*, this social insect preys primarily upon honeybees (*Apis mellifera* Linnaeus, 1758), but also upon other pollinators and insects (Rojas-Nossa & Calviño-Cancela, 2020; Rome et al., 2011). Consequently, the establishment of this IAS is a major concern because of its potential negative impact on pollinators and biodiversity. Major impacts will likely cause high economic losses due to the death of honeybee colonies and therefore a reduction in honey production (Barbet-Massin et al., 2020; Requier et al., 2019). Further, *V. velutina* may have negative impacts on the natural ecosystem and local biodiversity by causing declines in insect populations (Beggs et al., 2011). In addition, attacks on humans are possible, but rare (de Haro et al., 2010), when colonial nests are established in urban areas or when people get to close to the colonies without noting their presence in natural areas. In the particular case of Majorca, this invasion could be devastating, considering the situation of the populations of honeybees, the fragility of the ecosystem (typical of the island ecosystems) (Blondel et al., 2010; Traveset et al., 2019) and the impact on endemic insects (Ikegami et al., 2020).

Control/eradication plans for *V. velutina* around Europe include methods such as trapping of adults and queens, poisoned baits, nest destruction or mechanical passive traps (Beggs et al., 2011; Feás-Sánchez & Charles, 2019; Leza et al., 2018; Monceau et al., 2014; Turchi & Derijard, 2018). However, there is no clear coordination between countries and there are no uniform methods for eradication measures.

Here we present the first field study of the strategy of eradication of the IAS *V. velutina* that has been established in the westernmost Mediterranean archipelago. It could be an example of the detailed planning and execution of the operation of an eradication program for the yellow-legged hornet in regions recently invaded. We report here all the steps we followed since the detection of the first specimen, the establishment of a multidisciplinary working group, and the field and laboratory methodologies. More specifically, we investigated the combination of the following methods: (i) spring trapping of queens; (ii) detection of the presence of adults using traps; (iii) use of citizen science data for detection of presence; (iv) detection of nests using the triangulation method following adults (from artificial feeding points); and (v) destruction of nests using a mechanical method. To complete the study, we discuss the improvements of the methods used here, field work campaign costs and the next steps of the plan. In this study, we analyse and discuss the results obtained and the need to enforce preventative measures by establishing stringent biosecurity policies at the regional, national, and global scale. These activities contribute to the effective implementation of the current legislation at the EU (Regulation 1143/2014) and national level (RD 630/2013) that regulates the prevention and management of the introduction

and spread of IAS such as *V. velu*tina. The findings we present here could contribute to advance the 'science of eradication'.

Material and methods

Detection and identification of the organism: establishment of a multidisciplinary working group

In October 2015 the Laboratory of Zoology of the University of the Balearic Islands received a sample from an apiary of a beekeeper located in Sóller (39°47′45.3″ N, 2°42′53.9″ E) suspected to be *V. velutina*, according to the morphology of the insect. The specimen was classified according to an identification key (Archer, 2012).

The first step after the confirmation was the establishment of a multidisciplinary working group. The working group was created to coordinate the following aspects: (i) assessment of risks and impacts; (ii) to determine the extent of the infestation and affected geographical area; (iii) evaluation of appropriate treatment options available and their impact on nontarget fauna; (iv) to revise and acquire funds; (v) communication with stakeholders, including the citizenship; (vi) to decide which management options (eradication, control) are mostly like achievable; and (vii) to detail the planning methods. The working group was formed by specialists from the administration (technical staff from government and environmental agents), specialist technicians of the health and wildlife management department from the Consortium for the Recovery of Fauna in the Balearic Islands (COFIB) and specialist personnel of the University of the Balearic Islands. Moreover, the working group had the assistance of the beekeepers and local councils concerned.

Study site

Majorca (39°30′ N, 03°0′ E) has an area of 3.667 km^2 and is climatically considered Mediterranean (based on Emberger's Index). However, subclimatic divisions are defined for the mountains areas (mountains up to 1300 m in the north of the island, known as *Serra de Tramuntana*, and the southeast mountains, known as *Serres de Llevant*), the coastal area and the central area (known as *Es Pla*). The central depression where the following areas are outstanding are *Raiguer* (connected to *Serra de Tramuntana*) and *Migjorn* (which extends from the extreme east of Palma to the Eastern coast of the island) (Grimalt-Gelabert, Rodríguez-Perea, Servera-Nicolau, & Rodríquez-Gomila, 1991). The first detection was in *Sóller*, in the mountain area in the north of the island known as *Serra de Tramuntana*. This area is characterized by specific climatic and geomorphological conditions compared to the rest of the regions in Majorca, such as the highest precipitation rate (mean of 1400–1600 mm annual) and cooler temperatures (16.5 °C annual mean temperature). Consequently, the vegetation of the area is quite different from that in the rest of the island, and is dominated by pine trees (*Pinus halepensis* Miller), holm oaks (*Quercus ilex* L.), garrigue (mainly *Rosmarinus officinalis* L. and *Erica multiflora* L.), wild olive trees (*Olea europaea* var. *sylvestris* L.), orchards of orange trees (*Citrus sinensis* (L.) Osbeckand) and olive trees (*Olea europaea* L.). The other areas of the island are semi-arid. The meridional coast does not surpass the 300–350 mm annual precipitation rate, which is basically due to orographic factors.

Field work

Trapping: spring trapping of queens and detection of the presence of workers

From October 2015 to October 2020 intensive surveys were implemented in an area of 486 km^2, included in a radius of 28 km around the first detection in *Sóller*.

All the trapping areas comprise rural zones, urban zones, and forests, where traps were placed every 300 m in easily accessible points along nearby secondary roads or private paths (Figure 3). The traps were hung at heights ranging from 1.5 to 2 m, usually from a tree or any other high point. On average, traps were emptied and refilled every 14 days and all captured hornets were classified as queen or workers using morphological differences (Pérez-De-Heredia et al., 2017). Finally, the hornets were stored in vials with ethanol 70% for further research.

Every year the trapping design was adapted to the new scenario. The type of trapping (spring queen trapping and detection of the presence of adults), period, number and type of traps, characteristics of trapping, monitored area and trap density are shown in Table 1.

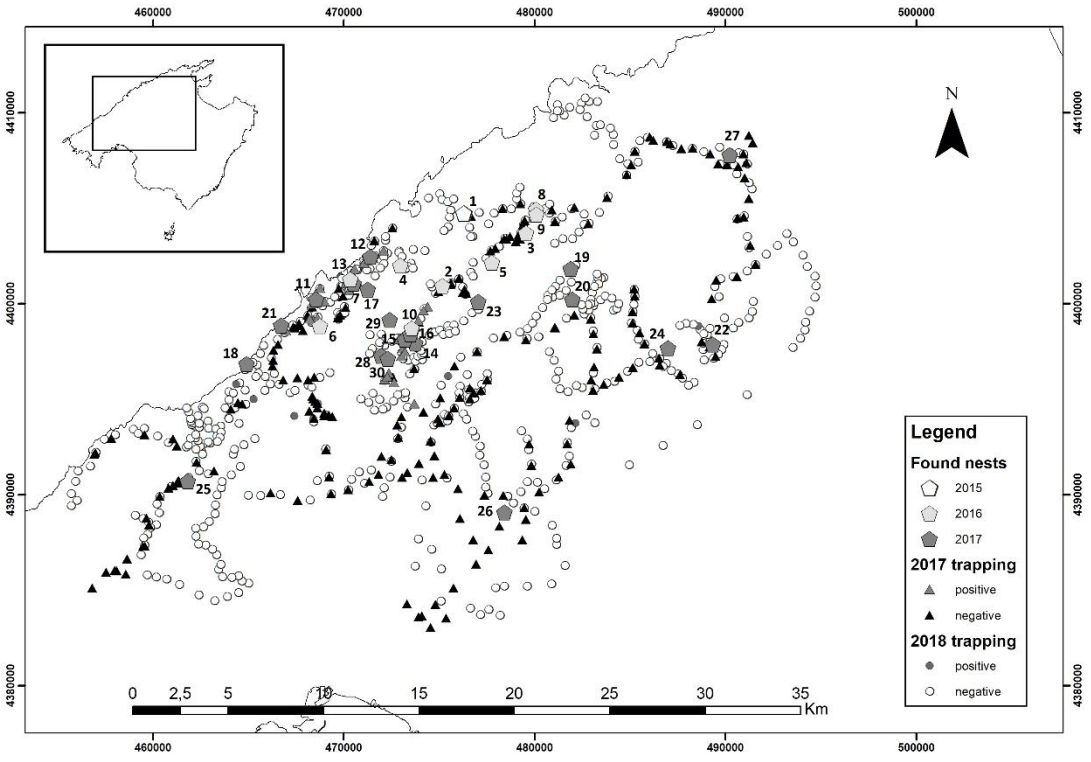

Figure 3.- Locations of the thirty *Vespa velutina* secondary nests (numbered from 1 to 30) and food traps (n = 1693) used during 2015 to 2020 on the Majorca island. 2015 = 1 nest, 2016 = 9 nests, 2017 = 20 nests and 17 positive spring traps, 2018 = 8 positive spring traps.

Table 1.- Main characteristics of the trapping: spring trapping of queens (Queens), detection of the presence of workers (Workers); number of traps (N); Type of traps and characteristics (sticky or bottle, attractant, and location); monitored area (km^2) and trap density (trap/km^2).

Year	Type of trapping	Period (days)	N traps	Type of trap and characteristics of trapping	Monitored area (km^2)	Trap density (trap/km^2)
2015	Workers	25th Oct - 1st Dec (38 days)	87	Sticky traps (20x20 cm) with protein attractant (raw fish). Traps located at ground level	7.1 km^2 (1.5 km ratio around the positive apiary in *Sóller*)	12.25
2016	Workers	5th Sept - 29th Nov (85 days)	75	At the same sampled point (x25), 3 types of traps: Sticky traps (20x20 cm) with protein attractant (raw fish) + Bottles traps filled with protein + Bottles traps filled with Avispa'clac©.	18 km^2 (around the hotspot area[1], which comprised three municipalities: *Sóller*, *Deià* and *Fornalutx*. Some of them were also placed further as a way to span the radius of action)	4.17
2017	Queens	3rd April - 20th June (78 days)	79	Craft-made traps following the classical funnel trap design, with an additional 10 cm wide yellow tape placed in the middle of the bottle (Figure 4). Traps filled with Avispa'clac©.	78.68 km^2 (area included the areas where nests had been found in previous years)	~1
	Workers	21st June - 11th Dec (173 days)	250		324.4 km^2 (area included the areas where nests had been found in previous years, plus farther sections as new nests were found)	0.77
2018	Queens	28th March - 20th June (84 days)	561	Bottles traps filled with (Avispa'clac©) or with home-made mixture based on beer. Traps were baited with sucrose attractant that was randomly alternated between the commercial and the home-made type in 5 different periods (Avispa'clac© was used during these periods: 28th March - 24th June (88 days).	425 km^2 (area included the areas where nests had been found in previous years, plus approximately 5 km* radius from the outlying nests in order to cover the buffer areas[2])	1.32

	Workers	21st June - 21st Nov (153 days)	582	Bottles traps filled with (Avispa'clac©) or with home-made mixture based on beer. Traps were baited with sucrose attractant that was randomly alternated between the commercial and the home-made type in 5 different periods (Avispa'clac© was used during these periods: 23rd July - 20th August (28 days), 27th September - 8th November (43 days), and the mix in between the periods). Also, Avispa'clac© was vaporized around the traps in two different periods (25th July - 2nd August (8 days) and 30th August - 8th November (70 days)) in order to give hornets an advantage.	451.1 km^2 (area included the areas where nests had been found in previous years, plus approximately 5 km* radius from the outlying nests in order to cover the buffer areas[2]) * It seemed to us to be cautious to select only 5 km to balance advantages and drawbacks of trapping method.	1.29
		4th Oct – 21st Nov (48 days)	28	Traps with protein lure (raw fish) were placed near the area where the hornet had been previously found.		
2019	Queens	18th March - 20th June (94 days)	578	Bottles traps filled with (Avispa'clac©).	425 km^2 (traps were placed in the same locations as 2018, optimising the distribution)	1.36
	Workers	21st June - 31th Oct (132 days)				
2020	Queens	15th April – 20th June (66 days)	283	Bottles traps filled with (Avispa'clac©).	298 km^2 (area included the buffers areas[2] where nests with males had been found in 2017 and	0.95

	Workers	21th June – 24th Oct (125 days)			where positive traps had been found in 2017 and 2018)	

[1]Hotspot area: area where *V. velutina* (adults or nests) have been detected.
[2]Buffer area: area around where *V. velutina* has been detected to provide additional protection and mitigate its negative impacts.

Citizen science data: detection of the presence of adults

An important community information task was carried out with leaflets, informational brochures, and local media. Also, two 24-h phone numbers were available for citizens to call or send a message to notify a possible positive. In 2016, a year after the first detection, a software called Vespapp was developed to let the public send photos to detect individuals or nests of *V. velutina*. This software involved a website (http://vespapp.uib.es/) and a free android app (https://play.google.com/store/apps/details?id=com.habitissimo.vespapp&hl=es), and citizens could send a picture of a suspicious observation (hornet) to a database and then experts confirmed or discarded it.

Moreover, visual observation was carried out by the beekeepers and 54 environmental agents in the apiaries and in natural zones throughout the island.

Figure 4.- Trap used during the trapping campaign.

Detection of nests

When an adult was detected (from public report or trap), feeding points with protein attractant (raw fish) were set in the area to identify the direction these adults took to the nest. At least three flight routes departing from three different feeding points were necessary to define the location of the nest by means of triangulation (Leza et al., 2018). Binoculars were used to track the adult hornets.

Destruction of nests

The nests were removed manually during the night. For this purpose, the technicians had to reach the nest, usually by climbing a tree. The nest was entirely removed and placed in a bespoke tight-fitting sack, which for safety reasons was placed within a bigger plastic bag. After that, if any individual was observed nearby domestic spray insecticide (permethrin) was applied. The nests were frozen at −20 °C for at least 48 h. A revisit to the location was made after 1 week to check if the hornets rebuilt the nests, and, if that was the case, they were removed.

Data and statistical analysis

Trapping: spring trapping of queens and detection of the presence of workers

To compare the spring queen trapping between 2017 and 2018, and the detection of the presence of workers, the rate of *V. velutina* individuals captured per trap per week and the standard error were calculated. Moreover, trap density (trap/km^2) per year was calculated. This descriptive statistical analysis was performed with Microsoft Excel.

The map was produced with ArcMap (ArcGIS Desktop 10.3.1).

Citizen science data: detection of the presence of adults

Reports from citizens made by web and Vespapp were combined and analysed.

Regarding Vespapp, data were obtained by Google play. The number of reports was analysed from 9 June 2016 to 15 March 2020 (1377 days). For each day, the numbers of reports and downloads were calculated (Graham et al., 2011). Peaks with more than six reports and downloads per day were related to social media communications (newspapers, TV and radio programs, and conferences). Finally, the percentage of positive reports was calculated yearly and the number of reports per download was calculated monthly in order to relate it to the life cycle of *V. velutina*.

Detection of nests

Regarding detection of nests the following parameters were calculated: colonised area each year, mean spread rate, the percentage of nests destroyed before the emergence of males in the autumn and its variation over the years, and the distance of nests from the starting points of triangulation. The average rate of species expansion was calculated by determining the distance between the

expansion front at period t and the expansion front at period t_{-1} (t_{2015}–t_{2016} and t_{2016}–t_{2017}) (Carvalho et al., 2020).

Field work campaign costs

We calculated the total costs of the field work campaign for the years 2015 to 2018. To do this we kept an account of the costs related to (i) bait for trapping, (ii) material for removing the nests, (iii) vehicle mileage and (iv) salaries of the specialist technicians of the health and wildlife management department of the COFIB.

Results

Detection and identification of the organism: establishment of a multidisciplinary working group

The first specimen sent by a beekeeper was identified as *Vespa velutina nigrithorax* according to an identification key (Archer, 2012). After the first identification, the working group decided to apply a contingency plan and detailed the planning. The working group met several times over the years.

Trapping: spring trapping of queens

Captures of *V. velutina* in 2017 reached 17 queens, while 2018 scored just eight positives (see Figure 3). In 2017, 79 traps were used for 11 weeks, yielding 0.0308 females per trap per week. This value ranges from 0.086 to 0.356 if just positive traps are taken into account. In contrast, in 2018, 561 traps were placed on the field for 12 weeks which overall yielded 2.36×10^{-3} females per trap per week (ranging from 0.002 to 0.012 among positive traps). On average, during the 2017 campaign traps were active for 41.8 ± 2.1 days while in 2018 they were active for 44.5 ± 1.1 days. During 2019 and 2020 no queens were captured.

Trapping: detection of the presence of workers

There were five captures of *V. velutina* in 2016, 56 in 2017 and in 2018 just two. The last adult was captured on 27 June 2018. In 2016, 75 traps were placed for ~12 weeks, while in 2017, 250 traps baited with sucrose were placed on the field for 25 weeks and in 2018 there were 610 (see Figure 3). Of these, 582 traps were baited with sucrose for 22 weeks and 28 with protein for 4 weeks. Overall, 2016 yielded 0.0055 adults per trap per week and in 2017 traps were active for 43.0 ± 2.5 days, yielding 0.0365 adults per trap per week. This value ranges between 0.001 and 0.055 if just positive traps are considered. In contrast, in 2018 traps baited with sucrose were active for 145.3 ± 0.6 days, yielding 0.0002 females per trap per week, while no protein-baited trap captured any specimen. During 2019 and 2020 no adults were captured.

Citizen science data: detection of the presence of adults

The total number of reports from Vespapp and web was 1024 (322 in 2016, 148 in 2017, 300 in 2018, 247 in 2019 and 7 in 2020). The proportion of positive reports was less than 8% (2016 4.3%, 2017 7.4%, 2018 3.3%, 2019 0%, 2020 0%), although in 2017 the number of positive reports was double that in 2016 and 2018, approximately. Moreover, spring and summer were the seasons with highest numbers of reports, and the lowest number of reports was detected in winter (Figure 5).

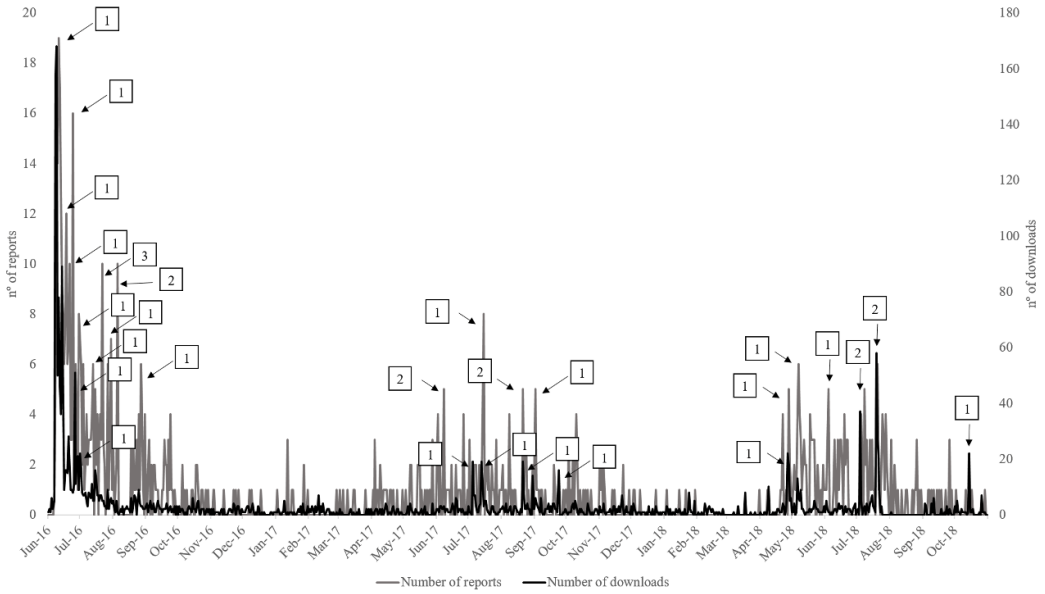

Figure 5.- Temporal relationship between number of Vespapp downloads and the number of reports of this application. The peaks of reports or downloads were related with social events which have been divided in three categories: (1) awareness sessions (in to out), (2) news in means of communication (out to in) and (3) scientist talks.

For Vespapp, a total of 3685 downloads was recorded. Specifically, there were 1435 downloads in 2016, 615 in 2017, 657 in 2018, 926 in 2019 and 52 in 2020. The number of downloads decreased by 57.2% in 2017, compared to 2016, and in 2018 and 2019 increased from 6.8% to 50.6% compared to 2017. In Fig. 3, the temporal relationship between the numbers of reports and downloads is shown. All peaks of both reports and downloads can be related to social media events which fall into three categories: (i) awareness sessions; (ii) news in the media; and (iii) scientist talks. Apart from these events, other workshops were performed such as '*Fira de sa Mel'*, a local beekeeping fair which is celebrated each November, and a special edition of '*Balears Fa Ciència'*, which is a local scientific program that dedicated time to talk about Vespapp and its importance to control this invasive species. Most social media events were of the first category: awareness sessions such as press conferences or interviews to inform and advise citizens.

The highest numbers of reports/number of downloads were in July and August 2016 when Vespapp was submitted. In 2017, April, May and June had the highest values, coinciding with the increase in hornet activity. In 2018, two peaks were observed, the first one from May to June, similar to 2017, and the other one from August to September (predation time). Finally, in 2019 the highest values were obtained between March to June.

Detection and destruction of nests

A total of 30 secondary nests were located and removed from 2015 to 2017 (see Figure 3 and Table 2). During July 2018 just one embryo nest was detected and removed, the first occupied embryo nest of *V. velutina* active and since then no more nests have been detected in the Balearic Islands. The distance of nests from the starting points of triangulation was between 0.7 and 3 km.

The colonized area increased from 33.63 km^2 in 2016 to 314.53 km^2 in 2017. No more nests were found in the following years. Moreover, the mean spread rate was 5.03 ± 0.73 km in 2016, with 9.57 km as the maximum value, and it was lower in 2017, rising to 4.00 ± 0.91 km with 11.40 km as maximum. To sum up, since *V. velutina* arrived in Majorca in 2015 until 2017, the last year when a secondary nest was found, it reached a mean spread rate of 4.52 ± 0.51 km/year.

A total of 56.67% of secondary nests were removed before male emergence in autumn, after checking every individual inside the nests to distinguish females and males by morphological differences (Monceau et al., 2014).

Table 2.- Main characteristics of the nest's findings: date of location, altitude, detection method (adult records received from members of the public or found in trap, or notification of citizen), days elapsed to find the nest, type of tree where nests were found or if the nest were found in rock, height of nest and if the nest was analysed.
ID= Identification; PR= Public Report; NA= no data; Sept.= September; Oct.= October; Nov.= November.
* Embryo nest.

ID Nest	Date of location	Altitude (m)	Detection method	Days to find the nest	Host tree or rock	Height of nest (m)
2015						
ID1	30th Oct.	303	PR	56	*Pinus halepensis*	14
2016						
ID2	19th August	100	PR	36	*Pinus halepensis*	12
ID3	26th August	525	PR	18	*Pinus halepensis*	25-30
ID4	6th Sept.	412	PR	1	*Pinus halepensis*	10
ID5	9th Sept.	155	PR	61	*Pinus halepensis*	15-20
ID6	13th Sept.	464	PR	8	*Quercus ilex*	8
ID7	19th Sept.	216	PR	14	*Pinus halepensis*	10-15

ID8	14th Oct.	642	PR	13	*Cupressus sempervirens*	15
ID9	31st Oct.	464	PR	NA	*Pinus halepensis*	12.5
ID10	22nd Nov.	375	PR	18	*Quercus ilex*	20-25
2017						
ID11	22nd June	320	Trap	41	*Pinus halepensis*	12 - 15
ID12	28th June	110	Trap	40	*Pinus halepensis*	15 - 18
ID13	4th July	336	Trap	12	Rock	25
ID14	6th July	687	Trap	20	*Quercus ilex*	6
ID15	8th July	509	Trap	22	*Pinus halepensis*	10
ID16	18th July	492	Trap	48	*Quercus ilex*	5
ID17	10th August	560	Trap	63	*Quercus ilex*	6
ID18	11th August	40	PR	6	Rock	8
ID19	14th August	621	PR	5	*Pinus halepensis*	12 - 15
ID20	22nd August	426	Trap	12	*Pinus halepensis*	12 - 15
ID21	28th August	89	PR	5	*Pinus halepensis*	15 - 18
ID22	5th Sept.	175	PR	11	*Ceratonia siliqua*	4 - 5
ID23	8th Sept.	689	PR	11	*Quercus ilex*	10
ID24	11st Sept.	220	PR	18	*Pinus halepensis*	10 - 12
ID25	11st Sept.	318	PR	5	*Quercus ilex*	10 - 12
ID26	13rd Sept.	114	PR	2	*Pinus halepensis*	15 - 18
ID27	15th Sept.	490	PR	0	*Prunus spinosa*	0
ID28	11st Oct.	750	Trap	2	*Pinus halepensis*	10 - 12

ID29	19th Oct.	716	Trap	5	*Pinus halepensis*	10 - 12
ID30	23rd Oct.	587	Trap	5	Rock	5
2018						
ID31*	10th June	60	PR	0	House	2

Field work campaign costs

The total field work campaign cost was 134123.14 euros for 4 years. In terms of salary, this analysis only includes the personal cost of one working group: the technicians of the health and wildlife management department from COFIB.

Discussion

It is important to note that the ecological niche model developed for the Iberian Peninsula and the Balearic Islands, based on Villemant et al., (2011) shows the northwest of the island with similar suitability values to the north of the Iberian Peninsula where this species has spread and established (RD 630/201322). It is known that invasive species often do not present a static niche. These species can expand, contract or change, reducing the predictive capacity of distribution models based on niche conservatism for species with a dynamic niche (Becerra López et al., 2017; Fitzpatrick et al., 2006; Pearman et al., 2008). Broennimann et al., (2007) provided the first empirical evidence that an invasive species can occupy climatically distinct niche spaces following its introduction into a new area. These results support the hypothesis that a climatic niche shift occurs during biological invasions. In fact, we detected an exponential growth in the number of nests, 1 in 2015, 9 in 2016 and 20 in 2017, as in other colonized areas (Bertolino et al., 2016; Monceau & Thiéry, 2016), showing the potential spreading of this IAS. On the other hand, Arca et al., (2015) concluded that *V. velutina* appears to be able to establish even after a severe genetic bottleneck resulting from the introduction of very few or possibly a single female. IAS are a major threat to native insular species and eradication may be feasible when a rapid response is ensured, and adequate resources are devoted. Eradication may become impracticable once the IAS reaches a certain population level and/or range expansion (Working Group on Invasive Alien Species). So, from the first detection of this invasive alien species in 2015 an intensive detection and control programme was implemented immediately.

From our point of view, the decision to establish a multidisciplinary working group and the application of a combination of various methodologies was essential to succeed. Planning was dynamic and evolved according to the contingencies, resulting in the team's broader expertise.

To limit the impact of *V. velutina*, methods such as the trapping of foundresses during spring were used. Comparing our spring trapping data with previous studies one can observe that our capture rate was quite low. We captured 0.0308 females per trap per week in 2017 and 2.36×10^{-3} in 2018, while (Monceaut et al., 2012) reached 0.71 females per trap per week, (Rojas-Nossa et al., 2018) 2.59 females per trap per week and (Monceau & Thiéry, 2016) an average of 0.78 females

per trap per week. Different variables have been used to determine the effect of bait trapping, such as mean minimal temperature (Monceau et al., 2012), land use (Rojas-Nossa et al., 2018), and proximity to apiaries (Rojas-Nossa et al., 2018) and to water bodies (Monceau et al., 2012). During 2019 and 2020 no queens were captured. On the one hand, our periods of trapping cover 11, 12, 13 and 9.4 weeks of trapping (for 2017, 2018, 2019 and 2020, respectively) compared with 7 weeks (Monceau et al., 2012) and 9 weeks (Rojas-Nossa et al., 2018) in the other studies, which has been suggested to have a strong effect on yields (see Monceau et al., 2012). The lower number of captures reached in 2018 could be explained by the higher number of traps that we used, 561 traps compared to 79 in 2017, 16 traps used by Monceau et al., (2012), 60 analysed by Monceau & Thiéry (2016) and 253 used by Rojas-Nossa et al., (2018). In addition, in 2018 almost half of the traps were placed out of the hotspot area, considering the buffer areas. It is likely that this contributed to the lower number of queens trapped that year.

On the other hand, these differences between study results could be explained by a lower *V. velutina* population in regions recently invaded compared to others where the population was already well established (Monceau et al., 2012; Rojas-Nossa et al., 2018). In our case we were still working in an early stage of invasion, where the density of nests was very low compared to other regions (see Monceau & Thiéry, 2016). Thus, if we consider that we are working towards eradication, the lowest number of captures in 2018 and no captures during 2019 and 2020 could be also explained by a decrease in the *V. velutina* population in relation to 2017, as in 2018 we just found one embryo nest compared to the 20 secondary nests found in 2017.

In addition, weather variability and the proximity of traps to water sources or apiaries (Monceau et al., 2012) could have played an important role. No captures were observed below a temperature of 10 °C and the highest numbers were reached at 15 °C. Thus, queen trapping is not necessary when the temperature is below this limit (Monceau et al., 2012). 2019 was the warmest year on record in Europe, very closely followed by 2014, 2015 and 2018. There is no clear trend in annual precipitation for Europe, and 2019 values were close to average (Copernicus Climate Change Service (C3S)/ECMWF/KNMI). Unfortunately, we have not found published literature for the numbers of queens during 2019 around Europe. However, we found that in Portugal the number of nests detected decreased during 2019 compared to 2018 (Carvalho et al., 2020). It could be that 2019 was not a favourable year for this species. However, the fact we did not find any nests during 2019 and 2020 seems to be due to the contention measures applied.

Even though the presence of water near the trapping area was constant (sea, dams, or torrents), no data was collected to determine the influence of water body proximity on the yield or the proximity to beehives.

Overall, it is widely thought that using spring queen trapping is controversial or even useless as it does not reduce significantly the population level (Monceau et al., 2012, 2014; Monceau & Thiéry, 2016), negatively affects the entomofauna (Haxaire & Villemant, 2010; Monceau et al., 2014), and may be less effective than intraspecific competition among foundresses in spring (Haxaire & Villemant, 2010; Monceau & Thiéry, 2016). However, in our case the invasion of foundresses from adjacent locations is not possible (thanks to insularity), and the presumed low population of *V. velutina* in our data encourages the likelihood of eradication. Thus, even though the efforts and costs assumed were high, we consider this method was justified and worthwhile at this stage of control of *V. velutina*.

On the other hand, regarding the trapping of workers, the objective was to have a starting point to find a nest and destroy it before the colony size reached its full maturity. According to Keeling

et al. (2017) the probability of such an event depends on two independent probabilities: detection of hornets feeding on an apiary and discovery of the nest by back-tracing the hornet flight path. These two procedures are an approximation to the method that we followed to control a rapidly expanding invasion in Majorca. In our case, a trapping campaign of workers was preferred over controlling the apiaries to reduce the random detection of nests.

Most studies are restricted to spring queen trapping and only (Demichelis et al., 2014) undertook trapping campaigns for adults for 6 years with a single capture during the whole period (1 male on 19 November 2012). With 0.0055 adults per trap per week in 2016, 0.0365 in 2017 and 0.0002 in 2018, our results show a higher capture rate from 2016 to 2017, which was crucial as it directly led us to detect 11 of the 20 nests found in 2017. Indeed, Monceau & Thiéry (2016) point out that intensifying surveys in those areas more likely to host *V. velutina* nests is one of the most promising techniques to limit its population.

We are especially interested in the positive response from the public. Citizen science can be defined as the collection of data relating to the natural world by members of the public. Public participation is increasing. They are collecting data for monitoring invasive species to detect early alerts, and also in not accessible areas by scientists, such as private ground or large landscapes {Formatting Citation}. Many citizen science programs are focused on invasive plants (Graham et al., 2011), marine animals (Clusa et al., 2018), birds (Klemann-Junior et al., 2017) and insects (Kobori et al., 2016). These studies confirm that citizen collaboration in control programs is essential for the best management of invasive species. Malek et al. (2018) compared the monitoring of stink bug by traditional methods as pheromone traps and by citizen observations. A higher number of reports was obtained through citizen science than with traditional methods of capture. In the case of *V. velutina*, a specific trap for this species has not yet been developed and for this reason citizen collaboration is crucial for its control. For example, Rome & Villemant (2017) showed how the surveillance system, based on citizen science and local networks, has made it possible to follow its spread in Europe. Nowadays, the use of smartphones is increasing and their advantages for sharing data make them a useful tool for invasive species detection (Jambeck & Johnsen, 2015; Kress et al., 2018). Vespapp has proven to be a useful means of sending out reports of possible individuals. The results of the present study show a decrease in the number of downloads in recent years, as in (Kress et al., 2018) although in 2019 there was an increase of 40.94% compared to the previous year (2018). However, after a social event, a peak of reports or Vespapp downloads was observed. This means that it is necessary to divulge the results of a control campaigns, for example in the media, workshops or scientist talks, to involve citizen in scientist studies (Newman et al., 2012). During 2017 the number of positive reports was the highest, coinciding with the highest number of removed nests since the first detection 2 years ago. These results suggest that most Majorcan people are now able to recognise individuals of *V. velutina*. When a positive report was received, the nest detection protocol was started, and nest removal was performed. Citizen reports have been essential in this campaign to control and eradicate the species as 58% of removed nests were found from citizen reports.

Another important point is the detection and destruction of nests. From our point of view, in the scenario of the early stage of invasion, active searching for nests is crucial, and the triangulation method from artificial food points was used. Recently, new methods for active searching for nests of *V. velutina* were developed and tested. Some of these are based on the radar system (Kennedy et al., 2018; Maggiora et al., 2019), thermal imaging (Lioy et al., 2021) and dron-assisted tracking (Reynaud & Guérin-Lassous, 2016). The appropriate method of destruction is essential to succeed.

The early intensive detection and control programme implemented could explain the lowest expansion rate registered for this invasive alien species worldwide (4.52 ± 0.51 km/year) in comparison with other studied areas: France (78 km/year) (Robinet et al., 2017), Portugal (37.4 km/year) (Carvalho et al., 2020), Great Britain (28 km/year) (Keeling et al., 2017), Italy (18.3 km/year) (Bertolino et al., 2016) and Korea (9.4 km/year) (Jung, 2012). Seventeen nests were removed before males emerged, reducing the number of colonies which were able to spread. Moreover, with the spring trapping we collected 25 queens, avoiding the foundation of new nests. Thus, the presence of a topographic barrier (*Serra de Tramuntana*) may represent a constraint to *V. velutina* (Robinet et al., 2017). Likewise, Carvalho et al. (2020) discuss how river valleys might be important corridors for *V. velutina*. In Majorca there are no rivers, so that can be another crucial factor for the lower expansion rate. Another important point for the success of the control plan is the fact that Majorca is an island and is situated 176 km off the mainland.

If we check the most important items that had the highest frequency of occurrence in unsuccessful actions for management of IAS (Dana et al., 2019), we can verify the viability of the eradication plan presented here. In this plan, (i) the funds are guaranteed during the necessary time frame; (ii) the risk of reinvasion is low because Majorca is an island; (iii) sufficient nest removal rate to achieve the eradication objective; (iv) evidence reporting that the methodology applied is effective; and (v) there is adaptation of the methodology every year since the IAS arrived.

Because absolute certainty of the absence of a species can only be attained by the passage of time without detection (Dahlsten & Garcia, 1989), eradication managers face a decision regarding when to dismantle an eradication program must be made despite the lack of certainty that elimination has been achieved because absolute certainty of the absence of a species can only be attained by the passage of time without detection (Morrison et al., 2007). Posteradication monitoring is an important components of success (Courchamp et al., 2011). To do this, different actions could be implemented in subsequent years: (i) sentinel traps in the same monitored area and the same density as 2020 from March; and (ii) a community information task.

Finally, the presence of *V. velutina* could lead to large economic costs related to a reduction in honey production (Monceau et al., 2013, 2014; Requier et al., 2019) and pollination (Rojas-Nossa & Calviño-Cancela, 2020), and due to the control effort (Barbet-Massin et al., 2020). In most countries the economic cost of IAS has not been quantified. In this paper we have provided information about the economic impact based on the field work campaign costs, including control costs as well as surveillance measures. Research costs and the environmental awareness campaign for public and technical analysis of the data were not taken into account in this study. The cost per year was around 33000 euros, cheaper than the cost of eradicating the species at a later stage or than the cost of removing nests in a species establishment scenario (Barbet-Massin et al., 2020; Russell et al., 2017). Early and rapid detection of individuals is a more cost-effective outcome for conservation (Holden et al., 2016). Barbet-Massin et al. (2020) provided the first estimate of economic costs resulting from the yellow-legged hornet. For example, more than 5 million euros, related to nest destruction, was estimated yearly in Germany, where *V. velutina* arrived in 2015. In France, yearly costs have increased by ~450 000 euros each year as the hornet keeps spreading and invades new areas (Barbet-Massin et al., 2020).

Conclusion

In conclusion, our findings reveal promising results about the possibility of eradicating this species in the early stages following a strategy of an active search for nests. Early detection of the invasive species is crucial to minimise its effects, and citizen science may offer an important source of information to determine the presence and distribution of *V. velutina*. The active search for nests and triangulation are essential for success in the control of *V. velutina.* Even though the efforts and costs assumed of spring trapping were high, we consider this method was justified and worthwhile at this stage of control of *V. velutina.* For the future, it is vital to work in three areas: (i) post-eradication monitoring; (ii) genetic characterization of the Balearic population of *V. velutina* to determine the path of introduction; and (iii) enforcement of preventative measures by establishing stringent biosecurity policies at the national, regional and global scale. These activities will contribute to the effective implementation of the current legislation at the EU (Regulation (EU) No 1143/2014) and national level (RD 630/2013). The findings we present here could contribute to advance the science of eradication of IAS.

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AUTHOR CONTRIBUTIONS

All authors conceived the ideas and designed methodology. ML, GP, TM, and VC collected the data. ML, CH, and GP analysed the data. ML, CH, and GP led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Chapter 2

Invasion genetics of the yellow-legged hornet *Vespa velutina* in the Westernmost Mediterranean archipelago

Content of this chapter is submitted as:

Herrera, C., Ferragut, J.F., Leza, M., & Jurado-Rivera, J.A. Invasion genetics of the yellow-legged hornet *Vespa velutina* in the Westernmost Mediterranean archipelago. *Paper submitted*.

Abstract

The yellow-legged hornet (*Vespa velutina*) is a social Hymenoptera native from Asia and an invasive species in Europe, where it was first detected in France in 2004. Since then, the species has spread across the continent invading mainland Spain and Mallorca island (Balearic archipelago, Western Mediterranean) in 2010 and 2015, respectively. Yellow-legged hornets cause severe damage to ecosystem by predating over a wide variety of pollinators including honeybees. Such a threat situation requires the development of effective management and prevention plans, which can greatly benefit from knowing both the origin and the genetic structure of the invading populations. Here we conduct a genetic study to shed light on both the origin and the phylogenetic relationships of *V. velutina* populations from Mallorca and mainland Spain using nuclear (STRs) and mitochondrial (cytochrome oxidase c subunit 1) gene markers. Our results show that Mallorca populations originated from invasive European specimens. Moreover, F_{ST} values, DAPC and genetic structure analysis suggest two independent incursions in the island with bottleneck and founder effect signatures. Finally, we contribute additional genetic evidence of the polyandrous behaviour of this invasive species based on the inference of a mean number of mattings per nest of 3.94 (range 2 – 6.5). This study supports the human-mediated pathways of this species and highlights the importance of implementing effective biosecurity measures to prevent the spread of invasive alien species in island habitats.

Keywords: *Vespa velutina*, cox1, STRs, invasion genetics, Mallorca, Spain.

Introduction

Social insects have high invasion capacities (Beggs et al., 2011) mainly due to the ability of single foundress to establish a huge colony and to produce many future queens which in turn offer a large dispersal capacity. Such colonization abilities can be further enhanced by global trade and human mobility (Arca et al., 2015; Mikheyev et al., 2009). The main ecological effects of invasions include the impact on biodiversity (Vilà et al., 2011) and ecosystem functioning (Pejchar & Mooney, 2009), with island environments being more vulnerable than continental systems due to their lower complexity, high endemicity rates and less diversity levels (Sax & Gaines, 2009; Traveset & Richardson, 2006, 2014).

The yellow-legged hornet (*Vespa velutina*) displays unique biological and life history traits that increase the probability of success in establishing and spreading in new territories (Moller, 1996), including excellent dispersal capacities, high reproductive rates, broad diets and habitat ranges, effective predator defences, and superior competitive skills (Beggs et al., 2011; Moller, 1996). Consistently, this species is known to be the first invasive Vespidae predator accidentally introduced from Asia to Europe (Monceau et al., 2014). It was detected in France in 2004 from where the species has successfully spread and stablished in neighbouring countries (Laurino et al., 2020). It was first reported from Spain in 2010 (López et al., 2011) where their populations have colonized all north Iberian peninsula arriving to Mallorca island (Balearic Islands, Western Mediterranean, Spain) in 2015 (Leza et al., 2021) probably due to an accidental introduction by humans (Robinet et al., 2019).

The first report of *V. velutina* in Mallorca leaded to the implementation of an intensive control protocol that allowed the removal of thirty-one nests and the capture of sixteen queens using spring trapping between 2015 to 2018 (Leza et al., 2021). After these interventions, no more nests were found and the species was declared officially eradicated in November 2020 (CAIB, 2019) until July 2021 when a new secondary nest was detected and removed in the island. Since then, neither adults nor nest have been detected in the archipelago.

While there is a wealth of studies on different aspects of the biology, ecology and management of *V. velutina* in Europe, only a few of them have focused on the genetics of the invasion (Arca et al., 2015; Budge et al., 2017; Granato et al., 2019; Jones et al., 2020; Quaresma et al., 2022). These studies have evidenced that the origin of the European invasion by yellow-legged hornets represented a genetic bottleneck event that explains their low levels of genetic diversity, probably as the result of a single queen introduction from southeast China (Arca et al., 2015). Nonetheless, this has not meant any limitation for the species to expand throughout the European continent. Such invasive capacity was also observed in Mallorca, where both the number of reported nests and the colonized area gradually increased since its detection until its eradication, after a potential population bottleneck event (Leza et al., 2021).

In this study, we expand the current knowledge on the invasion genetics of *V. velutina* in Europe based on mitochondrial (*cox1*) and nuclear (STRs) DNA analyses of the invading populations in the westernmost Mediterranean archipelago.

Material and methods

Sampling and DNA extraction

A total of 335 adults of *V. velutina* were collected in Spain: Asturias (N = 11), Basque Country (N = 20), Catalonia (N = 16), Extremadura (N = 2), Galicia (N = 12) and Mallorca (N = 274) (Figure 6, Table S1). Among sampled populations, only two individuals could be obtained from Extremadura (Spain) being the first detection of this invasive species in Extremadura. This particular sample was however retained in the analyses to increase the coverage of hornet invasive range. Queen individuals were collected from traps, workers were obtained from both traps and nests, while males were obtained directly from nests. Samples were stored in absolute ethanol and/or at -20ºC until molecular analysis.

Genomic DNA was individually extracted and purified from insect legs using the Qiagen DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's instructions and RNA was removed using 60 µg of RNAse A (Promega).

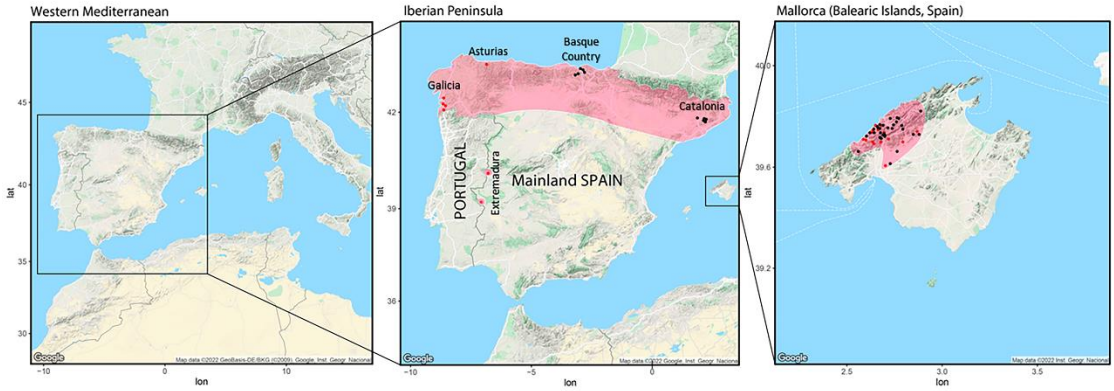

Figure 6.- Maps of the Iberian Peninsula and Mallorca showing the location of the *Vespa velutina* individuals sampled from nests (black dots) and from traps (red dots), respectively. Pink-shaded areas indicate the invaded territories in Spain according to Laurino et al., (2020). Maps were obtained with Google Maps (Map data 2020, Google) using the function 'get_map' in the package 'ggmap' version 3.0.0.902 in R version 3.6.3.

Mitochondrial DNA sequencing and analysis

Genetic analyses based on mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene sequences were conducted on a sub-sample of 103 *V. velutina* individuals from Asturias (n = 10), Basque Country (n = 15), Catalonia (n = 15), Extremadura (n = 2), Galicia (n = 12), and Mallorca (n= 49). Such analyses included all individuals from mainland Spain, at least one individual from each nest detected in Mallorca and all trapped queens. Therefore, we cover all potential *cox1* diversity present on the island. For each specimen, a *cox1* gene fragment was PCR-amplified and sequenced using a custom designed *V. velutina* specific primer-pair Vespa_LCO (ATTCAACAAATCACAAAGATATTGG) / Vespa_HCO (TAAACTTCTGGATGTCCAAAGAATCA) targeting the standard DNA barcoding 658 bp region. Primers were designed based on available *V. velutina* complete mitochondrial genome

sequences in GenBank (accession codes AP017943, AP018460, AP018461, AP018483, AP018484 and KY091645). PCR conditions used 0.2 µM of each primer and 3.5 mM $MgCl_2$ (50 mM) using a standard protocol with an initial denaturation stage at 94ºC (4 min.), followed by 40 cycles with annealing temperature at 48 ºC (30 sec.), denaturation at 94 °C (30 sec.) and elongation at 72 °C (60 sec), and a final extension step of 10 min. at 72ºC. PCR products were checked for amplification by electrophoresis on 1% agarose gel stained with ethidium bromide and subsequently purified using the MSB Spin PCRapace kit (Invitek). Sanger sequencing was performed with the same primers as above using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Electropherograms were assembled and edited with CodonCode Aligner (CodonCode Corporation) and their respective identity as *V. velutina cox1* sequences was assessed by comparing them with those available in GenBank using the blastn online tool (https://blast.ncbi.nlm.nih.gov/).

Newly generated *cox1* sequences were aligned using MAFFT 7 (Katoh et al., 2018) under default options with all available *V. velutina cox1* sequences in GenBank. A median joining haplotype network was computed using the popART 1.7 software (Leigh & Bryant, 2015). In addition, the same DNA alignment was also used to infer a maximum likelihood (ML) phylogenetic tree in IQ-TREE v 1.6.12 (Nguyen et al., 2015) by simultaneously searching for the best evolutionary model according to Bayesian Information Criterion (TN+F+G4) and specifying 1000 bootstrap replicates. The resulting tree was visualized with FigTree v1.4.4 (Rambaut, 2018).

STR amplification and analysis

All sampled individuals (335) were genotyped for 15 microsatellite loci using oligo combinations developed by previous studies on *V. velutina* population genetics (Arca et al., 2012; Daly et al., 2002; Hasegawa & Takahashi, 2002). Forward primers were labelled with 6-FAM (blue), VIC (green), NED (yellow) and PET (red) dyes and the PCR amplifications were segregated into two multiplex reactions (Table S2). PCR reactions were performed in a total volume of 10 µL including 1 µL of *V. velutina* template DNA, 5 µL of Multiplex PCR kit (Qiagen), 3.6 µL of primer mix (10µM) and 0.4 µL of H_2O. The PCR conditions consisted of an initial denaturation step at 95 ºC (15 min.) followed by 40 cycles at 94 ºC (30 sec.), 50 ºC (90 sec.) and 72 ºC (60 sec.), followed by a final extension stage at 60 ºC (30 min.). Fragment analysis was performed on an ABI Prism 3130 DNA Genetic Analyzer (Applied Biosystems, Foster, California, USA), using GeneScan 500LIZ® as internal size standard. Fragment lengths were determined using GENEMAPPER 5.0 (Applied Biosystems) and checked manually.

The microsatellite dataset generated from the 335 *V. velutina* individuals was merged with an existing dataset containing genotypes from 417 individuals collected in other invaded territories (mainland Spain: Basque Country = 3 and Galicia = 42, France = 83, Italy = 11, Portugal = 191, and Korea = 8) and also from the native range of the species (Chinese provinces of Yunnan = 20 and Zhejiang/Jiangsu = 30, Indonesia = 21, and Vietnam = 8) (Arca et al., 2015; Quaresma et al., 2022). To enable dataset merging, allele scores of each microsatellite locus were harmonized between laboratories by genotyping 10 DNA samples used by Quaresma et al. (2022), which in turn were previously harmonized with Arca et al. (2015). Although the studies by Budge et al. (2017) and Jones et al. (2020) also generated *V. velutina* STR data, we opted for the conservative approach of not including them in our analyses since they were not harmonized with other studies.

Observed number of alleles (Na), observed heterozygosity (Ho) and expected heterozygosity (He) were computed per locus and region using ARLEQUIN 3.5.2.2 (Excoffier et al., 2005) whereas unbiased expected heterozygosity (uHe) was estimated using the formula uHe = He*(2*n_individuals/(2*n_individuals-1)) (Nei, 1978) and allelic richness (Ar) was estimated using the R package *pegas* (Paradis, 2010), using the rarefaction method described by (Haulbert, 1971). In addition, inbreeding coefficient (*f*) was calculated using the formula *f* = (uHe-Ho)/uHe, where positive values represent an excess of homozygotes and negative values represent excess of heterozygotes (Li & Horvitz, 1953). For each population, the mean and standard deviation of the above-mentioned genetic parameters were calculated to enable comparisons. Genetic differentiation among populations from the invaded and native ranges was estimated based on the pairwise F_{ST} values computed in ARLEQUIN 3.5.2.2 and using 10000 random permutations to assess significance.

Population structure and relationships

The population structure was analysed using the fast maximum-likelihood genetic clustering approach (Beugin et al., 2018). This approach is very similar to the model implemented by STRUCTURE (Pritchard et al., 2000) but allows for much faster estimation of genetic clusters thanks to the use of the Expectation-Maximization (EM) algorithm. We initially investigated the number of clusters by using the k-means algorithm. The preferred number of clusters was evaluated using the Bayesian information criterion (BIC) scores.

A discriminant analysis of principal components (DAPC) was also used to infer population structure, sugin the *adegenet* 2.1.6 R package (Jombart, 2008). This multivariate analysis combines principal component analysis (PCA) and discriminant analysis to determine the number of genetic clusters in the sample. Moreover,

The program GeneClass v.2.0 (Piry et al., 2004) was used to assign or to exclude reference populations from either the invaded or the native ranges as possible sources of the Mallorcan individuals on the basis of multilocus genotypes by using the standard criterion described by Rannala and Mountain (1997). The Monte Carlo resampling method (Paetkau et al., 2004) was also applied to identify the accurate critical values of exclusion/inclusion by simulating 10.000 genotypes for each population with a threshold probability value of 0.05.

Bottleneck detection

The occurrence of recent genetic bottlenecks in Mallorca populations was explored with the test of heterozygosity excess implemented in BOTTLENECK 1.2.0.2 (Cornuet & Luikart, 1996; Piry et al., 1999). Heterozygosity excess is expected in populations that have experienced a significant reduction in size because rare alleles are lost (Cornuet & Luikart, 1996). Two mutation models were tested: the infinite allele model (IAM) and the two-phase model (TPM), the latter of which incorporates elements of the IAM and stepwise mutation model (SMM) (variance = 12, SMM = 95 %, Piry et al. 1999). The Wilcoxon sign- rank test was used to test for a statistically significant bottleneck (Luikart & Cornuet, 1998).

Mating statistics

Both queen and mate genotypes were inferred from worker offspring genetic data using Colony 2.0.1.1 (Wang & Santure, 2009) based on the 15 STR loci from Mallorca population. When available, males were also genotyped and known queen alleles were inferred since the haploid genotypes of males are the direct product of queen meiosis. Four trials with assumed genotyping error rates of 0.001, 0.01, 0.05, and 0.1 were performed. The allele frequencies inferred from Mallorca population between 2015 and 2021 were used as input.

Results

Mitochondrial DNA sequencing and analysis

All specimens sampled from Mallorca and mainland Spain (Asturias, Basque Country, Catalonia, Extremadura and Galicia) shared the same *cox1* haplotype (GenBank accession codes: ON094523 to ON094624), which was identical to the haplotype shared by all European *V. velutina* invaders sequenced to date and also by the specimens sampled in Jiangsu and Zhejiang (China) (Haplotype H1, Figure 7). Moreover, our phylogenetic analysis also revealed a possible new haplotype from China (GenBank: MN716845) (Figure 7). The possibility that the number of haplotypes is greater than that currently available in GenBank is consistent with the study by Perrard et al. (2014), which describe additional mtDNA haplotypes of *V. velutina* raising the total number to 25. However, these haplotypes are not available in GenBank and therefore have not been included in our analyses.

The phylogenetic analyses based on *cox1* sequences showed *V. velutina* divided into two main clades (Figure 7-A) in agreement with previous reports (Granato et al., 2019; Perrard et al., 2014; Takeuchi et al., 2017). The inferred tree supported the results derived from the network analysis (Figure 7-B) and showed the Spanish samples nested within the European invasion clade.

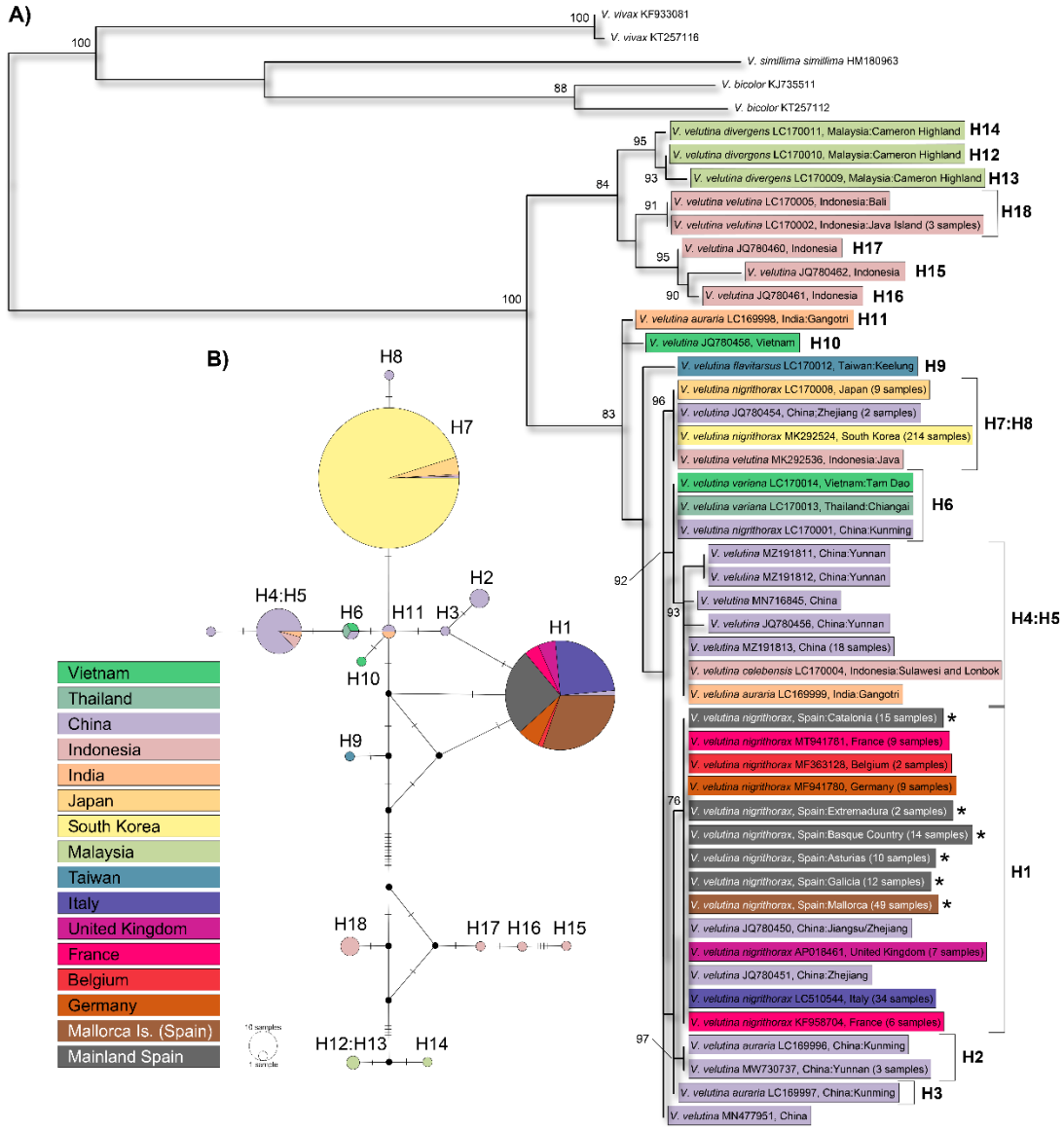

Figure 7.- A) Maximum Likelihood tree (log-likelihood = -1942.5559) computed using the TN+F+G4 evolutionary model selected by the ModelFinder algorithm (Kalyaanamoorthy et al., 2017) implemented in the IQ-TREE software. Node numbers represent ultrafast bootstrap support (only values >75 are shown). Samples contributed by this study are marked with asterisk symbols. B) Median joining network of *V. velutina* cox1 haplotypes inferred in PopART. The pie charts display haplotype frequencies in each sampled region with size proportional to the number of sequences included (see scale at the bottom left). Missing haplotypes and mutational steps are indicated with solid black circles and hatch marks, respectively.

STR amplification and analysis

The mean (± SE) number of alleles (Na), allelic richness (Ar), and heterozygosities (Ho, He) computed for each sampled region of the invaded and native ranges in Europe and Asia are shown in Figure 8.

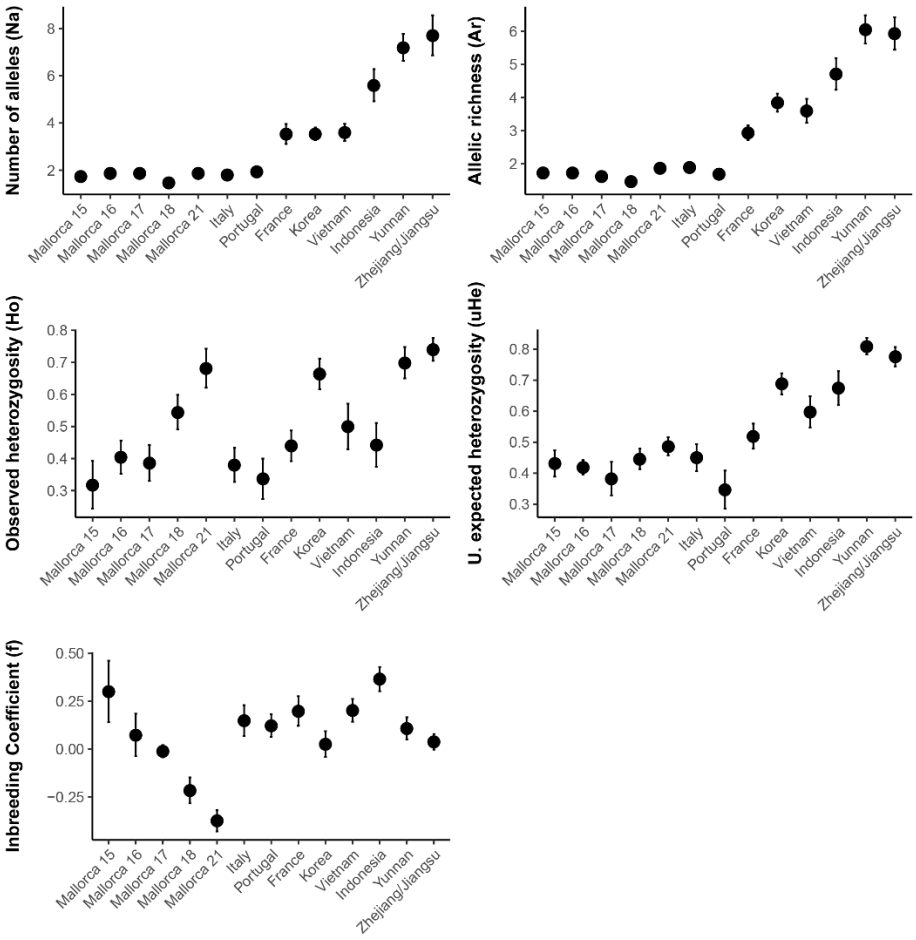

Figure 8.- Mean and standard error of number of alleles (Na), allelic richness (Ar), heterozygosities (Ho, uHe) and inbreeding coefficient (*f*) for each studied population from native and invaded range of *V. velutina*. Numbers following Mallorca codes refer to the year of collection (e.g., Mallorca 15 stands for samples collected in Mallorca in 2015).

The 15 microsatellite loci represented 180 different alleles, of which 174 were present in Asia and 64 in Europe. The number of private alleles was 116 and 6 in the native range and Europe, respectively. All loci from Asia were polymorphic, whereas in mainland Europe two loci were monomorphic (D2-142 in Portugal, mainland Spain, Italy and France, and R1-77 in mainland Spain), five loci were monomorphic in Mallorca between 2015 and 2018 (D2-142, D2-185, R1-77 and R1-169) and three loci were monomorphic in Mallorca 2021 (R1-80, D2.142 and R1-77).

Population structure and relationships

There was considerable divergence among populations: pairwise F_{ST} ranged from 0.0001 to 0.6366 and all values were statistically significant (*p-value* < 0.05), except between Mallorca 2015 and 2016 which was not different from zero (*p-value* = 0.43) (Figure 9). Generally, higher F_{ST} values corresponded to comparisons between native and invasive populations (excepting the pair Korea - Zhejiang/Jiangsu). Regarding the Mallorcan populations, all pairwise comparisons between samples from 2015 to 2018 yielded low F_{ST} values, in contrast with the higher F_{ST} values

retrieved from the analyses between them and Mallorca 2021 (range: 0.4378 to 0.465). According to F_{ST} values, the closest European population to the group conformed by Mallorca 2015 to 2018 was the Italian one (range: 0.1791 to 0.3392), while Mallorca 2021 was retrieved as closely related to *V. velutina* individuals from Catalonia (0.1822).

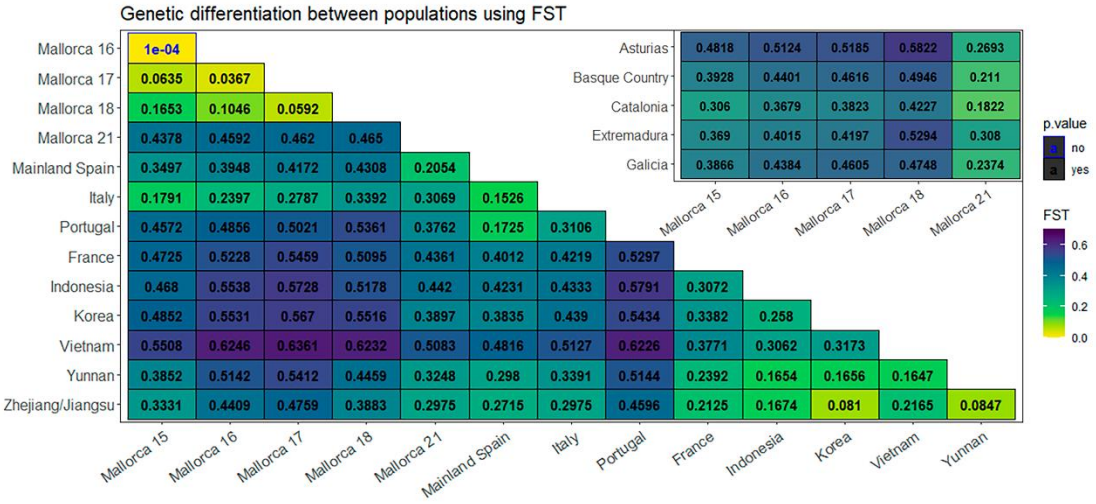

Figure 9.- Multi-locus estimates of pairwise F_{ST} genetic distances between *V. velutina* populations from both invaded and native ranges (lower diagonal) and between Mallorca populations and Mainland Spain populations (top right). Numbers following Mallorca codes refer to the year of collection (e.g., Mallorca 15 stands for samples collected in Mallorca in 2015).

DAPC analyses clearly separated Asian and European populations (Figure 10). Within the latter, the French populations were retrieved as the closest samples to the Asian ones (Figure 10A). Consistent with F_{ST} estimates, Mallorcan populations between 2015 and 2018 were located near to the Italian samples, and the Mallorcan population 2021 near to *V. velutina* individuals from mainland Spain (Figure 10B). The DAPC plot explained 93% of the total variance.

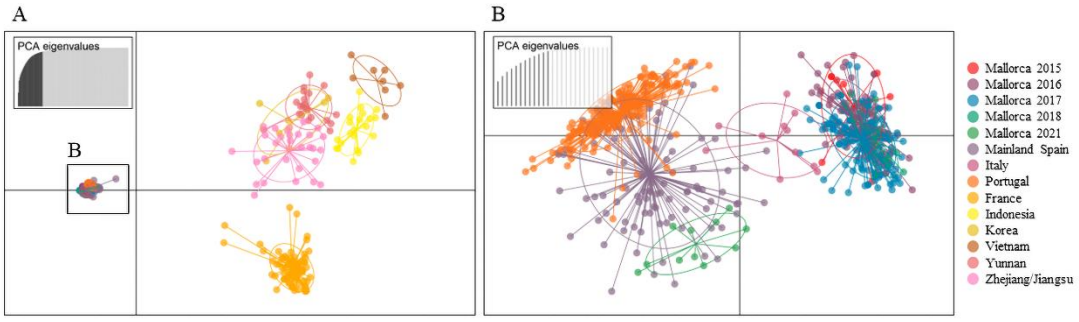

Figure 10.- Scatterplot of the discriminant analysis of principal components (DAPC) of A) Asian populations, France, and B) magnified view of other European countries. Individuals genotypes are represented in dots, while populations are distinguished by colours and 95% inertia ellipses. The DAPC plot explained 93% of the total variance.

A pattern of genetic heterogeneity within the European range was detected by the Maximum-Likelihood genetic clustering using the expectation-maximization algorithm implemented in the *adegenet* package (Fig. 6). At K = 2, there was a clear separation between native plus France populations from the rest of the invasive populations in Europe. Further partitioning of variation (K from 3 to 5) revealed different genetic structures in Europe (Figure 11). K = 5 showed that the invasion of Mallorca between 2015 and 2018 had a differentiated genetic structure (mean Q-value ± SE = 99.99 ± 0.0006%) that partially includes the Italian population (mean Q-value ± SE = 40.66 ± 12.47%). In the other hand, the Mallorcan population from 2021 (mean Q-value ± SE = 99.99 ± 0.0000007%) clustered with samples from mainland Spain (mean Q-value ± SE = 67.22 ± 4.42%). In addition, Portuguese (mean Q-value ± SE = 97.87 ± 1.16%), French (mean Q-value ± SE = 99.99± 0.00003%) and Asian populations (mean Q-value ± SE = 99.99 ± 0.00007%) conformed three distinct clusters. Likewise, DAPC analyses identified the Asian, French, Atlantic (Portugal + Spain), Mediterranean (Spain + Italy) and Mallorca as differentiated genetic entities (Figure 11).

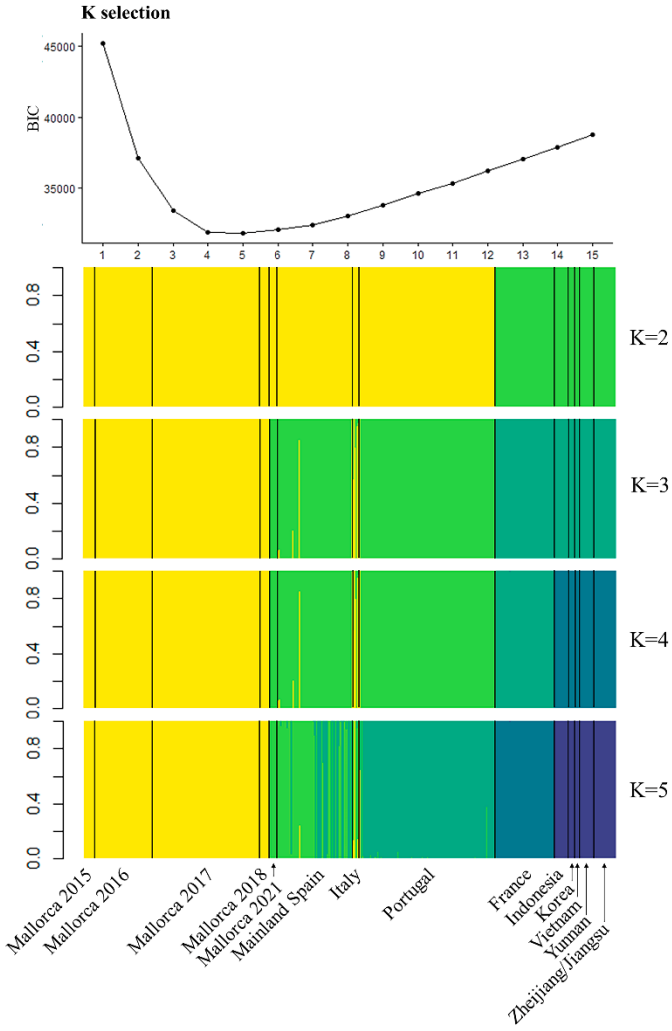

Figure 11.- Genetic structure of *V. velutina*. Populations admixture ancestry components are shown from K = 2 to K = 5 for the 752 individuals. The lowest Bayesian information criterion (BIC) was obtained at K = 5. Populations are delimited by black borders.

Notably, both DAPC (Figure 10) and admixture (Figure 11) analyses supported the genomic distinctiveness of Mallorca population between 2015 and 2018 from all other *V. velutina* populations.

One individual from Mallorca 2015 was detected by GENECLASS as first-generation migrant (*p-value* = 0.004) with a putative origin in Italy (log(L) = -6.804). Likewise, an individual from Mallorca 2017 was also detected as first-generation migrant (*p-value* < 0.001) with a putative origin located in mainland Spain (log(L) = -8.926).

Since F_{ST} values, DAPC and genetic structure analyses suggest two different introductions from mainland Europe in Mallorca, invasive populations sampled in the island were assigned by GENECLASS to two different regions: Mallorcan samples between 2015 and 2018 were assigned to Italy (score = 100%), and those from 2021 were assigned to mainland Spain, specifically Catalonia (score = 100%).

Bottleneck detection

The decrease in genetic diversity observed in the invasive populations may be indicative of a bottleneck event (Arca et al., 2015). In this regard, the heterozygosity excess indicating a genetic bottleneck was tested in Mallorca populations between 2015 and 2021. The results were consistent with a bottleneck episode according to the significant value of heterozygosity excess estimated under both IAM and TPM models (*p-value* < 0.05).

Mating statistics

Depending on the assumed genotyping error rate, the total inferred number of fathers varied from 1 to 10 over the Mallorcan nests studied. On the one hand, all nest from 2015 to 2018 (first invasion) presented different patrilines resulting in a mean number of observed mattings per nest of 3.94 (range 2 – 6.5). On the other hand, a single patriline was detected in the only nest found in 2021 (second invasion). Diploid males were detected in two nests collected in 2017 and 2021, respectively.

Discussion

Our results are compatible with a scenario in which *V. velutina* populations in both mainland Spain and the Balearic archipelago could have derived from the spreading southward of the *V. velutina* population initially established in France, rather than from multiple independent introductions from the native range. This view is supported by the identification of a single mitochondrial haplotype shared by all our samples and those previously reported from the remainder of the invaded territories in Europe (Arca et al., 2015; Budge et al., 2017; Dillane et al., 2022; Granato et al., 2019; Husemann et al., 2020; Jones et al., 2020; Quaresma et al., 2022). Furthermore, the European origin of the invasions of both mainland Spain and the Balearic archipelago is reinforced by nuclear STR data revealing the existence of a shared allelic pool and similar genetic diversity levels (Na and Ar) (Arca et al., 2015; Budge et al., 2017; Jones et al., 2020; Quaresma et al., 2022). Indeed, the F_{ST} values reported here indicate that Mallorca populations are closely related to representatives from Italy (samples Mallorca 2015 to 2018) and

Catalonia (single nest found in Mallorca in 2021), whereas mainland Spain colonies show closer genetic affinities with those from Italy and Portugal.

The invasion of Mallorca Island by *V. velutina* is compatible with a bottleneck event. The estimated excess of observed heterozygosity (Ho) and reduced inbreeding coefficient (*f*) can lead to a decrease in terms of both adaptive potential and fitness of a founding population, therefore compromising its establishment and spread across new ranges (Willi et al., 2006). However, there exists evidence that *V. velutina* populations can establish successfully even after a severe genetic bottleneck resulting from the introduction of very few or even a single female (Arca et al. 2015). The case of the invasion of Mallorca reported here reinforce this view, since even after a potential second bottleneck the invasive population of *V. velutina* kept growing after the first detection despite eradication efforts (Leza et al., 2021). In fact, the increase in observed heterozygosity detected on consecutive populations between 2015 and 2018 could be explained by the control and eradication efforts applied in the island (Leza et al., 2021). The eradication plan focused on entire nest removal before male emergences, therefore preventing reproduction and lowering the genetic diversity levels of the population and increasing the bottleneck event and founder effect on the next generations.

Genetic diversity of founders is critical for the adaptative response to new environments (Drake & Lodge, 2006; Lockwood et al., 2005). In this regard, the polyandrous mating system of *V. velutina* allowed the introduction of an important fraction of the genetic diversity of the native population into Europe (Arca et al., 2015). Here we contribute additional genetic evidence of the polyandrous behaviour of this invasive species based on the inference of up to 10 potential males fecundating a single queen. High allelic diversity in the founding individuals coupled with other extraordinary life history traits common to social Hymenoptera (Beggs et al., 2011; Moller, 1996) probably helped spread of this invasive species after a new bottleneck event in Mallorca. According to our genetic results and ecological niche models developed specifically for the island (Herrera et al., 2023), if an early detection and eradication plan would not have been implemented (Leza et al., 2021) the species probably would have colonized and stablished in part of the island, as predicted by Robinet et al. (2019).

Bottleneck events, inbreeding, limited gene flow and genetic drift reduce sex allele diversity (Darrouzet et al., 2015). As well as other Hymenoptera species, *V. velutina* sex is determined by a multiallelic locus in which females are diploid (heterozygous genotype) and males are haploid (hemizygous genotype) (Heimpel & De Boer, 2008). When allelic diversity is low at the sex locus, there is a higher chance that the queen will produce homozygous eggs, which will develop into diploid males (Darrouzet et al., 2015; Quaresma et al., 2022) that are usually sterile and do not contribute to colony tasks. In this study we report two nests hosting diploid males (7.69% of all sampled nests) after a bottleneck event. This value is considerably lower than the ratios reported by studies conducted in United Kingdom and France, where diploid males represented 75% and 48% of all nests genotyped, respectively (Budge et al., 2017; Darrouzet et al., 2015).

Our case study represents the first time that the yellow-legged hornet reaches a Mediterranean island after its arrival to Europe in 2004 (Monceau et al., 2014). In fact, the genetic results presented in this study shows that this species has reached Mallorca in two different moments from two different European regions, respectively: Italy (2015) and Mainland Spain (2021). Therefore, all nests identified and eliminated in Mallorca during the eradication efforts can be attributed to a single introduction that was initially detected in 2015. Fortunately, thanks to an observant citizen, the second introduction in 2021 was swiftly detected, enabling the competent administration to promptly locate the nest and sent it to the University of the Balearic Islands for

subsequent analyses. Given that the nest hosted male specimens, two possible scenarios were confronted: there was already a reproductive caste established indicating that the species was already dispersing, or the male specimens were sterile. Each of these scenarios could have distinct implications for the management tasks associated with controlling this invasive species. Consequently, a genetic analysis was conducted on male hornets using 15 STRs microsatellite markers revealing that all specimens were diploid, and therefore sterile (Heimpel & De Boer 2008; Darrouzet et al. 2015). The employed methodology provided valuable insights into the potential dispersal of this species, enabling us to predict whether the competent administration should be prepared to detect and eradicate new nests in the upcoming years in the way described by (Leza et al. 2021).

Robinet et al. (2019) explored the human-mediated dispersal of *V. velutina* concluding that the Mediterranean islands could not be naturally colonized, and only an anthropogenic introduction could favour the colonization of such territories. Mallorca island is 176 km away from the mainland nearest point, so the most probably introduction could be through shipping traffic. The genetic structure analyses performed here support the pathways of this species and assign French populations to a cluster that is more closely related to Asian populations than to the rest of European populations, in contrast with other genetic studies (Arca et al., 2015; Budge et al., 2017; Quaresma et al., 2022). Interestingly, the individuals sampled in Mallorca clustered into a well differentiated position from the rest of European or Asian clusters, which is consistent with the bottleneck event inferred by our analyses. This result may indicate that the diversity loss of invasive populations of *V. velutina* may vary across geographic range as documented for numerous animal and plant invasions (Forsman, 2014).

Our findings reveal the pathways used for this species to reach new isolated territories as the Mediterranean islands. Such a threat situation requires the development of effective management and prevention plans, which can greatly benefit from knowing both the origin and the genetic structure of the invading populations.

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AUTHOR CONTRIBUTIONS

CH, ML and JAJ-R conceived the study; CH and ML provided *Vespa velutina* samples; CH, JF and JAJ-R designed the experiments; CH, JF and JAJ-R performed the experiments and analysed the data; CH and JAJ-R performed phylogenetic analyses, analysed the data and created the figures; CH wrote the original draft, and all authors commented and contributed to the final manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

Table S1.- Sampling data and genotypes for the 335 samples analysed in this study. Basque C. = Basque Country.

Area	Sampled year	ID Nest	ID Queen	Sexe	ID Sample	R1_80	R4_33	R1_36	R4_100	D2_142	D2_185	R4_114	VMA_8	R1_75	VMA_6	D3_15	R4_26	LIST2020B	R1_77	R1_169
Mallorca	2015	1D	-	Female	WASP_149	110	209	99	180	153	214	128	251	144	240	160	250	195	253	158
						110	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2015	1	-	Female	WASP_002	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2015	1	-	Female	WASP_005	110	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	107	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2015	1	-	Female	WASP_008	110	209	99	180	153	214	128	251	144	244	160	250	189	253	158
						110	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2015	1	-	Female	WASP_007	110	209	99	180	153	214	128	251	144	244	160	250	189	253	158
						110	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2015	1	-	Female	WASP_150	110	209	99	180	153	214	128	251	144	244	160	252	189	253	158
						110	209	107	184	153	214	128	251	152	244	170	252	195	253	158
Mallorca	2015	1	-	Male	WASP_151	108	209	99	184	153	214	128	251	144	242	170	250	189	253	158
						108	209	99	184	153	214	128	251	144	242	170	250	189	253	158
Mallorca	2015	1	-	Male	WASP_152	110	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	180	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2015	1	-	Male	WASP_153	108	209	99	180	153	214	138	267	144	240	170	250	189	253	158
						108	209	99	180	153	214	138	267	144	240	170	250	189	253	158
Mallorca	2015	1	-	Male	WASP_154	110	209	99	180	153	214	128	251	144	240	170	250	195	253	158
						110	209	99	180	153	214	128	251	144	240	170	250	195	253	158
Mallorca	2015	1	-	Male	WASP_155	110	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						110	209	99	180	153	214	128	251	144	242	170	250	189	253	158
Mallorca	2016	1	-	Female	WASP_009	110	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						110	209	99	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_156	110	209	99	184	153	214	128	251	144	240	160	250	195	253	158
						110	209	107	184	153	214	138	251	144	242	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_157	110	209	99	180	153	214	128	251	144	240	160	250	195	253	158
						110	209	99	184	153	214	138	267	152	242	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_158	110	209	99	184	153	214	128	251	144	240	170	250	195	253	158
						110	209	99	184	153	214	138	251	152	242	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_159	110	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_160	110	209	99	184	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	138	267	144	244	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_161	110	209	99	184	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_162	?	209	?	184	153	214	128	251	144	242	170	250	195	253	158
						?	209	?	184	153	214	138	251	144	244	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_163	110	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	184	153	214	138	251	144	244	170	250	195	253	158
Mallorca	2016	1	-	Female	WASP_164	110	209	99	184	153	214	128	251	144	242	162	250	195	253	158
						110	209	99	184	153	214	138	251	144	244	170	250	195	253	158
Mallorca	2016	2	-	Female	WASP_010	110	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						110	209	99	184	153	214	138	251	152	244	170	250	189	253	158
Mallorca	2016	2	-	Female	WASP_165	110	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	2	-	Female	WASP_166	110	209	99	180	153	214	128	251	144	242	160	250	189	253	158

						110	209	99	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2016	2	-	Female	WASP_167	110	209	99	180	153	214	128	251	144	242	162	250	189	253	158
						110	209	99	184	153	214	138	251	144	244	170	250	189	253	158
Mallorca	2016	2	-	Female	WASP_168	110	209	99	184	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2016	2	-	Female	WASP_169	110	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	138	251	152	244	170	250	189	253	158
Mallorca	2016	2	-	Female	WASP_170	110	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	2	-	Female	WASP_171	110	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						110	209	99	184	153	214	128	267	152	244	170	250	189	253	158
Mallorca	2016	2	-	Female	WASP_172	110	209	99	180	153	214	128	251	144	242	162	250	189	253	158
						110	209	107	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	2	-	Female	WASP_173	110	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	128	267	144	244	170	250	189	253	158
Mallorca	2016	3	-	Female	WASP_006	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	99	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_174	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						110	209	99	180	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_175	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_176	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_177	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_178	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_179	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						108	209	107	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_180	108	209	99	180	153	214	128	251	144	242	162	250	189	253	158
						108	209	107	180	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_181	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2016	3	-	Female	WASP_182	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	180	153	214	138	267	144	244	170	250	195	253	158
Mallorca	2016	4	-	Female	WASP_004	110	209	99	180	153	214	128	267	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	4	-	Female	WASP_183	110	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	4	-	Female	WASP_184	110	209	99	180	153	214	128	267	144	242	160	250	189	253	158
						110	209	107	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2016	4	-	Female	WASP_185	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	267	144	242	170	250	195	253	158
Mallorca	2016	4	-	Female	WASP_186	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	4	-	Female	WASP_187	110	209	99	180	153	214	128	267	144	242	160	252	189	253	158
						110	209	99	184	153	214	128	267	152	242	170	252	195	253	158
Mallorca	2016	4	-	Female	WASP_188	110	209	99	180	153	214	128	251	144	242	160	252	195	253	158
						110	209	99	180	153	214	128	267	152	244	170	252	195	253	158
Mallorca	2016	4	-	Female	WASP_189	108	209	99	180	153	214	128	251	144	242	160	252	189	253	158

						110	209	107	184	153	214	128	267	144	244	170	252	195	253	158
Mallorca	2016	4	-	Female	WASP_190	108	209	99	180	153	214	128	267	144	242	170	250	189	253	158
						110	209	107	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	4	-	Female	WASP_191	110	209	99	180	153	214	128	267	144	242	160	252	195	253	158
						110	209	99	184	153	214	128	267	152	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_001	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						108	209	99	200	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2016	5	-	Female	WASP_192	108	209	99	180	153	214	128	251	144	242	160	252	195	253	158
						110	209	99	184	153	214	128	267	152	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_193	108	209	99	180	153	214	128	251	144	242	160	252	195	253	158
						110	209	99	180	153	214	138	267	144	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_194	108	209	99	180	153	214	128	251	144	242	170	252	195	253	158
						110	209	107	180	153	214	128	267	152	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_195	108	209	99	180	153	214	128	251	144	242	160	252	195	253	158
						110	209	107	180	153	214	138	267	144	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_196	108	209	99	180	153	214	128	251	144	242	160	252	195	253	158
						110	209	107	184	153	214	138	267	144	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_197	108	209	99	180	153	214	128	251	144	242	160	252	195	253	158
						110	209	107	184	153	214	128	267	152	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_198	108	209	99	180	153	214	128	251	144	242	160	252	195	253	158
						110	209	99	180	153	214	138	267	152	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_199	108	209	99	180	153	214	128	251	144	242	160	252	195	253	158
						110	209	107	184	153	214	128	267	144	244	170	252	195	253	158
Mallorca	2016	5	-	Female	WASP_200	110	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	107	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	6	-	Male	WASP_003	108	209	107	180	153	214	128	267	144	244	160	250	189	253	158
						108	209	107	180	153	214	128	267	144	244	160	250	189	253	158
Mallorca	2016	6	-	Female	WASP_087	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	107	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2016	6	-	Female	WASP_088	108	209	99	180	153	214	128	251	144	244	170	250	189	253	158
						110	209	107	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2016	6	-	Female	WASP_201	108	209	99	180	153	214	128	251	144	244	160	252	189	253	158
						108	209	99	180	153	214	128	267	152	244	170	252	195	253	158
Mallorca	2016	6	-	Female	WASP_202	108	209	99	180	153	214	128	251	144	244	160	252	195	253	158
						110	209	99	180	153	214	128	267	144	244	170	252	195	253	158
Mallorca	2016	6	-	Female	WASP_203	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	107	180	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2016	6	-	Male	WASP_204	110	209	107	180	153	214	128	251	152	244	170	250	189	253	158
						110	209	107	180	153	214	128	251	152	244	170	250	189	253	158
Mallorca	2016	6	-	Male	WASP_205	110	209	107	180	153	214	128	251	152	244	160	250	195	253	158
						110	209	107	180	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2016	6	-	Male	WASP_206	110	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	180	153	214	128	251	144	244	160	250	195	253	158
Mallorca	2016	6	-	Male	WASP_207	108	209	99	180	153	214	128	251	152	244	170	250	195	253	158
						108	209	99	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_011	108	209	99	180	153	214	128	251	144	244	160	250	189	253	158
						110	209	107	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_208	108	209	99	180	153	214	128	251	144	244	160	250	189	253	158
						110	209	107	180	153	214	138	267	144	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_209	108	209	99	180	153	214	128	251	144	244	170	250	189	253	158

						110	209	99	180	153	214	138	267	144	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_210	108	209	99	180	153	214	128	251	144	244	170	250	189	253	158
						108	209	99	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_211	108	209	99	180	153	214	128	251	144	244	170	250	189	253	158
						108	209	107	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_212	108	209	99	180	153	214	128	251	144	244	170	250	189	253	158
						108	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_213	108	209	99	180	153	214	128	251	144	244	170	250	189	253	158
						108	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_214	108	209	99	180	153	214	128	251	144	244	170	250	189	253	158
						110	209	99	180	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_215	110	209	99	180	153	214	128	251	144	244	160	250	189	253	158
						110	209	107	180	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2016	7	-	Female	WASP_216	108	209	99	180	153	214	128	251	144	244	160	250	189	253	158
						110	209	99	180	153	214	138	267	144	244	170	250	195	253	158
Mallorca	2016	8	-	Female	WASP_012	108	209	99	180	153	214	128	251	144	244	170	250	189	253	158
						110	209	107	180	153	214	138	267	144	244	170	250	195	253	158
Mallorca	2016	8	-	Female	WASP_217	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	138	267	144	244	160	250	195	253	158
Mallorca	2016	8	-	Female	WASP_218	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	138	251	144	244	170	250	195	253	158
Mallorca	2016	8	-	Female	WASP_219	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	138	251	152	244	160	250	195	253	158
Mallorca	2016	8	-	Female	WASP_220	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	8	-	Male	WASP_221	110	209	99	180	153	214	138	251	152	244	170	250	195	253	158
						110	209	107	180	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2016	8	-	Male	WASP_222	108	209	99	180	153	214	128	251	144	244	170	250	195	253	158
						108	209	107	180	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2016	8	-	Male	WASP_223	110	209	99	180	153	214	128	251	152	244	170	250	195	253	158
						110	209	99	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2016	8	-	Male	WASP_224	110	209	99	180	153	214	128	251	152	244	160	250	195	253	158
						110	209	107	180	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2016	8	-	Male	WASP_225	108	209	107	180	153	214	138	251	144	244	160	250	195	253	158
						108	209	107	180	153	214	138	251	144	244	160	250	195	253	158
Mallorca	2016	9	-	Female	WASP_013	108	209	99	184	153	214	128	251	144	242	170	250	189	253	158
						108	209	107	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	9	-	Female	WASP_226	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						108	209	99	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2016	9	-	Female	WASP_227	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						110	209	99	184	153	214	128	267	144	242	170	250	195	253	158
Mallorca	2016	9	-	Female	WASP_228	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						110	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2016	9	-	Female	WASP_229	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						108	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2016	9	-	Male	WASP_230	108	209	107	180	153	214	128	251	144	242	170	250	195	253	158
						108	209	107	180	153	214	128	251	144	242	170	250	195	253	158
Mallorca	2016	9	-	Male	WASP_231	110	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	180	153	214	128	251	144	244	160	250	195	253	158
Mallorca	2016	9	-	Male	WASP_232	108	209	107	180	153	214	128	251	152	242	170	250	195	253	158

						108	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2016	9	-	Male	WASP_233	108	209	107	180	153	214	128	251	152	242	170	250	195	253	158
						108	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2016	9	-	Male	WASP_234	108	209	99	180	153	214	128	251	152	242	160	250	195	253	158
						108	209	99	180	153	214	128	251	152	242	160	250	195	253	158
Mallorca	2017	2	-	Female	WASP_014	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	2	-	Female	WASP_235	108	209	99	180	153	214	128	251	152	242	170	250	189	253	158
						110	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	2	-	Female	WASP_236	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						110	209	107	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	2	-	Female	WASP_237	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						110	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	2	-	Female	WASP_238	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	2	-	Male	WASP_239	110	209	99	180	153	214	128	251	152	242	170	250	195	253	158
						100	209	99	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	2	-	Male	WASP_240	110	209	107	180	153	214	128	251	152	244	170	250	195	253	158
						110	209	107	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	2	-	Male	WASP_241	108	209	99	180	153	214	128	251	152	244	160	250	189	253	158
						108	209	99	180	153	214	128	251	152	244	160	250	189	253	158
Mallorca	2017	2	-	Male	WASP_242	108	209	99	184	153	214	128	267	152	242	160	250	189	253	158
						108	209	99	184	153	214	128	267	152	242	160	250	189	253	158
Mallorca	2017	2	-	Male	WASP_243	110	209	107	180	153	214	128	251	152	242	170	250	195	253	158
						110	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_015	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_244	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_245	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_246	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	184	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_247	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_248	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_249	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_250	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_251	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	4	-	Female	WASP_252	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_016	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_253	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_254	108	209	99	180	153	214	128	251	144	244	160	250	189	253	158

						110	209	99	184	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_255	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_256	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_257	108	209	99	180	153	214	128	251	144	244	160	250	189	253	158
						110	209	99	184	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_258	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_259	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	184	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_260	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	7	-	Female	WASP_261	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	184	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	8	-	Female	WASP_017	108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
						108	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	8	-	Female	WASP_262	108	209	107	180	153	214	128	251	152	242	160	250	195	253	158
						108	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	8	-	Female	WASP_263	108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
						110	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	8	-	Female	WASP_264	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						108	209	107	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	8	-	Female	WASP_265	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						108	209	107	184	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	8	-	Male	WASP_266	108	209	107	180	153	214	128	251	152	242	170	250	189	253	158
						108	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	8	-	Male	WASP_267	108	209	107	180	153	214	128	251	144	242	170	250	189	253	158
						108	209	107	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	8	-	Male	WASP_268	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	8	-	Male	WASP_269	108	209	107	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	8	-	Male	WASP_270	108	209	107	180	153	214	128	251	152	242	160	250	195	253	158
						110	209	107	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	10	-	Female	WASP_018	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	180	153	214	128	251	152	242	160	250	195	253	158
Mallorca	2017	10	-	Female	WASP_271	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	10	-	Female	WASP_272	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	107	180	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2017	10	-	Female	WASP_273	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	107	184	153	214	128	267	152	244	160	250	195	253	158
Mallorca	2017	10	-	Female	WASP_274	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	99	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	10	-	Female	WASP_275	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	10	-	Female	WASP_276	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	10	-	Female	WASP_277	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158

						108	209	107	184	153	214	128	267	152	242	160	250	195	253	158
Mallorca	2017	10	-	Female	WASP_278	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	184	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	10	-	Female	WASP_279	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_019	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_280	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_281	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						108	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_282	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	107	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_283	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						108	209	107	184	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_284	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	107	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_285	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_286	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						108	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_287	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	11	-	Female	WASP_288	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	184	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	12	-	Female	WASP_020	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	152	244	160	250	195	253	158
Mallorca	2017	12	-	Female	WASP_289	110	209	107	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2017	12	-	Female	WASP_290	108	209	107	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2017	12	-	Female	WASP_291	108	209	107	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	12	-	Female	WASP_292	110	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2017	12	-	Female	WASP_293	110	209	107	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2017	12	-	Female	WASP_294	108	209	107	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	12	-	Female	WASP_295	110	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	12	-	Female	WASP_296	110	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	12	-	Female	WASP_297	110	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2017	13	-	Female	WASP_021	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						108	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	13	-	Female	WASP_298	108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
						108	209	107	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	13	-	Female	WASP_299	108	209	99	180	153	214	128	251	152	242	170	250	195	253	158

						108	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	13	-	Male	WASP_300	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
Mallorca	2017	13	-	Male	WASP_301	108	209	99	180	153	214	128	251	152	242	170	250	189	253	158
						110	209	99	180	153	214	128	251	152	242	170	250	189	253	158
Mallorca	2017	13	-	Male	WASP_302	108	209	99	180	153	214	128	267	144	242	170	250	195	253	158
						110	209	99	180	153	214	128	267	144	242	170	250	195	253	158
Mallorca	2017	13	-	Male	WASP_303	108	209	99	184	153	214	128	?	?	242	?	250	189	253	158
						108	209	99	184	153	214	128	?	?	244	?	250	189	253	158
Mallorca	2017	13	-	Male	WASP_304	108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
						110	209	99	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	13	-	Male	WASP_305	108	209	99	180	153	214	128	267	152	242	170	250	195	253	158
						108	209	99	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	14	-	Female	WASP_022	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	14	-	Female	WASP_307	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	251	144	244	160	250	195	253	158
Mallorca	2017	14	-	Female	WASP_308	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	14	-	Female	WASP_309	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	251	144	242	170	250	195	253	158
Mallorca	2017	14	-	Female	WASP_310	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	144	242	170	250	195	253	158
Mallorca	2017	14	-	Female	WASP_311	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	267	152	244	160	250	195	253	158
Mallorca	2017	14	-	Female	WASP_312	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	128	267	144	242	170	250	195	253	158
Mallorca	2017	14	-	Female	WASP_313	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	144	242	160	250	195	253	158
Mallorca	2017	14	-	Male	WASP_314	110	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						110	209	99	180	153	214	128	251	144	242	170	250	189	253	158
Mallorca	2017	14	-	Male	WASP_315	108	209	107	180	153	214	128	267	144	242	170	250	189	253	158
						108	209	107	180	153	214	128	267	144	242	170	250	189	253	158
Mallorca	2017	15	-	Female	WASP_023	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	138	267	152	244	160	250	195	253	158
Mallorca	2017	15	-	Female	WASP_317	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	138	251	152	244	160	250	195	253	158
Mallorca	2017	15	-	Female	WASP_318	108	209	99	180	153	214	128	251	144	242	160	?	189	253	158
						110	209	107	184	153	214	138	251	152	244	170	?	195	253	158
Mallorca	2017	15	-	Female	WASP_319	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	138	251	144	244	170	250	195	253	158
Mallorca	2017	15	-	Female	WASP_320	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	138	251	144	244	160	250	195	253	158
Mallorca	2017	15	-	Female	WASP_321	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	138	267	144	244	160	250	195	253	158
Mallorca	2017	15	-	Female	WASP_322	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	138	251	144	244	170	250	195	253	158
Mallorca	2017	15	-	Female	WASP_323	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	138	267	152	244	160	250	195	253	158
Mallorca	2017	15	-	Female	WASP_324	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158

						110	209	107	180	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2017	15	-	Female	WASP_325	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	180	153	214	138	267	152	244	160	250	195	253	158
Mallorca	2017	16	-	Female	WASP_024	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	16	-	Female	WASP_326	108	209	99	180	153	214	128	251	152	242	160	250	195	253	158
						110	209	107	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	16	-	Female	WASP_327	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	16	-	Female	WASP_328	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	107	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	16	-	Female	WASP_329	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	16	-	Male	WASP_330	108	209	99	180	153	214	128	251	152	244	160	250	189	253	158
						108	209	99	180	153	214	128	251	152	244	160	250	189	253	158
Mallorca	2017	16	-	Male	WASP_331	108	209	99	184	153	214	128	267	152	242	170	250	195	253	158
						108	209	99	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	16	-	Male	WASP_332	108	209	99	184	153	214	128	251	144	244	170	250	189	253	158
						108	209	99	184	153	214	128	251	144	244	170	250	189	253	158
Mallorca	2017	16	-	Male	WASP_333	110	209	99	184	153	214	128	267	144	242	160	250	195	253	158
						110	209	99	184	153	214	128	267	144	242	160	250	195	253	158
Mallorca	2017	16	-	Male	WASP_334	108	209	99	184	153	214	128	251	152	242	170	250	189	253	158
						108	209	99	184	153	214	128	251	152	242	170	250	189	253	158
Mallorca	2017	17	-	Female	WASP_025	108	209	107	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_335	108	209	107	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_336	108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
						110	209	107	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_337	108	209	99	180	153	214	128	251	152	242	170	250	189	253	158
						110	209	107	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_338	108	209	107	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	107	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_339	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_340	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	107	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_341	108	209	107	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_342	108	209	107	180	153	214	128	251	152	242	170	250	189	253	158
						110	209	107	184	153	214	138	267	152	244	170	250	195	253	158
Mallorca	2017	17	-	Female	WASP_343	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	184	153	214	138	251	152	244	170	250	195	253	158
Mallorca	2017	18	-	Female	WASP_026	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	18	-	Female	WASP_344	108	209	99	180	153	214	128	251	152	242	170	250	189	253	158
						108	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	18	-	Female	WASP_345	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	18	-	Female	WASP_346	108	209	99	180	153	214	128	251	152	242	160	250	195	253	158

						108	209	99	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	18	-	Female	WASP_347	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	18	-	Male	WASP_348	110	209	99	180	153	214	128	267	152	242	170	250	189	253	158
						110	209	99	180	153	214	128	267	152	242	170	250	189	253	158
Mallorca	2017	18	-	Male	WASP_349	108	209	99	184	153	214	128	267	144	244	170	250	189	253	158
						108	209	99	184	153	214	128	267	144	244	170	250	189	253	158
Mallorca	2017	18	-	Male	WASP_350	108	209	99	184	153	214	128	251	144	244	170	250	195	253	158
						108	209	99	184	153	214	128	251	144	244	170	250	195	253	158
Mallorca	2017	18	-	Male	WASP_351	110	209	99	184	153	214	128	267	152	242	170	250	195	253	158
						110	209	99	184	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2017	18	-	Male	WASP_352	110	209	99	180	153	214	128	267	144	242	170	250	195	253	158
						110	209	99	180	153	214	128	267	144	242	170	250	195	253	158
Mallorca	2017	20	-	Female	WASP_027	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	20	-	Female	WASP_353	108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
						108	209	99	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	20	-	Female	WASP_354	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	20	-	Female	WASP_355	108	209	99	180	153	214	128	251	152	242	160	250	195	253	158
						108	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	20	-	Female	WASP_356	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						110	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	20	-	Male	WASP_357	108	209	99	180	153	214	128	251	152	244	160	250	189	253	158
						108	209	99	180	153	214	128	251	152	244	160	250	189	253	158
Mallorca	2017	20	-	Male	WASP_358	108	209	99	184	153	214	128	267	144	244	170	250	189	253	158
						108	209	99	184	153	214	128	267	144	244	170	250	189	253	158
Mallorca	2017	20	-	Male	WASP_359	108	209	99	184	153	214	128	251	152	242	170	250	195	253	158
						108	209	99	184	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2017	20	-	Male	WASP_360	110	209	99	184	153	214	128	251	152	244	170	250	195	253	158
						110	209	99	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	20	-	Male	WASP_361	110	209	99	184	153	214	128	267	152	242	170	250	189	253	158
						110	209	99	184	153	214	128	267	152	242	170	250	189	253	158
Mallorca	2018	1	-	Female	WASP_028	108	209	99	180	153	214	128	251	144	244	160	250	195	253	158
						110	209	99	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2018	1	-	Female	WASP_362	108	209	99	184	153	214	128	251	144	242	160	250	195	253	158
						110	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2018	1	-	Female	WASP_363	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						110	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2018	1	-	Female	WASP_364	108	209	99	180	153	214	128	251	144	242	160	250	195	253	158
						108	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2018	1	-	Female	WASP_365	108	209	99	184	153	214	128	251	144	242	160	250	195	253	158
						108	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2018	1	-	Female	WASP_366	108	209	99	184	153	214	128	251	152	242	170	250	195	253	158
						108	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2018	1	-	Female	WASP_367	108	209	99	180	153	214	128	251	152	242	160	250	195	253	158
						108	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2018	1	-	Male	WASP_368	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						108	209	99	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2021	1	1	Female	WASP_141	108	205	99	180	153	208	128	251	144	242	166	250	189	253	158

						108	209	107	184	153	214	134	267	156	242	170	252	189	253	158
Mallorca	2021	1	-	Female	WASP_133	108	205	99	180	153	208	134	267	144	242	166	250	189	253	158
						108	209	99	184	153	214	138	267	156	244	170	252	195	253	163
Mallorca	2021	1	-	Female	WASP_134	108	205	99	184	153	208	128	267	156	242	166	250	189	253	158
						108	205	99	184	153	208	138	267	156	244	170	252	195	253	163
Mallorca	2021	1	-	Male	WASP_135	108	205	99	184	153	208	134	251	156	242	166	250	189	253	158
						108	209	107	184	153	214	138	267	156	244	170	252	195	253	163
Mallorca	2021	1	-	Male	WASP_136	108	205	99	184	153	208	128	251	156	242	166	250	189	253	163
						108	209	99	184	153	214	138	267	156	244	170	250	195	253	163
Mallorca	2021	1	-	Male	WASP_137	108	205	99	184	153	208	128	267	144	242	166	250	189	253	158
						108	205	99	184	153	208	138	267	156	244	170	252	195	253	163
Mallorca	2021	1	-	Male	WASP_138	108	205	99	184	153	208	134	267	156	242	166	250	189	253	158
						108	205	107	184	153	208	138	267	156	244	170	252	195	253	163
Mallorca	2021	1	-	Female	WASP_139	108	205	99	184	153	208	128	251	156	242	166	250	189	253	158
						108	209	99	184	153	214	138	267	156	244	170	250	195	253	158
Mallorca	2021	1	-	Female	WASP_140	108	205	99	184	153	208	134	267	144	242	166	250	189	253	158
						108	209	107	184	153	208	138	267	156	244	170	252	195	253	163
Mallorca	2021	1	-	Female	WASP_369	108	205	99	180	153	208	128	251	144	242	170	250	189	253	158
						108	209	107	184	153	208	138	267	156	244	170	252	195	253	163
Mallorca	2021	1	-	Male	WASP_370	108	205	99	180	153	208	128	267	144	242	170	250	189	253	158
						108	205	107	184	153	208	138	267	156	244	170	252	195	253	163
Mallorca	2017	-	1	Female	WASP_058	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						110	209	99	184	153	214	128	267	144	244	170	250	195	253	158
Mallorca	2017	-	2	Female	WASP_060	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	107	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	-	3	Female	WASP_062	108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
						110	209	107	184	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2017	-	4	Female	WASP_064	108	209	107	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	184	153	214	128	251	152	244	160	250	195	253	158
Mallorca	2017	-	5	Female	WASP_061	108	209	99	184	153	214	128	251	152	242	170	250	189	253	158
						110	209	107	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	-	6	Female	WASP_063	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	107	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2017	-	7	Female	WASP_059	108	205	99	184	153	214	128	?	152	242	166	250	195	253	158
						108	209	107	184	153	214	128	?	152	242	170	250	195	253	158
Mallorca	2018	-	1	Female	WASP_029	108	209	99	180	153	214	128	251	144	244	160	250	189	253	158
						110	209	99	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2018	-	2	Female	WASP_030	108	209	99	180	153	214	128	251	144	242	160	250	189	253	158
						108	209	99	184	153	214	128	251	152	242	160	250	195	253	158
Mallorca	2018	-	3	Female	WASP_031	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						108	209	99	180	153	214	128	251	152	242	170	250	195	253	158
Mallorca	2018	-	4	Female	WASP_032	108	209	99	180	153	214	128	251	144	242	170	250	195	253	158
						108	209	99	184	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2018	-	5	Female	WASP_033	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158
						110	209	99	180	153	214	128	267	152	244	170	250	195	253	158
Mallorca	2018	-	6	Female	WASP_034	108	209	99	180	153	214	128	251	152	242	160	250	189	253	158
						110	209	99	180	153	214	128	251	152	244	170	250	195	253	158
Mallorca	2018	-	7	Female	WASP_035	108	209	99	180	153	214	128	251	152	242	160	250	195	253	158
						110	209	99	180	153	214	128	267	152	242	170	250	195	253	158
Mallorca	2018	-	10	Female	WASP_038	108	209	99	180	153	214	128	251	144	242	170	250	189	253	158

						110	209	99	180	153	214	128	251	152	244	170	250	195	253	158
Catalonia	-	-	-	Female	WASP_039	110	209	99	180	153	208	128	267	152	242	160	250	189	253	163
						110	209	99	180	153	214	128	267	156	242	160	250	195	253	163
Catalonia	-	-	-	Female	WASP_040	108	209	99	180	153	208	128	267	152	242	160	250	189	253	158
						110	209	99	180	153	214	138	267	156	244	160	250	195	253	163
Catalonia	-	-	-	Female	WASP_041	108	205	99	180	153	208	134	267	144	242	166	?	189	253	158
						108	209	107	180	153	208	138	267	144	242	166	?	195	253	163
Catalonia	-	-	-	Female	WASP_042	108	?	99	180	?	208	128	267	152	242	160	?	189	253	158
						110	?	107	180	?	208	128	267	152	242	168	?	195	253	163
Catalonia	-	-	-	Female	WASP_043	110	205	99	?	153	208	128	?	156	?	166	250	?	253	158
						110	205	107	?	153	208	138	?	156	?	170	250	?	253	158
Catalonia	-	-	-	Female	WASP_044	108	199	99	?	153	208	128	251	144	?	160	?	?	253	158
						108	205	107	?	153	214	138	267	144	?	166	?	?	253	163
Catalonia	-	-	-	Male	WASP_051	110	209	99	184	153	208	128	267	144	242	170	250	189	253	163
						110	209	99	184	153	208	128	267	144	242	170	250	189	253	163
Catalonia	-	-	-	Male	WASP_052	110	205	107	180	153	208	128	251	156	244	170	250	189	253	158
						110	205	107	180	153	208	128	251	156	244	170	250	189	253	158
Catalonia	-	-	-	Female	WASP_101	108	209	107	180	153	208	128	267	144	242	166	250	189	253	158
						108	209	107	184	153	208	128	267	156	242	170	252	189	253	163
Catalonia	-	-	-	Male	WASP_102	110	209	99	180	153	214	128	251	152	242	166	250	189	253	163
						110	209	107	180	153	214	134	267	156	244	166	250	189	253	163
Catalonia	-	-	-	Female	WASP_103	108	205	99	180	153	208	128	267	144	242	166	250	189	253	158
						110	209	99	184	153	208	134	267	156	244	166	250	189	253	163
Catalonia	-	-	-	Female	WASP_104	110	205	99	180	153	208	128	251	144	242	166	250	189	253	158
						110	205	99	180	153	208	138	267	156	244	170	250	189	253	163
Catalonia	-	-	-	Female	WASP_105	108	205	99	180	153	208	128	267	144	242	160	250	189	253	158
						110	205	107	180	153	214	128	267	144	244	166	252	189	253	163
Catalonia	-	-	-	Female	WASP_106	110	205	99	180	153	208	138	251	152	242	166	250	189	253	158
						110	205	107	180	153	208	138	267	156	244	166	250	195	253	163
Catalonia	-	-	-	Male	WASP_107	110	205	107	180	153	208	128	267	156	242	166	250	189	253	158
						110	205	107	180	153	208	128	267	156	242	166	250	189	253	158
Catalonia	-	-	-	Female	WASP_108	108	205	99	180	153	208	128	267	144	242	166	250	189	253	158
						110	205	107	184	153	208	134	267	152	244	166	250	189	253	163
Basque C.	-	-	-	Female	WASP_142	108	205	99	180	153	208	128	267	144	242	166	252	189	253	163
						108	209	107	180	153	214	138	267	144	244	170	252	195	253	163
Basque C.	-	-	-	Female	WASP_143	110	205	99	180	153	208	128	251	144	244	166	252	189	253	163
						110	209	107	180	153	214	138	267	144	244	170	252	195	253	163
Basque C.	-	-	-	Female	WASP_144	110	205	99	180	153	208	128	251	144	242	166	250	189	253	158
						110	209	99	184	153	208	128	267	156	242	170	250	189	253	163
Basque C.	-	-	-	Female	WASP_145	110	205	99	180	153	208	128	251	156	244	160	250	189	253	163
						110	209	99	184	153	214	138	267	156	244	166	252	189	253	163
Basque C.	-	-	-	Female	WASP_146	108	205	107	180	153	208	128	267	144	242	160	250	189	253	158
						108	205	107	180	153	208	138	267	156	242	166	250	189	253	163
Basque C.	-	-	-	Female	WASP_147	108	205	99	?	153	208	138	?	152	?	160	252	189	253	158
						108	209	107	?	153	212	138	?	152	?	160	252	189	253	163
Basque C.	-	-	-	Female	WASP_148	108	205	99	180	153	208	134	267	144	242	170	250	189	253	158
						110	209	107	184	153	214	138	267	152	242	170	252	189	253	158
Basque C.	-	-	-	Female	WASP_109	108	205	99	180	153	208	138	251	144	242	160	250	189	253	163
						110	205	107	180	153	208	138	251	144	244	160	250	189	253	163

Basque C.	-	-	-	Female	WASP_110	110	205	99	180	153	208	138	251	144	242	160	250	189	253	163
						110	205	99	180	153	208	138	251	144	244	160	250	189	253	163
Basque C.	-	-	-	Female	WASP_111	110	205	99	180	153	208	138	251	144	242	160	250	189	253	163
						110	205	107	180	153	208	138	267	144	244	170	250	189	253	163
Basque C.	-	-	-	Female	WASP_112	108	205	99	180	153	208	138	251	152	242	166	252	189	253	163
						108	205	107	180	153	214	138	267	156	242	166	252	189	253	163
Basque C.	-	-	-	Female	WASP_113	108	205	99	180	153	208	128	251	152	242	166	252	189	253	163
						110	205	107	180	153	214	138	267	156	242	166	252	189	253	163
Basque C.	-	-	-	Female	WASP_114	108	205	99	180	153	208	128	267	144	242	166	250	189	253	163
						108	209	99	180	153	208	134	267	156	242	170	250	189	253	163
Basque C.	-	-	-	Female	WASP_115	108	205	99	180	153	208	128	267	144	242	160	250	189	253	158
						110	205	107	180	153	208	138	267	152	244	170	250	189	253	163
Basque C.	-	-	-	Female	WASP_116	108	205	99	180	153	208	128	267	144	242	160	250	189	253	158
						110	205	107	180	153	208	138	267	152	244	160	250	189	253	158
Basque C.	-	-	-	Female	WASP_053	108	209	107	180	153	214	128	267	156	244	170	250	189	253	158
						108	209	107	180	153	214	128	267	156	244	170	250	189	253	158
Basque C.	-	-	-	Female	WASP_054	108	205	99	180	153	208	138	251	144	244	160	250	189	253	163
						110	205	99	180	153	208	138	267	144	244	170	250	189	253	163
Basque C.	-	-	-	Female	WASP_055	108	205	99	180	153	208	138	251	144	244	160	252	189	253	163
						110	205	107	180	153	208	138	267	144	244	170	252	189	253	163
Basque C.	-	-	-	Male	WASP_056	108	205	99	180	153	208	138	251	144	242	160	250	189	253	163
						110	205	107	180	153	208	138	267	156	244	170	250	189	253	163
Basque C.	-	-	-	Male	WASP_057	108	205	99	180	153	208	138	251	144	242	160	250	189	253	163
						110	205	107	180	153	208	138	267	144	244	170	250	189	253	163
Asturias	-	-	-	Female	WASP_065	110	205	99	180	153	208	138	267	152	242	166	250	189	253	158
						110	205	107	180	153	208	138	267	156	242	166	250	189	253	163
Asturias	-	-	-	Female	WASP_066	110	205	107	180	153	208	128	251	152	242	160	250	189	253	158
						110	209	107	180	153	208	128	251	156	242	160	250	189	253	163
Asturias	-	-	-	Female	WASP_067	108	205	99	180	153	208	128	267	152	242	166	250	189	253	158
						110	205	99	180	153	208	138	267	156	244	170	250	195	253	163
Asturias	-	-	-	Female	WASP_068	110	205	107	180	153	208	128	251	156	242	160	252	189	253	163
						110	209	107	180	153	208	138	267	156	244	166	252	189	253	163
Asturias	-	-	-	Female	WASP_069	110	205	99	180	153	208	128	267	152	242	160	250	189	253	158
						110	205	99	180	153	208	128	267	156	242	166	250	189	253	163
Asturias	-	-	-	Female	WASP_070	108	205	99	180	153	208	128	267	152	242	166	250	189	253	163
						110	205	107	180	153	208	138	267	156	244	170	252	189	253	163
Asturias	-	-	-	Female	WASP_071	110	205	99	180	153	208	128	251	156	242	166	250	189	253	158
						110	209	107	184	153	208	128	251	156	244	166	250	189	253	163
Asturias	-	-	-	Female	WASP_072	110	205	99	180	153	208	128	251	156	242	160	250	189	253	158
						110	209	107	180	153	208	138	251	156	242	160	250	189	253	163
Asturias	-	-	-	Female	WASP_073	108	205	99	180	153	208	138	267	152	242	166	250	189	253	163
						108	209	99	180	153	208	138	267	156	244	170	250	189	253	163
Asturias	-	-	-	Female	WASP_074	110	209	99	180	153	208	128	251	152	242	160	250	189	253	163
						110	209	107	180	153	208	138	267	156	242	166	250	189	253	163
Asturias	-	-	-	Female	WASP_075	108	205	99	180	153	208	138	267	156	242	160	250	189	253	163
						108	205	99	184	153	208	138	267	156	242	166	250	195	253	163
Galicia	-	-	-	Female	WASP_089	108	205	99	180	153	208	128	267	156	244	166	250	189	253	158
						110	209	107	184	153	208	138	267	156	244	170	250	189	253	163
Galicia	-	-	-	Female	WASP_090	108	205	99	180	153	208	138	251	156	244	170	250	189	253	158

						110	209	107	184	153	208	138	267	156	244	170	250	189	253	163
Galicia	-	-	-	Female	WASP_091	110	205	99	180	153	208	138	251	156	242	166	250	189	253	158
						110	209	99	180	153	214	138	267	156	244	170	250	189	253	158
Galicia	-	-	-	Female	WASP_092	110	209	99	180	153	208	138	267	152	244	166	250	189	253	163
						110	209	99	184	153	214	138	267	156	244	166	250	195	253	163
Galicia	-	-	-	Female	WASP_093	110	205	99	180	153	208	128	251	156	244	166	250	189	253	163
						110	209	107	180	153	208	138	267	156	244	166	250	189	253	163
Galicia	-	-	-	Female	WASP_094	108	205	99	180	153	208	128	251	156	244	160	252	189	253	158
						110	209	107	180	153	208	138	267	156	244	166	252	195	253	163
Galicia	-	-	-	Female	WASP_095	110	209	99	180	153	208	128	251	156	244	166	250	189	253	158
						110	209	99	184	153	214	138	251	156	244	166	252	189	253	163
Galicia	-	-	-	Female	WASP_096	108	205	99	184	153	208	128	267	144	242	166	250	189	253	158
						110	205	107	184	153	214	128	267	152	244	170	250	189	253	163
Galicia	-	-	-	Female	WASP_097	108	205	99	180	153	214	128	251	156	244	166	250	189	253	158
						110	209	99	184	153	214	138	251	156	244	166	252	189	253	163
Galicia	-	-	-	Female	WASP_098	110	205	99	180	153	208	138	267	152	244	166	252	189	253	158
						110	205	99	180	153	214	138	267	156	244	166	252	189	253	163
Galicia	-	-	-	Female	WASP_099	110	205	99	180	153	208	138	251	156	244	166	250	189	253	163
						110	209	99	184	153	208	138	267	156	244	166	250	189	253	163
Galicia	-	-	-	Female	WASP_100	110	205	99	180	153	208	128	251	152	244	166	250	189	253	163
						110	205	99	184	153	208	138	267	156	244	166	252	189	253	163
Extremadura	-	-	-	Female	WASP_117	110	205	99	184	153	208	128	267	152	244	166	250	189	253	158
						110	209	99	184	153	214	138	267	156	244	166	252	189	253	158
Extremadura	-	-	-	Female	WASP_118	110	209	99	180	153	208	138	251	152	244	160	250	189	253	158
						110	209	99	180	153	214	138	267	152	244	166	250	189	253	163

Table S2.- Multiplex characteristics for the fifteen STRs genotyped in this study.

Multiplex	Locus	Label	Size (bp)	Primer sequence (5'-3')	Reference	Repeat motif
1st	R1-80	6-FAM	100-174	F: CATCATCGGCACATACAAAAA R: TGGAATGAAAATTAACGAGTTT	Arca et al. (2012)	$(GT)_{19}$
	R4-33	6-FAM	199-225	F: TTGTCTCTTCGGGGAACAAT R: TGTTGCGTGAAAGAGAGAGTG	Arca et al.. 2011	$(GA)_{20}$
	LIST2020B	VIC	183-217	F: TTCTTCTCTACCCACGAC R: AGGGAGGCAAAAGGAG	Arca et al. (2015)	$(CT)_{23}(N)_{37}(CA)_{11}$
	R1-77	VIC	241-255	F: ACGTTCTAAGAGCCGTGCAT R: AATTGGACAAATCCGCTCTG	Arca et al. (2012)	$(CT)_{15}(CTT)_{5}$
	R1-36	NED	99-119	F: GGATTATACACCTTCGACCATTTT R: TCGCGAAGGGTAAAAGCAAT	Arca et al. (2012)	$(CT)_{14}$
	R4-100	NED	154-194	F: TCGGTAATTCAGATTATTAAGTGAAAG R: CTCACGCATGATCCCTATCG	Arca et al. (2012)	$(AC)_{19}(CT)_{6}$
2nd	D2-142	PET	147-182	F: AATGATTTCCAACTCAAGCGTTA R: GGTACATTCGAGATAAAATGGACTA	Arca et al. (2012)	$(CT)_{14}$
	D2-185	PET	208-228	F: CCGATTAAAACCGCGATGTA R: GCGGACGACAACTTTCATTA	Arca et al. (2012)	$(CT)_{18}$
	R4-114	6-FAM	122-152	F: GACGGCACGTCGTGTTAAAT R: GCGAATAAAGTTCTTCCTTCCA	Arca et al. (2012)	$(TC)_{15}$
	VMA-8	6-FAM	230-271	F: TAGACACGTACACCACTAG CTGGCCAGGATATTCCAGT	Hasegawa & Takahashi. (2002)	$(CT)_{9}$
	R1-75	VIC	142-154	F: TCGATTCGTCGAAATTCACA R: TATCGGAAGGGTGAAACGAA	Arca et al. (2012)	$(AC)_{14}$
	R1-169	NED	148-165	F: GACGGTCGGCTGTTAGGATA R: CGAGCGCCTTCTTTAGTGAG	Arca et al. (2012)	$(CT)_{27}$
	VMA-6	NED	238-242	F: ACAGTTTCTTGATTCGTCG R: GATGCTATCGTCGGCATTT	Hasegawa & Takahashi. (2002)	$(CT)_{16}$
	D3-15	PET	157-180	F: CGAAGGATTTTCCTCGGACT	Arca et al. (2012)	$(TC)_{15}$

R4-26	PET	229-272	R: TGCGTCGAAACAAATAGGTG F: CCAGGACGATCAGTATCAAGG R: TCGAAAGACAATAAAAAGAAACGA	Arca et al. (2012)	$(CT)_{21}$

Chapter 3

Ensemble of small models as a tool for alien invasive species management planning: evaluation of *Vespa velutina* (Hymenoptera: Vespidae) under Mediterranean island conditions

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Abstract

Ecological niche models have proved to be a powerful tool in assessing invasiveness risk of alien species, allowing the optimization of control strategies. *Vespa velutina* (Hymenoptera: Vespidae) is an invasive species with strong ecological, economical and health impacts in Europe after it was first reported in France in 2004. It was detected for the first time on a Mediterranean island (Mallorca, Balearic Islands, Spain) in 2015, where a single nest was found in the northwest of the island. Immediately, a control plan was implemented. In this study, we analysed 30 occurrence data in Mallorca island to assess the suitability distribution predicted for Mediterranean island conditions using an ensemble of small models. We obtained high values of AUC (0.9165), Somers'D (0.8331), Boyce (0.7611) and TSS (0.7754) as quality parameters of the final ensembled model. We show for the first time that there are suitable areas where this species can expand and stablish, mainly in steeper slopes and low isothermality zones. Likewise, the distribution suitability of *V. velutina* for other Mediterranean islands (Ibiza, Formentera, Menorca, Corsica, Sardinia, Sicily, Crete, and Cyprus) was also explored, showing potentially suitable zones. This study provides valuable information regarding the areas in the Mediterranean islands under risk of invasion, and it could be used by both scientists and managers for an early detection and control of the invasive species due to its cost-effectiveness in terms of conservation.

Key message: Recent invasions constitute a challenge for the application of ecological niche models. Ensemble of small models represented a novel and robust strategy to predict the distribution of *Vespa velutina* recent invasion in Mallorca. Climate, human and topographic influences combined explained the distribution of *V. velutina*. Models calibrated in Mallorca allowed to predict potentially suitable areas of invasion in other Mediterranean islands

Keywords: Biological invasion, Ecological niche models, Ensemble of small models, *Vespa velutina*, Mallorca, Mediterranean islands

Introduction

The increases of human mobility and global trade over the last years have resulted in an unprecedented number of invasions and establishment of invasive species all around the world (Levine & D'Antonio, 2003; Sardain et al., 2019), with severe environmental and socioeconomic impacts (Beggs et al., 2011; Paini et al., 2016; Requier et al., 2019). In particular, social Hymenoptera have had great success in establishing populations after introduction due to excellent dispersal abilities, high reproductive rates, broad diets and habitat ranges, effective predator defenses, and superior competitive abilities, which enhance their ability to establish, spread and have a major impact on native ecosystems (Beggs et al., 2011; Moller, 1996).

The yellow-legged hornet (*Vespa velutina* Lepeletier 1836) is an invasive alien hymenoptera species which was accidentally introduced in Europe from Asia (Laurino et al., 2020). It was reported for the first time in south-west France in 2004 and rapidly spread to nearby European countries (Laurino et al., 2020).

The main impact of the yellow-legged hornet is the likely decrease in honeybee (*Apis mellife*ra) populations (Monceau, Maher, et al., 2013), preying also upon other pollinators and insects (Rojas-Nossa & Calviño-Cancela, 2020; Rome et al., 2021, 2011). This invasion could be devastating for island ecosystems, considering the situation of the populations of honeybees (Dainat, vanEngelsdorp, & Neumann, 2012; Ellis, Evans, & Pettis, 2010), the fragility of the ecosystem (Traveset et al., 2019) and the impact on endemic insects (Ikegami et al., 2020). In addition, it is possible that the yellow-legged hornets attack humans when colonial nests are established in nearby areas, producing poison and allergy cases (de Haro et al., 2010; Herrera et al., 2020; Vidal et al., 2021).

In the case of Mallorca (Balearic Islands, Spain) an individual of this species was detected in 2015, being the first report of this invasive hornet in a Mediterranean island (Laurino et al., 2020). This individual was taken to the department of Biology of the University of the Balearic Islands where it was identified. Together with the local authorities, an intensive protocol to detect and remove nests was implemented, as described in Leza et al. (2021). In 2015, only one nest was found in the northwest of the island. During 2016, nine additional nests were detected and removed, and in 2017 the number of reported nests increased to twenty. Since then no more secondary nests were found (Leza et al., 2021), being the first territory where *V. velutina* has been officially eradicated (CAIB, 2019).

A recent spread modelling study (Robinet et al., 2019) suggests that *V. velutina* cannot naturally colonize Mediterranean islands (such as Sicilia, Sardinia, Mallorca or Corsica) by their own but can be introduced it by human-mediated dispersal. Hence, the incursion in Mallorca was probably due to an accidental introduction by humans (Robinet et al., 2019).

It is known that invasive species often do not present a static niche; Broennimann et al. (2007) provided the first empirical evidence that an invasive species can occupy climatically distinct niche spaces after its introduction into a new area. Therefore, anticipating potential distributions of invasive species is essential for prioritization, early detection and control (Broennimann et al., 2007), and indispensable for a conservation plan (Peterson & Robins, 2003).

Ecological niche models (Guisan & Thuiller, 2005) have been used to predict the invasion extents from a great diversity of invasive organisms, such as plants (Broennimann et al., 2007), fishes (Zengeya et al., 2013), birds (Ancillotto et al., 2016), snakes (Pyron et al., 2008) and insects

(Alaniz et al., 2021; Muirhead et al., 2006). The precision of such models increases with sample size, the higher the number of presences, the better the niche description (Hernandez et al., 2006). This is one of the main drawbacks of the application of this approach in recent invasions where the occurrences may be scarce, which can lead to model overfitting and thus limiting its applicability (Vaughan & Ormerod, 2005). Under this scenario, the ensemble of small models has arisen as a novel and robust strategy to predict the distribution of species accounting for few occurrences (Breiner et al., 2015, 2018). In spite of its usefulness, this methodology has been rarely applied in the context of invasive species, where just two studies were found in the literature: modelling an invasive squirrel (Di Febbraro et al., 2019) and a chytrid fungus (Beukema et al., 2018).

Here we aim to identify the ecological factors determining the presence of *V. velutina* and to predict potentially suitable areas of invasion under Mediterranean island conditions. To do so, we compiled the distribution of all secondary nests in Mallorca to calibrate ecological niche models using an ensemble of small models. In addition, we expand our calibrated ecological niche models to other Mediterranean islands still free from *V. velutina* (e.g. Ibiza, Formentera, Menorca, Corsica, Sardinia, Sicily, Crete and Cyprus) to identify areas susceptible to being colonized. This case study might assist the management and control of this alien invasive species by using a novel ecological niche modelling strategy for species with few occurrences.

Material and methods

Study site, presence data of *V. velutina* and environmental variables

Mallorca (39º30′ N, 03º0′ E) is the biggest island of the westernmost Mediterranean archipelago (Balearic Islands) and is climatically considered Mediterranean (based on Emberger's Index). The first specimen of *V. velutina* was detected in the northwest of the island (Leza et al., 2018). This area is characterized by specific climatic and geomorphological conditions compared to the rest of the regions in Mallorca, such as the highest precipitation rate (1400-1600 mm annual) and cooler temperatures. Consequently, its vegetation is quite different from the rest of the island, and is dominated by pine trees (*Pinus halepensis* Miller), holm oaks (*Quercus ilex* L.), garrigue (mainly *Salvia rosmarinus* (L.) Schleid and *Erica multiflora* L.), wild olive trees (*Olea europaea* var. *sylvestris* L.), orchards of orange trees (*Citrus sinensis* (L.) Osbeckand) and olive trees (*Olea europaea* L.) (Leza et al., 2021).

Since the first detection of *V. velutina* in Mallorca, intensive surveys have been implemented in order to detect all nests in the area based on bait traps and public reports on the presence of adults. When an adult was detected, feeding points with protein attractant (raw fish) were set in the area to identify the direction that these adults took and to triangulate the precise nest location with GPS for subsequent removal at night (Leza et al., 2018, 2021). This work program was implemented for 6 years (2015-2020) resulting in the record of 30 secondary nest's locations.

A total of 42 environmental belonging to five main categories were included in the analysis: climatic, topographic, remote sensing, hydrography and anthropogenic (Table S3, Supplementary material) (Barbet-Massin et al., 2012; Bertolino et al., 2016; Bessa et al., 2016; de Medeiros et al., 2018; Fournier et al., 2017; Lioy et al., 2019; Robinet et al., 2019; Rodríguez-Flores et al., 2019; Villemant et al., 2011a, 2011b). Mallorca island was divided into cells of 1 km^2 and all variables were aggregate by average to a resolution of 1 km^2. In the specific case of landcover, it

is a variable available at a resolution of 300m^2. We have assumed the loss of fine information (300 m^2) to work with a lower resolution variable (1km^2) in order to include this one in the model. Multicollinearity among the 42 variables was assessed and eight uncorrelated predictors, and VIF less than 4, were selected: continentality, human footprint, isothermality, land cover, precipitation of the coldest quarter, precipitation of the warmest quarter, precipitation seasonality and slope (Table S3 and Figure S1, Supplementary Material).

Ecological suitability modelling

It is recommended to include different models from different modelling techniques as it cannot be guaranteed that a particular method is the best for all situations (Hirzel & Le Lay, 2008; Johnson & Gillingham, 2005). Hence, ten different models were selected in this study: Surface Range Envelope (SRE), Generalized linear models (GLM), Generalized additive models (GAM), Multivariate adaptive regression spline (MARS), Classification tree analysis (CTA), Flexible Discriminant Analysis (FDA), Artificial neural network (ANN), Random forest (RF), Generalized boosting method (GBM) and Maxent available in *biomod2* and *ecospat* R packages (Broennimann et al. , 2020; Di Cola et al., 2017; Thuiller et al., 2009).

Thirty *V. velutina* occurrence points obtained from intensive fieldwork during active nest searches (Leza et al., 2018, 2021), plus a random sample including the 25% of all non-invaded Mallorca cells to be used as weighted background points, were used to fit and evaluate models as described in Barbet-Massin et al. (2012). One divided by the number of cases of presences and background will provide the weight of each group (presence: n = 30, weight = 0.0333; background: n = 1258, weight = $7.9491 \cdot 10^{-4}$). The random sample was taken from the entire Mallorca island, since the maximum spread distance registered for this species (78 km) (Robinet et al., 2017) indicates that the species could reach any point of the island from the initial invasion point. A general rule is that the sample size (number of presences) should be 10 times greater than the number of predictors included in the models (Harrell et al., 1996), meaning that we would need more than 80 occurrences points to avoid overfitting when including our eight environmental predictors. To circumvent this limitation we implemented the ensemble of small models (ESMs) (Breiner et al., 2015, 2018; Scherrer et al., 2019; Song et al., 2019; Virtanen et al., 2018) included in *ecospat* R package (Broennimann et al., 2020; Di Cola et al., 2017). This strategy involves building and evaluating many models with a small subset of predictors (Breiner et al., 2018). Finally, all simple models generated were ensembled including our eight predictors and avoiding overfitting (Breiner et al., 2018). Considering our data, 28 simple bivariate models combining the eight predictors in groups of two were generated. Each small model was fitted with 2/3 of data and evaluated with 1/3 not used to calibrate the model, cross-validation based on AUC index (Benito et al., 2017; Pearson et al., 2007) and using *ecospat* R package (Broennimann et al., 2020; Di Cola et al., 2017). We applied this methodology 100 times to each single bivariate model, and we used the following parameters to evaluate model performance: Area under the curve (AUC), Somers'D ($D = 2 \cdot (AUC - 0.5)$), Boyce Index (Boyce) and True skill statistic (TSS) (Allouche et al., 2006; Boyce et al., 2002; Newson, 2006; Somodi et al., 2017). Finally, we ensembled each small model using Somers'D weighted average (Breiner et al., 2015), where those models with AUC lower than 0.5 (Somer's D = 0) were not included in the final ensemble.

Response curves

For each climate variable, the predicted suitability over all values in the gradient of the variable was generated, while the other climate variables were kept at their average values. Then, the predicted values against the climate gradient were plotted, which represents the response of the species to the climate variable (Elith et al., 2005).

Importance of environmental factors

The sum of weights of bivariate models for each variable was calculated. This value was compared with the sum of weights of all bivariate models to determine the importance of each environmental factor. This procedure was performed for each method applied. Finally, for ensemble models, the contributions were weighted means of the different methods (Broennimann et al., 2020).

Measuring ecological niche

In order to measure the ecological niche occupied by *V. velutina* from the invaded range along the three years with invasion (2015-2017) under Mediterranean island conditions, sets of environmental variables of the 25% random background study area and *V. velutina* occurrences were subjected to 2D-PCA. The final 2D-PCA was plotted with ellipses (95% confidence) and marginal box plots which represent spatial niches offered and occupied (Janekovi & Novak, 2012). Finally, a Kruskal-Wallis test was used to check for statistical differences between background and *V. velutina* niches.

Transferability assessment

We explored the potential suitability of *V. velutina* in the largest Mediterranean islands (Corsica, Crete, Cyprus, Formentera, Ibiza, Menorca, Sardinia, and Sicily). For this purpose, the same environmental variables to a resolution of 1 km^2 used for model's calibration, were obtained for each island. Consecutively, the ecological niche model calibrated in Mallorca was projected in each island with its own environmental data. On the other hand, maritime ports were indicated for each island in order to detect nearby suitable areas where *V. velutina* could expand if it was detected. Likewise, Multivariate Environmental Similarity Surface (MESS) was applied in the final ensemble model for each island. This analysis determines how similar are the conditions of the other Mediterranean islands, in relation to the calibration conditions of the model in Mallorca (Elith et al., 2010).

Results

Ecological suitability modelling

After fitting and evaluating 100 times each single bivariate model from 28 different combinations of predictors and using 10 different methods, we obtained a final dataset of 28000 single bivariate

models. Then, a total of 694 single bivariate models were removed because AUC was lower than 0.5. Finally, the final ensemble model was obtained from 27306 models using Somers'D weighted average.

We obtained high values of AUC for all models (AUC_{min} = 0.673) as well as for Somers'D (Somers' D_{min} = 0.345), a rescaled version of the AUC. Moreover, models showed high values of TSS (TSS_{min} = 0.395) (Fig. 1) whereas Boyce Index showed more dispersal scores but always above 0, despite three SRE models which were equal or lower than 0. Finally, for the final ensemble model, we obtained a high value weighted mean of AUC (0.9165 ± 0.032), Somer's D (0.8331 ± 0.0631), Boyce (0.7611 ± 0.102) and TSS (0.7754 ± 0.0701) (Figure 12).

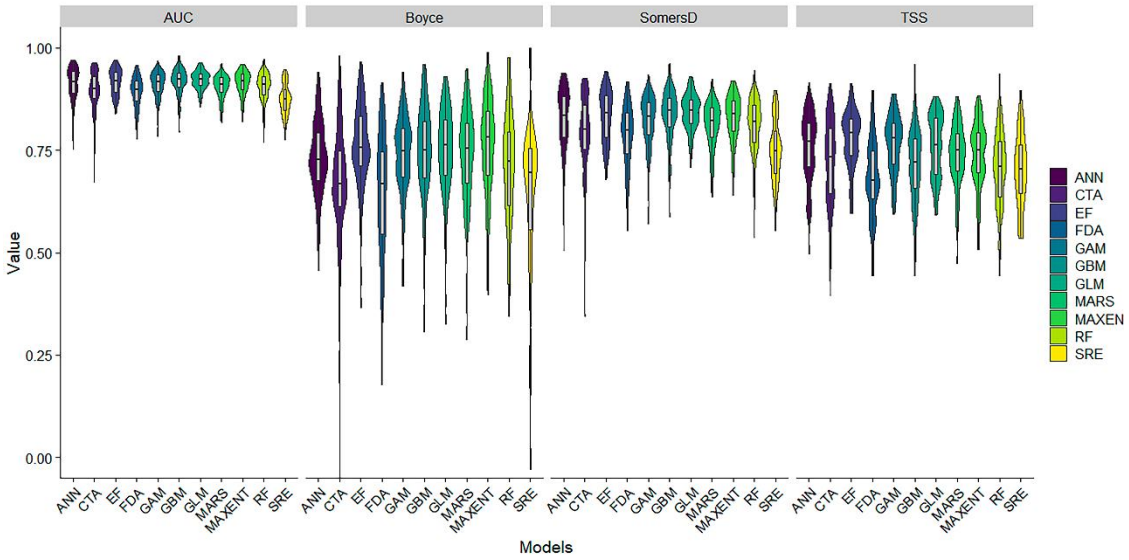

Figure 12.- Differences in accuracy (AUC, Boyce, SomersD and TSS) between the ten different models used in this study and the final ensemble model (EF). Artificial neural network (ANN), Classification tree analysis (CTA), Final Ensemble (EF), Flexible Discriminant Analysis (FDA), Generalized additive models (GAM), Generalized boosting method (GBM), Generalized linear models (GLM), Multivariate adaptive regression spline (MARS), Random forest (RF) and Surface Range Envelope (SRE).

The corresponding potential distribution obtained from the ensemble model (Figure 13) identifies Mallorca as an area of low suitability of *V. velutina* with a mean suitability of 0.045 ± 0.0656 (range 0.007 – 0.4530) for the entire island. Nonetheless, the westernmost mountain range presents higher values, reaching until 0.4530 as a maximum suitability. The suitability of the location where the first hornet nest was discovered in 2015 was estimated to be 0.239. Since then, the mean suitability for 2016 was 0.3344 ± 0.0784 (range 0.1780 – 0.4330) and 0.2994 ± 0.0897 (range 0.1100 – 0.4530) for 2017. As an average, a mean suitability for all located nests was 0.3046 ± 0.0864 (range 0.1100 – 0.4530) after three years of invasion.

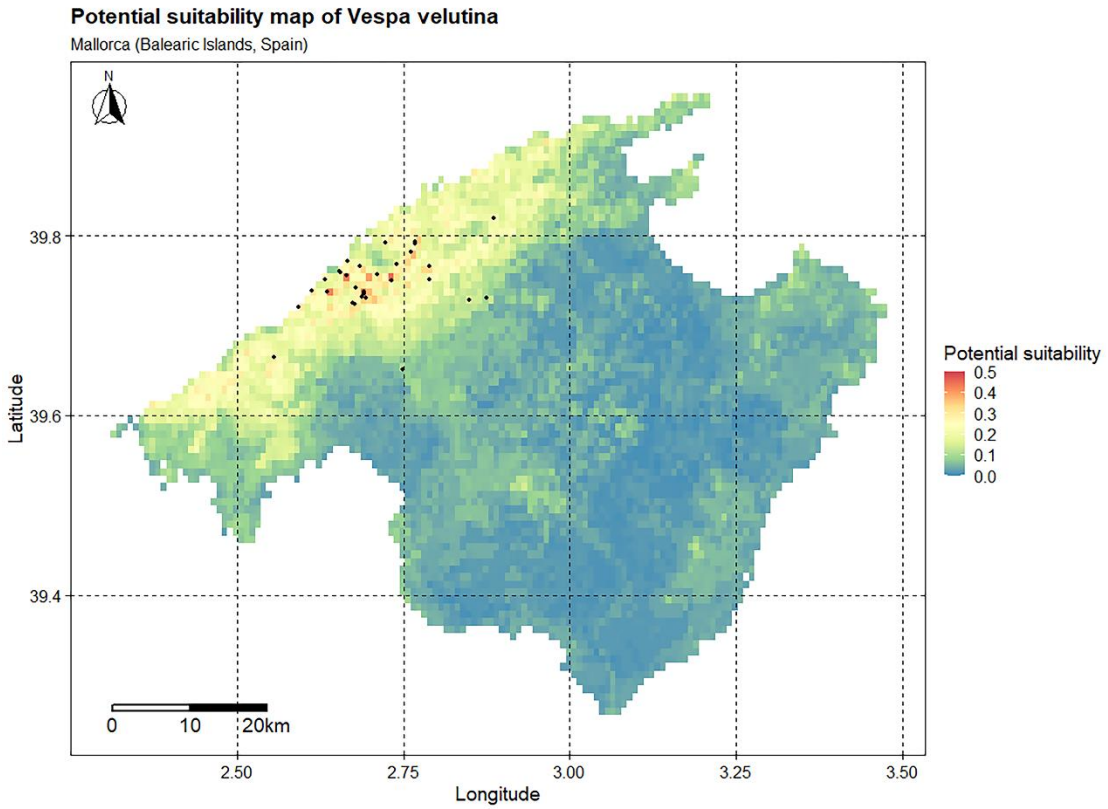

Figure 13.- *Vespa velutina* sites (black dots) used to calibrate the models and habitat suitability under Mediterranean island conditions. Potential suitability is represented in a colour scale from red (high values) to blue (low values).

Response curves

According to the response curves (Figure 14), land cover and the precipitation of the coldest quarter were centered. Slope and continentality showed right-skewed response curves, whereas precipitation of the warmest quarter, isothermality and precipitation seasonality showed left-skewed response curves. Finally, human footprint was stable across the gradient.

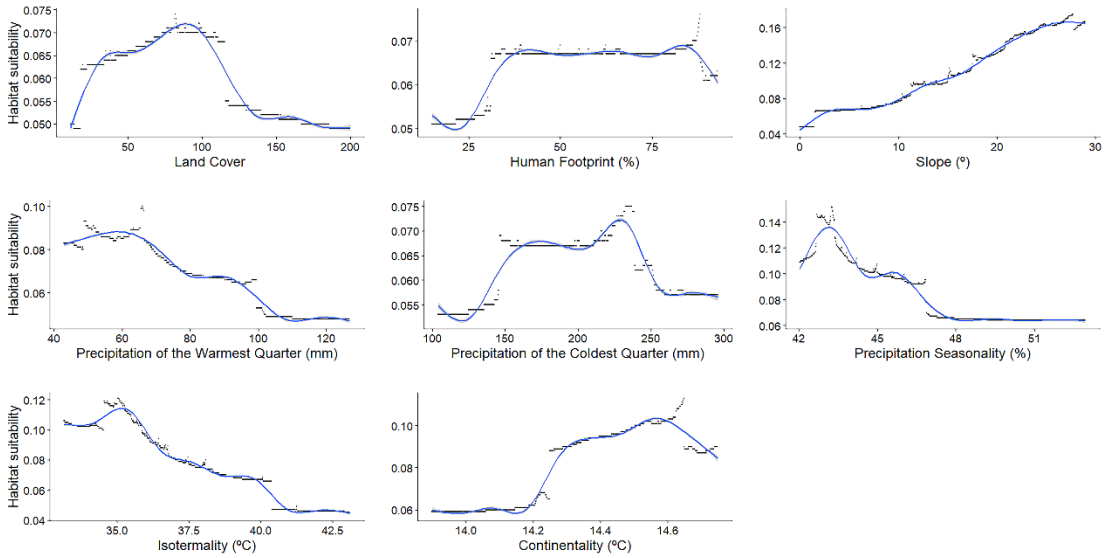

Figure 14.- Response curves of the habitat suitability final ensemble model of *Vespa velutina* under Mediterranean island conditions.

Sites occupied by *V. velutina* showed a land cover from rainfed croplands (GlobCover 2009 value: 14.00) to shrubland (GlobCover 2009 value: 130.62), and a human footprint from 30.29 to 88.32 %. Slope varied between 0.69 and 28.87º, characterized by a high slope diversity. The range of the precipitation of the warmest quarter was around 53.00 mm and the range of the coldest quarter was 100.00 mm. The range of isothermality was around 7.00 ºC and precipitation seasonality was 4.23 %. Finally, the continentality varied 0.4 ºC (Table 3). The comparison between the distributions of the presence and the background data showed high use versus availability in sites with higher average human footprint and precipitation of the coldest quarter (Fig. 2, Supplementary material). Nonetheless, the other ecological factors present a lower use versus availability, suggesting that these variables could limit its distribution (Fig. 2, Supplementary material).

Table 3-. Environmental variables used to determine the ecological niche of *Vespa velutina* under Mediterranean island conditions.

Predictor	Min	1st Quartile	Median	3rd Quartile	Max	Var. import
Slope (º)	0.69	12.31	15.74	20.50	28.87	0.1452
Isothermality (ºC)	34.29	35.44	36.24	37.26	41.25	14.24
Pre Seasonality (%)	42.65	43.24	44.02	45.59	46.88	0.1370
Continentality (ºC)	14.25	14.35	14.47	14.55	14.65	0.1256
Pre Warmest Q (mm)	48.00	66.00	71.50	83.25	101.00	0.1247
Pre Coldest Q (mm)	146.00	161.50	193.00	215.20	246.00	0.1090
Land Cover	14.00	63.75	76.88	88.75	130.62	0.1087
Human footprint (%)	30.29	39.34	52.72	62.35	88.32	0.1074

Importance of environmental factors and measuring ecological niche

The analysis of variable importance showed that the most important predictor was slope (0.1452), followed by isothermality (0.1424) (Table 3). Moreover, the 2D-PCA analyse revealed that the Dim 1 and the Dim 2 explained 38.2% and 22.9% of the total variance. Hence, the final 2D-PCA explained 61.1% of the total variance with statistical differences between background and *V. velutina* niches in Dim 1 (*p-value* < 0.05) (Figure 15). The most important variables in the Dim 1 were slope (27.70) and isothermality (23.81), as in the analysis of variable importance during modelling.

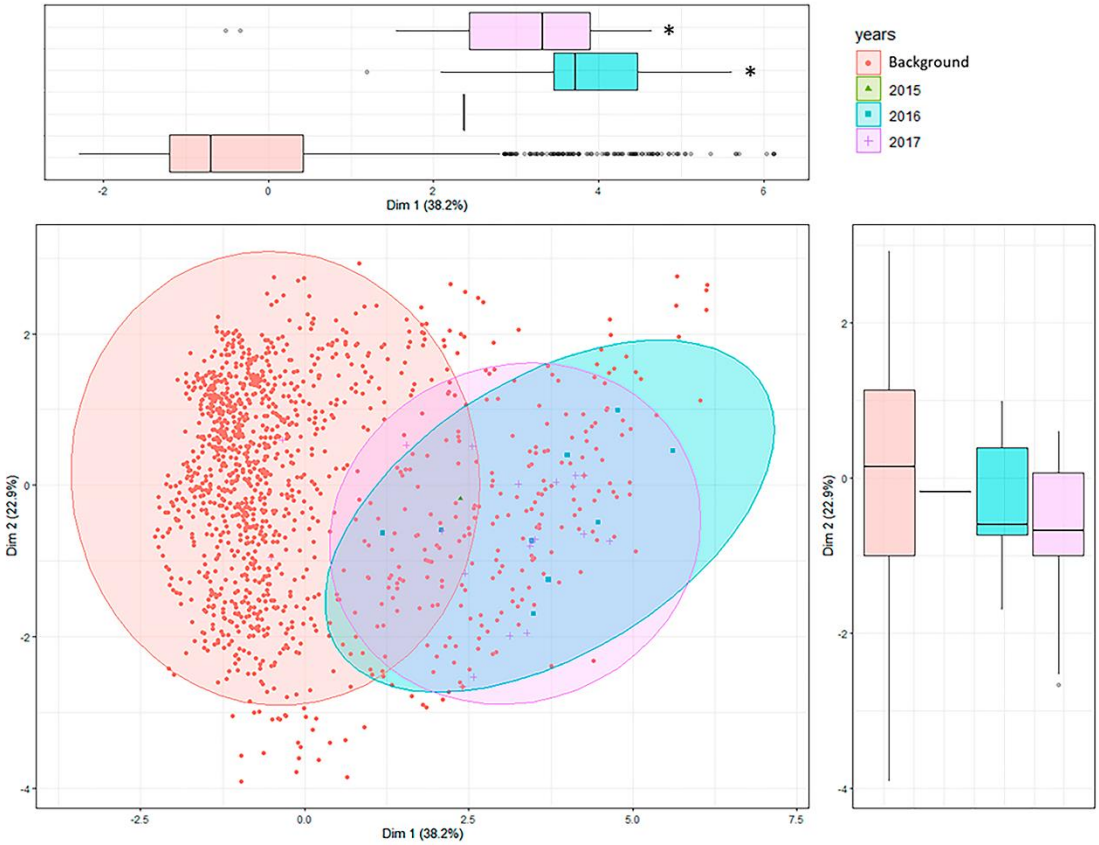

Figure 15.- Principal components analysis (2D-PCA) with ellipses (95% confidence) and marginal box plots showing *Vespa velutina* niche under Mediterranean island conditions. The position of occurrences, from the invaded range along the three years with invasion is indicated in green (2015), blue (2016) and pink dots (2017) and the background is indicated in red dots, respectively. Statistically significant difference between *V. velutina* niche and background in marginal box plots (Kruskal-Wallis test, **p-value* < 0.05). 2015 based in only one nest.

Transferability assessment

The results show a potential mean suitability higher in the other largest Mediterranean islands than in Mallorca for *V. velutina*. Specifically: Ibiza 0.07437 ± 0.02666 (range 0.0100 – 0.1910), Menorca 0.07139 ± 0.01665 (range 0.0300 – 0.1270), Corsica 0.1182 ± 0.04053 (range 0.0180 – 0.2740), Sardinia 0.1252 ± 0.03668 (range 0.0240 – 0.3150), Sicily 0.1251 ± 0.04433 (range 0.0300 – 0.3180), Crete 0.1375 ± 0.03444 (range 0.0640 – 0.2650) and Cyprus 0.1347 ± 0.02671

(range 0.0520 – 0.2550) (Fig. 5). However, any of the potential suitability values in these islands reached the high values found in some points of Mallorca (maximum suitability value 0.4530; Fig. 3). Finally, MESS analysis reported similar ecological conditions to Mallorca in Ibiza, Corsica and Sardinia (Figure 16).

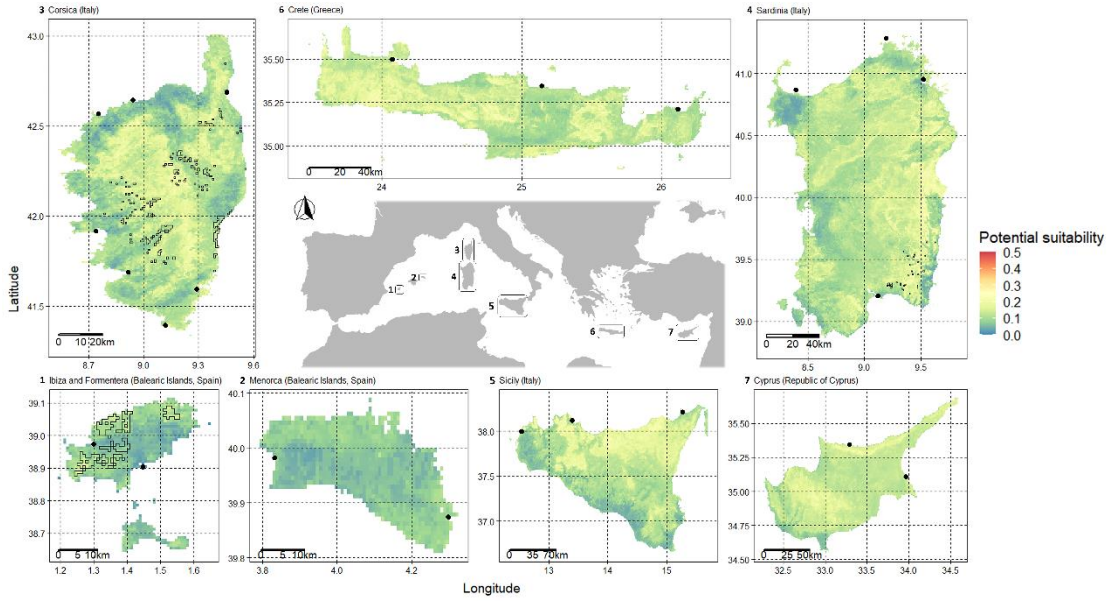

Figure 16.- Transferability of the potential suitability in the biggest Mediterranean islands for *V. velutina*. Potential suitability is represented in a colour scale from red (high values) to blue (low values) and ports are represented as black dots. Multivariate Environmental Similarity Surface (MESS) results are represented in dark polygons, determining areas with similar conditions to the calibration conditions of the model in Mallorca.

Discussion

It is known that invasive species often do not present a static niche, partly due to its ability to adapt and to the selective response of genes that facilitate its transition into invasive ranges (Favreau et al., 2023). This niche can expand, contract or change, reducing the predictive capacity of distribution models based on niche conservatism for species with a dynamic niche (Becerra López et al., 2017; Broennimann et al., 2007; Fitzpatrick et al., 2006; Pearman et al., 2008). For this reason, we integrally georeferenced the population of *V. velutina* from Mallorca (Balearic Islands) (Leza et al., 2021) and developed a predictive model for the entire island to obtain a distribution map of the habitat suitability with management purposes (Peterson et al., 2003). Furthermore, the high population density and dispersion rate of this invasive species highlight the real necessity for an early detection and eradication measures. This need is even more pronounced in biodiversity hotspots like Mallorca, which account for high endemicity levels that may be threatened by alien invaders (Bessa et al., 2016).

To achieve it, we used a specific strategy for the species distribution model with few occurrences (Breiner et al., 2018) which has been rarely used in the management of invasive species (Beukema et al., 2018; Di Febbraro et al., 2019). These studies cited before showed this methodology had good results for assessing the invasiveness of an alien species in a territory, representing an

important step forward to define their potential spread and provide a sound base to prioritize management actions.

Our case study represents the first time that *V. velutina* species reaches a Mediterranean island after its arrival to Europe in 2004 (Haxaire et al., 2006), and despite accounting for few occurrences it has been possible to determine its optimum niche in the first stages of invasion for an area that, a priori, seems to be low suitable (Villemant et al., 2011).

As a general view a great part of the island appears less suitable for *V. velutina* (Fig. 2), in agreement with previous studies reporting the low suitability of the Mediterranean environments (Barbet-Massin et al., 2013; Villemant et al., 2011). Nonetheless, our distributional model calibrated under Mediterranean island conditions proves that there are suitable areas for the establishment of *V. velutina* (most part of "*Serra de Tramuntana*", 307.45 km^2) (Figure 13). The ecological niche model developed for the Iberian Peninsula and the Balearic Islands, based on Villemant et al. (2011), shows similar suitability values from *Sóller* (Mallorca) to the north of the Iberian Peninsula where this species first stablished and spread (MAGRAMA, 2015). Likewise, the spread modelling developed by Robinet et al. (2019) concludes that *V. velutina* could effectively establish in Mallorca. The comparison among the above mentioned studies and our model reflects that analysing at different scales results in different patterns, with regional scales providing more suitable areas than continental ones (as in Bessa et al., 2016). Furthermore, this hornet and other invasive terrestrial pests are mainly translocated as a by-product of shipping, where the characteristics at arrival ports could influence the invasive spread rates across territory (Hudgins et al., 2017; Sardain et al., 2019). In the Mallorcan suitable area there is the port of *Sóller* with commercial connections with the Iberian Peninsula and France, both areas invaded by *V. velutina* (Laurino et al., 2020). Likewise, literature cites the invasion of the Argentine ant (*Linepithema humile*) in the Balearic Islands across the same port (Bernard, 1956).

Since November 2020, *V. velutina* is officially eradicated on the island after two years of active searching with no detections (CAIB, 2019). However, according to our results and if an early detection and eradication plan would not have been implemented, the species probably would have colonized and stablished in the entire northwest part of the island (Figure 13), as predicted by Robinet et al. (2019). In fact, we detected an increase in the number of nests, 1 in 2015, 9 in 2016 and 20 in 2017, as in other colonized areas (Bertolino et al., 2016; Monceau & Thiéry, 2016) showing the potential spreading of the pest.

Among the environmental factors used in this study (continentality, human footprint, isothermality, land cover, precipitation of the coldest quarter, precipitation of the warmest quarter, precipitation seasonality and slope), slope and isothermality were retrieved as the most important. In addition, our results show that the environmental suitability for *V. velutina* is positively correlated with slope. This topographic variable is correlated with a high number of environmental variables (Figure S1, Supplementary material) and is known to be very useful to develop ecological models at local scale (Lassueur et al., 2006), since the slope affects the conditions in an organism's microclimate (Sears et al., 2011). For example, steeper slope have been correlated with warmer core-body temperatures in butterflies (Barton & Terblanche, 2014). To check the effect of the slope in *V. velutina* it will be necessary to study the development of *V. velutina* immature stages inside the nest. Moreover, installing their nest in high trees plus steeper slopes provides a higher protection against potential predators and competitors, and make it difficult to apply control measures against them. One the other hand, the great range in land cover values used by *V. velutina* shows the high adaptation capability to construct their nests in many different Mediterranean areas, such as forests, shrublands or croplands (Figure 14) as well as in

other invaded areas where this species has been successfully stablished (Carvalho et al., 2020; Franklin et al., 2017; Monceau & Thiéry, 2016). Such high adaptation capability is a drawback to propose management and control measures mainly due to the great fieldwork effort needed to locate *V. velutina* colonies (Leza et al., 2021). In addition, the relationship between human footprint and suitability values suggests that disturbed habitats and human-mediated dispersal benefits this species, as reported in previous studies on similar species (Alaniz et al., 2021; Monceau et al., 2014; Robinet et al., 2019, 2017). The precipitation in summer (warmest quarter) was negatively correlated with suitability (Figure 14), thus playing an important role in the pest ecology. During summer pest growths exponentially and demands high amounts of proteins (Monceau et al., 2014), but high precipitation values could limit its predation activity (Bista et al., 2020a, 2020b). After wintering, foundress queens emerge and start building a delicate primary nest, where site selection is closely linked to protection from adverse weather (Monceau & Thiéry, 2016). Thus, the low precipitation rates typical of Mallorca during the coldest quarter benefits the spread of *V. velutina* queens in comparison with other continental areas where this species has successfully stablished (Figure 14) (AEMET, http://www.aemet.es/). Added to all the above, response curves show minimum values in terms of seasonal variables (isothermality and precipitation seasonality) and a reduced range for continentality in terms of use *vs* available (Figure S2, Supplementary material), pointing to a preference for stable environments that require lower energy efforts to regulate the nest conditions (e.g., temperature and humidity). This uncertain assumption, however, needs to be addressed.

Future studies should include density data of honeybee hives because honeybees are a quite important food resource (Bessa et al., 2016), as well as freight traffic (Lester & Beggs, 2018; Pusceddu et al., 2019).

Principal component analysis (2D-PCA) of the environmental data revealed one significant axis of environmental variation, defining the suitable ecological space for *V. velutina* establishment (Figure 15). It is important to note that since 2015 (Figure 15, green dot) until 2017 (Figure 15, pink dots) the ecological niche has shifted, moving away from the prevailing ecological niche offered by the rest of the island, despite the overlap between background and *V. velutina* niches. This niche shift occurs along the first axis, which is associated with topography slope and isothermality gradient across the island. Supporting this idea, *V. velutina* will be able to spread and establish in specific areas under Mediterranean conditions (Robinet et al., 2019).

Another important point is the approach of using niche models to predict the spread of potential invaders into new areas (Broennimann et al., 2007). Our results show that other Mediterranean islands are potentially suitable for the establishment and spread of *V. velutina* (Figure 16), zones where this species has not been recorded yet (Laurino et al., 2020), making it necessary to implement preventive actions to avoid a possible invasion. Robinet et al. (2019) explored the human-mediated dispersal of *V. velutina* concluding that the Mediterranean islands could not be naturally colonised, this invasive species could only reach the Mediterranean islands by an accidental introduction by humans. Although the probability calculated of introducing the hornet in the Mediterranean islands is relatively low in this study: to Sicilia was 0.925%, to Sardinia was 0.206%, to Mallorca was 0.095% and to Corsica was 0.032% (Robinet et al., 2019). *V. velutina* reached Mallorca in spite of the low probability of introduction, so these model extrapolations provide a valuable information regarding the areas in the other Mediterranean islands that might be invaded next. This could be used by scientists and managers for an early detection of the invasive species (Figure 16) (Broennimann et al., 2007), because it is more cost-effective outcome for conservation and can be most efficiently controlled (Holden et al., 2016; Monceau et al., 2014;

Robinet et al., 2017). There are detection and control methods for *V. velutina* across Europe, however, little has been done to date to limit its progression (Turchi & Derijard, 2018) and there is no clear coordination between countries or uniform methods for eradication since the first detection (Leza et al., 2021). Poor coordination between managers and stakeholders in France was thought to be one of the important reasons for the species continues to spread (Monceau et al., 2014). Hence, long-term monitoring of ports and nearby areas should be implemented for an early detection of *V. velutina* to be in time to implement a control and eradication protocol, as in Leza et al. (2021). Moreover, many studies have confirmed that citizen collaboration in control programs is essential for the best management of invasive species (Clusa et al., 2018; Graham et al., 2011; Klemann-Junior et al., 2017). In the case of *V. velutina*, a specific trap for this species has not yet been developed and for this reason citizen collaboration is crucial for its detection and control.

Future research will focus on the genetic characterization of the Balearic population of *V. velutina* to determine the path of introduction and to assess the role of shipping networks in spreading *V. velutina* across the Mediterranean Sea, which could help in promoting preventative measures such as the establishment of stringent biosecurity policies at the national, regional, and global scales

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AUTHOR CONTRIBUTIONS

C. Herrera contributed to conceptualization; data curation; formal analysis; methodology; software; validation; visualization; writing—original draft; writing—review and editing. J. A. Jurado-Rivera contributed to supervision, validation, visualization, writing—review and editing.

M. Leza contributed to conceptualization, supervision, validation, visualization, writing—review and editing.

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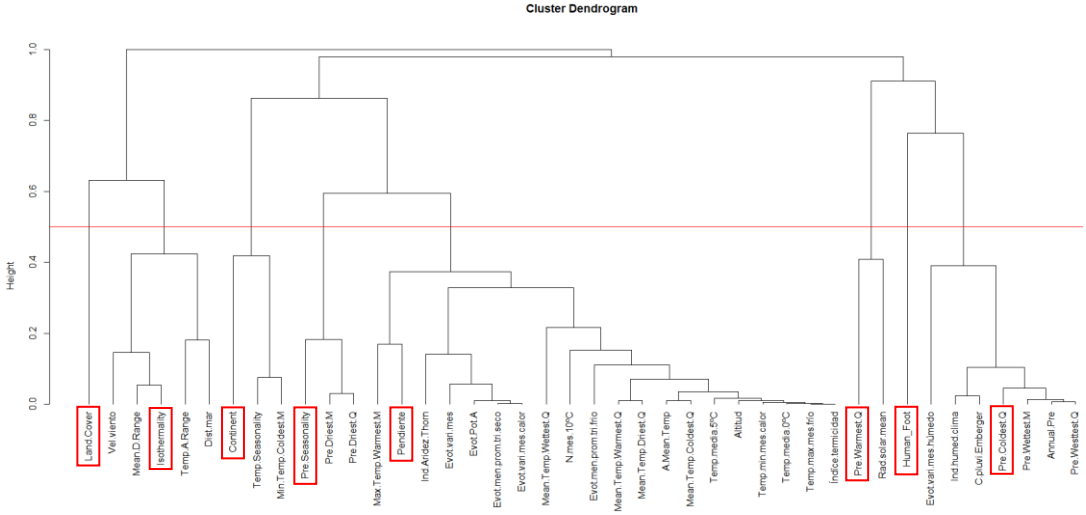
SUPPLEMENTARY MATERIAL



Land Cover, *Vel.viento* = Wind speed, *Mean.D.Range* = Mean Diurnal range, ***Isothermality***, *Temp.A.Range* = Temperature Annual Range, *Dist.mar* = Distance to the sea, ***Continent*** **= Continentality**, T*emp.Seasonality* = Temperature Seasonality, *Min.Temp.Coldest.M* = Min Temperature of Coldest Month, ***Pre.Seasonality*** **= Precipitation Seasonality**, *Pre.Driest.M* = Precipitation of Driest Month, *Pre.Driest.Q* = Precipitation of Driest Quarter, *Max.Temp.Warmest.M* = Max Temperature of Warmest Month, ***Pendiente*** **= Slope**, *Ind.Aridez.Thorn* = Thornthwaite aridity index, *Evot.vari.mes* = Monthly variability in potential evapotranspiration, *Evot.Pot.A* = Annual potential evapotranspiration, *Evot.men.prom.tri.seco* = Mean monthly precipitation of driest quarter, *Evot.vari.mes.calor* = Mean monthly precipitation of warmest quarter, *Mean.Temp.Wettest.Q* = Mean Temperature of Wettest Quarter, *N.mes.10ºC* = Months with a mean temperature greater than 10ºC, *Evot.men.prom.tri.frio* = Mean monthly precipitation of coldest quarter, *Mean.Temp.Warmest.Q* = Mean Temperature of Warmest Quarter, *Mean.Temp.Driest.Q* = Mean Temperature of Driest Quarter, *A.mean.Temp* = Annual Mean Temperature, *Mean.Temp.Coldest.Q* = Mean Temperature of Coldest Quarter, *Temp.media.5ºC* = Mean monthly temperature for months with mean temperature greater than 5ºC multiplied by number of days, *Altitud* = Altitude, *Temp.min..mes.calor* = Min temperature of the warmest month, *Temp.media.0ºC* = Mean monthly temperature for months with mean temperature greater than 0ºC multiplied by number of days, *Temp.max.mes.frio* = Max temperature of the coldest month, *Indice.termicidad* = Compensated thermicity index, *Pre.Warmest.Q* = Precipitation of Warmest Quarter, *Rad.solar.mean* = Solar radiation, *Human_foot* = Human footprint, *Evot.vari.mes.humedo* =

Mean monthly precipitation of wettest quarter, *Ind.humed.clima* = Climatic moisture index, *C.pluvi.Emberger* = Emberger's pluviothermic quotient, *Pre.Coldest.Q* = Precipitation of Coldest Quarter, *Pre.Wettest.M* = Precipitation of Wettest Month, *Annual.Pre* = Annual Precipitation, *Pre.Wettest.Q* = Precipitation of Wettest Quarter

Figure S1.- Cluster dendrogram used to check multicollinearity between variables. One predictor (in red) for each cluster below the 0.5 threshold (in red) was selected for climate suitability modelling.

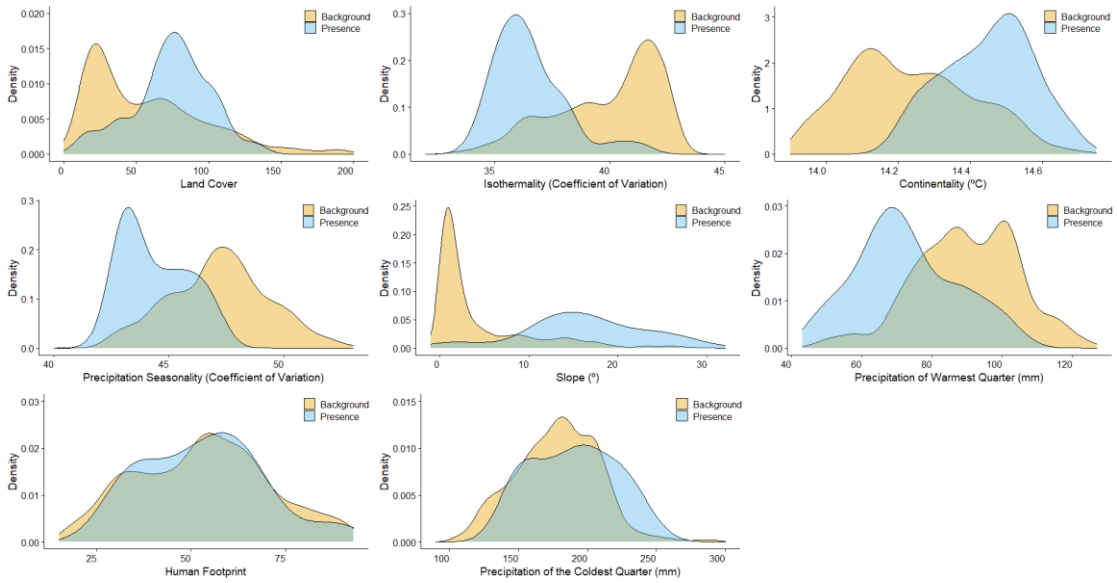


Figure S2.- Comparing the distributions of *V. velutina* presence with environmental background over the environmental predictors.

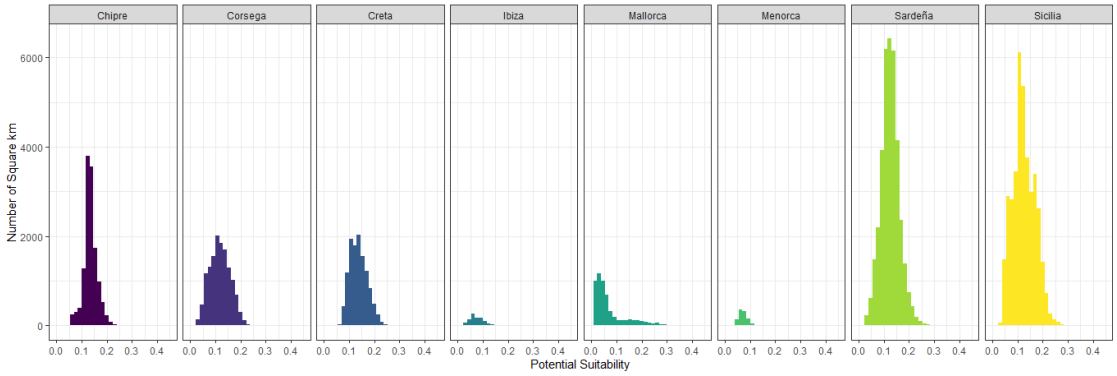


Figure S3.- Density plot representing number of km^2 for each potential suitability value in the Mediterranean islands used in this study.

Table S3.- Variables included in the analysis and variables used in the study are in bold. Average values in Mallorca shown together with the values where species presence was recorded. Source of data and references are included.

Type of variable	Variables	Mallorca min-max	Presences min-max	References
Climatic (n=37) Data source: - Worldclim 2.0 (Fick and Hijmans, 2017) - ENVIREM (Title and Bemmels, 2018)	Annual Mean Temperature (ºC)	10.79 – 17.23	12.74 – 16.40	Barbet-Massin et al., 2013; Bessa et al., 2016; de Medeiros et al., 2018; Fournier et al., 2017; Rodríguez-Flores et al., 2019; Villemant, Barbet-Massin, et al., 2011; Villemant, Muller, et al., 2011
	Mean Diurnal Range	7.61 – 11.53	8.38 – 10.73	
	Isothermality	33.17 – 43.10	34.29 – 41.25	
	Temperature Seasonality	521.60 – 620.00	545.10 – 606.00	
	Max Temperature of Warmest Month (ºC)	23.40 – 30.60	25.50 – 29.60	
	Min Temperature of Coldest Month (ºC)	-1.60 – 6.70	0.50 – 4.80	
	Temperature Annual Range	21.70 – 27.00	23.40 – 26.00	
	Mean Temperature of Wettest Quarter (ºC)	7.67 – 18.53	9.70 – 14.05	
	Mean Temperature of Driest Quarter (ºC)	18.95 – 24.05	20.68 – 23.33	
	Mean Temperature of Warmest Quarter (ºC)	18.95 – 24. 20	20.68 – 23.70	
	Mean Temperature of Coldest Quarter (ºC)	4.20 – 11.48	6.15 – 10.27	
	Annual Precipitation (mm)	404.00 – 934.00	505.00 – 796.00	
	Precipitation of Wettest Month (mm)	69.00 – 120.00	80.00 – 105.00	
	Precipitation of Driest Month (mm)	4.00 – 17.00	8.00 – 14.00	
	Precipitation Seasonality	42.04 – 53.62	42.65 – 46.88	
	Precipitation of Wettest Quarter (mm)	164.00 – 348.00	197.00 – 301.00	
	Precipitation of Driest Quarter (mm)	32.00 – 92.00	48.00 – 78.00	
	Precipitation of Warmest Quarter (mm)	43.00 – 127.00	48.00 – 101.00	
	Precipitation of Coldest Quarter (mm)	104.00 – 296.00	146.00 – 246.00	
	Wind speed (km/h)	2.63 – 5.00	2.98 – 4.27	This study
	Solar radiation (kJ/m2*day)	15320 – 16009	15592 – 15901	
	Compensated thermicity index (ºC)	193.00 – 381.00	258.00 – 377.00	
	Annual potential evapotranspiration (mm/year)	801.50 – 1010.00	864.60 – 994.80	
	Thornthwaite aridity index	51.80 – 73.34	56.00 – 70.11	
	Climatic moisture index	-0.56 – -0.13	-0.52 – -0.1	
	Continentality (ºC)	13.85 – 14.75	14.25 – 14.65	
	Emberger's pluviothermic quotient	67.14 – 144.38	74.78 – 36.53	
	Mean monthly temperature for months with mean temperature greater than 0ºC multiplied by number of days	46134 - 73422	55476 - 72918	
	Mean monthly temperature for months with mean temperature greater than 5ºC multiplied by number of days	35760 - 73422	55476 - 72918	
	Max temperature of the coldest month (ºC)	7.70 – 14.20	9.90 – 14.00	
	Min temperature of the warmest month (ºC)	14.90 – 20.60	16.90 – 20.50	
	Months with a mean temperature greater than 10ºC	6.00 – 12.00	7.00 – 12.00	
	Mean monthly precipitation of coldest quarter (mm/month)	26.79 – 37.33	30.00 – 36.53	
	Mean monthly precipitation of driest quarter (mm/month)	117.10 – 139.10	123.10 – 136.50	
	Monthly variability in potential evapotranspiration (mm/month)	3739.00 – 4254.00	3870.00 – 4167.00	
	Mean monthly precipitation of warmest quarter (mm/month)	106.60 – 126.30	112.10 – 123.60	
	Mean monthly precipitation of wettest quarter (mm/month)	34.40 – 69.42	37.96 – 67.74	
Topography (n=2) Data source: - SRTM 90m Digital Elevation Database v4.1 (Jarvis et al. 2008)	Altitude (m)	0.00 – 1229.50	78.50 – 825.50	Bertolino et al., 2016; de Medeiros et al., 2018; Rodríguez-Flores et al., 2019
	Slope (º)	0.00 – 35.34	0.69 – 28.87	This study

Remote sensing[4] (n=1) Data source: - GlobCover 2009	**Land cover**	11.00 – 200.00	14.00 – 130.62	de Medeiros et al., 2018; Fournier et al., 2017; Lioy et al., 2019
Hidrography (n=1)	Distance to the sea (km)	0.00 – 24.00	0.50 – 15.80	This study
Anthropogenic (n=1) Data source: - Human Footprint, 2018 Release (2009) (Venter et al., 2009; 2016)	**Human footprint**	15.00 – 93.00	30.29 – 88.32	de Medeiros et al., 2018; Robinet et al., 2017

Chapter 4

Niche modelling and landscape genetics of the yellow-legged hornet (*Vespa velutina*): an integrative approach for evaluating central–peripheral population patterns in Europe

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Abstract

Genetic diversity is an important biological trait for a successful invasion. During the expansion across a new territory, the invasive species may face unprecedented ecological conditions that will determine its demography and the genetic diversity. The first record of the yellow-legged hornet (*Vespa velutina*) in Europe dates back to 2004 in France, from where it has successfully spread through a large territory in the continent, including Italy, Spain, and Portugal. Integrative approaches offer a powerful strategy to detect and understand pattens of genetic variation in central and peripheral populations. Here, we have analysed the relationship between genetic diversity inferred from 15 *V. velutina* nuclear DNA microsatellite loci, and geographic and environmental drivers, such as the distance to the introduction focus, environmental suitability, and distance to native and invasive niche centroid. Our results revealed a central-peripheral pattern, where allelic richness decreased towards the edge of the expansion range. The low environmental suitability of the territories invaded by marginal populations could prevent a diverse population from establishing and reducing the genetic diversity in populations at the expansion edge. Moreover, Markov chain Monte Carlo analysis showed both geographic and environmental distances were influencing population genetic differentiation. This study highlight the importance of combining genetic analysis with geographical and environmental drivers to understand potential adaptation of this invasive species.

Keywords: environmental suitability, genetic structure, invasive species, microsatellite, single introduction, *Vespa velutina*

Introduction

Biological invasions are a key component of global change and represent one of the main causes of biodiversity loss (Carpenter et al, 2018), large economic costs for society (Bacher et al., 2018), and a threat to human health (Mazza et al., 2014). Consequently, alien species introductions are a problem of increasing concern (Sardain et al., 2019) and constitute a priority research area in international global change programs (Essl et al., 2020; Latombe et al., 2022; Roura-Pascual et al., 2021).

Understanding why some introduced alien species succeed and become invasive while others fail constitutes a major challenge in invasion ecology. Some of the most commonly studied traits underlying the success of an invasive event are related to community structure (David et al., 2017), niche conservationism (Broennimann et al., 2007), resource availability and competition (González et al., 2010), biotic resistance and community diversity (Guo et al., 2015), phylogenetic, functional, or ecological originality (David et al., 2017), and founding propagule as a source of genetic diversity (Lockwood et al., 2005).

Genetic diversity plays a key role in alien species persistence and resilience in a new environment (Hoffmann & Willi, 2008). Propagule pressure during the introduction stage (including the number of introduction events and the number of introduced individuals) as well as selection pressure acting during the establishment stage can affect the genetic diversity of an invasive population (Lockwood et al., 2005). Moreover, factors related to demographic history or ecological conditions may determine habitat suitability for species spread (Bacon et al., 2017; Unglaub et al., 2015; Williams et al., 2021) and establishment and could leave a genetic footprint in populations (Hewitt, 2000; Stuart et al., 2021).

Ecological niche modelling represent a powerful tool to obtain quantitative spatial data regarding the habitat suitability of given region and, hence, to assess how the potential occurrence of a given species varies across the territory (Alvarado-Serrano & Knowles, 2014). In addition, it can be applied to detect patterns of genetic variation based on population distributions (Moussalli et al., 2009), estimate the similarity of niches between populations with different genetic clusters (Acevedo-Limón et al., 2020), and detect potential local adaptation (Stuart et al., 2021). Moreover, habitat suitability information can be useful to detect potential migration paths or isolation events (McRae, 2006), and therefore determine gene flow between populations.

The central-peripheral hypothesis predicts that reduced habitat suitability and small effective population sizes at marginal ranges will lead to a decline of genetic diversity from the centre to the range edges of a population (Eckert et al., 2008; Guo, 2014; Trumbo et al., 2016). Therefore, genetic diversity values are expected to decrease along the expansion front (Excoffier et al., 2009) due to cumulative genetic drift effects linked to successive bottleneck events (van Boheemen et al., 2017). Hence, the central-peripheral hypothesis attempts to describe the genetic consequences of the abundant centre hypothesis (Eckert et al., 2008), where habitat suitability, population abundance, and connectivity among populations will decrease towards the edges of the expansion range. In this regard, biological invasions represent a unique opportunity to test evolutionary hypotheses within a species expansion range limits (Guo, 2014).

The yellow-legged hornet (*Vespa velutina*) is the first invasive Vespidae predator introduced from Asia to Europe (Monceau et al., 2014), where it has successfully spread from a single introduction in France in 2004 (Laurino et al., 2020). This invasive organisms provides a unique study model for testing the central-peripheral hypothesis (Lira-Noriega & Manthey, 2014) for several reasons

including that (i) its arrival in Europe is very recent (Haxaire et al., 2006), (ii) the location of the first introduction is known (Lot-et-Garonne, France) (Haxaire et al., 2006), (iii) there is evidence of a single founder event (Arca et al., 2015; Quaresma et al. 2022), and (iv) it has successfully spread and established in neighbouring countries, such as Italy, Spain, and Portugal (Laurino et al., 2020). Here we test the central-peripheral hypothesis in invasive *V. velutina* populations from France, Italy, Spain, and Portugal. In addition, we identify the geographical and environmental drivers that better explain the observed genetic patterns. To that end, we assess the relationship between genetic diversity parameters, inferred from 15 nuclear DNA microsatellite loci, and the geographic and environmental drivers such as the distance to the introduction focus, environmental suitability, and distance to native and invasive niche centroid.

Material and methods

Sample collection, DNA extraction, and microsatellite genotyping

A total of 333 adults of *V. velutina* were collected in mainland Spain from Asturias (N = 11), Basque Country (N = 20), Catalonia (N = 16), Galicia (N = 12), and on the island of Mallorca (N = 274). Samples were stored in absolute ethanol and/or at -20ºC until molecular analyses.

Genomic DNA was individually extracted and purified from hornet legs using the Qiagen DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's instructions, with RNA digested using 60 µg of RNAse A (Promega). All sampled individuals (333) were genotyped for 15 microsatellite loci using the oligo combinations developed by previous population genetic studies on *V. velutina* (Daly et al., 2002; Hasegawa & Takahashi, 2002; Arca et al., 2012). Forward primers were labelled with 6-FAM (blue), VIC (green), NED (yellow) and PET (red) dyes and the PCR amplifications were segregated into two multiplex reactions (Table S2). PCR reactions were performed in a total volume of 10 µL, including 1 µL of *V. velutina* template DNA, 5 µL of Multiplex PCR kit (Qiagen), 3.6 µL of primer mix (10µM), and 0.4 µL of H2O. The PCR conditions consisted of an initial denaturation step at 95 ºC (15 min.) followed by 40 cycles at 94 ºC (30 sec.), 50 ºC (90 sec.) and 72 ºC (60 sec.), followed by a final extension at 60 ºC (30 min.). Fragment analysis was performed on an ABI Prism 3130 DNA Genetic Analyzer (Applied Biosystems), using GeneScan 500LIZ® as internal size standard. Fragment lengths were determined using GENEMAPPER 5.0 (Applied Biosystems) and checked manually.

The microsatellite dataset generated from the 333 *V. velutina* individuals was merged with an existing one containing genotypes from 330 individuals collected in other invaded territories (mainland Spain: Basque Country = 3 and Galicia = 42, France = 83, Italy = 11, Portugal = 191) (Arca et al. 2015; Quaresma et al. 2022). To enable dataset merging, allele scores of each microsatellite locus were harmonized between laboratories by genotyping 10 DNA samples used by Quaresma et al. (2022), which in turn were previously harmonized with the samples analysed by Arca et al. (2015). Although the studies of Budge et al. (2017) and Jones et al. (2020) also generated *V. velutina* microsatellite data, we opted for the conservative approach of not including these data in our analyses because they were not harmonized with other studies.

Genetic structure and diversity

Population genetic analyses were based on the 15 microsatellite loci obtained from the 663 individuals sampled in France, Italy, Spain, and Portugal (this study; Arca et al., 2015; Quaresma et al., 2022). Population structure was analysed using the fast maximum-likelihood genetic clustering approach (Beugin et al., 2018). This method is similar to the model implemented by STRUCTURE (Pritchard et al., 2000) but allows for a much faster estimation of genetic clusters by means of the Expectation-Maximization (EM) algorithm. We initially investigated the number of cluster by using the k-means algorithm, where the preferred number of clusters was evaluated using the Bayesian information criterion (BIC) score. The Mallorca population sample was only used to assess the genetic structure across Europe since the geographical isolation of this territory might restrict gene flow among populations, thereby influencing the genetic parameters due to genetic drift (Wright, 1938).

Based on clustering analyses, and excluding Mallorca population for further analysis, the 389 individuals were segregated into 10 populations, distributed as follows: France (1), Italy (2), Spain (5), and Portugal (2). To alleviate the problem of the unbalanced sample size, we further divided the Portuguese sample (n = 191) into three populations, following the north-south spread: Portugal N (north), Portugal C (centre), and Portugal S (south). Accordingly, the ensuing analyses were based on twelve different populations (Table 4), whose allelic richness (Ar) was estimated using the R package *pegas* (Paradis, 2010), using the rarefaction method described by (Hulbert, 1971). Finally, genetic differentiation among populations was estimated based on the pairwise F_{ST} values computed in ARLEQUIN 3.5.2.2 (Excoffier et al., 2005), and using 10000 random permutations to assess significance.

Collection of occurrence data

We obtained species occurrence data from the native Asian and invaded European ranges available in GBIF (accessed on November 22, 2022) and from Verdasca et al. (2022), which is so far the most complete dataset used to model *V. velutina*. Moreover, we included the occurrences of the individuals used in previous genetic studies (Arca et al., 2015; Quaresma et al., 2022) and in this study (Table S1). We deliberately excluded occurrences from South Korea and Japan, as these also represent invaded ranges (Takeuchi et al., 2017).

We applied spatial thinning, a process in which a subset of locations is selected to reduce spatial autocorrelation and sampling bias, especially at larger geographical scales. Spatial thinning has been shown to decrease model overfitting and improve performance in studies where the presence records exhibit selection bias (Boria et al. 2014). In particular, we removed presence records based on a minimum neighbour distance (~4.5 km, half the resolution of environmental variables explained in the next section) between the remaining records in each grid. This procedure resulted in a total of 6473 occurrences for the invasive population in Europe and 127 occurrences for the native population in Asia.

Geographic peripherality and environmental suitability

The first field record of *V. velutina* was reported from the French department of Lot-et-Garonne in 2004 (Haxaire et al., 2006). The Euclidean distance (in km) between each sampled individual

and the first record in Lot-et-Garonne was then averaged to obtain a population-level distance to the introduction focus.

We used ecological niche models to infer the distribution of suitable environments for *V. velutina* in Europe (Villemant et al., 2011). Climatic variables were included in our modelling analyses since they are considered the main contributors to species niche delimitation at large scales (Luoto et al., 2007). Climatic data was extracted from the WorldClim 2.1 database (Fick & Hijmans 2017) as 5 arcmin grids (~9.3 km2).

Random points were sampled as background points around where *V. velutina* occurs, applying a 100 km buffer. The background buffer was selected since the maximum spread distance registered for this species has been 78 km (Robinet et al., 2017). This procedure resulted in a total of 6049 background points for the invasive population in Europe and 7022 background points for the native population in Asia. We merged both invasive and native population datasets for further analysis.

Multicollinearity among the variables was assessed, and six uncorrelated predictors with a variance inflation factor (VIF) lower than four were selected: Annual Mean Temperature (bio1), Isothermality (bio3), Temperature Seasonality (bio4), Mean Temperature of the Wettest Quarter (bio8), Annual Precipitation (bio12), and Precipitation of the Driest Month (bio14). Ten percent of the dataset was used for automated hyperparameter optimization of the models (tuning) (Feurer & Hutter, 2019). To assure that a particular method is the best for all situations, it is recommended to include different models from different modelling techniques (Johnson & Gillingham, 2005; Hirzel & Le Lay, 2008). Hence, we selected ten different models: Surface Range Envelope (SRE), Generalized Linear Models (GLM), Generalized Additive Models (GAM), Multivariate Adaptive Regression Spline (MARS), Classification Tree Analysis (CTA), Flexible Discriminant Analysis (FDA), Artificial Neural Network (ANN), Random Forest (RF), Generalized Boosting Method (GBM), and Maxent available in *biomod2* R package (Thuiller et al., 2009). We fitted and evaluated the models in Europe using weighted background, as described in Barbet-Massin et al. (2012). Each model was fitted with 70% of the data and evaluated with the remaining 30% using ROC cross-validation. We applied this methodology 50 times to each single model. Finally, we ensembled each single model using ROC > 0.7. The final ensemble model at the sampled-individual level was then averaged to obtain population-level environmental suitability.

We performed niche quantification, comparison, and equivalency and similarity tests using *ecospat* R package (Di Cola et al., 2017; Broennimann et al., 2020). Niche availability was defined using background environmental data, while niche occupancy was defined using occurrence data for invasive European populations and native Asian populations with a 400-pixel resolution for the grid of environmental space. Moreover, we estimated Schoener's D as an index of niche overlap between populations using *ecospat* R package, which ranges between 0 (no overlap) and 1 (full overlap). Likewise, we performed niche equivalency and similarity tests using 1000 replications. The niche equivalency test determines whether niches of two entities in two geographical ranges are equivalent (i.e., whether the niche overlap is constant when randomly reallocating the occurrences among the native and invasive ranges). On the other hand, the niche similarity test addresses whether the environmental niche occupied in one range is more similar to the one occupied in the other range than would be expected by chance (Warren et al., 2008). This test differs from the equivalency test because it examines whether the overlap between observed niches in two ranges is different from the overlap between the observed niche in one range and niches selected at random from the other range (Broennimann et al., 2012). We determined the invasive and native centroids of both niches and calculated the distance between

each sampled individual and each centroid, and then values were averaged to obtain population-level distance. Finally, environmental distance among populations was calculated using a niche plot. It is important to highlight that unequal availability of environmental occurrences between native and invasive ranges may add uncertainty to overlap niche measurements (Beukema et al., 2018). Nonetheless, this unbalanced data have been widely used in niche modelling of other Vespa species (Villemant et al., 2011; Zhu et al., 2020; Lioy et al., 2023).

Geographic and environmental drivers of genetic diversity

The relationship between genetic diversity parameters (Ar) and Euclidian distance, environmental suitability, and distance to both invasive and native niche centroids was assessed using a bivariate linear regression model, whose *p-value* was corrected using Bonferroni correction. Moreover, we explored the relationship between genetic variation (F_{ST}) and both geographic (km) and environmental distances using a Markov chain Monte Carlo (MCMC) with one million simulations, available in the *Sunder* package (Bradburd et al., 2013; Guillot & Rousset, 2013; Guillot et al., 2014; Botta et al., 2015). This approach uses a likelihood value to examine whether geographic distance, environmental distance, or a combination of both contributes to genetic differentiation. Statistical significance was set at $p\text{-value} < 0.05$.

Results

Genetic structure and diversity

Four different genetic clusters were detected in Europe by structure analysis using the expectation-maximization algorithm implemented in the *adegenet* package (Figure 17A). There was a clear and consistent genetic cluster in the French population (cluster 1, mean Q-value = 100%), which was differentiated from the rest of the invasive populations in Europe. Seven populations were assigned to cluster 2 (mean Q-values: Mallorca 2 = 100%, Catalonia = 93.75%, Basque Country = 95.65%, Asturias = 100%, Galicia = 44.44%, Italy = 54.54%, Portugal = 2.62%), four populations to cluster 3 (mean Q-values: Galicia = 55.56%, Portugal N = 100%, Portugal C = 100%, and Portugal S = 100%), and two populations to cluster 4 (mean Q-values: Mallorca 1 = 100%, and Italy = 45.46%). Cluster 2 was the most widespread in southern Europe, ranging from Italy to Portugal. In contrast, cluster 3 was detected only in the westernmost part of the Iberian Peninsula, and cluster 4 in Italy and Mallorca. After excluding Mallorca from further analysis, we calculated the genetic parameters for 12 different populations (Table 4). The highest diversity was detected in French population, and the lowest was most often detected in Portugal C (Table 4).

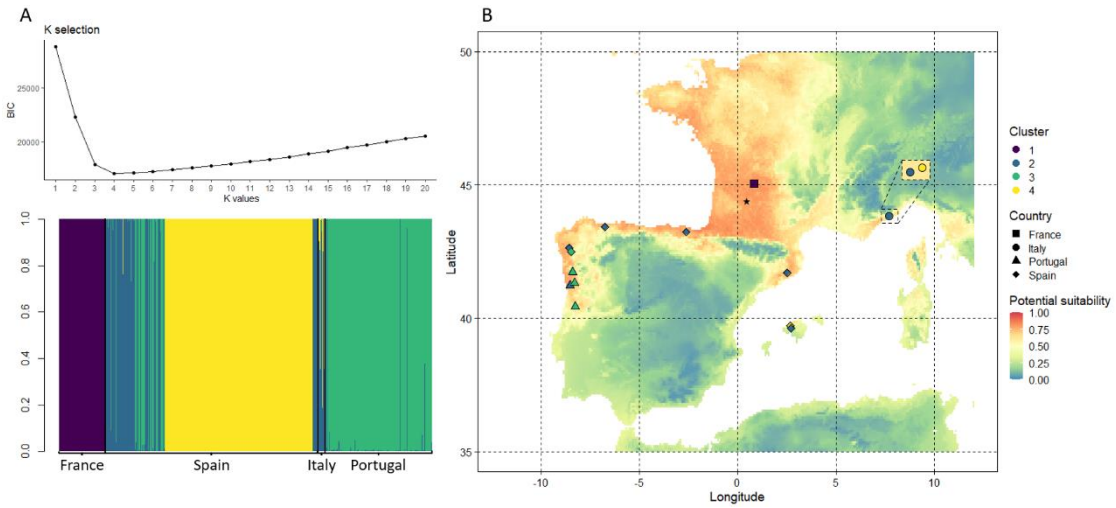

Figure 17.- Genetic structure and environmental suitability of *V. velutina* in Europe. A) Populations admixture ancestry components are shown for the 665 individuals, where different countries are delimited by black borders. The lowest Bayesian information criterion (BIC) was obtained at K = 4. B) Potential suitability is represented in a colour scale from red (high values) to blue (low values). The location of each population centroid included in the study and their genetic cluster are represented on the map. The black star represents the first field record of this species in Europe.

Table 4.- Genetic parameters, geographical and environmental drivers from *V. velutina* populations studied. Number of individuals (N.ind), allelic richness (Ar), the distance to the introduction focus (Dist.km), environmental suitability (Suitab.), and distance to both native (Dist.nat) and invaded niche centroids (Dist.inv).

ID	Country	Cluster	N.ind	Ar	Dist.km	Suitab.	Dist.nat	Dist.inv
France1	France	1	83	2,775	119.215	0.749	3.218	0.949
Catalonia2	Spain	2	16	2,526	350.507	0.810	2.707	0.647
BasqueC2	Spain	2	23	2,398	280.327	0.798	2.631	0.814
Asturias2	Spain	2	11	2,032	586.792	0.714	2.374	0.625
Galicia2	Spain	2	24	2,238	755.695	0.721	2.113	0.538
Galicia3	Spain	3	30	1,908	752.652	0.707	2.144	0.506
Italy4	Italy	4	6	1,933	584.225	0.587	3.236	1.393
Italy2	Italy	2	5	1,933	583.018	0.606	3.254	1.375
Portugal2	Portugal	2	5	2,133	820.374	0.725	1.885	0.568
Portugal N	Portugal	3	65	1,749	779.770	0.657	2.155	0.439
Portugal C	Portugal	3	62	1,731	795.073	0.652	2.092	0.463
Portugal S	Portugal	3	59	1,747	844.486	0.606	2.065	0.335

Geographic peripherality and environmental suitability

After fitting and evaluating each single model 50 times using 10 different methods, we obtained a final dataset of 500 models. A high weighted mean value using ROC scores (0.82) was observed for the final ensemble model, indicating good performance during model construction. The corresponding potential distribution identifies the centre of Europe, part of the Mediterranean coast, and the north and west of the Iberian Peninsula as high environmental suitability areas for

V. velutina (Figure 17B). The environmental suitability values ranged between 0.59 (Italy cluster 4) and 0.81 (Catalonia cluster 2) for the populations included in this study (Table 4).

According to the equivalence test carried out between both Asian and European distributional ranges, the invaded niche was equivalent to the native niche (*p-value* = 1.00, Figure 18), likewise the similarity test showed that the European niche was not more similar to the native niche than expected by chance (*p-value* = 0.07). In fact, the niche centroid of *V. velutina* shifted from its native range to the invaded range in the same direction as the background (Figure 18). There was a niche overlap between both ranges of 1.90%, a niche expansion of 51.23%, a niche stability of 48.77%, and a niche unfilling of 28.60%. The Italian populations were the farthest away from the native centroid, and the rest of the populations were expanding towards the native niche centroid, except the French population. In this regard, the environmental distance to the native niche centroid ranged between 1.89 (Portugal cluster 2) and 3.25 (Italy cluster 2). Similarly, Italian populations were the farthest away from the invasive niche centroid, and Portuguese populations were the closest to both the invaded and native niche centroids (Figure 17). The distance to the invaded niche centroid ranged between 0.33 (Portugal S cluster 3) and 1.39 (Italy cluster 4) (Table 4).

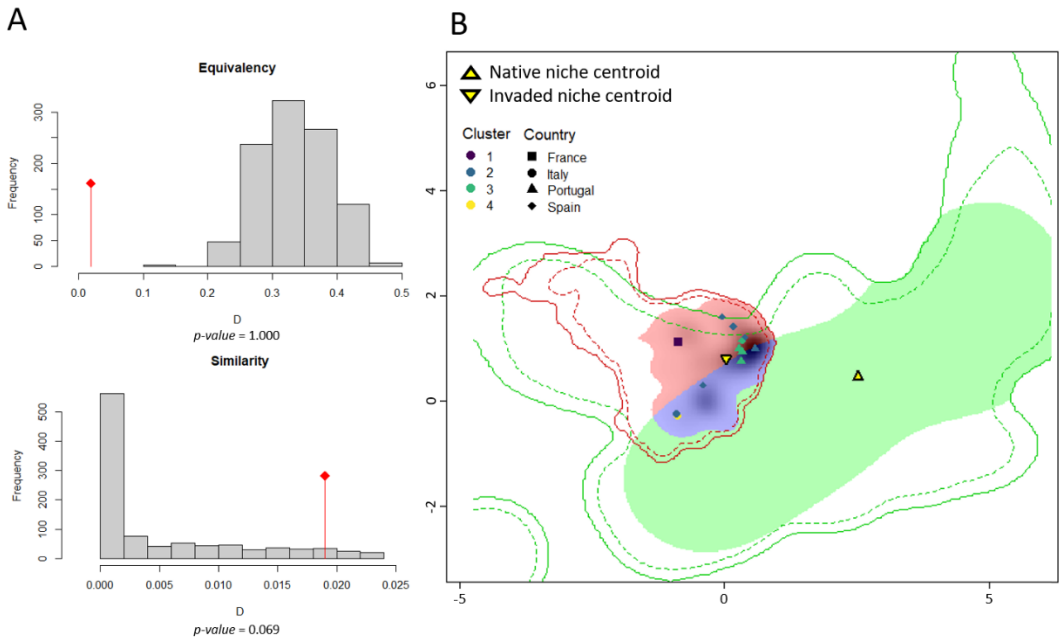

Figure 18.- A) Equivalency and similarity tests between invaded niche (red shading) and native niche (green shading) with observed niche overlap between the two ranges (bars with a diamond) and simulated niche overlaps (grey bars). Tests of niche equivalency were run using 1000 replications. B) Overlap between niches are represented in blue, while solid and dashed lines show 100% and 75% of the available (background) environment, respectively. Mallorca populations were excluded from the plots.

We found a significant association between allelic richness (Ar) and distance to the introduction focus (km) (*p-value* < 0.002, R^2 = 0.692) and environmental suitability (*p-value* = 0.011, R^2 = 0.568). On the other hand, there no was significant association between Ar and distance to the native niche centroid (*p-value* = 0.676, R^2 = 0.098) and distance to the invaded niche centroid (*p-*

value = 1.000, R^2 = -0.049) (Figure 19). Finally, according to MCMC analysis, both geographic and environmental distances influenced population genetic differentiation (likelihood = -1821.189), rather than geographic distance (likelihood = -1826.467), or environmental distance separately (likelihood = -1901.335) (Figure 20).

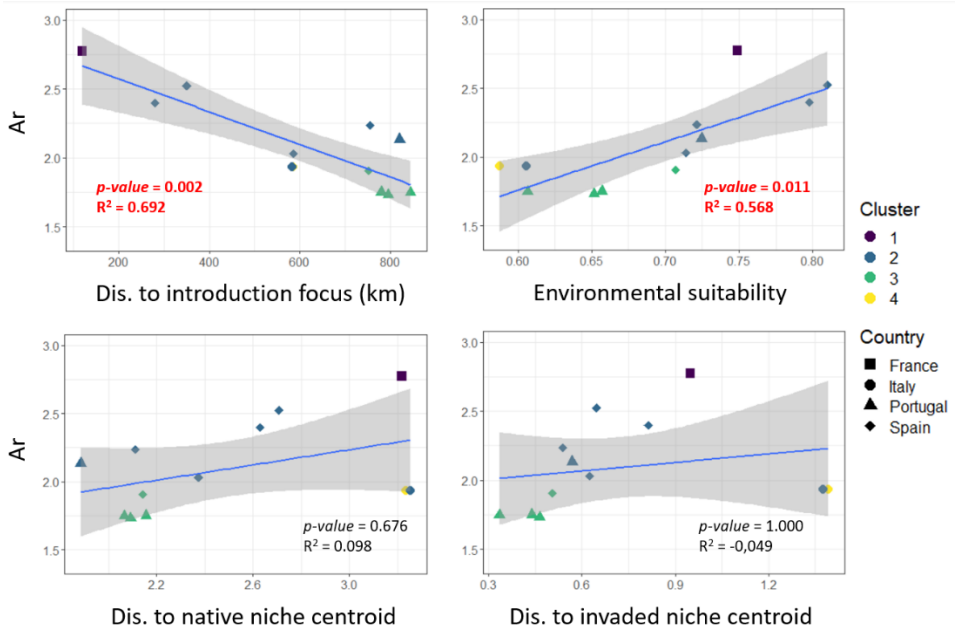

Figure 19.- Bivariate linear regression models between genetic diversity and geographic and environmental drivers. The genetic parameters included allelic richness (Ar), while geographic and environmental drivers included distance to the introduction focus, environmental suitability, and distance to both native and invaded niche centroids. The *p-values* and R^2 are shown in each plot. The red colour in bold represents a *p-value* < 0.05. Mallorca populations were excluded from the plots.

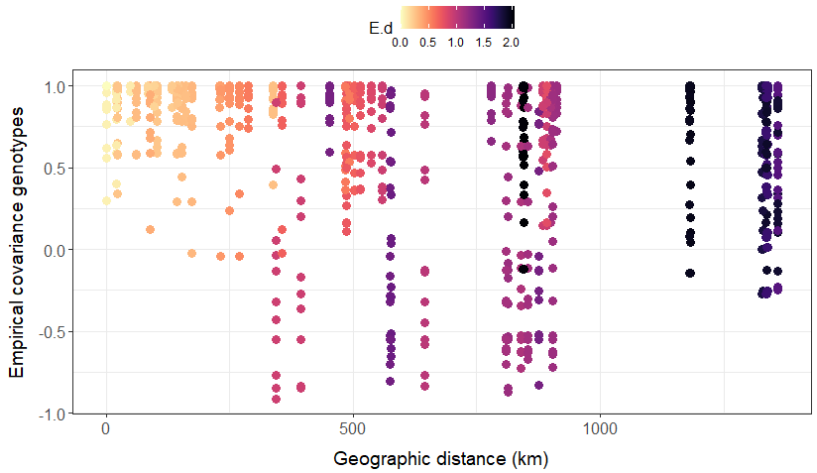

Figure 20.- Empirical covariance genotypes for all populations pairs (n = 12) and loci (n = 71) based on the geographic distance (km) and coloured by environmental distance (E.d). Mallorca populations were excluded from the plot.

Discussion

Expansion range limits of species can be shaped by a combination of ecological and evolutionary factors, including demography, genetic diversity, habitat heterogeneity, and gene flow (Sexton et al., 2009). A single introduction of *V. velutina* has been documented for Europe, with very few or possibly only one single multi-mated female as the source of all genetic diversity on the continent (Arca et al., 2015). The genetic patterns observed for *V. velutina* seem to be associated mainly with the natural dispersion of the species throughout the invaded territory, following the chronology of the new records in European countries (Laurino et al., 2020). Evidence for natural dispersion comes from the recovery of new genetic clusters along the expansion front, with an exclusive genetic cluster in the founder population (cluster 1 in the French population) and in marginal populations (cluster 3 in Portuguese populations and cluster 4 in the Italian population), and an intermediate genetic cluster with a more widespread distribution (cluster 2 in Italy, Spain, and Portugal). This scenario is most likely the result of serial founder events across the expansion range (Böhme et al., 2007), where newcomers are the offspring of the individuals living near the expansion front (Klopfstein et al., 2006).

Further support for a natural dispersion scenario comes from the gradual loss of allelic richness (Ar) as the front moves away from the introduction focus in France (Haxaire et al., 2006; Quaresma et al., 2022). However, human-mediated dispersal as well as habitat disturbance have also been alluded to as beneficial for range expansion in this and other species of the genus *Vespa* (Alaniz et al., 2021; Monceau et al., 2014; Robinet et al., 2017). This is the case of the geographically isolated islands of Great Britain and Mallorca (Budge et al., 2017; Jones et al., 2020; Leza et al., 2021), in which *V. velutina* invasions resulted from human-mediated dispersion, most probably due to shipping traffic from the mainland (Robinet et al., 2019). Long-distance jumps have also been reported for *V. velutina* invasions in the continent, such as in Portugal (Quaresma et al., 2022). This combination of short and long distance dispersal is a common feature in several invasive insects (Sharov & Liebhold, 1998; Gilbert et al., 2004; Meats & Smallridge, 2007). While long-distance jumps may lead to a lack of relationship between genetic and spatial distances (Acevedo-Limón et al., 2020), we detected that both geographic and environmental distances were influencing population genetic differentiation (Figure 20).

The reduction of genetic diversity across the expansion range from the introduction focus has been well described in invasive species (van Boheemen et al., 2017) and it is here further documented for *V. velutina*, as revealed by the negative association between allelic richness and distance from the introduction focus. Interestingly, we also detected a positive association between allelic richness and environmental suitability, highlighting the importance of a suitable environment that enables the successful establishment of a diverse population. Hence, the low environmental suitability at the expansion limits could prevent a diverse population from establishing itself and reduce the genetic diversity in edge populations. Similar patterns have been reported for other invertebrate species (Acevedo-Limón et al., 2020; Ortego et al., 2015). Unsuitable environmental conditions and lower genetic diversity in marginal populations translate into higher potential for genetic differentiation, which could affect local adaptation (Aguirre-Liguori et al. 2017; Leimu & Fischer, 2008). Moreover, genetic drift can be an important force in shaping the genetic structure of expanding populations (Excoffier & Ray 2008). For example, Bélouard et al. (2019) found that genetic drift dominated in the evolution of allele frequencies in isolated populations of the invasive red swamp crayfish during its spread in a wetland area. Furthermore, it seems that founder effects rarely limit the fitness of invasive alien insects and may

even benefit populations (Garnas et al., 2016), although the detrimental impact of founder events on the genetic variability of edge populations (Sherpa et al., 2020) or on sex determination (Hagan & Gloag, 2021), has also been documented particularly on *V. velutina* (Darrouzet et al., 2015).

The relevance of the suitability–genetic diversity relationships for the management of biological invasions can be modulated by climate change. Barbet-Massin et al. (2013) predicted a potential increase in environmental suitability and therefore a range expansion of *V. velutina* by 2100, towards the southwest region of the Iberian Peninsula. Based on our results, this potential expansion could lead to low genetic diversity at the expansion edges while still supporting locally adapted invading populations. This scenario is based on the observation that dispersal rates can evolve in response to natural selection (Ronce, 2007). For example, Simmons & Thomas (2004) detected increased frequencies of dispersive and long-winged individuals in a recently colonized bush cricket populations in comparison with longer-established populations in the range core, however, after a decade of colonization individuals showed wing morphology similar to those of core populations. This equilibrium has been observed in other insect species, such as butterflies (Hughes et al., 2003) and sand crickets (Roff & Fairbairn, 2007).

The empirical covariance genotype values from local (< 100 km) to continental level (> 1000 km) reflect the effect of geographical and environmental distances on genetic differentiation among *V. velutina* invasive populations. These results agree with the annual cycle and high dispersal rates registered for this species, ranging between 18 and 78 km per year in continental populations (Bertolino et al., 2016; Robinet et al., 2017). Based on previous discussion, historical processes seem to be the main factors shaping this pattern as for other invasive species (Ackiss et al., 2018; Chung et al., 2018; Eckert et al., 2008).

In summary, we found that populations at the edge of the *V. velutina* distribution range have lower allelic richness than populations at the range core. This pattern could be shaped by life history, population sizes, or gene flow between populations. Nonetheless, Arca et al. (2015) highlights that other biotic and abiotic factors could compensate the loss of genetic diversity of invading peripheral populations, such as the abundance of honeybees or reduced competition with other hornets (Villemant et al., 2011). Hence, we might not underestimate the invasiveness of this species on the European continent.

Our results highlight the importance of combining genetic analysis with geographical and environmental drivers to further understand the genetic patterns of *V. velutina* to the newly invaded environments. In addition, international coordination, and the implementation of preventative measures by all invaded countries are necessary to more efficiently control the spread of invasive species at the European level.

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AUTHOR CONTRIBUTIONS

C.H. designed the study. C.H., M.A.P., and M.L. performed sampling of biological material. C.H., M.A.P., and J.A.J.R. generated the data. C.H. analysed data and led the writing, with contributions from M.A.P., M.L., and J.A.J.R. Final manuscript was approved by all co-authors.

CONFLICT OF INTEREST STATEMENT

There are no conflicts to declare regarding the contents of this publication.

Chapter 5

Automated detection of the yellow-legged hornet (*Vespa velutina*) using an optical sensor with machine learning

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Abstract

BACKGROUND

The yellow-legged hornet (*Vespa velutina*) is native to Southeast Asia and is an invasive alien species of concern in many countries. More effective management of populations of *V. velutina* could be achieved through more widespread and intensive monitoring in the field, however current methods are labor intensive and costly. To address this issue, we have assessed the performance of an optical sensor combined with a machine learning model to classify *V. velutina* and native wasps/hornets and bees. Our aim is to use the results of the present work as a step towards the development of a monitoring solution for *V. velutina* in the field.

RESULTS

We recorded a total 935 flights from three bee species: *Apis mellifera*, *Bombus terrestris* and *Osmia bicornis*; and four wasp/hornet species: *Polistes dominula*, *Vespula germanica*, *Vespa crabro* and *V. velutina*. The machine learning model achieved an average accuracy for species classification of 80.1 ± 13.9% and 74.5 ± 7.0% for *V. velutina*. *V. crabro* had the highest level of misclassification, confused mainly with *V. velutina* and *P. dominula*. These results were obtained using a 14-value peak and valley feature derived from the wingbeat power spectral density.

CONCLUSION

This study demonstrates that the wingbeat recordings from a flying insect sensor can be used with machine learning methods to differentiate *V. velutina* from six other Hymenoptera species in the laboratory and this knowledge could be used to help develop a tool for use in integrated invasive alien species management programs.

Keywords: automated detection; Hymenoptera; pest management; *vespa velutina*; wingbeat frequency

Introduction

The yellow-legged hornet *Vespa velutina* Lepeletier, 1836 is an invasive alien species accidentally introduced in Europe from Asia in 2004 (Laurino et al., 2020). For the affected regions, the control of *V. velutina* represents both an economic cost (Barbet-Massin et al., 2020; Monceau et al., 2014), and an ecological impact whose full extent is still being investigated (Leza et al., 2019; Rojas-Nossa & Calviño-Cancela, 2020). The monitoring of populations of *V. velutina* can assist the management of incursions and provide a better understanding of the spatio-temporal patterns of the species (Reaser et al., 2020) .

The population distribution and dynamics of *V. velutina* are currently assessed through the trapping of adults (Leza et al., 2021; Monceau et al., 2012) or by nest location (Leza et al., 2021) although these approaches are labour intensive, and trapping can have a negative impact on non-target insects (Rodríguez-Flores et al., 2019; Rojas-Nossa et al., 2018). Various automated methods are being trialled to monitor *V. velutina*, including radiotelemetry (Kennedy et al., 2018), harmonic radar (Maggiora et al., 2019), thermal imaging (Lioy et al., 2021) and drones (Reynaud & Guérin-Lassous, 2016).

In general terms, a wide range of methods is available to monitor biological diversity, such as: analysis of environmental DNA (Sakata et al., 2021) or RNA from terrestrial hematophagous parasites (Weiskopf et al., 2018), remote sensing (Rocchini et al., 2018) or citizen science projects (Carvalho et al., 2020; Leza et al., 2021). Automated monitoring approaches using sensors have the potential to be more cost effective and provide more timely results than existing manual or laboratory methods and could also be used to complement existing methods.

Acoustic and vibrational-based methods are an important tool for monitoring biodiversity (Blumstein et al., 2011; Buxton et al., 2018) and might also be used to monitor *V. velutina*. The approach generally uses a digital recorder in the field to collect animal sounds that are species specific, to derive estimates of species abundance and diversity at spatial and temporal scales (Blumstein et al., 2011; Potamitis, 2014). Acoustic technology has been used in studies of marine mammals (González-Hernández et al., 2017), birds (Acevedo et al., 2009), frogs (Khalighifar et al., 2021) and insects (Kawakita & Ichikawa, 2019; Khalighifar et al., 2019; Towsey et al., 2014). In many cases, remote monitoring of animal sounds can outperform skilled observers (Celis-Murillo et al., 2009). It has also been used to monitor invasive alien species, such as red-billed leiothrix *Leiothrix lutea* (Scopoli, 1786) (Farina et al., 2013), cane toad *Bufo marinus* (Linnaeus, 1758) (Hu et al., 2009), coconut rhinoceros beetle *Oryctes rhinoceros* (Linnaeus, 1758) (Mankin & Moore, 2010), red palm weevil *Rhynchophorus ferrugineus* (Olivier, 1791) (Potamitis et al., 2009) and tiger mosquito *Aedes albopictus* (Skuse, 1894) (Balestrino et al., 2016).

In the case of flying insects which emit a sound as they fly, acoustic methods are often used to determine the insect wingbeat frequency, since wingbeat frequency can be species specific (Blumstein et al., 2011). However, it can be difficult to obtain acceptable quality audio recordings of free flying insects in the field due to the presence of background noise (Li et al., 2017; Mukundarajan et al., 2017) and where swarms of insects are present.

To address the limitations of acoustic methods, optical methods have been employed in which a light source and a light sensor are used to illuminate an individual flying insect and to detect the light reflected and scattered, or attenuated, by the insect in flight (Brydegaard, 2015; Kirkeby et

al., 2016; Mullen et al., 2016). Under similar conditions, the fundamental wingbeat frequency reported by an optical sensor and by an acoustic sensor should be similar when both sensors are designed to detect the wingbeat of the flying insect.

Our study is designed to assess the hypothesis that the flights of *V. velutina* may be automatically differentiated from the flights of other Hymenoptera species based on features derived from the wingbeat recordings of a flying insect sensor. The other Hymenoptera species are: *Apis mellifera* Linnaeus, 1758, *Bombus terrestris* (Linnaeus, 1758), *Osmia bicornis* (Linnaeus, 1758); *Polistes dominula* (Christ, 1791), *Vespula germanica* (Fabricius, 1793) and *Vespa crabro* Linnaeus, 1758 which are likely to coexist in the field with the invasive *V. velutina*. Our overall aim is to contribute to the development of an automatic system to monitor populations of *V. velutina* in the field.

Material and methods

Data collection

Individuals from seven Hymenopteran species were collected in the field: three bee species (*A. mellifera*, *B. terrestris* and *O. bicornis*) and four wasp/hornet species (*P. dominula*, *V. germanica*, *V. crabro* and *V. velutina*) as shown in Table 5.

The insects were collected during 2019 and 2022 in the Balearic Islands and Catalonia, Spain. For the six social Hymenoptera species, only individuals of the worker caste were collected because it is the most populous caste and the one whose members are most likely to be found outside the nest. For the solitary Hymenoptera species (*O. bicornis*) only males were collected because they are more abundant than females during breeding season. The insects were carefully collected and transported to the laboratory in an entomological tent (25 x 25 x 25 cm) in less than one hour.

In the laboratory, individuals were transferred to a larger entomological tent (60 x 60 x 60 cm), after rejecting any insects with signs of damage to their body or wings. The tent contained a flying insect optical sensor developed by Irideon S.L. (Barcelona, Spain) which automatically recorded the wingbeat waveform of each insect as it flew through the sensor (Figure 21A). For practical reasons, all individuals of the same species were introduced into the tent at a time, so each individual could give rise to zero, one, or more than one recording per session. *Vespa velutina* and *V. crabro* were each recorded in two separate sessions, but each individual was used in one recording session only. Each recording session lasted about one hour and was ended when all individuals in the tent had stopped flying.

The sensor contained an optical emitter panel and an optical receiver panel, which faced each other across a distance of 19.7 cm, through which insects could fly. The optical emitter comprised a two-dimensional (2D) array of 940 nm wavelength infrared light emitting diodes (LEDs), and the optical receiver comprised a 2D array of 940 nm photodiodes. The emitter and receiver panels each had an active area of 10.2 x 7.1 cm. The volume of space bounded by the emitter and receiver panels was equal to 10.2 x 7.1 x 19.4 cm (or 1405 cm^3) and is referred to as the sensing volume. The sensor had the following design attributes: a well-defined sensing volume with a relatively even response to an insect flying anywhere within the sensing volume, negligible sensitivity to

insects flying outside of the sensing volume, and good immunity to background acoustic noise. The basic operating principle of the optical sensor is illustrated in Figure 21B. Further details about the sensor technology can be found in the references (González-Pérez et al., 2022; Patent No. Num. 5904, ISSN: 1889-1292, NIPO: 088170165, 30/4/2021, 2021).

The analog output signal of the optical sensor was acquired by an analog to digital converter (ADC) to digitize the signal. When a flying insect entered the sensing volume, it automatically triggered a single recording of 106.7 milliseconds in duration, comprising 1024 discrete ADC samples taken at a rate of 9603 samples per second. The duration of each recording was long enough to provide acceptable frequency resolution. Longer recording times would have increased the possibility of more than one insect being recorded simultaneously. The sensor automatically added a timestamp to each recording.

After each recording session, each wingbeat recording was downloaded from the sensor and processed using a Python script (Python version 3.7.9) written by Irideon to produce a playable audio (WAV) file from which a series of features were extracted by the same script. Figure 21C shows the plot for a typical *V. velutina* WAV file, by way of example. The WAV waveform is not very informative on its own but can be processed to yield more informative data as will be described. The complete dataset contains 935 recordings as shown in Table 5.

Table 5.- Main characteristics of the seven Hymenopteran species used in this study, including the number of insects collected and the number of recordings made for each species.

	Species	Sex	Caste	No. individuals	No. flight records	Date of collection
Bees	*Apis mellifera*	Female	Worker	126	178	24/11/2021
	Bombus terrestris	Female	Worker	11	121	26/02/2019
	Osmia bicornis	Male	-	79	120	23/02/2021
Wasps/Hornets	*Polistes dominula*	Female	Worker	42	108	10/02/2020
	Vespa crabro	Female	Worker	10	91	20/10/2021
	Vespa velutina	Female	Worker	30	117	11/10/2021
	Vespula germanica	Female	Worker	22	200	09/10/2019

Feature extraction

Using the Python script, each WAV file recording was processed to extract its power spectral density (PSD). A PSD is the measure of the signal's power content versus frequency in which the measurable frequency range is segmented into a series of discrete ranges referred to as bins. PSDs are used in numerous applications including the analysis of vibration and noise (Norton & Karczub, 2003). Each PSD was calculated using Welch's method (Villwock & Pacas, 2008) with a segment length of 512 ADC samples and an overlap of 50 % to give the power per bin from 0 Hz to 4801.5 Hz in 256 bins, with a bin width of 18.756 Hz. As part of Welch's method, a window function (Hann window) was applied to each segment to reduce spectral leakage (side lobes) in the PSD due to the segmentation. The window function minimized spectral leakage due to a recording being terminated whilst the insect was still flying in the sensing volume. The powers in the PSD were corrected to compensate for the non-flat frequency response of the sensor

and for insects which flew through the sensing zone before the end of the recording length (106.7 milliseconds). The PSD plot for a typical *V. velutina* recording is shown in Figure 21D.

From each PSD, the Python script extracted a series of machine learning features. A feature refers to an individual measurable property or characteristic extracted from a recording. The concept of feature is related to that of an explanatory variable used in statistical techniques. The features used in this work are illustrated in Figure 21D and are described below.

1. Wingbeat fundamental frequency in Hertz (Hz), referred to as F1 (Hz) was estimated using a combination of the following pitch determination methods (Cai, 2013): autocorrelation, cepstrum and harmonic product spectrum. The wingbeat fundamental frequency is the frequency at which the insect flaps its wings. In cases where F1 (Hz) could not be determined with confidence, the recording was rejected from the data set.
2. Fundamental peak power in decibels (dB) referred to as F1 (dB) is the power at F1 (Hz).
3. A 14-value "PSD peak and valley feature" comprising the frequencies and powers of the wingbeat fundamental frequency and the 2^{nd}, 3^{rd}, and 4^{th} harmonics, and the frequencies and powers of the PSD valleys, which lie midway between each of the peaks, as depicted in Figure 21D. By definition, the harmonics frequencies are at integer (whole number) multiples of the fundamental frequency. The harmonic frequencies were estimated by calculating: F2=2 x F1; F3 = 3 x F1; and F4 = 4 x F1. The PSD was then searched to find the maximum (peak) power within a small frequency range close to each of the estimated harmonic frequencies. The final values for each harmonic frequency were taken as the frequency at the corresponding peak power. The valley frequency and powers were calculated by searching the PSD for the minimum power (valley) approximately midway between the peaks on either side of the valley.
4. A 2-value feature comprising only F1 (Hz) and F1 (dB).
5. An 8-value feature comprising F1 (Hz) and the seven peak and valley powers, referred to as "F1 (Hz) with peak and valley powers".

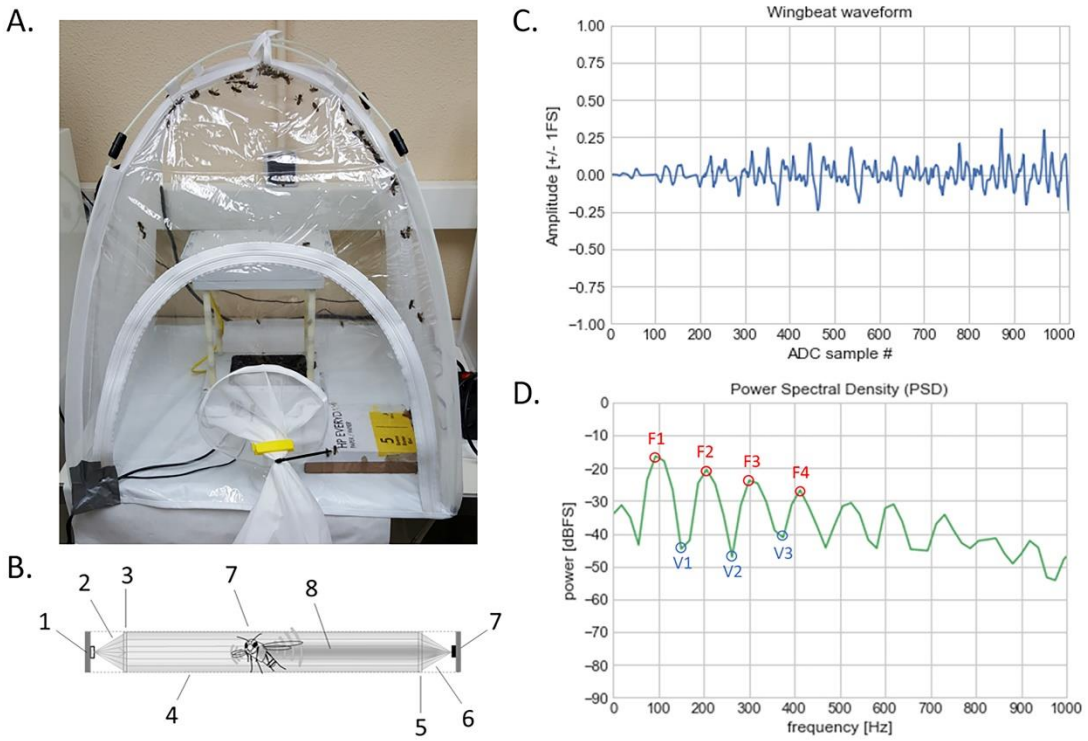

Figure 21.- A) Photo showing the optical sensor inside of the entomological tent. The optical sensor comprises an optical emitter module facing upwards and an optical receiver module facing downwards, with the modules separated by four white plastic posts, each with a length of 20 cm, such that insects may fly between the emitter and receiver modules. The black plastic cover on the emitter module is transparent to the infrared wavelengths used by the sensor. Beneath that cover is a 2D array of collimated optical emitters which project their light beams upwards towards the optical receiver module. The optical receiver module contains a 2D array of downward facing optical receivers also covered by black plastic which is not visible in the photo. The sensing volume is given by the 2D area common to both the emitter and receiver (71 cm2) multiplied by the distance between the black plastic covers (19.4 cm). B) illustrates the basic operation of each optical emitter-receiver in which a LED (1) emits a diverging beam of light (2), which falls upon a lens (3) that form a collimated beam of light (4), which after a certain distance, falls upon a second lens (5) that forms a converging beam (6), which focuses onto a photodiode (7). When an insect (7) flies through the collimated beam (4), it casts a shadow (8) upon the photodiode (7) i.e., the sensor employs the so-called optical extinction mode of operation. As the insect flaps its wings within the collimated beam (4), the light falling on the photodiode (7) is modulated, allowing determination of various wingbeat features of the flying insect. In the sensor, 24 optical emitter-receiver pairs are employed to cover the 2D area described. C) Example of a recorded flight with the ADC sample number (0 to 1023) on the x-axis, and amplitude on the y-axis with a range of [-1, 1] which corresponds to the full-scale range of the analog to digital converter (ADC) used to digitize the analog output of the optical receiver. The low frequency signal which corresponds to the body of the insect is not visible in the waveform due to a high-pass filter in the sensor, which also attenuates the impact of ambient light and electronic offset voltages. D) The power spectral density (PSD) plot for the recording shown in B), with frequency (Hz) on the x-axis and power (dB) on the y-axis. The fundamental,

2nd harmonic, 3rd harmonic, and 4th harmonic peaks for this PSD plot are indicated as F1, F2, F3, F4 respectively, and the 1st, 2nd and 3rd valleys are indicated as V1, V2, V3 respectively.

Statistical analysis and classification of species using machine learning

For each species, the mean and standard deviation of the fundamental frequency and power were calculated to enable comparisons to be made. Differences in fundamental frequency and power between the seven species were assessed using a Kruskall-Wallis test and a Pairwise Wilcoxon Rank Sum Test with Holm adjustment, post hoc.

We also assessed the performance of a machine learning model to classify each of the seven Hymenopteran species using each of the five features described. The Random Forest machine learning algorithm with permutation parameter importance was used to develop the model. This algorithm generates multiple decision trees on a set of training data, each of them built over a random extraction of the observations from the dataset and a random extraction of the features, and the results obtained are combined in order to obtain a single model that is more robust and less prone to overfitting compared to the results of each tree separately (Breiman, 2001). The *ranger* package (Wright & Ziegler, 2017) implementation of the Random Forest algorithm was used.

We adopted a train-test split procedure, in which the dataset was divided into two subsets: the training set (70 % of the full dataset) which was used to train the model; and the test set (the remaining 30% of the full dataset, and not used previously for training) which was used to evaluate the performance of the model, i.e; to determine how well the model classifies the species on new data. This procedure was repeated in 100 random train-test splits, which enables calculation of the mean and standard deviation of the performance metrics and of the feature importance (Breiman, 2001).

The performance of each model was evaluated using the following metrics: out-of-bag (OOB) error (estimated error resulting from the model prediction using the observations from the training set that are not used to create the trees) (Breiman, 2001); precision (proportion of correctly classified positives among all the samples classified as positive); recall (proportion of correctly classified positives among all the positives); f1 (which strikes a balance between precision and recall, in a single value metric) (Fernandes et al., 2021); and accuracy (which describes the percentage of outcomes that the model has classified correctly). The equations for these metrics are:

$$Precision = \frac{TP}{TP + FP} \qquad Recall = \frac{TP}{TP + FN} \qquad f1 = 2 \cdot \frac{Precision \cdot Recall}{Precision + Recall}$$

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN}$$

Where TP (true positives) is the number of outcomes that the model correctly classifies as positive, TN (true negatives) is the number of outcomes that the model correctly classifies as negative, FP (false positives) is the number of outcomes that the model incorrectly classifies as positive, and FN (false negatives) is the number of outcomes that the model incorrectly classifies

as negative. All statistical analyses were performed with R (v4.0.2) (Team, 2020) and statistical significance was set at *p-value* < 0.05.

Results

The mean and standard deviation of the fundamental frequency and power for each species are shown in Figure 22 (and are listed numerically in Table S4 and Table S5, Supporting Information). A high degree of overlap is apparent between *V. velutina*, *P. dominula* and *V. crabro* in one group, and between *V. germanica*, *B. terrestris* and *O. bicornis* in another. *A. mellifera* did not overlap with any of the other species studied. The standard deviations observed for each species indicate that the described methodology has yielded a reasonably rich and varied dataset for each.

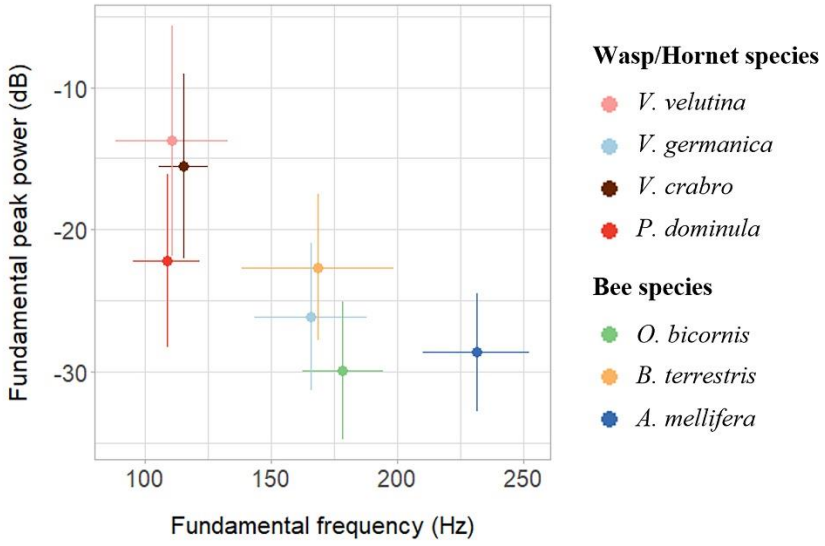

Figure 22.- Mean fundamental frequency and fundamental peak power and standard deviation for the seven Hymenoptera species in this study.

V. velutina presented a fundamental frequency F1 (Hz) which was statistically lower than that of the other Hymenoptera species (Kruskal-Wallis chi squared = 707.9, df = 6, *p-value* < 0.001; Pairwise Wilcoxon Test, *p-value* < 0.001), except for *P. dominula* whose fundamental frequency was not significantly different to that of *V. velutina* (Pairwise Wilcoxon Test, *p-value* = 0.7). *V. velutina* also presented a fundamental power (F1 dB) which was statistically higher than those of the other Hymenoptera species (Kruskal-Wallis chi squared = 462.63, df = 6, *p-value* < 0.001; Pairwise Wilcoxon Test, *p-value* < 0.05).

The classification accuracy achieved using each of the different features is shown in Table 6. The highest accuracy for all species (80.1 %) was achieved using the 14-value PSD peak and valley feature. The next highest accuracy (68.9 %) was for the 8-value F1 (Hz) with peak and valley power feature. Consequently, under the conditions of this work, which includes the use of the

Random Forest algorithm, the PSD harmonic and valley frequencies are seen to make a substantial contribution to model accuracy even though they are known to be highly correlated with F1 (Hz).

Table 6.- Mean classification accuracy (± standard deviation) for all species and for *V. velutina*, for the different features, indicating the number of PSD values comprising each feature.

Feature	PSD values	Mean accuracy for all species	Accuracy for *V. velutina*
1 Fundamental frequency, F1 (Hz)	1	50.9 ± 20.9 %	41.7 ± 8.0 %
2 Fundamental peak power, F1 (dB)	1	27.2 ± 8.9 %	43.4 ± 8.2 %
3 PSD peak and valley feature	14	80.1 ± 13.9 %	74.5 ± 7.0 %
4 F1 (Hz) and F1 (dB)	2	60.0 ± 15.9 %	58.4 ± 8.4 %
5 F1 (Hz) with peak and valley powers (dB)	8	68.9 ± 13.8 %	67.5 ± 8.4 %

The performance of the machine learning model using the PSD peak and valley feature is considered good: OOB error = 16.9 ± 1.1 %, Precision = 81.3 ± 2.5 %, Recall = 80.1 ± 2.4 % and f1 = 80.0 ± 2.5 %.

The confusion matrices (Figure 23) show the relationship between the true species identity and the species predicted by the trained model and includes the classification accuracy for each species. The highest classification accuracy was for *V. germanica* (93.9 ± 3.1 %) and the lowest was for *V. crabro* (52.5 ± 8.4 %). The accuracy for *V. velutina* was 74.5 ± 7.0 % and the mean accuracy for all seven species was 80.1 ± 13.9 %. Figure 23 indicates that *V. velutina* was mostly confused with *P. dominula*, and to a lesser degree with *V. crabro* and *V. germanica*. It also indicates that *V. crabro* was the species most often misclassified, confused mainly with *V. velutina* and *P. dominula*, which may be due to the relatively high overlap of the fundamental frequencies between these three species (Figure 22). Figure 23. also shows a low rate of misclassification between *B. terrestris*, *O. bicornis* and *V. germanica* even though their fundamental frequency and power features are highly overlapped (Figure 22). Figure 23 also indicates that the model shows very little confusion between the bee species and the wasp/hornet species.

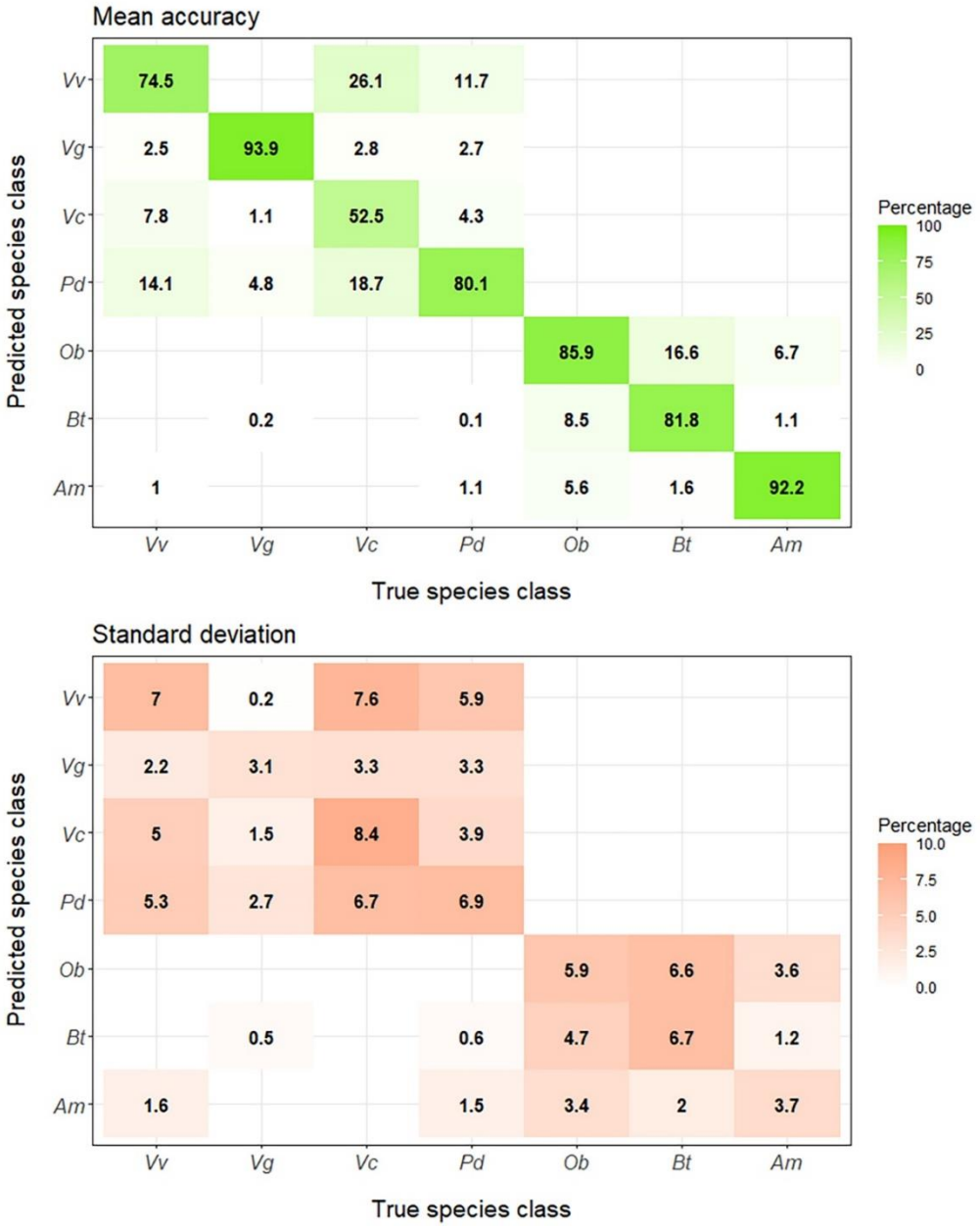

Figure 23.- Confusion matrices assessing the performance of the machine learning model that predicts the identity of seven Hymenoptera species. The x-axes indicate the actual or "true" species in the test set and the y-axes indicate the species predicted by the model. For each axis: Vv = *V. velutina*, Vg = *V. germanica*, Vc = *V. crabro*, Pd = *P. dominula*, Ob = *O. bicornis*, Bt = *B. terrestris*, Am = *A. mellifera*. In the upper plot, the value in each column indicates the percentage of samples in the true species (x-axis) which are classified into each of the predicted classes (y-axis). Consequently, each value on the diagonal, from the top left corner to the bottom right corner, indicates the percentage of samples in the class which is correctly classified. Values which do not lie on the diagonal indicate the percentage of confusion, or misclassification for the indicated classes. In the lower plot: the value in each cell indicates the standard deviation for the classification accuracy given in the corresponding cell of the upper plot. Zero values were removed for ease of visualization.

All PSD peak and valley values made a positive contribution to the performance of the machine learning model (Figure 24). The most important value was fundamental frequency F1 (Hz) and the next five most important values were also related to frequency. The most important power value was F1 (dB) which was in 7th place overall.

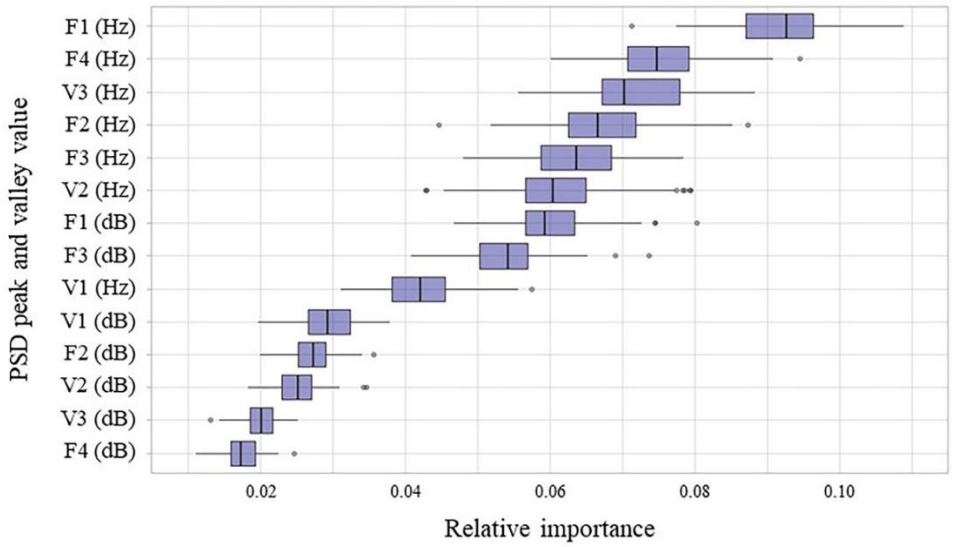

Figure 24.- Box and whisker plots for the relative importance of each of the PSD peak and valley value. The boxes indicate the interquartile range (IQR) from Q1 to Q3. The vertical line within each box indicates the median. The horizontal lines show the "minimum" to "maximum" range, from Q1 – 1.5 x IQR to Q3 + 1.5 x IQR, with outliers shown as dots.

Discussion

We recorded the flights from seven species of Hymenoptera, including *V. velutina*, using an optical sensor and extracted five wingbeat frequency and power features from the PSD of each recorded waveform. We performed a statistical analysis of the fundamental wingbeat frequency and power of each species. We also used the features in a machine learning model and assessed the performance of the model to classify each species.

There is good agreement between the wingbeat fundamental frequencies of our work and that of previous works for: *A. mellifera* (251 Hz) (Kawakita & Ichikawa, 2019), *B. terrestris* (170 Hz) (van Roy et al., 2014), *V. germanica* (148 Hz) (Tercel et al., 2018) and other *Vespa* (*V. crabro* = 100 Hz; *V. simillima xanthoptera* = 100 Hz and *V. orientalis* = 125 Hz) (Byrne et al., 1998; Ishay, 1975; Kawakita & Ichikawa, 2019). Our study was performed using insects flying in an entomological tent, which may have affected insect flight characteristics compared to natural flight in the field. However, the fundamental frequency we obtained for *A. mellifera* (231.5 ± 21.1 Hz) is similar to that reported in another work (251.2 Hz ± 45.0 Hz) (Kawakita & Ichikawa, 2019) in which the recordings were made outdoors in a rural area. To the best of our knowledge, the present work is the first to report the wingbeat frequencies of *V. velutina*, *P. dominula*, and *O. bicornis*.

Previous studies have shown that Hymenoptera wingbeat frequencies are generally inversely proportional to body size (which depends on caste and sex) (Burkart et al., 2012; Gradišek et al., 2016) and to wing length (Miller-Struttmann et al., 2017; van Roy et al., 2014). This is consistent with our findings, in which the lowest fundamental frequencies correspond to *V. velutina* and *V. crabro* which are predators of insects and are larger than bees (Kawakita & Ichikawa, 2019).

In the present work, we assessed classification performance using frequency and power values from the PSD in addition to fundamental frequency. The 14-value peak and valley feature gave the best classification performance, which was 80.1 ± 13.9 % on average for all species and 74.5 ± 7.0 % for *V. velutina*. Other studies show comparable accuracy results for the classification of Hymenoptera species (Gradišek et al. (2016) = 82.7 %; Kawakita & Ichikawa (2019) = 85.3 %), and for other insects, such as mosquitoes (Fernandes et al. (2021) = 78.1 %).

Wingbeat fundamental frequency is the feature most used in works pertaining to insect species classification using audio recordings (González-Pérez et al., 2022; Kawakita & Ichikawa, 2019) with wingbeat harmonics used to a lesser extent (Cator et al., 2009; Raman et al., 2007). In the present work, wingbeat fundamental frequency F1 (Hz) was found to be the most important single-value feature.

Previous studies have also assessed other features as an alternative to, or in combination with wingbeat fundamental frequency. For example Eyben et al. (2010) provide a feature extraction tool (openSMILE) for audio analysis, which was used by Gradišek et al. (2016) to compute 1582 numerical features for bumblebee species classification. Features studied in comparable species classification works include: minimum and maximum frequencies (Acevedo et al., 2009); maximum power (Acevedo et al., 2009); frequency and power modulations (Arthur et al., 2014); full spectrogram (Becker et al., 2018; Fernandes et al., 2021); raw waveforms (Becker et al., 2018); spectrum octave analysis (González-Hernández et al., 2017); and wingbeat harmonics (Cator et al., 2009; Raman et al., 2007).

In this study, only a relatively small dataset was available to train and test the machine learning model, but it was large enough to achieve good levels of classification accuracy. To further improve model performance, planned future work includes collecting and recording more insects to increase the size of the dataset and to improve the numeric balance within the species, caste, and sex classes. Consideration of wingbeat frequency and recording time stamp could be made to provide a rough indicator of individual insects which fly though the open sensor multiple times.

The results of the present work indicate potential for the development of an automated system to monitor populations of *V. velutina* in the field to assist localized management actions and provide information about the ecology of the species to better understand its spatio-temporal patterns across environmental gradients. Classification accuracy for *V. velutina* would probably be higher than 74.5% if a sensor were placed in an apiary since there would be no confusion of *V. velutina* with *A. mellifera*. Nevertheless, the presence of *P. dominula* or *V. crabro* could result in reduced accuracy in localities where they coexists with *V. velutina*.

A range of attractants could be considered for use with the sensor in the field, to attract target insects towards the sensor, where needed. Potential recruitment substances for *V. velutina* include baits which have been tested with that species (Lioy et al., 2020; Rojas-Nossa et al., 2018) and hive products, protein sources, and chemical substances (Couto et al., 2014). Likewise, a sex

pheromone for attracting *V. velutina* males has been identified, which could be used to attract males in autumn (Wen et al., 2017) although it is not yet available for field use.

Furthermore, given that Hymenoptera wingbeat frequency is generally inversely proportional to insect age (Parmezan et al., 2021); and is a strong indicator of insect rate of metabolism and physical structure (Santoyo et al., 2016); and changes during bumblebee buzz pollination (Burkart et al., 2012), it might be possible to develop new machine learning models for the optical sensor to classify such attributes and behaviour, in addition to species. In addition, differences in wing shape have been found between haploid and diploid males in *B. terrestris* (Bortolotti et al., 2022). As such, it would be interesting to determine if there are differences in wing shape between haploid and diploid males of *V. velutina* which may be detectable using the sensor. This could be of interest in an integrated invasive alien species management program because, in Europe, diploid males of *V. velutina* are sterile and haploid males are fertile (Darrouzet et al., 2015).

The present study could be a first step in multiple avenues of research, with potential application of the sensor in the field with machine learning models for automated monitoring of the biodiversity of Hymenoptera and possibly insects in other orders, and the monitoring of flying invasive alien and pest species. A further potential application could be in the agro-food industry to monitor pollination performed by bees and other pollinating insects.

Conclusion

Our study demonstrates the effectiveness of the optical sensor and machine learning methods to identify seven common hymenopteran species which demonstrated an average classification accuracy of 80.1 ± 13.9 % and an accuracy of 74.5 ± 7.0 % for the invasive alien *V. velutina*, which was the primary target species for this work. The insects were collected in the field and recorded in an entomological tent in the laboratory. We conclude that the approach shows promise for the development of a system for automatic detection of the invasive *V. velutina* in the field, in the presence of common wasp and bee species.

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AUTHOR CONTRIBUTIONS

CH, MW, JE and ML conceived the ideas and designed methodology. CH collected, analyzed the data and led the writing of the manuscript. MW, JE, NRP, BF, JAJR and ML contributed critically to the draft. All authors gave final approval for publication.

CONFLICT OF INTEREST STATEMENT

There are no conflicts to declare regarding the contents of this publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Table S4.- Mean and standard deviation of the peak and valley frequencies (Hz).

Species	F1	F2	F3	F4	V1 (F1 -> F2)	V2 (F2 -> F3)	V3 (F3 -> F4)
Apis mellifera	231.5 ± 21.1	462.0 ± 42.2	692.1 ± 64.2	925.3 ± 83.7	351.8 ± 41.1	581.2 ± 57.8	815.7 ± 76.4
Bombus terrestris	168.6 ± 30.1	336.7 ± 58.9	503.3 ± 91.4	672.1 ± 125.4	252.0 ± 39.6	421.5 ± 72.4	589.5 ± 107.0
Osmia bicornis	178.5 ± 16.0	356.5 ± 32.7	535.6 ± 49.6	706.7 ± 85.8	267.1 ± 30.0	451.7 ± 44.6	626.4 ± 55.9
Polistes dominula	108.8 ± 13.3	217.1 ± 28.2	324.8 ± 43.6	432.3 ± 58.2	161.9 ± 19.1	274.2 ± 31.1	381.2 ± 51.6
Vespa crabro	115.4 ± 9.8	229.0 ± 20.3	345.2 ± 32.0	460.9 ± 40.4	174.0 ± 16.8	289.6 ± 26.3	404.0 ± 36.6
Vespula germanica	165.8 ± 22.3	330.4 ± 44.3	496.2 ± 67.1	661.9 ± 89.4	248.9 ± 33.4	413.9 ± 54.6	579.8 ± 75.9
Vespa velutina	110.8 ± 22.2	219.8 ± 41.1	331.4 ± 68.3	439.2 ± 87.2	168.0 ± 35.9	281.5 ± 66.4	390.0 ± 66.4

Table S5.- Mean and standard deviation of the peak and valley powers (dB)

Species	F1	F2	F3	F4	V1 (F1 -> F2)	V2 (F2 -> F3)	V3 (F3 -> F4)
Apis mellifera	-28.63 ± 4.2	-34.1 ± 6.6	-41.9 ± 6.0	-43.6 ± 7.1	-65.0 ± 5.2	-65.8 ± 5.5	-66.8 ± 6.0
Bombus terrestris	-22.7 ± 5.2	-31.9 ± 9.5	-37.9 ± 9.1	-41.9 ± 10.0	-60.2 ± 6.2	-62.6 ± 9.8	-63.8 ± 11.0
Osmia bicornis	-29.9 ± 4.8	-33.4 ± 5.2	-41.3 ± 4.0	-42.6 ± 5.9	-58.3 ± 7.1	-59.7 ± 6.7	-62.1 ± 7.1
Polistes dominula	-22.2 ± 6.0	-26.3 ± 6.9	-32.5 ± 6.0	-33.6 ± 7.7	-44.9 ± 8.4	-47.1 ± 8.3	-48.72 ± 7.7
Vespa crabro	-15.5 ± 6.5	-21.2 ± 6.3	-27.9 ± 6.3	-30.4 ± 7.9	-42.9 ± 8.4	-44.9 ± 8.0	-45.8 ± 8.3
Vespula germanica	-26.1 ± 5.2	-32.6 ± 5.1	-39.1 ± 5.2	-41.0 ± 6.7	-61.1 ± 5.9	-61.7 ± 6.6	-62.4 ± 7.1
Vespa velutina	-13.8 ± 8.1	-20.7 ± 7.8	-26.2 ± 7.9	-29.3 ± 10.2	-39.9 ± 8.7	-41.1 ± 9.5	-42.8 ± 9.4

GENERAL DISCUSSION

Invasive alien species constitute a major social challenge due to the high rate of biodiversity extinction around the world, which represents a risk to the functioning of ecosystems and human society (Bacher et al., 2018; Carpenter et al., 2018; Mazza et al., 2014). In fact, invasive alien species are the second biggest cause of loss of biodiversity in the world (Chapin III et al., 2000), and the main threat for the biological diversity of islands and evolutionary-isolated ecosystems (Brooke et al., 2007). Since the early 1980s the European Union has been working to prohibit and prevent the introduction of alien species into the natural European environments (Genovesi & Shine, 2004). Nonetheless, accidental introductions due to global network shipping are still occurring (Sardain et al., 2019).

This PhD thesis has focused on the study of the alien species *Vespa velutina nigrithorax* Buysson 1905 (Hymenoptera: Vespidae) in Europe through a multidisciplinary perspective to (i) propose effective eradication methods in new invaded areas, (ii) identify introduction pathways to island ecosystems, (iii) determine general patterns that help us to predict, prevent and manage this biological invasion in the main Mediterranean islands, (iv) test the central-marginal hypothesis in invasive *V. velutina* populations from France, Italy, Spain, and Portugal, and (v) improve our detection capacity through an automated optical sensor. The results from this PhD thesis are presented in three peer-reviewed and published articles (Herrera et al., 2022, 2023; Leza et al., 2021) plus another two papers currently submitted to scientific journals.

On the one hand, we have discussed the combination of different methods to assess a successful eradication of this invasive alien species for the first time in Europe. We have used different methodologies including trapping, the implementation of the citizen science data for detection of presence, the active search of nests, and their removal using mechanical methods. Some of these methodologies has been criticized by some authors based on their low success rate on *V. velutina* and their high impact on non-target species. For example, it has been reported that trapping has low efficiency to reduce hornet density but has a high impact on entomofauna (Monceau et al., 2012; Rojas-Nossa et al., 2018; Turchi & Derijard, 2018). Likewise, these studies also conclude that the triangulation method for the active search of nests is difficult to implement in practice, mainly due to landscape structure may be complex and therefore the directions of hornets are often difficult to follow (Turchi & Derijard, 2018). However, most of the studies that have drawn these conclusions have worked with established populations of *V. velutina*. Our case represents an initial invasion stage, with a relatively low number of nests detected and where eradication has been possible.

Since 2018, neither nests nor individuals have been found in the island. Hence, the *Ministerio de Medio Ambiente y Energía para la Transición Ecológica* from Spain, as the competent authority on the management of invasive alien species, declared *V. velutina* eradicated in the island in 2020. Despite having been declared eradicated, the team did not stop raising awareness about this species since if it has invaded once, it could be detected again. Indeed, the following year (2021) a new nest was detected in Mallorca. Based on genetic analyses, we have concluded two independent introductions of this invasive hornet in Mallorca in two different moments from two different European regions: Italy (2015) and Mainland Spain (2021). That means all nests detected and removed in Mallorca during eradication tasks are the result of the first and a single introduction detected in 2015. Fortunately, the second introduction (2021) was detected quickly thanks to a citizen, which allowed the competent administration to quickly locate the nest and sent it to the University of the Balearic Islands for subsequent analyses. Since the nest hosted males, we could face two scenarios: there was already a reproductive caste, and the species was already dispersing, or they were sterile males. Each of these scenarios could have had different

implications to management tasks of this invasive species. Therefore, male hornets were genetically analysed using 15 STRs microsatellite markers. Since sterile males of the yellow-legged hornet are characterized by having two sets of chromosomes per cell instead of just one (they are diploid), the genetic load was used as an indicator of their sterility (Darrouzet et al., 2015; Heimpel & De Boer, 2008). All males sampled from the nest were diploid, and therefore sterile, where the research team concluded that the following year there would be no new nests derived from this one. This case is very interesting, since the applied methodology has allowed us to predict if there will be a dispersal of this species, and therefore, if the competent administration should prepare to detect and remove new nests in future years in the way described by Leza et al. (2021). Here, we shed light on the pathways used for this species to reach new isolated territories and the necessity of effective management and prevention plants for the spread of invasive alien species.

Previous results are very important because they indicate that the eradication tasks assessed have been successful since the first detection of this species in 2015. Likewise, we determined that the nest found in 2021 constitutes a new independent introduction of this invasive species on the island, and it is not a nest remaining from the previous invasion (2015-2018). This aspect is discussed as Lazarus effect, the reappearance of biota through to be extinct (Morrison et al., 2007), where absolute certainty of the absence of a species can only be attained by the passage of time without detection. In this case, after the last nest detected in 2018, there were two years without detection of this invasive alien species. In addition, we assessed genetic analyses to confirm and give scientific rigor to the fieldwork done during eradication tasks.

Both introductions presented bottleneck and founder effect signatures. Nonetheless, Arca et al. (2015) concluded that this species can establish successfully even after a severe genetic bottleneck resulting from the introduction of very few or even a single female. The case of the invasion of the first introduction in Mallorca reinforce this view, since even after a second bottleneck the invasive population of *V. velutina* kept growing after the first detection.

Anticipating the possible distribution of an invasive species is essential for prevention, early detection, and control, and indispensable for a conservation plan. For this reason, we applied the ensemble of small models with our experience with *V. velutina* nests detected in Mallorca during the first introduction. It was possible to determine its ecological niche in Mallorca and to identify areas susceptible to being colonized across other Mediterranean islands (Corsica, Crete, Cyprus, Formentera, Ibiza, Menorca, Sardinia, and Sicily), considered important biodiversity hotspots. Our results showed that there are suitable areas where this species could spread and stablish in Mallorca, indicating that if an early detection and eradication plan would not have been implemented with both introductions, the species probably would have colonized and stablished in part of the island, as predicted by Robinet et al. (2019). Furthermore, the nest detected in 2021, which constitutes a new introduction in the island, was found on the edge of suitable area of *V. velutina* in Mallorca (specifically, in Marratxi). This means that, if this single nest had not been detected and eliminated quickly, it could have reproduced and very probably expanded to the Serra de Tramuntana (area with higher values of habitat suitability). Moreover, we discuss the shipping traffic as potential pathway used to reach the island, an invasion strategy already used by the Argentine ant (*Linepithema humile*) to colonize Mallorca (Bernard, 1956). These pathways are also supported by our genetic analyses. The maximum dispersal recorded for this species has been 78 km in Europe (Robinet et al., 2017), while reaching Mallorca from Italy and Catalonia means spread 600 and 175 km respectively through the sea. The only explanation that allows this great spread difference is a human-mediated transport.

With the experience acquired in population genetics and in ecological niche model, we set out to integrate both methods to understand patterns of genetic variation in central and peripheral populations. Our results revealed a central-peripheral patterns, where allelic richness decreased towards the edge of the expansion range. Moreover, the low environmental suitability of the territories invaded by marginal populations could prevent a diverse population from establishing and reducing the genetic diversity in populations at the expansion edge. The relevance of the suitability–genetic diversity relationships for the management of biological invasions can be modulated by climate change. Barbet-Massin et al. (2013) predicted a potential increase in environmental suitability and therefore a range expansion of *V. velutina* by 2100, towards the southwest region of the Iberian Peninsula. Based on our results, this potential expansion could lead to low genetic diversity at the expansion edges while still supporting locally adapted invading populations. Nonetheless, Arca et al. (2015) highlights that other biotic and abiotic factors could compensate the loss of genetic diversity of invading peripheral populations, such as the abundance of honeybees or reduced competition with other hornets (Villemant et al., 2011). Hence, we might not underestimate the invasiveness of this species on the European continent.

To complete these studies in which we increase the knowledge and management of the species, we provide a new automated detection method to monitor this invasive alien species, along with radiotelemetry (Kennedy et al., 2018), harmonic radar (Maggiora et al., 2019), thermal imaging (Lioy et al., 2021) and drones (Reynaud & Guérin-Lassous, 2016). We used wingbeat features obtained by an optical sensor to distinguish the invasive alien species *V. velutina* from other seven Hymenoptera species. This automated method can greatly reduce the economic costs and human resources needed to detect this species. For example, all the fieldwork conducted in Mallorca could have been greatly benefited by implementing this method and represents an environmentally friendly strategy to replace traditional trapping, which has a negative impact on non-target entomofauna (Monceau et al., 2012; Rojas-Nossa et al., 2018; Turchi & Derijard, 2018). Furthermore, wing shape have been correlated with wingbeat features, and Bortolotti et al. (2022) detected differences in wing shape between haploid and diploid males of *Bombus terrestris*. As such, it would be interesting to determine if there are differences in wing shape between haploid and diploid males of *V. velutina* which may be detectable using the sensor, reducing the laboratory work and the economic cost of determining its genetic load by nuclear microsatellites. At the same time, we provide in which areas of Mallorca it would be most suitable to install this automated sensor, to direct the search and detection of this species based on wingbeat features.

This PhD thesis has generated new questions and challenges that we will intend to face in future studies, such as the analysis of the diversity of the hornet's diet through metabarcoding, the role of maritime traffic in the dispersal of this and other invasive species in the Mediterranean, and improvement of the automated sensor by increasing the spectrum of detected species. These new studies will help us better understand the impact of this invasive alien species on the biodiversity of wild insects, the role of maritime traffic in the spread of this and other invasive species, as well as reduce the economic and human costs for detection and management from *V. velutina*.

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CONCLUSIONS

1. Early detections of the invasive alien species were crucial to minimise their effects, and citizen science may offer an important source of information to determine the presence and distribution of *V. velutina*.
2. The active search for nests and triangulation were essential for success in the control of *V. velutina*.
3. Our results show that Mallorca populations originated from invasive European specimens.
4. F_{ST} values, DAPC and genetic structure analysis suggest two independent incursions in Mallorca with bottleneck and founder effect signatures.
5. 30 nests detected and removed in Mallorca during 2015 and 2018 were caused by a single introductions of *V. velutina* from Italy.
6. The single nest detected in Mallorca in 2021 was caused by a different single introduction of *V. velutina* from Catalonia.
7. We contributed additional genetic evidence of the polyandrous behaviour of this invasive species based on the inference of a mean number of mattings per nest of 3.94 (range 2 – 6.5).
8. We show for the first time that there are suitable areas where this species can expand and stablish in Mallorca, mainly in steeper slopes and low isothermality zones.
9. The main Mediterranean islands (Ibiza, Formentera, Menorca, Corsica, Sardinia, Sicily, Crete, and Cyprus) showed also potentially suitable zones for *V. velutina*.
10. We revealed a central-marginal dynamic in Europe, where allelic richness decreased towards the edge of the expansion range. Moreover, the low environmental suitability at the expansion limits could prevent a diverse population from establishing itself and reduce the genetic diversity in edge populations.
11. MCMC showed both geographic and environmental distances influenced population genetic differentiation.
12. *V. velutina* presented a fundamental frequency F1 (Hz) which was statistically lower than that of the other Hymenoptera species, except with *P. dominula*. While *V. velutina* presented a fundamental power (F1 dB) which was statistically higher than those of the other Hymenoptera species.
13. The highest classification accuracy was achieves using the 14-value PSD peak and valley feature, indicating a substantial contribution to model accuracy even though they are known to be highly correlated with fundamental frequency F1 (Hz).
14. We demonstrated that the wingbeat recordings from a flying insect sensor can be used with machine learning methods to differentiate *V. velutina* from six other Hymenoptera species in the laboratory.

ANNEXE

Annexe 1

The impact of the invasive species *Vespa velutina* on honeybees: A new approach based on oxidative stress

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Abstract

Honeybees have an essential role in ecosystems pollinating wild flowers and cultivated crops, representing an important cultural and economic benefit for humans. Honeybee populations are decreasing over the last decade, due to multifactorial causes. The aim of this field study was to investigate the effects of the presence of the invasive species *Vespa velutina*, a bee predator, in oxidative stress parameters of honeybee workers. To achieve this objective, positive or negative apiaries for the presence of the *V. velutina* were selected. Five honeybees from six hives of each apiary were sampled in spring, summer and autumn, analysing a total of 233 samples. Analysis of mRNA expression of oxidative stress-related genes, catalase enzymatic activity and lipid peroxidation were performed. An increase in sod2, tpx3, trxR1, gtpx1, gstS1, coxI, cytC and if2mt genes expression, as well as a raise in catalase activity and lipid peroxidation were observed in *V. velutina* positive samples. Thus, here we present a new methodology to analyze the impact of the predation pressure of the invasive species *V. velutina* on honeybees under field conditions. In conclusion, the results obtained in this study indicate the negative impact of the presence of the yellow-legged hornet on honeybees' health and the activation of their antioxidant system to protect them against this biotic stressor. Moreover, the redox status they present could increase the susceptibility of honeybees, essential insects that currently receive many inputs of different stresses, to another stressor.

Keywords: Honeybees' health, Yellow-legged hornet, Oxidative stress, Catalase, Lipid peroxidation, Redox status

Highlights:

- *Vespa velutina* presence increases mRNA expression of oxidative stress-related genes.
- Catalase activity and lipid peroxidation in honeybees increase with the predator presence.
- The presence of *Vespa velutina* has a negative impact on honeybees' health.

Abbreviations

CAT: catalase, CoxI: cytochrome C oxidase subunit I, Cox6b1: cytochrome C oxidase subunit 6b1, CytC: cytochrome complex, DWV: deformed wing virus, GPX: glutathione peroxidase, GST: glutathione S-transferases, GTPX: phospholipid-hydroperoxide GPX homologues with TPX activity, if2mt: translation initiation factor IF-2, MuLV: murine leukemia virus, PCR: polymerase chain reaction, ROS: reactive oxygen species, RT-PCR: reverse transcription polymerase chain reaction, SOD: superoxide dismutase, TPX: thioredoxin peroxidase, TrxR: thioredoxin reductase.

Introduction

Honeybees are one of the most important pollinators of wild flowers and cultivated crops (Aizen & Harder, 2009; Allen-Wardell et al., 1998), pollinating about 60–95% of plants in some areas (Garibaldi et al., 2011; Morse & Calderone, 2000). They also produce honey, wax, royal jelly, pollen and propolis which are important from a cultural and economic point of view. Honeybee populations are suffering from many threats including diseases caused by parasites such as *Varroa destructor* and *Nosema ceranae*, the excessive use of pesticides to control pests or the limited resources to feed (Goulson et al., 2015; Potts et al., 2010).

Predation plays an important role in honeybee colony losses. The yellow-legged hornet, *Vespa velutina nigrithorax* (Hymenoptera: Vespidae) is one of the most important predators. In Europe, it was reported for the first time in south-west France in 2004 (Haxaire et al., 2006; Rome et al., 2009; Villemant et al., 2011) and rapidly spread to nearby European countries: Spain (Castro & Pagola-Carte, 2010; López et al., 2011), Portugal (Grosso-Silva & Maia, 2012) , Italy (Demichelis et al., 2014), Belgium (Bruneau, 2011; Rome et al., 2013), Germany (Witt, 2015), Great Britain in 2016, Switzerland in 2017 (Budge et al., 2017). This species is also established in South Korea (Choi et al., 2012, 2013) and Japan (Ueno, 2014). Its presence was first detected in Majorca island in 2015 (Leza et al., 2018). The main impact of *V. velutina* is the likely decrease in honeybee populations (Moncea et al., 2013a, 2013b; Tan et al., 2007), as hornet larvae feed on the proteins of honeybees. The decrease of honeybee's population is anticipated to economic losses for the farmers due to the decrease of crop production (Arca et al., 2015; Villemant et al., 2011) and ecological impacts, such as biodiversity losses, due to the decrease of wild plants pollination (Morse & Calderone, 2000).

It is known that some abiotic, biotic and physiological stresses in animals can cause oxidative stress due to an imbalance between reactive oxygen species (ROS) production and detoxification (Abdel-Daim et al., 2018; Li et al., 2018). Mitochondria are the main source of ROS and, for this reason, mitochondrial biogenesis and functionality are linked to ROS generation (Gonçalves et al., 2012). ROS have the potential to react with macromolecules like lipids, proteins and nucleic acids, damaging cellular components and leading to cell degeneration and death (Birben et al., 2012)s ROS generation can be produced as a defence mechanism, for example in front to pathogens, or as a result of the own cellular metabolism (Farooqui & Farooqui, 2012; Nikolenko et al., 2012; Turrens, 2003). Honeybees' cells have antioxidant enzymes to detoxify ROS such as superoxide dismutase (SOD), catalase (CAT) or peroxidases that reduce hydrogen peroxide using thioredoxin or glutathione as an electron donor. In insects, this reduction is carried out by thioredoxin reductase (TrxR), thioredoxin peroxidase (TPX), phospholipid-hydroperoxide GPX homologous with TPX activity (GTPX) and glutathione S-transferases (GST) (Nikolenko et al., 2012).

It has been reported that factors like environmental factors and the season of the year (Orčić et al., 2017), diseases like varroosis or nosemosis (Aufauvre et al., 2014; Farjan et al., 2014; Zaobidna et al., 2015), pesticides (Aufauvre et al., 2014) or other factors related to lifestyle like flight (James & Xu, 2012; Korayem et al., 2012) cause oxidative stress.

Few studies have been performed about the impacts of *V. velutina* on honeybee colonies since this predator arrived in Europe. Here we present, for the first time, a molecular approach to define the impact of predation at colony level. Concretely, we analysed the effects on oxidative stress of

honeybees. Following this objective, parameters such as the expression of antioxidant genes, mitochondrial-related genes, lipid peroxidation and catalase enzymatic activity were performed.

Material and methods

Study sites

The study was conducted in Majorca, the largest island of the Balearic archipelago (3667 km^2) (Figure 25A). A total of 16 apiaries of *Apis mellifera iberiensis* were included in the study. The area studied was the "*Serra de Tramuntana*", a mountain range located in the north-east of the island of Majorca. This area is characterized by specific climatic and geomorphological conditions compared to the rest of the regions in Majorca, such as the highest precipitation rate (mean of 1400–1600 mm annual) of the island and cooler temperatures (climatically considered Mediterranean, sub-humid, based on Emberger's Index). Consequently, the vegetation of the area is quite different from the rest of the island, and it is dominated by pine trees (*Pinus halepensis* Miller), holm oaks (*Quercus ilex* L.), garrigue (mainly *Rosmarinus officinalis* L. and *Erica multiflora* L.), wild olive trees (*Olea europea* var. *sylvestris* L.) and orchards of orange trees (*Citrus sinensis* (L.) Osbeckand) and olive trees (*Olea europea* L.).

Sampling method and field experimental design

In order to evaluate the stress that can be generated by the presence of the invasive exotic species *V. velutina* on honeybee populations, an experimental design was proposed that includes sampling of a total of 16 apiaries of the same bioregion: 8 apiaries at zones with the presence and predation of *V. velutina* detected and confirmed since 2015 (positive apiaries) and 8 apiaries without *V. velutina* (control apiaries) (Figure 25B).

The distance from located *V. velutina* nests was the parameter used to select the apiaries in this study, so selected positive apiaries were located at 0,6 to 2,9 km away from nests and control apiaries were selected for being >6 km away from nests. Moreover, *Varroa* infestation was analyzed before to select the apiaries. The selected apiaries did not show significant differences between positive and control apiaries for the *Varroa* infestation (Student's t-test; $P = 0{,}974$).

In each apiary, wherever possible, six hives were monitored during three seasons of 2018 (Spring, Summer and Autumn). A total of 233 samples of 95 hives were analysed. 5 bees (selected avoiding the active foragers, in order to eliminate other external stressors) per hive were sampled with an analyses pot, directly introduced in nitrogen liquid and preserved in −80 °C until being processed to evaluate oxidative stress parameters.

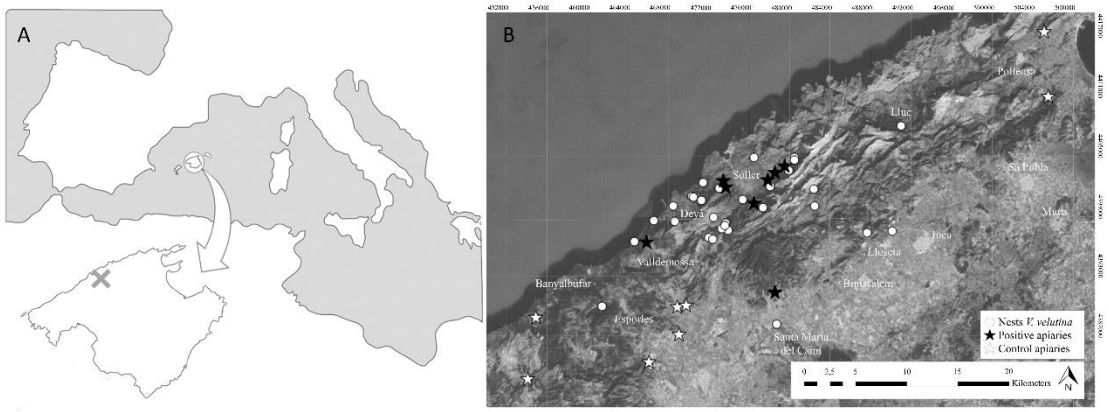

Figure 25.- Location of study area in Majorca Island A) on the east of Mediterranean Sea. B) Location of positive apiaries (black filled stars), which had predation of *Vespa velutina* nests (white filled circles), and control apiaries (white filled stars).

Reagents

Routine chemicals were supplied by Takara Bio Inc. (Kusatsu, Japan), Panreac (Barcelona, Spain), Sigma-Aldrich (St. Louis, MO, USA) and Bio-Rad Laboratories (Hercules, CA, USA). Primers were purchased from Metabion (Planegg, Germany).

RT-PCR

Total RNA was isolated from whole honeybees using Tri-Reagent® (Sigma-Aldrich) following the manufacturer's protocol and quantified using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Kyoto, Japan) set at 260 nm. One microgram of total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U of MuLV reverse transcriptase in a 10 µl volume of retrotranscription reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 2.5 mM random hexamers, 10 U RNase inhibitor, and 500 mM of each dNTP. Each resulting cDNA was diluted 1/10.

PCR was done for the target genes showed in Table 7 using SYBR green technology on a Light-Cycler 480 rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 µl, containing 7.5 µl Lightcycler® 480 Master SYBR Green I (with 0.5 µM sense and antisense specific primers) and 2.5 µl of the cDNA template. The amplification program consisted of a preincubation step for denaturation (10 min 95 °C) followed by 40 cycles consisting of a denaturation step (10s, 95 °C), an annealing step (10s, temperature depends on each pair of specific primers), and an extension step (12 s, 72 °C for all). Table 7 shows the primers and conditions used for RT-PCR. A negative control without cDNA template was run in each assay.

The Ct values of the real time PCR were analyzed, considering the efficiency of the reaction and referring these results to the mRNA expression levels of RPL8 as housekeeping, using the GenEx Standard Software (MultiDAnalises, Sweden).

Table 7.- Primers and conditions used for RT-PCR.

Gene	Forward Primer (5'-3') Reverse Primer (5'-3')	T. An. (°C)
RPL8	gCAACTgCTgCATCATCCTC ggAAAAgAgCCTCgggACAA	60
Catalase	TggATgTTCAACAgggTTCATA CTggTggTggACgTATTgATAA	60
SOD1	TAgCCTTCgATCCggCTCAT TgTTTgggCCAAgACgATgT	61
SOD2	gTgTgCgTTCTTCAgggTgA gggTTgAAATgTgCACCAgC	60
TrxR-1	ATCTTTCCCCAAATggCggT TgAgCggTTTgCgACCAATA	60
Tpx-4	gCAAgTACTgTTgCCCAggA ACgTAATCgATgATCggggC	60
Tpx-3	TgCTgATCATgACCgCACTT TgAAgAggCCTCgTAAAgCA	60
GstD1	CACCgAgAAAACAgggTggA CgCAAATggTCgTgTggATg	60
Gtpx1	TCCTTggAACgAggAgAggT gCCgCATTTTgACgCTACAT	60
Cox6b1	TgTgTTCTTTCAgTCACCAACg TggTgCTgTgTTTggTTTCAA	60
CytC	TTTTCAATCgCACggCTgAC TCgCgAgAgAgCACCAAAAT	60
CoxI	CgTCTggTACTCgAgCgTTT TACCACAAATTTCTgAACATTgACC	60
IF-2mt	CATgAACAgTTCCATCCTTAggT TgAAAgCAgCATgTCCAggT	60
GstS1	gggCATgTTgATCATggCAAA gCgATCAAgCgAgAAATAgCC	60

2.5. Measurement of 4-hydroxy-2-nonenal

Each whole honeybee was homogenate in 1 ml of homogenization buffer and protein content was determined with the Bradford's method (Bradford, 1976) and were kept at −20 °C until 4-hydroxy-2-nonenal levels determination.

For 4-hydroxy-2-nonenal (4-HNE) analysis, 40 µg of protein from honeybee homogenates were separated in an SDS-PAGE and transferred onto a nitrocellulose membrane. Antiserum against 4-HNE (HNE11-S) was used as primary antibody (Alpha Diagnostic International, San Antonio, TX). Bands were visualized by Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analysed with Quantity One software (Bio-Rad).

Catalase activity

Each whole honeybee was homogenate in 1 ml of homogenization buffer and protein content was determined with the Bradford's method (Bradford, 1976) and the enzymatic activity assay was run immediately.

Catalase activity was determined using the spectrophometric method based in the Purpald® oxidation described in 1988 by Johansson and Borg (1988).

Statistical analysis

The statistical analyses were performed with the Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM). The gene expression statistical differences were analysed using a Kolmogorov-Smirnov test to find out whether the gene expression data is normally distributed. The results obtained showed that the gene expression data did not fit to a normal distribution. To solve it, we have repeated all the gene expression statistical analysis using a non-parametric test for two independent samples, concretely a Mann-Whitney U test. The statistical differences in catalase activity and 4-HNE levels were analysed using a Student's t-test. Statistical significance was set at $p\text{-}value < 0.05$ and $p\text{-}value < 0.1$.

Results

Effects of *Vespa velutina* presence on honeybee workers' antioxidant genes expression

As shown in Figure 26, honeybee workers from apiaries positive to *V. velutina* presence presented a statistically significant augmented expression of some antioxidant enzymes genes. Concretely, sod2 showed an increased expression (1.00 vs. 1.12), as well as gtpx1 (1.00 vs. 1.18), tpx3 and trxR1 (1.00 vs. 1.15 and 1.00 vs. 1.17, respectively), showed in Figure 26A and B. Moreover, gstS1, an enzyme that recycle glutathione, showed an increase in their expression (1.00 vs. 1.14), as shown in Figure 26C.

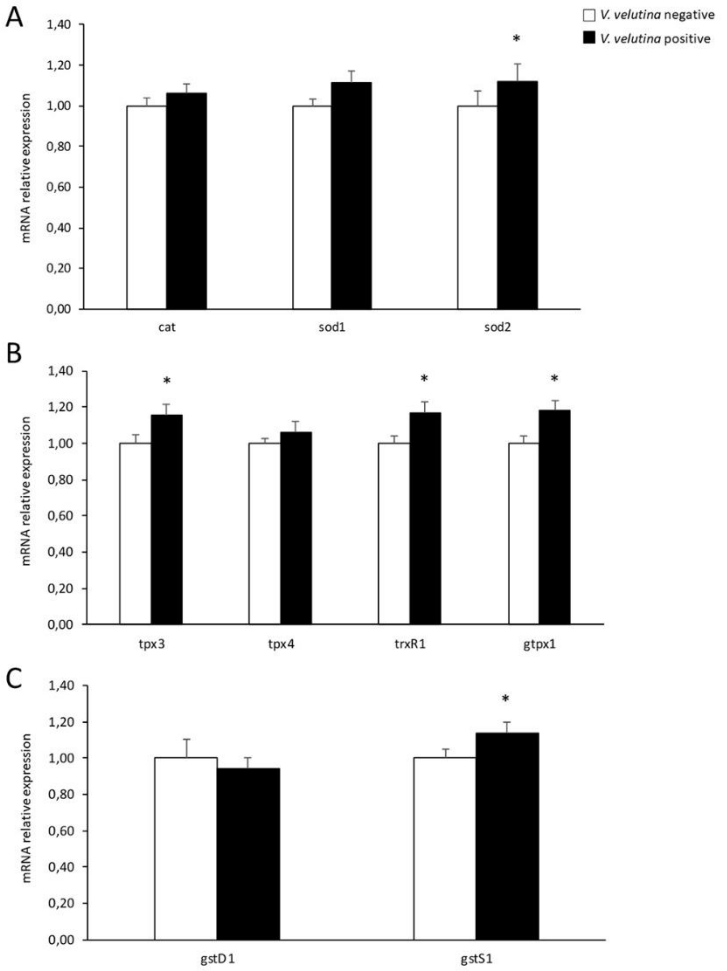

Figure 26.- Effects of *Vespa velutina* presence on antioxidant enzymes gene expression. Honeybees were sampled in apiaries which have been in direct contact with *V. velutina* and in control apiaries which had no contact with this predator. Data represent means ± SEM mRNA relative expression to RPL8 mRNA expression levels. A) Effects of *Vespa velutina* presence on cat, sod1 and sod2 gene expression. B) Effects of *Vespa velutina* presence on tpx3, tpx4, trxR1 and gtpx1 gene expression. C) Effects of *Vespa velutina* presence on gstD1 and gstS1 gene expression. Statistically significant difference between honeybee workers from *V. velutina* negative and *V. velutina* positive apiaries (Mann-Whitney U test, **p-value* < 0.05).

Effects of *Vespa velutina* presence on honeybee workers' mitochondrial-related genes expression

Figure 27 shown the expression of mitochondrial-related genes, which presented a statistically significant increase in honeybee workers from apiaries located near the *V. velutina* nests. *V. velutina* presence increased the expression of coxI (1.00 vs. 1.19), cytC (1.00 vs. 1.26) and if2mt (1.00 vs. 1.19).

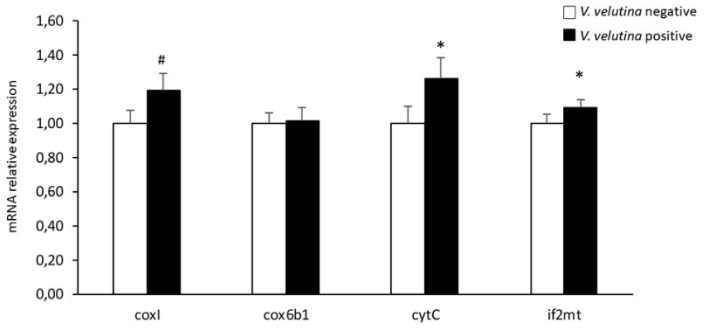

Figure 27.- Effects of *Vespa velutina* presence on mitochondrial-related gene expression. Honeybees were sampled in apiaries which have been in direct contact with *V. velutina* and in control apiaries which had no contact with this predator. Data represent means ± SEM mRNA relative expression to RPL8 mRNA expression levels. Statistically significant difference between honeybee workers from *V. velutina* negative and *V. velutina* positive apiaries (Mann-Whitney U test, **p-value* < 0.05 and $^{\#}$*p-value* < 0.1).

Vespa velutina presence causes an increase in lipid peroxidation and in catalase enzymatic activity

Table 8 indicates that honeybee workers from apiaries located near the *V. velutina* nests showed more lipid oxidative damage than control bees, as they had more 4-hydroxy-2-nonenal groups (+24%).

Moreover, honeybee workers from apiaries positive for the presence of *V. velutina* had an increased catalase enzymatic activity (+20%).

Table 8.- Catalase activity and 4-HNE quantification. Statistically significant difference between honeybee workers from *V. velutina* negative and *V. velutina* positive apiaries (Student's t-test, **p-value* < 0.05).

	V. velutina Negative	*V. velutina* Positive
Catalase activity (mUI/mg prot)	309 ± 26.9	371 ± 29.2*
4-HNE (A.U.)	100 ± 9.66	124 ± 7.61*

Discussion

In this work, we demonstrate that the presence of the yellow-legged hornet, *V. velutina nigrithorax*, in a concise area, produces an increase of oxidative stress in honeybee workers under field conditions. This leads to a higher expression and activity of antioxidant enzymes and mitochondrial-related genes and higher lipid oxidative damage in the individuals of the colony exposed to this predator.

In the last years, the oxidative stress in bees has been linked to the impact of negative factors. Several studies have analysed the impact of some abiotic stressors such as insecticide treatments over oxidative stress in insects (Candy, 2002; Kodrík & Socha, 2005). Moreover, beekeeping

management such as migratory activity induces oxidative stress in honeybees (Simone-Finstrom et al., 2016). Furthermore, biological factors such as the presence of parasite *Varroa* affect the antioxidant capability of *A. mellifera* cells (Badotra et al., 2013). Some authors have described that parasitic diseases increase free radical production in the host (Clarkson & Thompson, 2000; Sorci & Faivre, 2009). Here, for the first time, we present a field study where it has been analysed the impact of other biological stressor, the bee predator *V. velutina*, on honeybee colonies, through a molecular approach.

Insects have an open circulatory system and the free flow of ROS in the body could be very dangerous. Antioxidant system plays an important role neutralizing this ROS, preventing the insect organism from total intoxication (Nikolenko et al., 2012). Our work focalizes in several levels of this antioxidant system and in the lipid oxidative damage. It has been stablished that, in other insects such *Drosophila melanogaster*, the overexpression of gtpx1 could confer resistance to ROS inducing compounds, suggesting that this enzyme could play an important role detoxifying ROS (Missirlis et al., 2003). In honeybees, mated-queens present an increased expression of CAT, GST and SOD to maintain the redox homeostasis of the sperm, to avoid its oxidative degradation (Collins et al., 2004). These results agree with ours, since we have observed a general increased expression of the antioxidant system in those honeybee workers exposed to *V. velutina*.

Our results could indicate that honeybees may increase catalase activity to decrease oxidative stress detoxifying hydrogen peroxide, since in insects the regulation of catalase enzymatic activity seems to be more important than its expression (Diaz-Albiter et al., 2011). In this sense, Badotra et al., (2013) observed an increasing activity level of catalase and other antioxidant enzymes in honeybees with other stress factor like *Varroa* infestation.

Not only have we observed an increase in the gene expression and/or activity of the main antioxidant enzymes, but also, we have observed an increment in the lipid peroxidation in honeybee workers exposed to *V. velutina*. This could indicate that, despite the activation of the antioxidant system, the honeybee workers exposed to *V. velutina* could not be able to palliate the oxidative stress generated by the predation, so they present oxidative damage in lipids. Other authors have published that other stressors like herbicides or migratory management could increase lipid peroxidation in honeybees (Helmer et al., 2015; Simone-Finstrom et al., 2016), suggesting that these situations, as well as the presence of *V. velutina*, could affect honeybees' health.

It is worth to note that the effects over gene expression not only affect to primary detoxifying enzymes, such as catalase and SOD, but also another type of enzymes that maintain cofactors reduction to preserve redox homeostasis. The presence of *V. velutina* increases even the mitochondrial-related genes expression which affect, at the end, to the mitochondrial oxidative respiratory chain, the main source of ROS generation. In fact, our results show that this eusocial insect has a special multilevel regulation indicating that homeostasis redox is a mechanism that honeybees need to control, not only at individual level, but at colony level, as Nikolenko et al., (2012) suggest in their studies.

It is known that, in other insects like mosquito, mitochondrial biogenesis and function play an important role in ROS generation (Gonçalves et al., 2012). Mitochondria are the main source and target of ROS, so this organelle is more susceptible to oxidative damage (Nikolenko et al., 2012). There is a lack of studies of mitochondrial biogenesis and ROS production in insects. However,

it has been seen that yeast and mammalian cells that are subjected to oxidative stressors could increase their mitochondrial mass to generate non-damaged mitochondria, to palliate the degenerative effects of ROS in cells (Bouchez & Devin, 2019). Our results agree with this, because the predation stress of *V. velutina* over honeybee workers seems to increase the expression of mitochondrial-related genes, such as coxI, if2mt and cytC.

Conclusion

To sum up, here we present a new methodology which could be a useful tool to assess the impact of the predation pressure of the invasive species *V. velutina* on honeybees under field conditions. It is a new approach based in some molecular indicators related to oxidative stress that could help scientists, administrators and beekeepers to evaluate the colony sanitary status and manage the situation properly. In conclusion, the results obtained in this study indicate the negative impact of the presence of the yellow-legged hornet on honeybee's health and the activation of their antioxidant system to protect themselves against this biotic stressor. Moreover, the redox status they present could increase the susceptibility of honeybees, essential insects that currently receive many inputs of different stresses, to another stressor.

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Annexe 2

Diversity of compounds in *Vespa* spp. venom and the epidemiology of its sting: a global appraisal

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Abstract

Poisonous animals imply a risk to human life, because their venom is a complex mixture of low molecular weight components, peptides and proteins. Hornets use the venom for self-defence, to repel intruders and to capture prey, but they can cause poisoning and allergic reactions to people. In particular, they seem to be a health problem in the countries where they are native due to their sting, which in the most severe cases can lead to severe or fatal systemic anaphylaxis. But this situation is being an emerging problem for new countries and continents because hornet incursions are increasing in the global change scenario, such as in Europe and America. Furthermore, 55 detailed cases of hornet sting were found in 27 papers during the current review where 36.4% died due to, mainly, a multi-organ failure, where renal failure and liver dysfunction were the most common complications. Moreover, the great taxonomic, ecological diversity, geographical distribution and the wide spectrum of pathophysiological symptoms of hornets have been the focus of new research. Considering this, the present systematic review summarizes the current knowledge about the components of *Vespa* venom and the epidemiology of its sting to serve as reference for the new research focused on the development of techniques for diagnosis, new drugs and treatments of its sting.

Keywords: Venom, Sting, Hornet, *Vespa*, Health

Introduction

Social hymenoptera are one of the most diverse groups within poisonous animals that can sting, such as bees (Apoidea), wasps (Vespidae) and ants (Formicidae) (Figure 28) (Bazon et al., 2018; dos Santos-Pinto et al., 2018; Košnik et al., 2002; Kularatne et al., 2014; Lopes et al., 2017; Shin et al., 2012; Sookrung et al., 2014). Sting cases usually happen accidentally; however, one of the most dangerous characteristics of social hymenoptera is when the colony is disturbed, which can lead to a massive attack and produce an important poisoning (Spradberry, 1973).

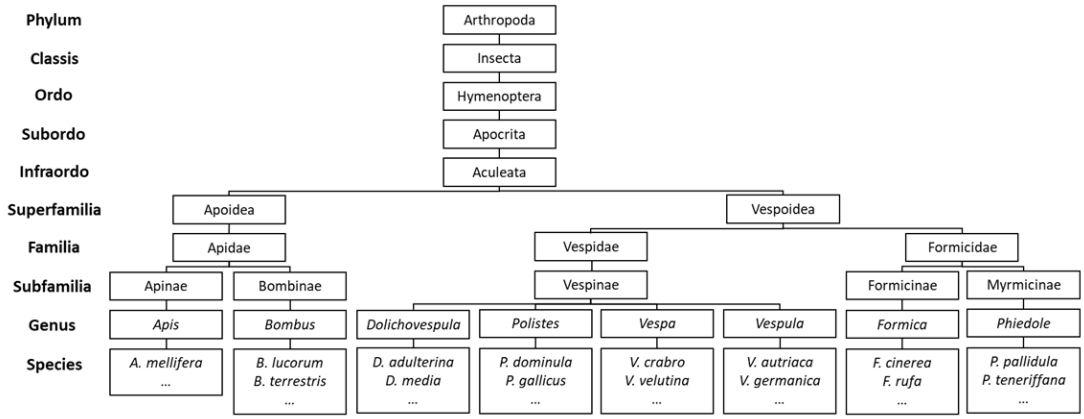

Figure 28.- Taxonomy of the main social Hymenoptera of sanitary importance.

Hymenoptera species which sting humans are less than 200, reducing to 100 those species whose sting is more common. Of these, only bees (*Apis* = 7 spp), yellow jackets (*Vespula* = ~ 12 spp.) and hornets (*Vespa* = ~ 12 spp.) are able to kill people through direct poisoning, adding to the cases of death due to allergy (Schmidt, 2018).

Several studies in recent decades have focused on the isolation and biological characterization of bioactive compounds of wasp venoms, mainly in hornets (Chen et al., 2008; Czaikoski et al., 2010). This is because, in those countries and continents where hornets are present, mainly Europe and Asia, many beekeepers, woodcutters, farmers or gardeners have been wounded or died due to stings every year (Abe et al., 2000; Liu et al., 2015; Witharana et al., 2015). Normally, a single hornet sting is not dangerous, but the situation can be aggravated in allergic people (Vetter et al., 1999; Yoon et al., 2015), and multiple stings cases can cause death due to poisoning (Baek et al., 2013; Schmidt et al., 1986). These are the main types of reactions in humans (Yanagawa et al., 2007).

The toxicity of hornet venom is species specific and varies depending on the components (Schmidt et al., 1986). The major venom components of hornets have been reported individually for each species, but there is a lack of information on the clinical manifestations of its sting, such as local, systemic reactions or anaphylaxis (Sookrung et al., 2014; Yoon et al., 2015).

Due to the deficiencies in diagnosis and lack of medical surveillance, the pathophysiological symptoms of hornet sting may be underestimated (An et al., 2012). This review aims at summarizing and discussing the diversity of compounds in *Vespa* spp. venom and the

epidemiology of hornet sting, to serve as reference for the new research focused on the development of techniques for diagnosis, new drugs and treatment of its sting.

Material and methods

Actually, there is scientific information available about *Vespa* venom, its composition, characterization and wide spectrum of pathophysiological symptoms experienced after the sting. We performed a scientific bibliographic search using different search engines such as Web of Science (WOS), PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and Scopus (https://www.scopus.com). We used a combination of the following words: (Venom AND Vespidae OR *Vespa* OR Hornet) and (Sting AND Hornet) until 2019, included (Table 9). Considering that many events are not published because they are chance or isolated encounters, we also performed a Google search (in English and Spanish). Finally, given the different and extensive terminology used to study this topic, we examined the references of articles we reviewed for additional reports not found in our searches. Additionally, we performed a search in NCBI Protein of the following words: (Venom AND *Vespa*) without excluding criteria. In the case of NCBI Protein, we found 75 results. A total of 242 articles were pertinent and included in the database.

Table 9.- Search strings and number of returned articles found on Web of Science (WOS), Pubmed and Scopus.

Search no.	1st Keyword	2nd Keyword	WOS	Pubmed	Scopus
1	Venom	Vespidae	1079	42	969
2	Venom	*Vespa*	403	173	1104
3	Venom	Hornet	572	1070	1681
4	Sting	Hornet	358	792	895

Results and discussion

Hornets are poisonous animals that endanger human life. Their venom, as the rest of social Hymenoptera, is a complex and rich mixture composed of low-weight molecules, a large number of peptides and proteins, acting as toxins and allergens, producing local and systematic allergic reactions, including death (Bazon et al., 2018; Farag & Swaby, 2018; Perez-Riverol et al., 2017; Rungsa et al., 2018; Silva et al., 2017). This complex mixture of compounds (synthesized, stored and secreted by the venom gland) represents a powerful biological arsenal that acts synergistically to defend, repel intruders and capture prey (dos Santos-Pinto et al., 2018; Farag & Swaby, 2018; Mendonça et al., 2017; Sadler et al., 2018; Silva et al., 2017).

There are 24 species (An et al., 2012), but only 4 species have been described for producing allergy. These species are *Vespa crabro*, *V. magnifica*, *V. mandarinia* and *V. velutina* (https://www.allergen.org, accessed December 2019). Due to the deficiencies in diagnosis and lack of medical surveillance, allergy to *Vespa* sting may be underestimated (An et al., 2012). Even so, there are documented cases of allergy to its sting, resulting in highly toxic poisoning (An et al., 2012; Chen et al., 2008; Han et al., 2008).

Venom composition

The wasp and hornet venom gland is a complex and specialized organ that performs a wide range of functions (Xu et al., 2006; Yang et al., 2009, 2013). During the sting, the contents of the venom sac are injected toward the body through the contraction of the muscle tissue that surrounds the poisonous sac. Most of the content can be injected, while a small portion is deposited in the skin (Jimenez et al., 2016).

Wasps and hornets mainly have three groups of components in their venom: (a) small molecular weight peptides, (b) high molecular weight proteins and (c) other components that act as enzymes, toxins and allergens (Cvetković-Matić et al., 2009; Li, et al., 2006; Yang et al., 2009; Yang et al., 2013).

Peptides

Most of the venom's peptide components are cationic helical amphipathic peptides from 12 to 50 aa, of which 50% consist of hydrophobic residues to interact with cell membranes, constituting 70% of the dry weight of the venom. These peptides produce cell and antimicrobial lysis, mast cell degranulation with inflammatory effects, neutrophil increase and polymorphonuclear leukocyte chemotaxis, smooth muscle contraction and even promote the administration of cell activators/mediators (Baek et al., 2013; Dias et al., 2015; Farag & Swaby, 2018; Jalaei et al., 2014; Liu et al., 2015; Monteiro et al., 2009; Perez-Riverol et al., 2017; Silva et al., 2017). Many peptides with anticoagulant, antiplatelet, anti-inflammatory and immunosuppressive activities were also isolated from hornet venom (An et al., 2012; Han et al., 2008; Kaushik, Thounaojam et al., 2014).

There are three main families of peptides, depending on their biological activity: (1) mastoparans (producing a degranulation of mast cells), (2) chemotactic peptides (producing chemotaxis of polymorphonuclear leukocytes) and (3) kinins (producing pain) (Arcuri et al., 2016; Chen et al., 2008; Monteiro et al., 2009; Xu et al., 2006; Yang et al., 2009).

Mastoparans

The mastoparans constitute the most abundant family of peptides in hornet venom, even in solitary wasps (Lee et al., 2016; Lin et al., 2011; Monteiro et al., 2009). They are active polycationic molecules of 14 (12) aa that contain two to four lysine residues (symbol of aa: K), whose position along the aa chain allows a stable helical structure, as well as a homogeneous hydrophobic surface, giving an amphipathic character (Figure 29a) and their lithic activity (dos Santos-Pinto et al., 2018; Jalaei et al., 2018; Lee et al., 2007; Lin et al., 2011; Lopes et al., 2017; Monteiro et al., 2009; Murata et al., 2009; Perez-Riverol et al., 2017; Silva et al., 2017; Xu et al., 2006).

Several studies have shown that mastoparans act on mast cells. These cells are the most important effector cells in inflammatory and allergic diseases of the immune system. They are responsible for releasing, mainly, histamine, as well as proteases, lipid mediators and cytokines, producing the characteristic symptomatology of a local inflammation (redness, oedema and pain) (Chen et

al., 2008; Fox & Aparicio, 2008; Jalaei et al., 2016; Lee et al., 2007; Lopes et al., 2017; Perez-Riverol et al., 2017). Therefore, they play a leading role in allergic and inflammatory reactions, due to mastoparans produce exocytosis of the granule content, even acting in defence against parasites and bacteria (Jalaei et al., 2016).

These peptides act on mast cells in two ways: (1) by competitive binding to specific sites of the α subunit of protein G, causing a transduction/cascade of signals and events, including an increase of intracellular Ca^{2+}, resulting in an exocytosis (Figure 29b) and (2) binding to specific proteins that, without an increase in intracellular Ca^{2+}, cause an exocytosis mediated by FcεRI (high-affinity IgE receptor) (Figure 29c). Both pathways release mainly histamine, causing dilation and an increase in capillary permeability (extravasation). While in the first case, the mastoparans act with the plasma membrane, the second case acts directly with the endosome membrane. It suggests they have to trespass the cell membrane somehow (Delazari dos Santos et al., 2012; Dias et al., 2015; dos Santos-Pinto et al., 2018; Lopes et al., 2017; Monteiro et al., 2009; Perez-Riverol et al., 2017; Silva et al., 2017; Yang et al., 2009).

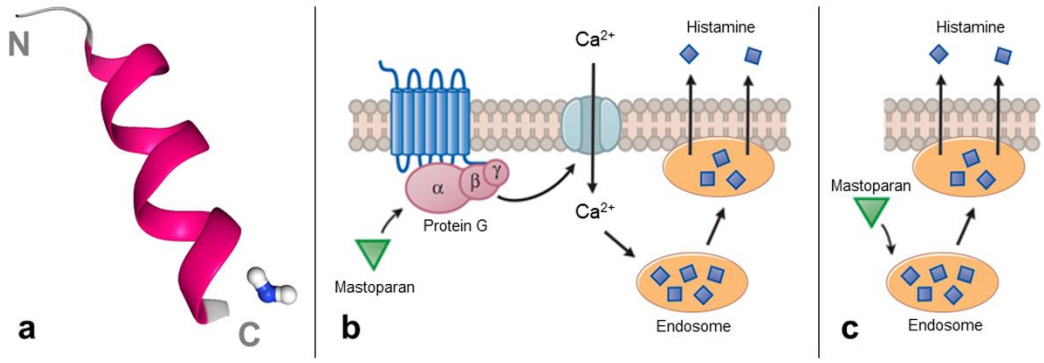

Figure 29.- Biochemistry of mastoparan. a) α-Helical secondary structure of mastoparan-X isolated from *Vespa xanthoptera* (NCBI-Protein). Histamine released by b) G protein binding and c) direct endosome binding

Its antimicrobial and cytolytic activity in animal cells is because they adopt a helix conformation in the presence of membrane lipids, interacting with the cationic domains of the peptide with the negatively charged surface of the cells. In addition, it has also been observed that they can block potassium channels, acting as an epileptogenic neurotoxin, lower blood pressure in rats and activate guanylate cyclase and phospholipase A2, C and D2 (Chen et al., 2008; Jalaei et al., 2018; Lee et al., 2007; Lin et al., 2011). All these functions make them useful for studying the mechanism of signal transduction (Lee et al., 2007).

Despite the research carried out, much remains to be known about the levels and diversity of biological activities of these peptides. However, it seems to indicate that mastoparans are key molecules in the poisoning process, causing the release of histamine at low concentrations, producing an oedema and an important physiological discomfort, all of them part of the immediate and innate inflammatory response (Chen et al., 2008; dos Santos-Pinto et al., 2018; Jalaei et al., 2016; Perez-Riverol et al., 2017; Silva et al., 2017). Avoiding mast cell degranulation and subsequent histamine release would prevent the activation of the immune system, so these peptides could be a source of new drugs (Jalaei et al., 2016).

Many studies have shown the presence of mastoparans in different species of *Vespa* (Table 10). On the one hand, Lin et al., (2011) tested the mastoparans of six different *Vespa* species and observed, at the same concentration of mastoparans, their capacity to act on mast cells and release their content, which was exponential and distinct between species. Therefore, the activity of this peptide depends not only on the concentration, but also on the species.

Table 10.- Mastoparans identified in *Vespa* spp. venom

Species	Peptide	Aa sequence	Da	Reference
V. affinis	Mastoparan-AF	INLKAIAALAKKLF	1514	Lin et al., (2011)
V. analis	Mastoparan-A	IKWKAILDAVKKVL	1369	King et al., (2003)
V. basalis	Mastoparan-B	LKLKSIVSWAKKVL	1613	Ho et al., (2001)
V. bicolor	Mastoparan-VB1	INMKASAAVAKKLL	1457	Chen et al., (2008)
V. crabro	Mastoparan-C	LNLKALLAVAKKIL	1508	Argiolas & Pisano (1984)
V. ducalis	Mastoparan-D	INLKAIAAFAKKLL	1514	Lin et al. (2011)
V. magnifica	Mastoparan-like peptide 12a	INWKGIAAMAKKLL	1557	Li et al. (2006)
	Mastoparan-like peptide 12b	INWKGIAAMKKLL	1486	
	Mastoparan-like peptide 12d	INLKAIAAMAKKLL	1498	Yang et al. (2006)
V. mandarinia	Mastoparan-M	INLKAIAALAKKLL	1480	Hirai et al. (1981)
V. orientalis	HR-I	INLKAIAALVKKVL	1494	Miroshnikov et al. (1981)
	Mastoparan-II	INLKALLAVAKKIL	1508	Nakajima et al. (1985)
V. tropica	Mastoparan-T	INLKAIAAFAKKLL	1514	King et al. (2003)
	Mastoparan-VT1	INLKAIAALAKKLL	1480	Yang et al. (2013)
	Mastoparan-VT2	NLKAIAALAKKLL	1367	
	Mastoparan-VT3	INLKAITALAKKLI	1510	
	Mastoparan-VT4	INLKAIAPLAKKLL	1506	
	Mastoparan-VT5	VIVKAIATLASKLL	1440	
	Mastoparan-VT6	INLKAIAALVKKLL	1508	
	Mastoparan-VT7	INLKAIAALARNY	1431	
V. velutina	Mastoparan-V	IAWKGIAAMAKKLL	1514	Lin et al. (2011)
V. xanthoptera	Mastoparan-X	INWKGIAAMAKKLL	1557	Todokoro et al. (2006)

On the other hand, Jalaei et al. (2016) observed in their study that the *V. orientalis* mastoparan did not cause histamine release, suggesting that this kind of peptides possesses both inflammatory and anti-inflammatory activity, such as with *Apis mellifera* mastoparan. Kaushik et al. (2014) support the same idea, suggesting that mastoparans could even be used as a treatment for better management of neuroinflammation and its related diseases, such as Parkinson's, Alzheimer's, and multiple sclerosis.

Also, Hsieh et al. (2011) and Ho et al. (2001) cite that mastoparan-B is the main isolate from *V. basalis* venom, the most dangerous wasp species in Taiwan, with a potent hemolytic and antibacterial activity, causing early symptoms of hypotension in rats.

Chemotactic peptides

The chemotactic peptides conform the second most important family of peptides. They act by promoting the massive recruitment of polymorphonuclear leukocytes, which release high amounts of hydrogen peroxide (H_2O_2) around the sting, producing local inflammation, cytolysis and pain (Palma, 2013; Perez-Riverol et al., 2017). These peptides act as pain mediators, intensifying the inflammatory effects caused by the other venom components (Li et al., 2006).

It is postulated, but it is not entirely known, that these peptides of the hornet venom act similarly to mastoparans but, instead of mast cells, on polymorphonuclear leukocytes. The chemotactic peptides would interact with the G-protein coupled receptors in the cell membrane of the leukocytes, causing a cascade of signals and, finally, a cellular migration and inflammatory response in the sting (Figure 30a) (dos Santos-Pinto et al., 2018; Perez-Riverol et al., 2017).

There are two types of chemotactic peptides with different biological activities: (1) a 10–12 aa peptide, with a single lysine residue that produces leukocyte chemotaxis and (2) a small peptide of just 4–8 aa that modulates leukocyte chemotaxis (Figure 30b). Both have an amphipathic character with positively charged aa and hydrophobic residues arranged along the peptide chain (Chen et al., 2008; Li et al., 2006).

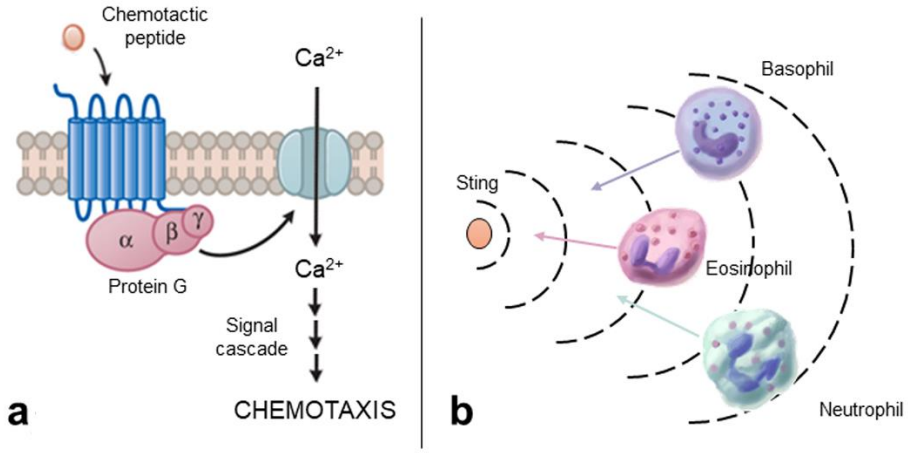

Figure 30.- Biochemistry of chemotactic peptides. a) G-protein binding in order to induce b) chemotaxis in polymorphonuclear leukocytes

Many studies have shown the presence of chemotactic peptides in different species of *Vespa* (Table 11). This family of peptides shows a molecular signature which allows a specific relationship to a genus or species (Dias et al., 2015), such as the FLP sequence in the N-terminal region in *Vespa* spp., present in most chemotactic peptides of this hornet genus.

Table 11.- Chemotactic peptides (type i) identified in *Vespa* spp. venom

Species	Peptide	Aa sequence	Da	Reference
V. analis	VesCP-A	FLPMIAKLLGGLL	1386	Nakajima et al. (1985)
V. bicolor	VESP-VB1	FMPIIGRLMSGSL	1421	Chen et al. (2008)

V. crabro	Crabrolin	FLPLILRKIVTAL	1497	Krishnakumari & Nagaraj (1997)
V. magnifica	VCP 5e	FLPIIAKLLGGLL	1369	Li et al. (2006)
	VCP 5f	FLPIPRPILLGLL	1462	
	VCP 5g	FLIIRRPIVLGLL	1523	
	VCP 5h	FLPIIGKLLSGLL	1384	Yu et al. (2007)
V. mandarinia	VesCP-M2	FLPILAKILGGLL	1368	Ombati et al. (2018)
	VesCP-M	FLPIIGKLLSGLL	1384	Nakajima et al. (1985)
V. orientalis	HR-II	FLPLILGKLVKGLL	1524	
V. tropica	VesCP-T	FLPILGKILGGLL	1354	
	VCP-VT1	FLPIIGKLLSGLL	1384	Yang et al. (2013)
	VCP-VT2	FLPIIGKLLSG	1158	
V. xanthoptera	VesCP-X	FLPIIAKLLGGLL	1368	Nakajima et al. (1985)

dos Santos-Pinto et al., 2018 classified HR-II as mastoparan, while the original article classifies it as a chemotactic peptide (Nakajima et al., 1985). Moreover, it presents the molecular signature FLP of a chemotactic peptide of *Vespa*.

The diversity of antimicrobial peptides suggests that the antimicrobial mechanisms are also diverse and complex (Li et al., 2006), because identical peptides are rarely found in two different species, even those closely related. However, antimicrobial peptides with identical sequences have been found in different hornets, suggesting that these molecules may perform similar crucial functions (Yang et al., 2013).

Additionally, chemotactic peptides and mastoparans have presented microbial activity against standard strains of *Bacillus subtilis*, *B. cereus*, *Candida albicans, C. parapsilosis, Citrobacter koseri, Enterococcus faecalis, E. faecium, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudemonas aeruginosa, Salmonella typhimurium, S. choleraesuis, Staphylococcus aureus, S. xylosus, Streptococcus alactolyticus, S. mutans* and *Vibrio parahaemolyticus*, as well as with drug-resistant strains (Aschi et al., 2019; Chen et al., 2008; Farag & Swaby, 2018; Jalaei et al., 2014, 2018; Lin et al., 2011; Li, et al., 2006; Yang et al., 2013; Yu et al., 2007). This is because both peptides present a positive net charge that interacts with negatively charged microbiological membranes, so they could be a source of new antibiotics (Chen et al., 2008; Jalaei et al., 2018; Lin et al., 2011; Li, et al., 2006; Yang et al., 2013).

Both kinds of peptides have a poor binding to mammalian cells and a selective binding to bacteria, because the bacteria membrane exposes lipoteicoic acid and negatively charged phospholipids, while in mammalian cells negatively charged lipids are not exposed and are found in the lipid bilayer (Farag & Swaby, 2018; Jalaei et al., 2018; Lin et al., 2011; Li, et al., 2006; Yang et al., 2013).

These results represent an important contribution to health, both human and animal, against infectious diseases, as the inappropriate use of antibiotics, together with genetic mutations, produces resistance, constituting a serious global problem for human health (Farag & Swaby, 2018; Jalaei et al., 2014, 2018; Yang et al., 2013).

Kinins

The kinins are the third most important family of peptides and perhaps the least known, since their toxicology is still not clearly understood. They are 9–18 aa molecules with a sequence and structure similar to bradykinin, a polypeptide secreted by the sweat glands and the endothelium of the blood vessels, which causes pain and vasodilation (dos Santos-Pinto et al., 2018; Fox & Aparicio, 2011).

Most of these kinins have longer chains and more lasting effects than bradykinin. They have been described as neurotoxic molecules of wasp venom, causing a presynaptic block in cholinergic transmission (acetylcholine) through irreversible depletion, which could be due to a non-competitive inhibition of choline absorption (dos Santos-Pinto et al., 2018; Monteiro et al., 2009; Mortari et al., 2016, 2012).

This family of peptides seems to produce a multitude of similar symptoms in different animals: such as hyper/hypotension or contraction/dilation of smooth muscle and pain, depending on the animal (dos Santos-Pinto et al., 2018). The last one seems to be a highly distributed effect on social wasps for defence against warm-blooded animals, mainly mammals and birds, because they constitute the predators of these colonies (De Castro E Silva et al., 2016; Dias et al., 2015; Jimenez et al., 2016).

In comparison with the rest of the peptides of relevant importance, the kinins have been identified in smaller number in *Vespa* (Table 12). These peptides appear to have the GRP molecular signature for this genus, similar to chemotactic peptides.

Table 12.- Kinins identified in *Vespa* spp. venom

Species	Peptide	Aa sequence	Da	Reference
V. analis	Vespakinin-A	GRPPGFSPFRVI	1330	Gobbo et al. (1995)
V. magnifica	Vespakinin-M	GRPPGFSPFRID	1346	Yoon et al. (2015)
V. mandarinia	Vespakinin-M	GRPXGFSPFRID	1346	Kishimura et al. (1976)
V. tropica	Vespakinin-T	GRPPGFSPFRVV	1316	Gobbo et al. (1995)
V. xanthoptera	Vespakinin-X	ARPPGFSPFRIV	1344	Yasuhara et al. (1977)

Chen et al., (2008) cite that precursors that encode mastoparans, chemotactic peptides and kinins have similar structures, with similar cleavage sites for enzymatic processing.

Proteins

In this complex mixture of components in wasp venom, the small molecules and peptides mediate reactions locally, causing discomfort (Han et al., 2008; Monteiro et al., 2009; Yang et al., 2008). Proteins are involved in tissue damage or induction of allergic reactions, including anaphylaxis (Cvetković-Matić et al., 2009; dos Santos-Pinto et al., 2018; Jakob et al., 2017; Perez-Riverol et al., 2017).

Most of the proteins characterized by wasp venom are allergenic toxins (dos Santos-Pinto et al., 2018). Currently, up to 44 venom proteins from 21 species of wasps have been officially described as allergens (https://www.allergen.org, accessed December 2019). On the one hand, the

predominant allergens are hyaluronidase, phospholipase A1 and antigen 5, which have been identified in the venom of all wasp species with clinical interest. On the other hand, other allergens have been less described as clinically relevant, such as serine protease and dipeptidyl peptidase IV (Han et al., 2008; Macchia et al., 2018; Rungsa et al., 2016a; Sukprasert et al., 2013; Yang et al., 2008). These proteins are reported to be life threatening and to accelerate fatal anaphylactic reactions (Sukprasert et al., 2013).

Hyaluronidase

The hyaluronidase is a 33–45 kDa glycosylated enzyme (glycoprotein) (Table 13) that hydrolyses hyaluronic acid, a high molecular mass polysaccharide and one of the main components of the vertebrate extracellular matrix that maintains cell adhesion (Bazon et al., 2018; dos Santos-Pinto et al., 2018; Jacomini et al., 2013; Monteiro et al., 2009; Rungsa et al., 2016b, 2018). Specifically, this enzyme is endo-N-acetylhexaminidase type, since it catalyses the release of N-acetylglucosamine reducing groups from hyaluronic acid (disaccharide polymer D-glucoronic acid and N-acetyl-D-glucosamine) (Monteiro et al., 2009; Yoon et al., 2015).

Table 13.- Best proteins identified in *Vespa* spp. venom

Protein	aa (kDa)	Species	Short name	Reference
Hyaluronidase	331 (39,04)	*V. affinis*	VesA2	Rungsa et al. (2019)
	331 (35)	*V. magnifica*	Vesp ma 2	An et al. (2012)
	357 (~39,6)	*V. tropica*	VesT2a	Rungsa et al. (2016b)
	356 (39,1)		VesT2b	
	341 (40)	*V. velutina*	Vesp v 2A	Monsalve et al. (2019)
	331 (39,1)		Vesp v 2B	
Phospholipase	334 (~34)	*V. affinis*	Vesp a 1.1	Sukprasert et al. (2013)
			Vesp a 1.2	
	300 (33,2)	*V. basalis*	vPLA2	Hou et al. (2016)
	301 (~33)	*V. crabro*	Vesp c 1	Hoffman et al. (2005)
	304 (~34)	*V. magnifica*	Magnifin	Yang et al. (2008)
	Unknown (29,5)	*V. mandarinia*	PLB α	Abe et al. (2000)
	Unknown (26,0)		PLB β	
	301 (33)	*V. tropica*	Ves t 1	Rungsa et al. (2018)
	152 (~17)	*V. orientalis*	Orientotoxin I	Korneev et al. (1989)
	139 (15,3)		Orientotoxin II	
	Unknown (~35)	*V. velutina*	VT 1	Ho et al. (1999)
	Unknown (~33)		VT 2a	
	Unknown (~33)		VT 2b	
	304 (33,9)		Vesp v 1	Monsalve et al. (2019)
	Unknown	*V. xanthoptera*	PLB I	Abe et al. (2000)
	Unknown		PLB II	
Antigen 5	Unknown	*V. affinis*	No name	Rungsa et al. (2016a)
	202 (~23)	*V. crabro*	Vesp c 5.01	Hoffman (1993)
	202 (~23)		Vesp c 5.02	

	202 (~23)	*V. mandarinia*	Vesp m 5	Hoffman and Schimdt [direct submission]
	225 (~25)	*V. magnifica*	Vesp ma 5	An et al. (2012)
	Unknown	*V. tropica*	No name	Rungsa et al. (2016a)
	202 (22,7)	*V. velutina*	Vesp v 5	Monsalve et al. (2019)
Serine Protease	54 (5,7)	*V. bicolor*	Bicolin	Yang et al. (2009)
	242 (27,4)	*V. magnifica*	Magnvesin	Han et al. (2008)
	Unknown (~42)	*V. orientalis*	Protease I	Haim et al. (1999)
	Unknown	*V. velutina*	No name	Liu et al. (2015)
Dipeptidyl Peptidase IV	Unknown	*V. affinis*	No name	Rungsa et al. (2016a)
	775 (~88,5)	*V. basalis*	No name	Lee et al. (2007)
	Unknown	*V. tropica*	No name	Rungsa et al. (2016a)

This enzyme, widely distributed in venomous animals, fragments hyaluronic acid, reduces the viscosity of the extracellular matrix and acts as a propagation factor for a better distribution and absorption of venom (Baek et al., 2013; Bazon et al., 2018; dos Santos-Pinto et al., 2018; Jacomini et al., 2013; Monteiro et al., 2009; Perez-Riverol et al., 2017; Rungsa et al., 2016b, 2016a, 2018; Yoon et al., 2015). Also, it has been suggested that it is essential for a paralytic activity (Baek et al., 2013; Rungsa et al., 2016a). It has also been described as an allergenic factor in several genera and species of wasps, capable of inducing severe and fatal IgE-mediated anaphylactic reactions in humans (An et al., 2012; Jacomini et al., 2013; Moawad et al., 2005; Monteiro et al., 2009; Perez-Riverol et al., 2017; Perez-Riverol et al., 2015; Rungsa et al., 2016b, 2016a, 2018; Shin et al., 2012).

Rungsa et al., (2016a, 2016b) cite that at least two types of hyaluronidase are known, depending on the substitutions of two catalytic residues along the chain: hyaluronidase A (Asp 107 and Glu 109), as active isoform, and hyaluronidase B (Hys 107 and Hys 109). As an exception, *V. tropica* presents Asn in these two positions (Rungsa et al., 2016a).

It presents homology between the sequences and structures in different Hymenoptera venom, being one of the most phylogenetically preserved allergens. Therefore, it is one of the main proteins causing cross-reactivity in tests to detect the presence of specific IgE between wasps and bees (Jacomini et al., 2013, 2014; Monteiro et al., 2009; Rungsa et al., 2016a; Sturm et al., 2011).

Their biochemical and structural characterization is crucial for the development of new tools for treatment in sting cases (Rungsa et al., 2016a). It has been proposed that the development of antivenoms or specific hyaluronidase inhibitors could limit the venom spread, preventing or delaying the appearance of significant tissue damage and better clinical management (Perez-Riverol et al., 2017; Rungsa et al., 2016a). For example, Rungsa et al., (2016b) cite an anti-hyaluronidase serum with an efficiency against crude venom of *V. tropica* in a 1:12 ratio (venom: antiserum).

It is assumed that the high proportion of proteins and their activity contribute to the allergenic and toxic potential of the venom (Rungsa et al., 2016a; Yoon et al., 2015). For example, *V. tropica* presented 2.5 times more hyaluronidase than *V. affinis*. In turn, there was a greater activity of

hyaluronidase, phospholipase and dipeptidyl peptidase, the last two with similar proportions, suggesting that *V. tropica* venom is more potent than *V. affinis* venom (Rungsa et al., 2016a, 2016b).

Phospholipase

The phospholipases have been well characterized in wasp venoms (Bazon et al., 2018). These proteins are responsible for the membrane phospholipids hydrolisis that contain diacylphospholipids, such as phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine, producing free chain fatty acids and phospholipids. That allows a better diffusion of the rest of the toxins, causing symptoms like enemas (Bazon et al., 2018).

Phospholipase A1 is a protein, normally non-glycosylated, of ~ 34 kDa known as the toxin that hydrolyses the sn-1 fatty acids of phospholipids, producing 2-acyl-lysophospholipid and free fatty acids (e.g. arachidonic acid) (Figure 31). This enzyme belongs to the pancreatic lipase gene family, together with human pancreatic lipase (HPL), lipoprotein lipase (LPL), endothelial lipase (EL), pancreatic lipase-realted protein-2 (PLRP2) and phosphatidylserine-specific phospholipase A1 (PS) (PS-PLA1) (dos Santos-Pinto et al., 2018; Perez-Riverol et al., 2016; Rungsa et al., 2016b, 2018; Santos et al., 2007; Sukprasert et al., 2013; Yang et al., 2008; Yoon et al., 2015). The PLA1 of wasps are part of subgroup number eight. PLA1 from different sources exhibit an important similarity of regions conserved with HPL and PLRP2. All these enzymes contain the typical residues of the Ser-His-Asp catalytic triad (Aoki et al., 2007), increasing significantly the enzyme activity with Ca^{2+} presence (Rungsa et al., 2016b; Sukprasert et al., 2013).

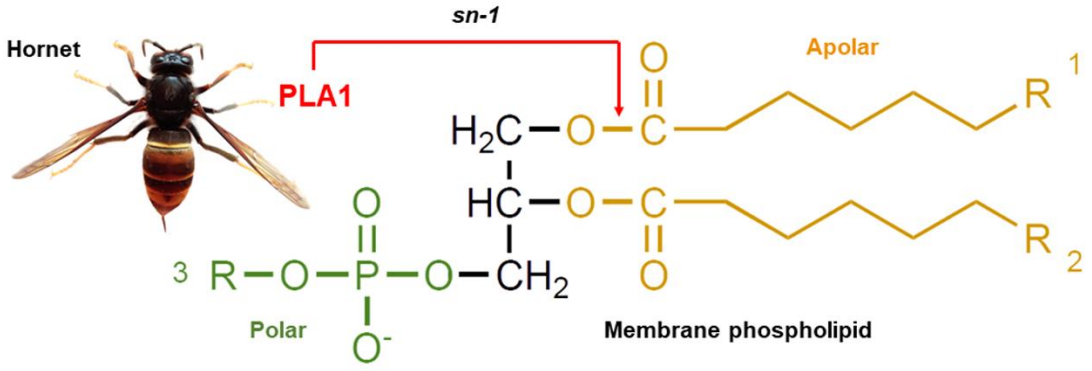

Figure 31.- Specificity of PLA1 of hornet venom on membrane phospholipids (amphipathic molecule). The red arrow indicates the ester link where nucleophilic substitution 1 (sn-1) occurs.

It is assumed phospholipase can disrupt the phospholipid packaging of many types of biological membranes, causing severe haemolysis that leads to cardiac dysfunction and is responsible for animals and humans lethality (dos Santos-Pinto et al., 2018; Monteiro et al., 2009; Perez-Riverol et al., 2016, 2017; Rungsa et al., 2016b, 2018; Santos et al., 2007; Sukprasert et al., 2013).

It can cause local inflammatory reactions, such as severe haemolysis with subsequent cardiac dysfunction and death. Furthermore, it has been reported that these enzymes activate platelet

aggregation, smooth muscle contraction, stimulation of cell proliferation and induce thrombosis in vivo (Monteiro et al., 2009; Rungsa et al., 2016b; Sukprasert et al., 2013; Yang et al., 2008). At the same time, it acts as an allergen, constituting one of the main proteins responsible for IgE-mediated allergic reactions (Moawad et al., 2005; Monteiro et al., 2009; Perez-Riverol et al., 2016, 2017, 2015; Rungsa et al., 2018; Shin et al., 2012; Sukprasert et al., 2013), causing fatal anaphylaxis (Yoon et al., 2015). This protein has been identified in many hornets (Table 13) with offensive and defensive role for *Vespa*.

Abe et al., (2000) describe phospholipase B (activity A1 and A2) of *V. mandarinia*. These authors conclude that these are more universal digestive enzymes than phospholipase A, appearing to be an adaptation of carnivorous insects (*Vespa*) against vegetarian insects (*Apis*), which contain only phospholipase A in their venom.

On the other hand, Sukprasert et al., (2013) identified four phospholipase A1 isoforms of *V. affinis* (Ves a 1), highlighting that the toxicity of these enzymes did not appear to be correlated with their activity. In that sense, greater knowledge of these proteins would be valuable for therapeutic and commercial applications (Sukprasert et al., 2013).

Antigen 5

It is a 23 kDa non-glycosylated protein identified as a potent allergen, often the greatest of all, since it induces an acute hypersensitivity response in humans after the sting of almost all species of the Vespidae family, such as those that belong to the genus *Dolichovespula*, *Polistes*, *Polybia*, *Vespa* and *Vespula* (An et al., 2012; Bazon et al., 2018; dos Santos-Pinto et al., 2018; Moawad et al., 2005; Monteiro et al., 2009; Perez-Riverol et al., 2017, 2015; Rungsa et al., 2016b, 2018; Shin et al., 2012).

The antigen 5 protein belongs to the superfamily of catabolite activated proteins (CAP) formed by secretory proteins rich in cysteines (CRISP). It has been studied that these proteins participate in many biological processes such as reproduction, cancer and allergic reactions (Bazon et al., 2017, 2018; dos Santos-Pinto et al., 2018; Rungsa et al., 2016b), but its biological action as a component of venom of this insects family is not known. Some studies have shown that it has not toxic action, but it may be associated with hypersensitivity responses (Bazon et al., 2018).

According to the list of allergens of the International Union of Immunological Societies (IUIS), this allergen is present in practically all the venoms of the Vespidae family, as well as in the Hymenoptera order (https://www.allergen.org, consulted in February 2019), with a 44–90.3% sequence similarity between *Vespula*, *Vespa* and *Polistes* (Monteiro et al., 2009; Rungsa et al., 2016b). This allergen may have varying degrees of cross-reactivity, due to the similarity between sequences (Rungsa et al., 2016b).

The first hornet species where this protein was purified and characterized was in *V. magnifica* (An et al., 2012). It is likely that antigen 5, together with hyaluronidase, are the main and strongly conserved allergens in *Vespa* venom (Table 13), as in horseflies (Tabanidae) (An et al., 2012; Yoon et al., 2015).

Other proteins

Social insect venom does not contain significant amounts of proteases. These catalyse the breakdown of peptide bonds in proteins (Bazon et al., 2018).

Han et al., (2008) studied a *V. magnifica* anticoagulant protein. This protein was named magnvesin and was catalogued as a serine protease, presenting a 52% identity with the serine protease of *Polistes dominulua*. This protein acts by hydrolysing the coagulant factors TF, VII, VIII, IX and X. This same study cites a proteolytic enzyme in *V. orientalis*, named protease I, which presented anticoagulant activity, identified later in *V. tropica* (Baek et al., 2013).

Two proteins were isolated from *V. tropica* and *V. analis* which matched with dipeptidyl peptidase IV (DPP-IV) from *V. basalis* (Rungsa et al., 2016b). DPP-IV also belongs to serine proteases, which selectively removes dipeptides from the N-terminal of the peptide chains with proline or alanine in the penultimate position. Despite not knowing its function well, it is associated with exosomes and may be limited to activating or inactivating venom biomolecules, which may produces additional or synergistic effects to accelerate toxicity (Hsieh et al., 2011; Lee et al., 2007; Rungsa et al., 2016a).

Serine proteases are part of the S1 trypsin family of the SA clan, the largest family of peptidases. These affect the homeostatic system, affecting a wide range of coagulation components, fibrinolytic system and cells (Han et al., 2008). In addition, some of these proteases can trigger allergic reactions or inhibit melanization, acting as a neurotoxin (Han et al., 2008).

In the study conducted by Sookrung et al., (2014), several new hornets' allergenic proteins are cited for the first time, such as arginine kinase, enolase, heat shock proteins, GADPH and fructose bisphosphate aldolase. In this study, arginine kinase was isolated from *V. affinis* venom as a protein responsible to maintain ATP levels through phosphoarginine phosphorylation, which reacted with the IgE of 73.33% of allergic patients to wasp venom. In addition, this protein has been described as an allergen of the American cockroach *Periplaneta americana*.

Similarly, enolasa, a glycolytic enzyme described as allergen from pollen, mold, yeast, cockroach, shellfish, latex and in *V. affinis*, reacted with the IgE of 66.67% of patients allergic to wasp venom (Sookrung et al., 2014).

In addition to those already mentioned, thermal shock proteins (Hsp70) which reacted with the IgE of 73.3% of allergic patients to wasp venom. They are chaperones responsible for folding ATP-dependent proteins and have been cited as allergens from dust mite *Dermatophagoides farinae*, hazelnut pollen and fungus (Sookrung et al., 2014).

To these glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is added, an enzyme responsible for the conversion of glyceraldehyde 3-phosphate to glycerate 1,3-bisphosphate, cited as an allergen from indoor fungus, which reacted with 60% IgE of the study patients (Sookrung et al., 2014).

Finally, they cite fructose bisphosphate aldolase, an enzyme which catalyses the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. This enzyme was identified as an allergen in *Candida albicans* and reacted with 53.3% of allergic patients (Sookrung et al., 2014). This study has allowed a better knowledge about other

allergens not described in *Vespa* so far, with both diagnostic and clinical implications for a better diagnosis and design of specific therapies.

Nucleases have only been cited in Cohen-Armon et al., (2006), where *V. orientalis* venom showed deoxyribonuclease activity, causing rapid DNA breaks of cortical neurons in rat brain, and inhibition of poly-ADP-ribose-polymerase 1, responsible for DNA repair and mechanism of apoptosis. This leads to a slight idea about the mechanisms of cell death in neurons exposed to *Vespa* venom.

To sum up, it is important to stress that proteins have been less studied than peptides in hornet venom, considering proteins to be majorly responsible for poisoning and allergic reactions.

Other components

Amines

Acetylcholine has been identified in *V. crabro* and *V. orientalis* venom, with amounts far superior to any biological system studied to date. However, it is not known for sure if the venomous gland has choline acetyltransferase activity, an enzyme responsible for the actylcholine formation, so the origin of this neurotransmitter in the venom remains uncertain (Cohen-Armon et al., 2006).

Similarly, histamine has been found in extremely high concentrations in *V. crabro* venom. It is a tissue amine involved in the immune response, contributing to local pain and vasodilation after the sting (Cohen-Armon et al., 2006).

Another amine found has been serotonin, widely distributed in the arthropod venom, identified in the venom of *V. orientalis* (Cohen-Armon et al., 2006).

Finally, catecholamines are one of the last components in the hornet venom. This includes adrenaline, norepinephrine and dopamine, whose concentrations may vary between different species (Cohen-Armon et al., 2006).

Alarm pheromone

In social insects, such as hornets, alarm pheromones are volatile compounds and represent an important agent of information, providing an advantage by assuming an improvement in colony defence and external danger warming (Cheng et al., 2017; De Castro E Silva et al., 2016; Esteves et al., 2017; Jimenez et al., 2016; Thiéry et al., 2018). For the successful establishment of a nest, it depends on workers ability to prevent predation and allow future founders and males to reproduce (Jimenez et al. 2016). The information that is known about this type of pheromones is currently more limited in wasps in relation to bees and ants (Cheng et al., 2017), citing this type of behaviour in 11 species of 4 genera of social wasps (*Dolichvespula*, *Provespa*, *Vespa* and *Vespula*) (Jimenez et al., 2016).

In social wasps and hornets, nest guards can release these volatile molecules to recruit defenders, mark feeding points or to attack (Cheng et al., 2017; De Castro E Silva et al., 2016; Yoon et al.,

2015). It has been experimentally shown that alarm pheromones are released during the sting, as well as in the air before the attack. The release of these pheromones during the attack serves to attract more workers to defend the colony (Esteves et al., 2017).

In a large part of the social insects studied, this class of pheromones consists of mixtures of various compounds, which cause alarm behaviours (Cheng et al., 2017). In general, alarm pheromones are stored in the venom sack, along with toxins and allergens. Alcohols and aldehydes attract individuals outside the nest, while acids attract them in a spepcific direction and fatty acid methyl esters cause aggressive behaviour (De Castro E Silva et al., 2016; Esteves et al., 2017; Ono et al., 2003), volatile components containing nitrogen and ketones also produce an alarming role and behaviours oriented to attack (Jimenez et al., 2016). Esteves et al. (2017) suggest that all these compounds can be synthesized in the Dufour's gland, a structure associated with the bees and wasps venomous apparatus.

Dufour gland literature in Hymenoptera suggests that it may have various roles related to the synthesis and release of chemical markers for other different biological functions in Hymenoptera with different eusociality degrees, such as individual recognition from the same colony, egg marking, larval rearing and nest defence (Esteves et al., 2017).

Reported cases analysis

A total of 55 detailed cases of hornet sting were found in 27 papers during the systematic review and manual search of the references from the key articles. Of these, just one was an epidemiological study (Yanagawa et al., 2007), two studies showed unusual complications such as intracranial haemorrhages or ocular injuries (Maugeri et al., 2017; Ono et al., 2018) and the rest were isolated reported cases of patients who were stung by hornets. We summarized the cases in Table 14. Statistics extracted from hospitals and poison centres from all mentioned countries were not included due to their difficulty in obtaining them. Nevertheless, despite the number of cases presented, this health problem can be underestimated due to the lack of public information, so it is very important to facilitate the sharing of this information. This bias is also reflected in the number of species with reported cases (Table 14: 7 sp.) in comparison with the number of species whose venom has been characterized (i.e. Table 10: 12 sp.).

Table 14.- Reported cases of hornet stings

Hornet species	Country	Area	Age, Gender	Time from sting to hospital	Number of stings	Complications	Treatment	Time admitted to hospital	Outcome	Reference
V. affinis	Sri Lanka	Forest	46, F	90 minutes	40	ResF, HF	Crt, Anth, Loop, β2-ago, Vent	7.5 hours	Death	Kularatne et al. (2014)
		Forest	48, M	90 minutes	130	ResF, T		30.5 hours	Death	
		Na	38, M	Na	70	LD, MI	Dialysis	24 days	Survival	Kularatne et al. (2003)
		Urban	57, M	Na	50 – 60	RF, LD,		2 days	Death	
	Nepal	Urban	60, F	30 minutes	1	S, HF	Crt, Anth, Adr	2 days	Survival	Ralapanawa & Kularatne (2014)
		Forest	55, M	< 1 hour	150	RF	Crt, Anth, β2-ago, H2-ant, Vent, Blood, dialysis.	8 days	Death	Das & Mukherjee (2008)
		Forest	40, M	24 hours	25	RF	Crt, β_2-ago, Anth, H2-ant, Loop, diuretics, Dop, dialysis	21 days	Survival	
	Sabah	Forest	37, M	< 1 hour	250	RF, ResF, R, HF	Dialysis	9 days	Death	Shilkin et al. (1972)
		Na	19, M	< 1 hour	180	RF, R		28 days	Survival	
	Taiwan	Forest	50, M	< 1 hour	200	HF	Na	7 days	Survival	Tsai et al. (2005)
	India	Na	2, F	< 1 hour	10	RF, S, R, HF	Crt, Anth, Antb, Loop, Ino, Plasma, Ste, Vent	4 days	Death	Ravikira et al. (2019)
V. crabro	Greece	Urban	87, M	< 1 hour	1	MI	Crt, Thro	3 days	Survival	Korantzopoulos et al. (2006)

	Italy	Urban	55, M	< 1 hour	1	MI	Na	Na	Survival	Quercia et al. (2001)
	Turkey	Na	30, M	< 1 hour	1	HF	Antc, Anta	2 days	Survival	Okutucu et al. (2011)
	Germany	Urban	32, M	0	1	HepF, P, S, HF	None	0	Death	Blank et al. (2019)
	Serbia	Urban	45, M	2 hours	1	S, MI	Antc	20 days	Survival	Cvetković-Matić et al. (2009)
	EEUU	Urban	66, M	< 1 hour	15	MI	Adr, Crt, Xan	> 3 days	Survival	Levine (1976)
V. ducalis	Taiwan	Na	41, M	8 hours	1	RF, R	Anth, Crt	4 days	Survival	Lin et al. (2003)
V. magnifica		Fields	62, F	2 days	40	RF, R, LD	Anth, Crt, Antb, Ste, Dialysis	42 days	Survival	
	India	Urban	7, M	< 1 hour	25	RF, R, LD, S	Anth, Ste, Antb, Blood, Dialysis	3 days	Death	Vikrant et al. (2005)
		Na	38, F	24 hours	30	RF, LD	Antb, Ste, Anth, Dialysis	56 days	Survival	
V. mandarinia	Japan	Urban	57, M	< 1 hour	38	LD, T, R, S	Adr, Crt	10 days	Survival	Yanagawa et al. (2007)
		Na	80, M	60 minutes	78	RF, LD, ResF, DIC, S	Na	Na	Death	
		Na	80, M	< 24 hours	30	RF, LD, P, S	Na	Na	Survival	
		Na	61, M	60 minutes	17	LD, R, S	Na	Na	Survival	
		Na	55, F	30 minutes	100	RF, LD, ResF, DIC, R, S	Na	Na	Death	
		Na	74, M	24 hours	50	RF, HepF, HF, DIC, R	Na	Na	Death	
		Na	76, M	Na	20	RF, LD, R, S	Na	Na	Survival	
		Na	39, F	< 8 hours	> 50	RF, LD, ResF, DIC, R, S	Na	Na	Survival	

		Na	57, M	48 hours	27	RF, HepF, HF, R, S	Na	Na	Survival	
		Na	86, F	1 hour	30	LD, R, S	Na	Na	Survival	
		Na	78, M	2 hours	30 – 40	RF, LD, ResF, T, R	Na	Na	Death	
		Na	79, M	7 days	3	RF, LD, R	Na	Na	Survival	
		Na	71, M	5 hours	70	RF, HepF, ResF, HF, DIC, R, S	Na	Na	Death	
		Na	62, M	< 24 hours	17	RF, LD, ResF, DIC, R, S	Na	Na	Death	
		Na	36, M	4 days	30 – 40	RF, LD, R, S	Na	Na	Survival	
	China	Na	42, M	5 hours	66	RF	Crt, Plama, Dialysis	31 days	Survival	Li et al. (2015)
V. orientalis		Na	38	Na	55	RF		Na	Survival	
		Na	65	Na	46	RF		Na	Death	Sakhuja et al. (1988)
		Na	45	Na	22	RF, LD, T	Dyalisis	Na	Death	
	India	Na	8	Na	63	RF, R, LD, T		Na	Survival	
		Na	28	Na	24	RF, R		Na	Survival	
		Urban	18, M	2 days	27	RF, Tt,	Crt	28 days	Survival	Sharma et al. (2006)
	Israel	Urban	4, F	1 hour	120	RF, HF	Anth, Crt, Plasma, Blood	17 hours	Death	Korman et al. (1990)
		Na	3, M	< 1 hour	40	ResF, LD, RF, HF, S	Vent, Antb, Crt, dialysis	3 days	Death	Watemberg et al. (1995)
V. velutina	France	Na	46, M	Na	1	IH	Crt,	10 days	Survival	Ciron et al. (2015)
Unidentified hornet	Nepal	Na	39, M	2 days	50 – 60	RF, LD, R,	Dialysis, Crt, Anth, Antb, vit K	39 days	Survival	Dongol et al. (2012)
		Na	30, M	Na	Na	RF	Dialysis	1	Death	

	Na	35, M	20 days	~50	RF	Dialysis, Crt, Anth, Antb, vit K, vit D	16 days	Survival	
Singapore	Na	8, F	< 48 hours	20	RF, HF	Anth	Na	Death	Hoh & Soon (1966)
Italy	Forest	48, M	7 days	1	BI	Surgery	Na	Survival	Maugeri et al. (2017)
	Na	9, F	Na	1	OI	Str	Na	Survival	
Japan	Na	57, M	Na	1	OI	Na	Na	Survival	Ono et al. (2018)
	Na	42, F	Na	1	OI	Na	Na	Survival	
India	Forest	60, M	< 1 hour	Na	RF, P	Adr, Crt, dialysis	12 days	Survival	Sharma et al. (2006)

F: female; M: male; DIC: disseminated intravascular coagulopathy; LD: liver dysfunction; HF: heart failure; HepF: hepatic failure; R: rhabdomyolysis; RF: renal failure; ResF: respiratory failure; P: pancreatitis; S: shock; T: thrombocytopenia; Tt: thrombocythemia; IH: intracranial haemorrhages; MI: myocardial infarction; BI: bone infection; OI: ocular injuries. Na: Not available

Crt: Corticosteroids, Anth: Antihistamines, Loop: Loop diuretics, β2-ago: β2-adrenergic receptor agonist, Vent: Assisted ventilation, Blood: Blood transfusions, H2-ant: Histamine H2-receptor antagonist, Adr: Adrenaline, Dop: Dopamine, Antb: Antibiotics, Ino: Inotropes, Ste: Steroids, Thro: Thrombolytic treatment, Antc: Anticoagulant, Anta: Anti-arrhythmic, Xan: Xantines

The most common clinical manifestations were local reactions, while severe allergic reactions were less frequent (Ciron et al., 2015). Normally, after a sting, IgE levels increase transiently, returning to baseline levels past 1–3 months (Perez-Riverol et al., 2017). However, the venom can produce a type I hypersensitivity reaction, where the first exposure to the allergen triggers the blood plasma cells to release IgE in large quantities, which adhere to the high-affinity Fc receptor (FcεRI) in the mast cells and basophils membrane that are sensitized, causing degranulation and release of other inflammatory factors. A second exposure to the allergen causes an exacerbated response (Bazon et al., 2018; Ciron et al., 2015; Dongol et al., 2012; Jacomini et al., 2014; Khurana et al., 2017; Kularatne et al., 2014; Mendonça et al., 2017; Perez-Riverol et al., 2017, 2015). Moreover, an unexposed person may suffer poisoning after multiple stings (Dongol et al., 2012).

Bibliography cites sting allergies can occur at any age, being less frequent in children than in adults (Visitsunthorn, Kijmassuwan, Visitsunthorn, Pacharn, & Jirapongsananuruk, 2019). In central and western Europe, these types of allergies are mainly caused by *Vespula* and, less frequently by *Vespa* (i.e. 16–38 deaths per year in France) (Hayashi et al., 2014; Khurana et al., 2017; Macchia et al., 2018; Mingomataj et al., 2002; Nittner-Marszalska et al., 2015; Perez-Riverol et al., 2015; Shin et al., 2012).

In general, the components of venom acted on the renal system, the cardiorespiratory system and the immune system (Cvetković-Matić et al., 2009; Han et al., 2008; Monteiro et al., 2009; Mortari et al., 2012; Nittner-Marszalska et al., 2015; Perez-Riverol et al., 2015; Rungsa et al., 2016b; Yang et al., 2008). The classic symptoms of poisoning were local burns, followed by oedema and pain which can last for long periods of time. But, in cases of allergies, the most severe cases can lead to severe or fatal systemic anaphylaxis (Bazon et al., 2018; De Castro E Silva et al., 2016; Eno, Owo, Itam, & Konya, 2001; Monteiro et al., 2009; Mortari et al., 2012; Nittner-Marszalska et al., 2015; Perez-Riverol et al., 2016, 2018, 2015; Rungsa et al., 2018).

The toxicological effects depended on the time after sting and the dose injected. Inflammatory mediators are involved such as kinines, autacoids and lipoxygenase derivatives, which can cause haemolysis, coagulopathies, rhabdomyolysis, acute renal failure and hepatotoxicity, including aortic thrombosis and cerebral infarction in cases of massive stings (Ciron et al., 2015; Dongol et al., 2012; Eno et al., 2001; Han et al., 2008; Lopes et al., 2017; Monteiro et al., 2009; Rungsa et al., 2016a; Yang et al., 2008; Yoon et al., 2015).

Despite early reactions, the patient management at the hospital, insufficient monitoring, lack of clinical signs, delay in early care and therapeutic decisions and inappropriate medication can be crucial factors on the evolution of the patient (Kularatne et al., 2014). Unfortunately, in many cases patients die at the hospital hours or days after the sting/s, due to unpredictable complications such as heart attack or multi-organ failure (Kularatne et al., 2014).

Many personal physiological factors can influence such as previous cardiovascular and/or respiratory disease, age, mastocytosis, high levels of serum tryptase or previous use of angiotensin-converting enzyme inhibitors (Perez-Riverol et al., 2015). Furthermore, venom composition may be related to the species and its geographical distribution (Mendonça et al., 2017). That is why in the current study, we present a worldwide distribution of the hornet species which produced reported sting cases, reflecting hornet stings are a health problem in practically all continents (Figure 32). Most number of cases and responsible species are concentrated in Asia,

where hornets are native, but hornet incursions are increasing in the global change scenario, such as *V. velutina* in Europe or *V. crabro* in America (Dvořák, 2006; Kimsey & Carpenter, 2012; Laurino et al., 2020), increasing this health threat to other countries.

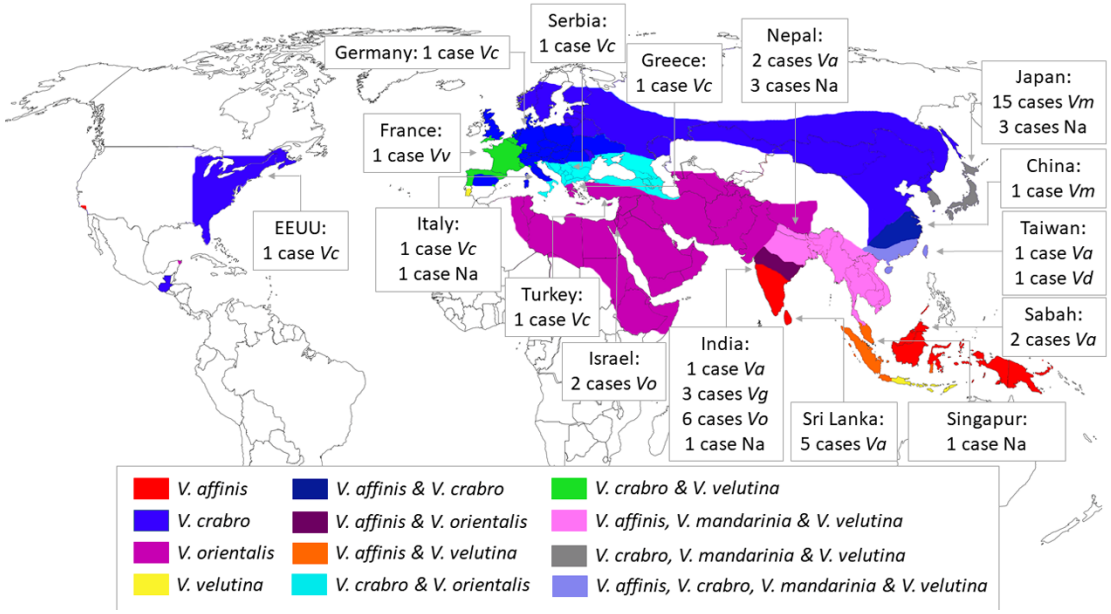

Figure 32.- Worldwide distribution of the main hornet species which reported sting cases (Table 13) per country. Va: *V. affinis*, Vc: *V. crabro*, Vd: *V. ducalis*, Vg: *V. magnifica*, Vm: *V. mandarinia*, Vo: *V. orientalis*, Vv: *V. velutina* and Na: Not available. *V. ducalis* and *V. magnifica* distribution was not available. Distribution references: *V. affinis* (Archer, 1997; Kimsey and Carpenter, 2012), *V. crabro* (Kimsey and Carpenter, 2012; Landolt et al. 2010; www.vespavelutina.eu), *V. mandarinia* (Archer, 1995), *V. orientalis* (Archer, 1998; Dvořák, 2006; www.vespavelutina.eu) and *V. velutina* (Laurino et al. 2020; www.vespavelutina.eu).

36.4% of the patients included in this study died due to, mainly, a multi-organ failure. Also, there were no significant differences between the number of intra-species stings and the outcome, contrary to Yanagawa et al., (2007) who found significant differences in *V. mandarinia*. This means more studies are needed to conclude if the number of hornet stings is a key factor to the patient outcome. Moreover, there were no significant differences between the number of stings and the outcome, without taking species into account.

All the above-mentioned have a negative impact not only on the health, but also on the economy. Choi et al., (2019) calculated the economic impact of Hymenoptera stings and medical costs due to their stings during 4 years in South Korea, a country where hornets are very common, resulted in an average of 0.64 million USD every year (0.57 million euro approximately).

Conclusion

The current available information indicates hornet venom contains a vast diversity of proteins and peptides with a wide range of pathophysiological health effects, including cross-reactivity with antigens from many animals and plants, giving to hornets a high clinical importance. Moreover, some compounds are still to be identified, characterized and studied due to the low amounts

present in the venom. This diversity of components and risks to human health summarized and discussed makes this study a reference for new research focused on human health.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by CH, ML and EM-L. The first draft of the manuscript was written by CH and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS APPROVAL

This contribution is a systematic review and does not warrant an ethical statement.