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Evaluation of the invasion dynamics of non-native species and associated socio-ecological impacts
— the case study of *Vespa velutina* —

"Documento Definitivo"

Doutoramento em Biodiversidade, Genética e Evolução

Maria João Caldeira Verdasca

Tese orientada por:
Professor Doutor Rui Miguel Borges Sampaio e Rebelo
Professora Doutora Luísa Gigante Carvalheiro
Doutor Hugo Emanuel Vitorino Rebelo

Documento especialmente elaborado para a obtenção do grau de doutor

2022

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- Doutora Ana Sofia Vaz, Investigadora de Pós-Doutoramento Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO – InBIO Laboratório associado) da Universidade do Porto;
- Doutor Mário Rui Canelas Boieiro, Investigador Grupo da Biodiversidade dos Açores da Universidade dos Açores;
- Doutor José Tadeu Marques Aranha, Professor Associado com Agregação Departamento de Ciências Florestais e Arquitetura Paisagista da Universidade de Trás-os-Montes e Alto Douro;
- Doutor César Dias Santos Capinha, Investigador Auxiliar Instituto de Geografia e Ordenamento do Território (IGOT) da Universidade de Lisboa;
- Doutor Rui Miguel Borges Sampaio e Rebelo, Professor Auxiliar Faculdade de Ciências da Universidade de Lisboa (orientador).

Documento especialmente elaborado para a obtenção do grau de doutor

Fundação para a Ciência e a Tecnologia
(PD/BD/128351/2017 & COVID/BD/151632/2021)

2022

This dissertation should be cited as:

Verdasca MJ (2021). Evaluation of the invasion dynamics of non-native species and associated socio-ecological impacts – the case study of *Vespa velutina*. PhD Thesis, Universidade de Lisboa, Portugal

This study was funded by Fundação para a Ciência e a Tecnologia, Portugal, through the PhD scholarships PD/BD/128351/2017 and COVID/BD/151632/2021.

Nota Prévia

A presente tese apresenta resultados de trabalhos já publicados ou aceites para publicação (capítulos 2, 3 e 4) em revisão (capítulo 1) ou em preparação para publicação (capítulo 5), de acordo com o previsto no nº 2 do artigo 25º do regulamento de Estudos Pós-graduados da Universidade de Lisboa, publicado no Diário de República II série 2.ª série — N.º 60 — 26 de março de 2018. Tendo os trabalhos sido realizados em colaboração, esclareço que participei integralmente na conceção dos trabalhos, obtenção dos dados, análise e discussão dos resultados, bem como na redação dos manuscritos.

Oeiras, novembro de 2021

Maria João Verdasca

Para o meu marido.
Para os meus filhos.

“*Biological invasions have more to do with the history of humans, and with our behaviours, than with the biology of species*”

Piero Genovesi
As invasões Biológicas em Portugal

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Acknowledgments

It's time to thank all of those who helped me to get here making this journey so much more enjoyable!

I start by thanking the financial support of **FCT** through the scholarships (PD/BD/128351/2017 and COVID/BD/151632/2021) in the scope of BIOdiv doctoral program. Without it, this work would not be possible.

I thank **Margarida Santos Reis** for opening to me the cE3c door and for the trust and help during my first year in Lisbon University.

I would like to express my immense gratitude to my exceptional main supervisor, **Rui Rebelo**. I was lucky to count on your guidance, experience, and permanent availability to answer my questions and doubts. I recognize you were tireless throughout the several revisions of our papers and all your valuable insights that help me to improve the quality of my PhD. I also thank the chance to go to several conferences, and particularly to the meeting of Velutina Task Force in Bilbao, where I had the opportunity to meet the work of experienced colleagues in my research area. My sincere Thank You!

My immense gratitude also goes to **Luísa Carvalheiro** and **Hugo Rebelo**, my two also exceptional co-supervisors. The first for accepting my research ideas with arms wide open, for given a broad vision to our papers, for being very present and available (even with the Atlantic Ocean between us) and for always seen the positive side of every paper rejection we had (and it were a lot!), always with encouraging words, even when I struggled the most. The second for teaching me all I know about species distribution models, for the insights in ecology and for the enthusiasm and positivity about my work. Thank you both!

Due the pandemic situation, my learning period about DNA molecular techniques at CIBIO in Porto was cancelled and the genetics lab work was carried out by **Raquel Godinho** and **Rita Rocha** from CIBIO. Both were also excellent mentors by the valuable suggestions to the paper presented in this thesis in Chapter 3 and for the pedagogical revision of the manuscript. Without both of you, the diet study would not be possible! Thank you.

To **José Pedro Granadeiro** and **Pedro Segurado** I thank for the good discussions we had about the different modelling techniques, for the advice in data analysis and for the help provided when I was struggling with R.

I am also grateful to **Marco Portocarrero** from “A Nativa – Association” and to **Graça Oliveira** (beekeeper in Aveiro) for all the help in sample collection and share of knowledge. It is always a pleasure to talk and learn from you both!

A huge thanks also goes to Eng. **Paulo Carmo** from Instituto para a Conservação da Natureza e das Florestas (ICNF) for the yearly updates regarding the spread of *Vespa velutina* in Portugal. A grateful word also goes to **Sofia Quintães** from Direção Geral de Alimentação e Veterinária (DGAV) for having provided the data related with beekeeping activity in Portugal.

To **Quentin Rome** from MNHN Paris e **Sébastien Puechmaille** from University of Montpellier, I thank respectively for having provided the French records of *Vespa velutina* and the script to inspect for niche shifts, respectively.

My sincere thank you also goes to **Mariana Alves** e **Rafael Galupa**, for the opportunity to join the **team** of the amazing project *Cartas com Ciência*, that aims to create spaces for dialogue between scientists and children in Portuguese speaking countries. It has been an incredible experience, and I am grateful for being part of this team!

A word of gratitude also goes to my **colleagues of BIODiv and cE3c** and to my **friends** with whom I shared good moments, chats, lunches, and picnics during these last years. These moments with you were and are a breath of fresh air!

I am deeply thankful to my family:

To **José, Arminda**, **Isabel**, **Sérgio**, **Sofia**, **Emanuel**, **Joana**, and **Francisco** for being part of my life and for all the enjoyable moments on the weekends, birthdays, holidays, and through zoom meetings during the pandemic situation. A special thanks goes to my father **José Verdasca** for its unconditional love and continuous support throughout my life.

To finish, my heart and profoundly gratitude goes to **António Sérgio**, my husband and main supporter, and to our lovely sons, **Tomás** and **Simão**, the joys of my life!! Doing a PhD is always challenging but doing it with two young children at home during a pandemic situation is even more challenging! If there's one thing, I'm proud of in this journey, it's having managed to do my work being at the same time a dedicated mother. You three were the best thing that ever happened to me! Love you all!

Abstract

Invasive species are considered one of the greatest threats to the conservation of biodiversity and ecosystem services, putting at risk human health and economic activities. An example of a successful invasive species is the yellow-legged hornet, *Vespa velutina nigrithorax* (hereafter *V. velutina*). Originally from China, this species was first found in Europe in 2004 in the Bordeaux region of France. Since then, it has been expanding, being now present in ten western European countries, including Portugal. Since 2016 *V. velutina* has been considered by the European Union an invasive that requires monitoring and control. Knowing the potential of *V. velutina* for further expansion and its probable dispersal routes is crucial to anticipate its arrival and define adequate management practices. This species readily adapts to urban and agricultural environments and, as a voracious predator of honeybees and wild pollinators, is causing serious impacts on human health, on beekeeping and agricultural economies, and on native biodiversity. Here, I intend to generate new knowledge about *V. velutina* and deliver novel recommendations for future action plans for this invasive. The main goals of this work were: (i) explore the niche dynamics and the potential for further expansion in Europe; (ii) explore the dispersal patterns in Portugal and assess which drivers have been influential for each; (iii) develop a new molecular method to identify the best sample to study predation on honeybees and other wild insects; (iv) assess the perception of Portuguese beekeepers about the impacts on their beekeeping activity; (v) assess whether *V. velutina* can have qualitatively different impacts at different densities as the invasion proceeds. In general, the results showed that *V. velutina* still has enormous potential for expansion in Europe, because a large area of the continent has favourable environmental conditions for the species, and that its dispersal is facilitated by human activities, namely along motorways. The results also confirmed *V. velutina* as an additional stress for native biodiversity and for beekeeping, agricultural and forestry activities. Indeed, metabarcoding analysis of *V. velutina* larvae faecal pellets revealed the presence of honeybee DNA in all analysed colonies, irrespective of their location. Moreover, at initial stages of invasion, *V. velutina* poses major risks more frequently in urban and rural areas; only later, when at high densities, it can become a risk to humans engaged with forestry activities (e.g., loggers). The results of this thesis have relevant implications for ongoing monitoring control actions that will help mitigate the impacts of *V. velutina* and hamper its ongoing expansion.

Keywords: Diet analysis, Dispersion pathways, Distribution models, Invasion dynamics, Invasive species, Socio-ecological impacts, *Vespa velutina*

Resumo

As espécies invasoras são consideradas uma das maiores ameaças à conservação da biodiversidade e dos serviços dos ecossistemas, pondo também em risco a saúde pública e as atividades económicas. Um exemplo de uma espécie invasora bem-sucedida é a vespa-asiática (*Vespa velutina nigrithorax*). Proveniente de um único episódio de invasão com origem em populações do leste da China, esta espécie foi encontrada pela primeira vez na Europa em 2004 na região de Bordéus, em França. Desde então tem vindo a expandir-se por vários países europeus estando agora presente em dez países, incluindo Portugal. Desde 2016 que a vespa-asiática é considerada pela União Europeia como uma espécie invasora que requer medidas para a sua monitorização e controlo. Conhecer o seu potencial de expansão e as principais vias de dispersão é fundamental para antecipar sua chegada e definir práticas de controlo adequadas. Por ser uma espécie que se adapta bem a ambientes urbanos e agrícolas e por ser uma voraz predadora da abelha-do-mel e de outros insetos polinizadores, a vespa-asiática está a causar sérios impactos na saúde pública, na apicultura, na agricultura e na biodiversidade nativa. Nesta tese de doutoramento, pretendo gerar conhecimento novo sobre a espécie e produzir recomendações que possam vir a incorporar os planos de ação e controlo da invasão atualmente em vigor na Europa. Para tal, os objetivos do trabalho foram: (i) explorar a dinâmica do nicho e o potencial de expansão de *V. velutina* na Europa; (ii) perceber os padrões de dispersão da espécie em Portugal e que variáveis os influenciam; (iii) desenvolver um novo método molecular que identifique a melhor amostra para o estudo da predação da vespa-asiática sobre a abelha-do-mel e outros insetos polinizadores; (iv) avaliar a perceção dos apicultores portugueses sobre os impactos da vespa-asiática na sua atividade apícola; (v) avaliar como varia a suscetibilidade a impactos na biodiversidade nativa, nas atividades humanas (apicultura e agricultura) e na saúde pública, com o desenrolar do processo de invasão. De uma forma geral, os resultados desta tese mostraram que a vespa-asiática ainda tem um enorme potencial de expansão na Europa, não só porque uma grande área do continente tem condições ambientais favoráveis à espécie, mas também porque a sua dispersão é facilitada pelas atividades humanas, nomeadamente ao longo das autoestradas. Os resultados confirmaram ainda a vespa-asiática como fator adicional de stress para a biodiversidade nativa e para as atividades apícola, agrícola e florestal. Por exemplo, a análise de *metabarcoding* dos conteúdos fecais das larvas da vespa-asiática revelou a presença de DNA de abelha-do-mel em todas as colónias analisadas, independentemente da sua localização. Além disso, os riscos colocados pela vespa-asiática em estágios iniciais de invasão estão mais associados com as áreas urbanas e rurais; apenas mais tarde, quando a espécie ocorre em elevadas densidades, pode representar um novo risco para os humanos envolvidos em atividades florestais (por exemplo, madeireiros). Vários dos resultados da tese têm implicações relevantes para as ações de controlo e mitigação dos impactos da espécie.

Para além da introdução e da discussão geral, a tese engloba 5 capítulos principais, referentes a: (i) três artigos já publicados ou aceites para publicação em revistas internacionais com revisão por pares (capítulos, 2, 3 e 4), (ii) um artigo em revisão (capítulo 1) e (iii) um artigo em fase final de preparação para submissão (capítulo 5). Segue-se um resumo de cada um.

1. Padrões contrastantes em duas frentes de invasão sugerem uma mudança de nicho de uma espécie invasora predadora de abelhas-do-mel

Como a vespa-asiática (*Vespa velutina nigrithorax*) é invasora em pelo menos duas regiões diferentes na Europa (França e Noroeste da Península Ibérica) e as duas populações são geneticamente muito semelhantes, podemos considerar esta invasora como um bom modelo para estudar a dinâmica do nicho ecológico ao longo do processo de invasão. Não estando a vespa-asiática em equilíbrio com o ecossistema na Europa, importa investigar o potencial de expansão futura da espécie e que outras regiões do continente estão em risco. Para melhor aferir este potencial é importante perceber até que ponto as condições em que a espécie vive na Europa são ou não semelhantes às que a espécie ocupa na Ásia, de onde é nativa, e se as mesmas se encontram disponíveis em regiões não invadidas na Europa. Para tal, no **capítulo 1** desta tese usei modelos de distribuição de espécies em conjunto com análises de dinâmica de nicho. Percebi que uma potencial mudança de nicho ocorreu no Noroeste da Península Ibérica, onde a espécie vive em condições ambientais distintas tanto das da população de França como das da sua área de distribuição nativa. O facto de ambas as populações europeias se encontrarem em expansão, apesar de se encontrarem em regiões com condições ambientais diferentes, realça a capacidade da vespa-asiática para a invasão de diferentes envelopes climáticos. Verifiquei também que existe ainda uma grande extensão da Europa que apresenta condições ambientais semelhantes às do nicho ecológico da espécie na Ásia e que até ao momento ainda não foi invadida. Como tal é de esperar que com o desenrolar do processo de invasão estas novas áreas possam também vir a ser ocupadas.

2. Vespas invasoras na estrada: a dispersão ao longo das autoestradas deve ser considerada nos planos de gestão da vespa-asiática

Tendo sido demonstrado no estudo anterior a importância do clima na distribuição da vespa-asiática (*Vespa velutina nigrithorax*) a uma escala continental, tornou-se importante perceber como a espécie se está a expandir em Portugal e que variáveis de paisagem estão a contribuir para o processo de invasão a uma escala regional. Para tal, **no capítulo 2** da tese reuni informação sobre a ocorrência da espécie em Portugal entre 2013-2018. Para cada ano foi mapeada a área de distribuição contínua da espécie e calculada a velocidade de expansão. Tendo verificado que um conjunto ainda largo de registos se situava longe da área de distribuição contínua (registos esses a que chamei "*outposts*"), foram criados dois conjuntos de dados. O primeiro com os pontos de ocorrência situados ao longo da linha da frente da invasão e o segundo com os "*outposts*". Verifiquei que a proximidade dos ninhos a zonas arbustivas e com maior grau de naturalidade levaram a uma diminuição da dispersão da vespa a partir da área onde já apresenta uma distribuição contínua. Surpreendentemente, as autoestradas foram identificadas como importantes potenciadoras da dinâmica de invasão, uma vez que detetei uma relação de proximidade entre os "*outposts*" e estas grandes vias. Este resultado permitiu-me definir uma área em torno das autoestradas onde o esforço de deteção precoce e de controlo de ninhos numa fase inicial do seu desenvolvimento deve ser reforçado.

3. Uma ferramenta de *metabarcoding* para detectar a predação da abelha-do-mel e outros insetos pela invasora vespa-asiática

Apesar da vespa-asiática ser referida com uma voraz predadora da abelha-do-mel e de outros polinizadores, o estudo da sua dieta tem sido feito até ao momento através da observação direta de eventos de predação ou através da captura de obreiras que regressam ao ninho com as partes mais nutritivas das suas presas. Para ultrapassar esta limitação, no **capítulo 3** desta tese, e com a colaboração de uma equipa interdisciplinar, foi usada a técnica de DNA *metabarcoding* para testar três diferentes tipos de amostras e escolher a mais informativa para detetar a ocorrência de predação da vespa-asiática (*Vespa velutina nigrithorax*) sobre a abelha-do-mel e para usar em estudos futuros que pretendam analisar mais amplamente a dieta desta invasora. Foram testadas as mandíbulas das obreiras (que contactam diretamente com as presas), o seu estômago (por poder conter proteína animal, acidentalmente consumida pela vespa aquando da preparação das presas para levar para o ninho), e ainda os conteúdos fecais das larvas que permanecem no fundo de cada alvéolo do vespeiro. Foram usados *primers* específicos para detetar a abelha e desenvolvido um *primer* bloqueador do DNA da própria vespa. O DNA de abelha-do-mel foi identificado em todos os tipos de amostras, mas os conteúdos fecais das larvas obtiveram o maior número de *reads* de abelhas e a maior diversidade de presas, em qualquer dos vários níveis taxonómicos analisados. A análise dos conteúdos fecais por *metabarcoding* revelou ser um método promissor para o estudo da dieta da vespa e de outros Hymenoptera. A presença da abelha-do-mel em todas as colónias analisadas (independentemente do local de recolha na zona já invadida pela vespa) e a diversidade de outras ordens de insetos detetadas, corrobora a perceção dos impactos negativos que a vespa-asiática tem nas abelhas melíferas e na provisão dos serviços de polinização.

4. Avaliação da perceção do impacto da vespa-asiática (*Vespa velutina nigrithorax*) na atividade apícola em Portugal

Apesar do risco potencial para a produção de mel e para a prestação de serviços de polinização, até ao momento ainda não foi feita uma avaliação do seu impacto na apicultura. No **capítulo 4**, e através de um questionário online lançado em 2018 aos apicultores das zonas norte e centro do país, pretendemos fazer uma primeira avaliação da perceção dos apicultores sobre o impacto da vespa-asiática (*Vespa velutina nigrithorax*) na sua atividade apícola e sobre as estratégias de controlo da espécie que estão atualmente a ser implementadas em Portugal. Verificou-se que a grande maioria dos apicultores que respondeu ao inquérito já está informado sobre a problemática associada à vespa-asiática, sendo que aqueles que desenvolvem a sua atividade nos concelhos onde o número oficial de ninhos reportados é maior foram os que percecionaram maiores impactos da espécie na sua produção de mel. Os apicultores realçaram a falta de conhecimento sobre quais as técnicas mais eficazes para combater a vespa-asiática e solicitaram uma maior aproximação da comunidade científica. Foi ainda demonstrada insatisfação com a atuação das autoridades competentes no combate e prevenção da expansão da vespa-asiática. Referiram também como urgente a implementação de uma estratégia nacional vinculativa que regule o modo de atuação perante a vespa-asiática entre todos os municípios.

5. Dinâmica dos impactos socioeconómicos e ecológicos de espécies invasoras ao longo do processo de invasão.

Com o desenrolar da expansão da vespa-asiática (*Vespa velutina nigrithorax*) ao longo dos últimos anos em Portugal, é expectável que a densidade de ninhos esteja a aumentar, levantando a hipótese de que novos impactos até agora não registados possam aparecer. No **capítulo 5** da tese avaliei para a região centro de Portugal como a densidade de ninhos se relaciona com potenciais impactos na saúde pública, na biodiversidade nativa e nas atividades económicas (apicultura, agricultura e silvicultura). A adequação do habitat para polinizadores, a densidade da população humana, a densidade de colmeias por freguesia, o coberto florestal e a necessidade de polinizadores nas culturas agrícolas foram mapeadas e usadas para investigar os impactos potenciais nos polinizadores selvagens, na saúde humana, na apicultura, silvicultura e na agricultura, respetivamente. Verifiquei que a vespa-asiática começa por se estabelecer em áreas urbanas e agrícolas, e somente quando esse habitat, provavelmente preferencial, já está colonizado, é que se dá a invasão de zonas mais florestadas. A associação inicial a habitats antrópicos poderá potenciar elevadas densidades, levando a um risco acrescido de picadas em humanos nas zonas urbanas e peri-urbanas. Há também riscos de redução da produção agrícola desde os estados iniciais da invasão, uma vez que áreas com agricultura tradicional e com culturas dependentes de polinizadores estão maioritariamente localizadas perto de áreas urbanas e semiurbanas. Por outro lado, a vespa-asiática só constitui risco para os humanos envolvidos em atividades florestais quando ocorre em altas densidades. Com este trabalho foi possível concluir também que as áreas adequadas para polinizadores selvagens e as colmeias parecem favorecer o estabelecimento da vespa, mas apenas quando localizadas perto de zonas rurais e numa fase inicial do processo de invasão. Como tal, os planos de controlo da espécie devem considerar ações diferenciadas de acordo com a fase de invasão (que se traduz na densidade dos ninhos) e com a localização dos ninhos da espécie.

Palavras-chave: Análise de dieta, Dinâmica de invasão, Dispersão, Espécies invasoras, Impactos socio-ecológicos, Modelos de distribuição de espécies, *Vespa velutina*

Outline and publications

This thesis is a compendium of publications, organized in four parts: I - Prologue, II - Main chapters, III - Epilogue, IV - Supplementary Materials and References. The prologue presents an overview of the invasive process by *V. velutina* in Europe, introduces relevant background information of the specific topics studied in this thesis, and highlights the general objectives. The second part consists of five independent chapters, each one corresponding to a different research article. Three of them are already published (or in press), one is under review and the last one is in prep for submission. The epilogue contains a general discussion of the main findings of the thesis, ideas for future research and the final considerations. Finally, the supplementary material and a comprehensive list of references are included.

List of accepted publications included in this thesis:

Verdasca MJ, Verdasca MJ, Carvalheiro L, Aguirre Gutierrez J, Granadeiro JP, Rome Q, Puechmaille SJ, Rebelo R, Rebelo H. 2022. Contrasting patterns from two invasion fronts suggest a niche shift of an invasive predator of native bees. *PeerJ* 10:e13269 https://doi.org/10.7717/peerj.13269 (IF2021 3,061; Q1 Agriculture and Biological Sciences)

Verdasca MJ, Rebelo H, Carvalheiro LG, Rebelo R (2021) Invasive hornets on the road: motorway-driven dispersal must be considered in management plans of *Vespa velutina*. NeoBiota 69: 177-198. https://doi.org/10.3897/neobiota.69.71352 (IF2020 3,684; Q1 Biodiversity Conservation)

Verdasca, MJ., Godinho, R., Rocha, R.G., Portocarrero, M., Carvalheiro, L., Rebelo, R., Rebelo, H. A metabarcoding tool to detect predation of the honeybee *Apis mellifera* and other wild insects by the invasive *Vespa velutina*. J Pest Sci (2021). https://doi.org/10.1007/s10340-021-01401-3 (IF2020 5,918; Q1 Entomology)

Verdasca MJ, Carvalheiro L, Rebelo H, Rebelo R. Avaliação da perceção do impacto da vespa-asiática (*Vespa velutina nigrithorax*) na atividade apícola em Portugal (2021). Lucanus – Revista de Ambiente e Sociedade, Volume V, Páginas 84-103. http://www.lucanus.cm-lousada.pt/2022/02/15/vespa-asiatica-em-portugal/

List of submitted publications included in this thesis:

Verdasca MJ, Rebelo R, Rebelo H, Carvalheiro LG. Dynamics of socioeconomic and ecological impacts of invasive species throughout the process of population expansion (submitted)

I

Prologue

General Introduction

Brief overview on biological invasions

Before human-driven transcontinental transactions, species were only able to colonize new environments and expand their distribution through natural processes of migration or drift caused by air or water currents (Vicente et al. 2018). The slow pace at which such expansions usually occurred allowed local species to co-evolve with the new-arrivals and adapt to the environmental changes that such arrivals could cause. Nowadays, human mobility and worldwide trade have been helping species transgressing those natural barriers to dispersal (Hulme 2009), leading to high rates of intentional and unintentional transfer of organisms among ecosystems (Perrings et al. 2010). Compared to the natural rates of range expansion, humans have massively increased the rate at which species reach new areas (Lockwood et al. 2007, Helmus et al. 2014). Some species introductions are considered positive by humans, such as those of crop plants, whose distribution is mostly dependent on human manipulation. Many others are able to establish even with no human intervention (other than the introduction per se), and from these a small proportion is able to spread successfully becoming invasive, some having important negative impacts (Kolar and Lodge 2001).

In the last decades there has been a growing concern about the negative impacts of biological invasions. Covering a broad taxonomic range, from viruses and bacteria to fungi, plants and animals (Keller et al. 2011), invasive species are putting at risk native biodiversity, provision of ecosystem services, human health, and economic activities (Le Maitre et al. 2004). Many invasive species eat, compete and hybridize with native species often in detriment of the natives (Lockwood et al. 2007), leading to biodiversity loss, and sometimes to the extinction of many native species. Invaders can disrupt an ecosystem’s equilibrium, alter its nature and affect the provision of services, for example, by leading to habitat loss, changing disturbance regimes with increased frequency of wildfires and flooding, altering water or soil quality, or interfering with pollination (Thorpe et al. 2015, CABI 2018). Some invasive species pose a threat to people as potential carriers of diseases and can also cause allergies and skin damage. Invasive species are also a major economic and social concern due, for example, to the large amount of money spent in control actions, the pollution resulting from the overuse of chemicals to control infestations, and to reduced food security.

Assessing whether the climatic niche of a species may change between different geographic areas or time periods has become increasingly important in the context of biological invasions (Guisan et al. 2014). This is important to help evaluate the relative accuracy of geographical projections of future distributions that are based on climatic modelling approaches. This could be done by evaluating the degree of climate matching between geographical distributions of species in their native and naturalized ranges (Sax et al. 2007). As preventive measures are less costly than attempts to control or eradicate invasive species (Broennimann and Guisan 2008), species distribution models (SDMs, also called Ecological niche models) have become an important tool to predict areas prone to invasion (Guisan and Zimmermann 2000, Medley 2010, Guisan et al. 2014, Rödder et al. 2017). This approach combines observations of species occurrence or abundance with environmental estimates to predict distributions across space and time (Elith and Leathwick 2009). Once developed for the native distribution, SDMs can be applied to another region to identify areas prone to invasion (Peterson and Shaw 2003). This approach is frequently referred as reciprocal distribution modelling. However, as species' niches are not static (can expand, contract or shift; Pearman *et al.*, 2008), and invasive species are not at equilibrium in the invaded range during the invasive process, the use of traditional spatial modelling approaches for predicting their future expansion (Broennimann et al. 2012) can be challenging.

Species can spread by self-mediated dispersal from an initial location (diffusion; Lockwood, Hoopes and Marchetti, 2007) or through jump dispersal (using different dispersal vectors like wind, water, other organisms or human-mediated transport). Distinguishing between these patterns is crucial to understand the dynamics of an invasive process. The dispersion of invasive species depends, among other factors, on the existence of suitable environmental conditions (climatic and land cover; Cabra-Rivas *et al.*, 2016) and geographic and abiotic barriers. Hence, landscape heterogeneity and different environmental conditions interfere with the dynamics of population growth and expansion, accelerating or hampering the invasive process (Hastings et al. 2005).

The cost to prevent, monitor, control and respond to biological invasions in Europe has been estimated to amount to more than 12 billion EUR per year (Kettunen et al. 2009). Another assessment available at the EU level showed that over 27 million EUR were spent managing invasive species through the LIFE programme from 1992 to 2002 (Scalera and Zaghi 2004). As invasive species are very difficult and expensive to eradicate or even control, it is important to find the most efficient management strategies. Prevention is usually more cost-effective than post-entry eradication or containment, but obviously it is already too late to use this option for the many established invasions (Taylor and Hastings 2004). The management of invasive species presents some particular challenges: (i) their impacts tend to increase over time as populations become larger and spread (Lodge et al. 2006); (ii) there is often a considerable time lag between introduction and spread; (iii) invasive species frequently travel as contaminants of valuable trade; (iv) controlling the spread of invasive species requires international cooperation that is often difficult to achieve (Perrings et al. 2010). These specificities must be accounted for an effective control of the invasive processes.

According to Roques *et al.* (2009), insects are the dominant group among non-native terrestrial invertebrates in Europe: of the 1522 established species, 1306 (86%) were insects (Roques et al. 2009). Social insects, in particular social Hymenoptera, are more likely to become pests than other insects, due to their close association with human transportation with relatively low probability of detection, and because only one fertilised queen is needed to establish a new population (Beggs et al. 2011). Moreover, their excellent dispersal abilities, high reproductive rates, broad diets and habitat ranges, effective predator defences and competitive abilities, enhance their ability to establish and spread into new regions, with the consequent major impacts on native ecosystems (Moller 1996). Among the vespid species identified by Beggs *et al.* (2011) in introduced ranges around the world, 68% are eusocial. This indicates that sociality favours invasiveness, giving the species a plasticity of responses that allow survival and establishment in new environments. This is the case of *Vespa velutina nigrithorax* Lepeletier, 1836 which displays all the characteristics thought to make organisms efficient invaders, including polyphagy, colony initiation by a single inseminated queen and an annual life cycle which allows for a rapid population increment (Muller et al. 2010, Monceau et al. 2014, Arca et al. 2015).

The case of Vespa velutina

Identification

Morphologically, the invasive *Vespa velutina nigrithorax* (*hereafter V. velutina*) can be easily distinguished from the native European hornet, *Vespa crabro*, due to its dark 'velvety' thorax, from which the subspecies *nigrithorax* derives its name. Its clearest distinguishing features are that *V. velutina* is slightly smaller (2.5–3 cm long), has yellow legs and only the third segment of abdomen is orange (Fig. I-A), while *V. crabro,* has a comparatively yellowish abdomen, dark legs being also longer (3 - 3.5 cm).

Fig. I-A – Dorsal (on the left) and ventral view (on the right) of *Vespa velutina* (adapted from (Espinosa et al. 2019)

The nests of these two species are also distinguishable. Despite some secondary nests of *V. velutina* can be found on soil, slopes with vegetation, walls or buildings, most of them are at the top of the trees (Carvalho et al. 2020). However, spotting them is often difficult because they remain hidden until leaf fall in autumn and winter reveals their position. The nests can reach large dimensions (80 cm – 1 m) with a pear like shape with a lateral entrance, while the nests of *V. cabro* are smaller (with a smaller number of individuals), located inside trees cavities or human infrastructures and with the entrance at the bottom (Fig. I-B). Depending on the latitude, hornet nests can be either annual, started by a new queen every spring, or perennial, where young queens take over from old ones (Matsuura and Yamane 1990). Colonies in warm tropical climates tend to be perennial.

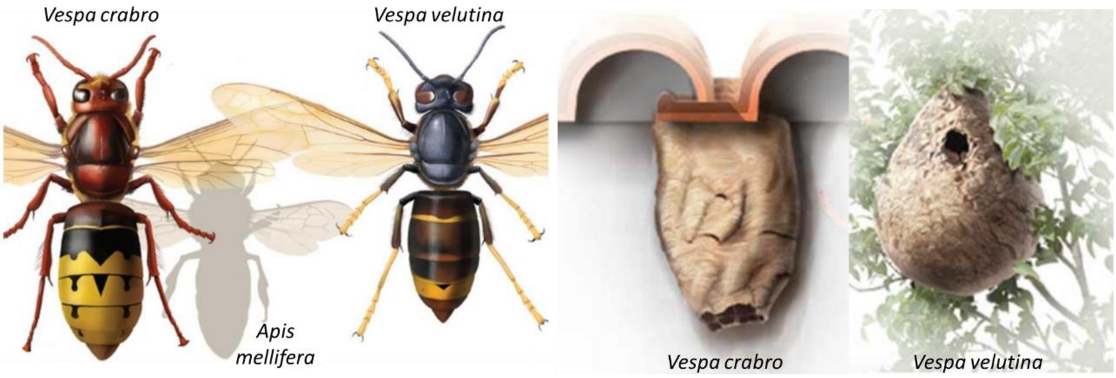


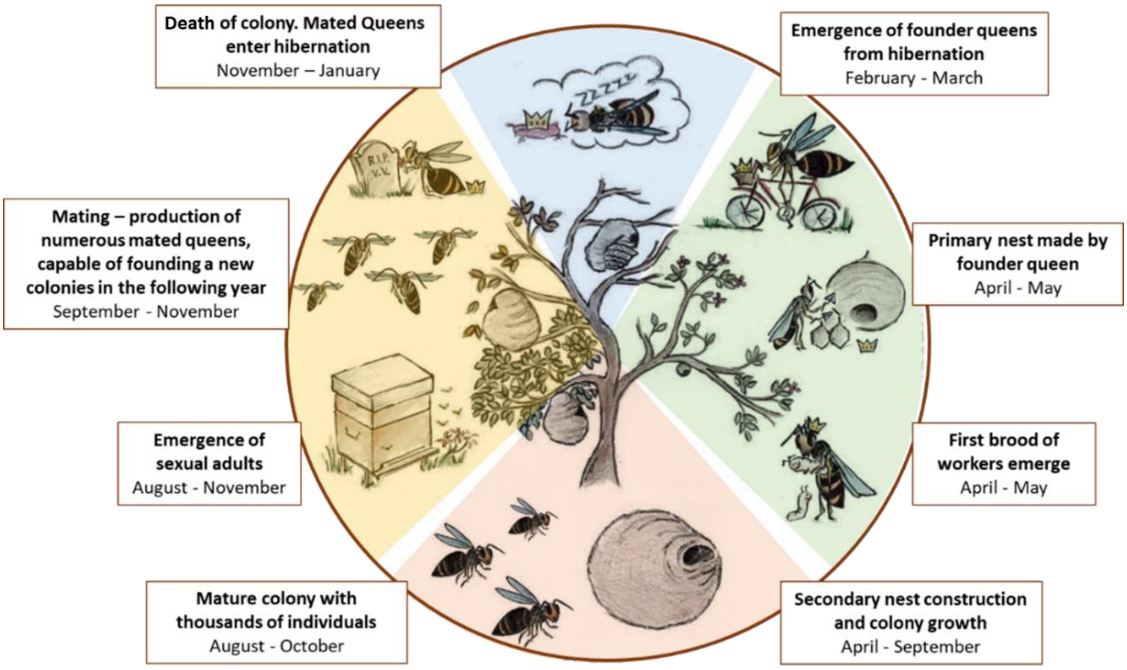
Fig. I-B – Comparison of morphology and nest appearance, between the two hornet species present in Portugal: *Vespa crabro* (on the left) and *Vespa velutina* (on the right). Credits: Anyforms Design (National Geographic - Portugal, Fev.2021).

By being a social Hymenoptera, *V. velutina* colonies are organized with a division of work based on three classes: queen, workers, and males. Besides the males (which are very similar to females in size and colour but lack a sting apparatus and have longer and thicker antennae; Monceau, Bonnard and Thiéry, 2014), there are no distinct morphological differences between the queen and workers (infertile females). Workers are usually smaller (notably in spring) but in autumn many workers are as big as the future queens (CABI 2018). Inside the colony, the laying queen can be recognized by her distended abdomen at the end of the season and by her damaged wings (CABI 2018).

Life cycle

In tropical regions *Vespa velutina* colonies can be found in all stages of development at any given time of the year, whereas in temperate zones colonies have an annual cycle (Espinosa et al. 2019). As it happens with other invasive species, the establishment of *Vespa velutina* in a given place plausibly results from a compromise between suitable climatic conditions that ensure colony survival, and suitable habitat structure providing resources such as shelter and food (Villemant et al. 2011b, Bessa et al. 2016). The association of *V. velutina* with urban and agricultural areas (Choi et al. 2012a, Rome et al. 2015) in detriment of natural areas (Fournier et al. 2017) can be explained by the protection that human infrastructures offer to primary nests and by the availability of nutritional resources such as food found in fish markets (Monceau and Thiery 2017).

In spring, the overwintered foundresses (fertilized females) initiate their new colony by starting to build a small round primary paper nest (+/-10 cm) in an enclosed and protected place, to raise the first generation of female workers (Fig. I-C). In this early phase (called ‘‘the queen colony phase’’), the queen is alone and vulnerable until the first workers emerge, being the only responsible for building the nest, laying the eggs, and feeding the brood.



adapted from https://www.vespavelutina.eu/it-it/, LIFE14 NAT/IT/001128 STOPVESPA

Fig. I-C – Annual life cycle of *Vespa velutina* (adapted from https://www.vespavelutina.eu/it-it/, a website from the project LIFE STOPVESPA (LIFE14 NAT/IT/001128 STOPVESPA “Spatial containment of *Vespa velutina* in Italy and establishment of an early warning and rapid response system”).

As the colony grows, most colonies abandon the primary nest, because it is either in a confined site or too close to the ground, to build instead a new, much larger secondary nest in branches of trees over 10 m tall shielded from detection by the leaves, but also in slopes with vegetation, in the soil, walls and buildings (Carvalho et al. 2020). The secondary nest will host the colony till the end of the season. One of the key factors that allows hornets to be highly successful predators, even if temperatures are lower, is their ability to thermoregulate their nests to a constant temperature of around 30°C (Martin 1990, Perrard et al. 2009). When temperatures reach maximum levels in summer months, hornets ventilate their nest. Workers achieve evaporative cooling by regurgitating water from their mandibles onto the nest (sometimes soaking the nest wall), and the evaporation of that water through the vibration of their wings (Perrard et al. 2009). Despite the ability to maintain a stable nest temperature, hornet larvae (unlike honeybee larvae) are able to tolerate a wide range of temperatures (Chauzat and Martin 2009). To construct the nest envelope, the workers prepare a paste by masticating wood fibres with their saliva (Monceau et al. 2014); therefore easy access to suitable wood fibres and water sources is crucial to build the nest. At this stage, the workers are responsible for nest enlargement (which may reach 80 cm to 1 m) and brood rearing, while the queen is exclusively dedicated to laying eggs. A mature colony may produce a mean number of 13.000 individuals over a season (Rome et al. 2015) which represents a consumption of an average of 11.32 kg of insect biomass (Rome et al. 2021). Around September the first reproductive adults (males and fertile female - gynes) emerge. These females destinated to be queens may mate with several males (Arca et al. 2015), increasing this way their offspring genetic diversity. After mating the males die, while mated females seek for a suitable place to overwinter (e.g., under the bark of trees, stones, or leaf litter). The queen lives for about one year and usually dies before her reproductive offspring emerge, whereas the rest of the colony dies of starvation at the onset of winter (CABI 2018). The nest will gradually be abandoned, and the colony dies at the end of winter. The life cycle restarts again in the following spring when the winter surviving foundresses emerge (Fig. I-C).

Distribution and invasion process

The species *Vespa velutina* is native from the tropical and subtropical regions of northern India to eastern China, Indochina, and Indonesia. Despite the existence of 13 different subspecies of *V. velutina* in this region, genetic exchanges are probably still occurring between native mainland populations (Perrard *et al.*, 2014), except for the Indonesian and Malaysian populations, that are clearly genetically different (Takeuchi et al. 2017). In 2004, one single fertilised female of the subspecies *V. velutina nigrithorax*, du Buysson 1905, (from temperate south-eastern China) was accidentally introduced into south-west France, probably via boat transport from the Zhejiang or Jiangsu provinces of eastern China (Arca et al. 2015). Since then, the species has spread to other European countries: Spain (López et al. 2011), Portugal (Grosso-Silva and Maia 2012), Italy (Demichelis et al. 2014), Germany (Witt 2015), Great Britain (Keeling et al. 2017), Belgium, Netherlands (Smit et al. 2018), Switzerland (Poidatz et al. 2018) and Luxembourg (Rome and Villemant 2021: MNHN-INPN). This hornet is now colonizing the Mediterranean Peninsulas, thus being exposed for the first time to Mediterranean-type climates. The species is also invasive in Korea (Kim et al. 2006) and Japan (Takeuchi et al. 2017) having most probably arrived to these Asiatic countries also via trade from China and Korea, respectively. According to Villemant *et al.* (2011), other suitable regions for *V. velutina*, located in north and south America and in Australia, may also be vulnerable to invasion if an accidental introduction occurs in these continents.

A stratified diffusion process, which includes a mixture of natural diffusion with jump dispersal events (Hengeveld 1989, Suarez et al. 2001) has been recorded in the spread of *V. velutina* in Europe (Bertolino et al. 2016, Robinet et al. 2017, Lioy et al. 2019). Once females destinated to be queens can fly over long distances and survive long periods hibernating, *V. velutina* is a serious candidate for long distance transport in shipments of goods, for example in containers, in pots, in building material, under the bark of trees, in decorative material or wooden items or in cars, boxes, trucks, farming equipment, etc (Marris et al. 2011, CABI 2018). Although more studies are needed to understand the drivers of dispersion of this invasive, it seems to take advantage of river valleys and major highways to disperse, and on the other hand, it seems to avoid pure stands of conifers (Rome and Villemant 2021: MNHN-INPN).

Recent studies showed that *V. velutina nigrithorax* is spreading at a rate of 78 km/year in France (Robinet et al. 2017), and of 10-20km/year in Italy (Bertolino *et al.*, 2016: probably hindered by high mountains ranges). A similar spread rate to that of Italy was registered for Korea (10-20 km/ year), in this case perhaps due to competition with six other hornet species (while in Europe the species only competes with *Vespa crabro*: Choi, Martin and Lee, 2012). The average rate of *V. velutina nigrithorax* expansion in Portugal was estimated by Carvalho et al. (2020) as 37.4 km / year. However, the western Iberian Peninsula encompasses different bioclimatic belts (Mesotemperate, Supramediterranean, Mesomediterranean and Thermomediterranean, Rivas-Martínez *et al.*, 2017), and these are more sprayed out over the North-South axis along the Atlantic coast, than over the West-East axis. Therefore, it is important to disentangle the spread rate along these different gradients, that partly coincide with mountain ranges. The climatically suitability for *V. velutina* is higher along the Atlantic coast than in the southern regions of Europe (Bessa et al. 2016), and therefore it is expected that the rate of expansion will be faster towards south than to east. However, even in the thermomediterranean regions of Europe, some climatic suitable “islands” may exist, which highlights the need of identification for these spots to better plan the management of this invasive species.

Diet

Adults of *Vespa velutina* feed on sugary substances (flower nectar, fruits, tree sap, depending on the environment and season, and on special regurgitations from their own larvae; Matsuura and Yamane, 1990), but prey on other insects, such as honeybees and wild pollinators (and also on waste from food markets: Beggs *et al.*, 2011) as a source of protein to feed their larvae. *Vespa velutina* is much faster and more agile than other hornet species in catching its prey (CABI 2018). Attacks of *V. velutina* to honeybee colonies are frequent, particularly from July to November (Monceau et al. 2012) when the production of future queens requires high provision by hornet workers (Mollet and Torre 2006). While other vespid species land on a hive and grab bees that try and attack them, the hunting behaviour of *V. velutina* consists of catching bees in flight in a characteristic hovering position backwards to the hive called bee-hawking (Tan et al. 2007). Being in front of the hive entrance in stationary flight, they catch the honeybees that return to the hive (Monceau et al. 2018). Once the prey has been caught, it is carried off to a tree branch where the head, wings, legs, and abdomen are removed and a meatball with the prey thorax (which contains the nutritious flight muscles) is made and taken back to the nest. Inside the nest, a worker–larvae trophallaxis occurs, i.e., reciprocal exchange of food between adults and larvae. Workers feed portions of prey to the larvae, after which the larvae regurgitate a highly energetic solution containing sugars, protein and free amino acids to feed adults (Matsuura and Yamane, 1990; Hunt, 1991).

Despite the well-known predation of honeybees by *V. velutina*, the hornet also consumes several other species of insects, with a preference for other social Hymenoptera (e.g. common wasps) and Diptera, such as hoverflies and houseflies (Williams 1988, Perrard et al. 2009, Villemant et al. 2011b, CABI 2018). To date, the accumulated knowledge about the diet of *V. velutina* has been undertaken via direct observation of wasps foraging in the wild or via examination of food pellets - body fragments of prey taken to the nest by the workers (Perrard et al. 2009, Beggs et al. 2011). However, these approaches are often time-consuming and generally limited to high taxonomic level assignment (i.e., Order, Family), so studies based on molecular approaches, like DNA metabarcoding, are needed to better infer about the diet of *V. velutina*.

Impacts

Vespa velutina nigrithorax is considered a serious threat to native biodiversity, crop pollination services, beekeeping activities and public health (Monceau et al. 2014, 2018, CABI 2018, Requier et al. 2019). Notably this invasive affects managed honeybee colonies by predation on foragers and causing a reduction in the collection of food resources. The mere presence of *V. velutina* near the hives induces stress on honeybees, activating their antioxidant system (Leza *et al.*, 2019), and decreases their foraging activity, thus reducing pollen and nectar collection, with impacts on winter survival of the colony (CABI 2018, Monceau et al. 2018, Requier et al. 2019). For the hornets, apiaries, where hives (with up to 50,000 bees each) are gathered in a relatively small area, represent an abundant, easy, and accessible source of food (Espinosa et al. 2019). If a honeybee colony becomes sufficiently deprived of workers, *V. velutina* will then enter the hive, feed on the honey and remove the brood (CABI 2018). Unlike *Apis cerana*, that co-evolved with *V. velutina* in Asia and developed effective defence techniques, resulting in suffocation and heating of the predator (balling), and the formation of a compact agglomeration of honeybees on the flying board: Ken *et al.*, 2005), *Apis mellifera* is unable to cope with the predation pressure of *V. velutina* and hence is much more vulnerable to *V. velutina* attacks. Despite the defence mechanisms of *A. cerana*, *V. velutina* can destroy up to 30% of a colony of this species (CABI 2018); so the potential impacts on a defenceless species can be much higher.

As *V. velutina* can attack a broad variety of wild pollinators (Hymenoptera, Diptera, Lepidoptera: (Rome et al. 2011)), it could also pose additional risks and have further negative effects on crop production as well as on biodiversity (Monceau et al. 2014). In fact, the negative impact of *V. velutina* on pollination services as a consequence of their hunting of pollinators in flower patches was recently addressed by Rojas-Nossa and Calviño-Cancela (2020). These authors found that the patch visitation rate of honeybees, the flower visitation rate of small hymenopterans and the flower visitation time of bumblebees (*Bombus* sp.) and syrphids was significantly reduced in patches with presence of the predator.

In Korea, Choi, Martin and Lee (2012) found a competitive displacement of the six species of *Vespa* that were known to live in Busan city, after the arrival of *V. velutina*. As 20% drop in *Vespa simillima* and a 10% drop in *Vespa mandarinia* was observed by the same authors. Recently a reproductive interference between *V. velutina* and the native populations of *V. simillima* was also reported in Japan (Yamasaki et al. 2019), where these authors showed that a large percentage of the analysed *V. simillima* queens had been inseminated by *V. velutina* males. However, a recent study in Italy showed that the presence of *V. velutina* is not leading to an evident replacement of *Vespa crabro* and *Vespula* species; instead native Vespidae are probably avoiding or minimizing the competition pressure (Carisio et al. 2020). On the other hand, the absence of true competitors for the same resources and nesting sites in Europe potentiates the occurrence of extremely high density of nests, as it was already reported in France (8 nests per km^2 in mixed land uses to 23 nests per km^2 in an urban area: Franklin *et al.*, 2017).

Concerning the potential danger to humans, *V. velutina* is not considered to be more aggressive than the European hornet, *Vespa crabro* (de Haro et al. 2010). Like other hornets, it can be dangerous by inducing a life-threatening allergic reaction or after multiple stings. Despite some concern about the risk of fruit pickers being stung while harvesting fruit, severe attacks only occur when nests are accidentally disturbed (e.g., during vegetation clearing activities). With increasing densities of nests in urban areas, to which *V. velutina* is well adapted (Choi et al. 2012a), people are likely to accidentally disturb nests more frequently (CABI 2018).

Despite the potential negative impacts of *V. velutina* on native biodiversity, crop pollination, beekeeping activities and public health (Monceau et al. 2014, 2018, CABI 2018, Requier et al. 2019), until now there is no accurate quantification and assessment of the real consequences and associated costs (Monceau, Bonnard and Thiéry, 2014; Espinosa, Franco and Chauzat, 2019), being also a topic for future research.

Predators and natural enemies

Natural population control may occur due to lack of prey, bad environmental conditions at key stages of the life cycle, limited nesting sites, usurpation (queen fighting) (Martin 1992) and predation. At the end of the season when the activity of *V. velutina*'s colonies start to decline, attacks by some native avian predators have been reported in France (CABI 2018). These species, like green woodpeckers (*Picus viridis*), jays (*Garrulus glandarius*) and tits (Paridae) are often seen pillaging nests and eating the remaining larvae, although they are not able to attack large active colonies. There have been rare records of more specialist predators, such as the European bee-eater (*Merops apiaster*), and the European honey buzzard (*Pernis apivorus*) (Macià et al. 2019). Additionally, in a very recent work, it was reported for the first time and in the late summer, the presence of *V. velutina* in the faeces of the yellow-throated marten (*Martes flavigula*) in South Korea (Kim and Choi 2021).

Like other wasps *V. velutina* is susceptible to various diseases and parasites, such as *Conops vesicularis* (a parasitic fly) (Darrouzet et al. 2015), and various nematodes and entomopathogenic fungi (Turchi and Derijard 2018). Recent studies shed light on the ability of some virus to jump between superfamily taxa (Apoidea-Vespoidea) in view of a possible natural prey/predator re-equilibrium between honeybees and invasive hornets (Mazzei et al. 2019, Marzoli et al. 2020), and on the role played by such viruses as potential biocontrol agents of honeybee predators (Dalmon et al. 2019). However, and comparing to *Vespa crabro*, a high pathogen resistance in reproductive females of *V. velutina*, was recently found (Cappa et al. 2021), which might represent a key factor contributing to the ecological success and spread of this invader.

Modeling Vespa velutina nigrithorax invasion

Since *Vespa velutina nigrithorax* became established in several countries, several studies emerged with different approaches and at diverse spatial scales to model its invasive range (Villemant *et al.*, 2011; Ibáñez-Justicia and Loomans, 2011; Barbet-Massin *et al.*, 2013, 2018; Bertolino *et al.*, 2016; Bessa *et al.*, 2016; Robinet, Suppo and Darrouzet, 2017; Fournier *et al.*, 2017; Franklin *et al.*, 2017; Keeling *et al.*, 2017; Monceau and Thiery, 2017; Rodríguez-Flores *et al.*, 2019; Robinet, Darrouzet and Suppo, 2019, this thesis - chapter 1). The amount and geographic origin of studies on this topic evidence the growing concerns of European countries about this species. To model *V. velutina* expansion, it is first necessary to identify the environmental variables that potentially affect its ecophysiology (see some examples in Villemant *et al.*, 2011; Bessa *et al.*, 2016; this thesis - chapter 1). The majority of studies about the potential spread of *V. velutina* in Europe rely only on partial data of the invaded area, failing to predict some regions where the species is currently thriving (Ibáñez-Justicia and Loomans 2011, Fournier et al. 2017, Barbet-Massin et al. 2018, 2020, Kim et al. 2021). In a recent study focused in Iberian Peninsula (Bessa et al. 2016) the authors analysed a data set of nest distribution in the north of Portugal and evaluated which variables best predict the species presence. They showed that besides temperature and precipitation, land-use also plays an important role in *V. velutina* expansion at regional scale. In another study, conducted by Bertolino *et al.* (2016), the authors used data from western Liguria in Italy (from 2013 to 2015) to evaluate the range area, the spread rate, and the modalities of dispersion (natural or human-mediated). They found that the distribution of nests in Italy was mainly explained by elevation and distance from source sites, while also highlighting the role of human mediated dispersal in the observed new records far from the continuous distribution range. Recently, Robinet, Suppo and Darrouzet (2017) simulated the spread of *V. velutina* in France under different control intensities and different human-mediated scenarios. They considered 2004-2009 occurrence data and showed that if effective control measures are applied it will be possible to reduce the hornet population density. In the same year Keeling *et al.* (2017) built a mathematical model to simulate the spread of *V. velutina* across the landscape of Great Britain, pointing that without control, *V. velutina* could colonise the British mainland rapidly.

Control methods

Although it is illusory to try to eradicate *Vespa velutina nigrithorax*, it is acknowledged that a targeted control would limit its threatening trend (Turchi and Derijard 2018). To date, control methods have been ineffective and non-specific (Goldarazena et al. 2015, Rojas-Nossa et al. 2018, Lioy et al. 2020, Sánchez and Arias 2021), highlighting the need for applied research in this topic. Engaged in the control of *V. velutina*, French, Spanish, Portuguese, and Italian local authorities have recently implemented action plans for the destruction of the nests with little success.

There are several techniques to control of this invasive (for detailed information see Turchi and Derijard (2018)):

i) Visual location and suppression of the nests (whenever possible, nests are burnt during the night, when all the colony is inside). However, as the nests are located preferentially in high trees, they remain hidden in spring–summer due to foliage (Monceau et al. 2014), such that <5% of hornet's nest are spotted (Robinet et al. 2017).
ii) Injection of biocides. When a nest is spotted, a pest control technician destroys the nest by injecting permethrin. As permethrin has a substantial remanence, technicians are obliged to unhook the nests after injection in order to avoid contamination of the environment and food chain (Turchi and Derijard 2018).
iii) Trapping. The most widespread traps are those intended to capture workers during the hunting season to limit predation on apiaries (Monceau et al. 2014). As queens work alone at the beginning of the spring, various beekeeping syndicates and associations promote this technique earlier in the season. The disadvantage of this kind of trap is its significant lack of selectivity with respect to *V. velutina* (Goldarazena *et al.*, 2015; Rojas-Nossa *et al.*, 2018: <1% of the total catches are *V. velutina*; INIAV, 2019; Lioy *et al.*, 2020; Sánchez and Arias, 2021), that results in many other wild pollinators being improperly captured, with the consequent impact on native biodiversity. Indeed, the indiscriminate use of traps (regardless of higher or lower density of *V. velutina*) seems to be a greater threat to insect biodiversity than *V. velutina* predation (Turchi & Derijard, 2018; also, four to six small traps may catch as many insects as a *V. velutina* colony can prey: Rome et al., 2021).
iv) Poison baits. While strictly regulated in Europe and banned in France, beekeepers widely use several types of poisoned bait devices in their apiaries to target *V. velutina*, although they all have the major disadvantage of releasing biocides into the environment and thus causing collateral damage (e.g., local contamination of the food web, or when a poisoned hornet enters a beehive, especially at the end of the season when their energy requirements are higher, etc.). However, these poisoned baits have the advantage of targeting and destroying nests remotely without having to locate them at advance (Turchi and Derijard 2018).
v) Electric harp. The electric harp is aimed to electrocute hornets passing through two wires powered by a current generator. The spacing of the wires is such that a honeybee can fly through without touching them, while a hornet inevitably touches both, electrocutes and falls into a water basin underneath (Turchi and Derijard 2018).

Recently there are promising nest location techniques, although very expensive and difficult to implement in all the invaded area. Some examples are:

i) Triangulations. This technique involves capturing at least three specimens, then releasing them at various locations and recording the direction of their flight. Captured and stressed hymenopteran tend to return in a straight direction to their nest. If the three individuals are from the same nest, there is a good chance that the three directions they took would intersect at a point that will give the nest position (Blot 2008).

ii) Harmonic radar. When *V. velutina* is equipped with a small passive transponder, this radar system can track its flight trajectory and locate nests to be destroyed (Maggiora et al. 2019). However, the radar operates only on a 500m range, which implies constant changes in its position to follow the hornets. It has the disadvantage of not being easy to transport because it is placed on the roof of a vehicle and has a large wingspan. Moreover, in the presence of some landscape barriers or obstacles, it is difficult to follow the signal.

iii) Radio telemetry. This method enables a faster detection of nests, than previous methodologies. It implies the attachment of an active radio tag to the hornet abdomen (up to 0.8 ratio of their weight) that is followed through a radio tracking receiver (Kennedy et al. 2018).

iv) Thermal imaging. This recent technique (only tested in Italy in a controlled environment) enables to scan the landscape using thermographic camera and the detection of nests by thermal imaging (Lioy et al. 2021). Further optimizations of this method are needed to overcome some limitations in the detectability of nests under high environmental temperatures, or when the tree canopy is very dense.

All the techniques described have pros and cons, either those that target *V. velutina*'s nest and larvae (nest localization, poisoned baits, biocides injection, etc..) or those that can only affect hornet workers (traps, electric harps, etc.). Depending on whether one takes the side of a beekeeper looking for simple, fast and inexpensive solutions, or a citizen bothered by *V. velutina* or sensitive to the current threat to pollinators, or even a decision-maker who must make strategic choices, the choice of the best control method will be different. In fact, a combination of several techniques is often the best choice (Turchi and Derijard 2018).

Policy and management

The invasion of *Vespa velutina nigrithorax* in Europe presents a practical challenge to current policies regarding management of invasive species (Monceau et al. 2014). Indeed, the importance of early reaction following a pest introduction is crucial to the success of pest management (Reaser et al. 2020). Recently *V. velutina* received significant media attention in Europe, probably because its preferred prey, the honeybee, is a symbol of biodiversity and because many people are afraid of wasps and hornets (Monceau et al. 2014). At the European level, invasive species policies are diverse, with little coordination within and between member states, and this may favour the proliferation of alien pests (Keller et al. 2011).

Vespa velutina was listed in the EU list of invasive alien species in 2014 (Regulation EU No 1143/2014 of the European Parliament and of the Council) and activities directed to its control can be carried out within a national management program of the Member States concerned. Recently, a working group "*Velutina Task Force*" was created to gather efforts between different countries to i) monitor the spread; ii) assess the impacts on beekeeping and biodiversity; iii) study the biology and ecology in native and invaded ranges; iv) develop sound control methods to hamper the invasion. In Portugal, to support the identification and control of the invasive process, an online platform was created in 2013 (managed by ICNF - Instituto da Conservação da Natureza e das Florestas), enabling the georeferentiation of *V. velutina* nests by citizens (currently available at http://stopvespa.icnf.pt/; where it is also possible to be informed on how to act in case of hornet/nest detection). Two years later an Action Plan for *Vespa velutina* was approved with a set of guidelines to promote the security of citizens, the protection of beekeeping activities and mitigation of the impacts on native biodiversity (DGAV, INIAV and ICNF, 2015, updated in 2018). Since 2015, passive surveillance (communication of every observation in the online platform) and active surveillance (set of permanent traps to detect the present of *V. velutina*, being the number of traps reinforced in regions that are acting as potential pathways for invasion) are ongoing strategies to hamper the invasive process. In 2017 a Commission for the Monitoring, Prevention and Control of *V. velutina* was created to implement a national strategy in collaboration with different stakeholders (decision makers, researchers, municipalities, and beekeepers). The growing concerns about the impacts of these invasive species led the Portuguese government to allocate, since 2019, a financial support to help campaigns of nest destruction and monitoring actions at a regional level. From 2012 to February 2020, 49013 nests were destroyed in Portugal, being most of them secondary nests located on treetops (70.3%), man-made infrastructures (e.g. indoor, roof, wall, 20.8%), shrubs (5.4%), and on the ground (3.6%) (Carvalho et al. 2020). In 2018, a good practices manual was produced, where different technics for nest destruction are explained (Marques et al. 2018). The most common practices involve mechanical removing of nests during the night, burning, or injection of authorized biocides. The methodological options are made by the local technicians and depends on the nest size, localization, and height.

General objectives of this thesis

In this thesis I aim to provide new information to incorporate management plans, about (i) the invasion dynamics of *Vespa velutina nigrithorax* in Europe (chapter one: "**Contrasting patterns from two invasion fronts suggest a niche shift of an invasive predator of native bees**"), (ii) the main colonization pathways in Portugal (chapter two: "**Invasive hornets on the road: motorway-driven dispersal must be considered on management plans of *Vespa velutina***"), (iii) the development of a new molecular method that enabled evaluating the usefulness of different types of samples to study the predation of *Vespa velutina* upon *Apis mellifera* (chapter three: "**A metabarcoding tool to detect predation of the honeybee *Apis mellifera* and other wild insects by the invasive *Vespa velutina***"*)*, (iv) the perception of Portuguese beekeepers about the impacts of *Vespa velutina* in beekeeping activities (chapter four: "**Beekeepers' perceptions of the impact of the yellow-legged hornet (*Vespa velutina nigrithorax*) on beekeeping activity in Portugal**" and (v) the dynamics of socio-ecological impacts of *Vespa velutina* at different stages of invasion *(*chapter five**: "Dynamics of socioeconomic and ecological impacts of invasive species throughout the process of population expansion"***)*

II

Main chapters

1

Contrasting patterns from two invasion fronts suggest a niche shift of an invasive predator of native bees

The content of this chapter is published as:

Verdasca MJ, Verdasca MJ, Carvalheiro L, Aguirre Gutierrez J, Granadeiro JP, Rome Q, Puechmaille SJ, Rebelo R, Rebelo H. 2022. Contrasting patterns from two invasion fronts suggest a niche shift of an invasive predator of native bees. *PeerJ* 10:e13269 https://doi.org/10.7717/peerj.13269 (IF2021 3,061; Q1 Agriculture and Biological Sciences)

Author Contributions

MJV, LGC, RR and HR conceived the study. QR provided data from MNHN Paris. SJP provided the R script. MJV, JAG and JPG ran SDMs. MJV wrote the manuscript. All authors contributed to the final version.

Contrasting patterns from two invasion fronts suggest a niche shift of an invasive predator of native bees

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Abstract

The accuracy of predictions of invasive species ranges is dependent on niche similarity between invasive and native populations and on our ability to identify the niche characteristics. With this work we aimed to compare the niche dynamics of two invasive populations of *Vespa velutina nigrithorax* (an effective predator of honeybees and wild pollinators), one in central Europe and another one in the north-western Iberian Peninsula, and hence to identify uninvaded regions susceptible to invasion.

Niche dynamics and shifts of *V. velutina* were assessed by comparing the environmental niches of the native and of the two invasive populations, using climatic, topographic and land use variables. We also ran reciprocal distribution models using different algorithms and records from both native and invasive ranges to compare model predictions and estimate which regions are at a greater risk of being invaded.

An apparent niche shift was detected in the population of the NW of Iberian Peninsula, where the species is living under environmental conditions different from the native niche. In central Europe, large suitable areas remain unoccupied. The fact that both invasive populations are well established, despite occupying environmentally distinct regions indicates that *V. velutina* has a high capacity to successfully invade different environmental envelopes from those existing in its native range. E.g., in north-western Iberian Peninsula the species is now thriving out of its native niche limits. Moreover, the large extent of still unoccupied environmental space with similar conditions to those used by the species in its native range suggests that there is still a large area of central and eastern Europe that can be potentially invaded by the species.

Keywords: Invasive species; Niche dynamics; Realized niche; Reciprocal distribution models; *Vespa velutina*

Introduction

The niche concept is broadly used to characterize requirements and impacts of species (Chase and Leibold 2003), and to predict ecological and evolutionary responses to environmental change (Lavergne et al*.* 2010). If the niche characteristics of a species in its native range are known to a large extent (they can never be completely inferred from occurrence data studies), it is possible to anticipate with greater precision the potential geographic course of its spread, should the species be introduced in a new area and become an invader (Peterson 2003). Niches are defined by the species' physiological tolerances (the fundamental niche which represents the conditions under which a species can live indefinitely), as well as by biotic interactions and dispersal barriers, which constrain the fundamental niche to the realized niche, i.e., the conditions under which a species actually lives (Tingley et al*.* 2014).

Non-native species, when introduced to new geographic areas, may establish in environmental conditions different from their native range because of the lack of natural enemies (i.e., occupying a larger realized niche) or local adaptation (i.e., changing their fundamental niche), or both. Therefore, the analysis of the changes in the niche depicted by an invasive species between its native and introduced ranges may be useful in understanding range expansion and invasion potential (González-Moreno et al*.* 2014; Kumar et al*.* 2015). Non-analogue climates represent a severe problem when calculating these niche change metrics, because no insight on the biology of the species in these non-analogue climates can be inferred from a comparison between ranges (Guisan *et al.* 2014). The colonization of parts of environmental space not present in the native range cannot be unambiguously considered as resulting from evolution (i.e., changes in fundamental niche) in the non-native range. To address this issue, Guisan et al*.* (2014) proposed a framework for niche comparison between native and invasive ranges with three basic components (Fig. 1.1): (i) niche unfilling, representing environmental conditions similar to the niche of the native population not (yet) occupied in the invasive range (quantifying niche unfilling areas in the invaded range is critically important because it suggests the likelihood of further spread: González-Moreno et al*.* 2014); (ii) niche stability, representing overlapping environmental conditions used by the species in both the native and invasive ranges; (iii) niche expansion, representing environmental conditions present in the invasive range that are not used by the native population, but fall within the available native climates (and not used either due to physical barriers or biotic interactions). This latter component can be used as an indicator of ecological or even evolutionary change.

Inspecting marginality is also important to draw inferences about a species invasion ability. Marginality can be defined as the distance between the mean habitat conditions used by the species and the mean environmental conditions over the entire area (Hernández Fariñas et al. 2015). Populations with marginal niches occur in less common environmental conditions in a given area, contrasting with those with non-marginal niches (Hernández Fariñas et al. 2015), which are closer to the centre of the niche hyperspace; see Fig. S1.1). This means that species with a marginal niche in a region usually have low tolerance to one or more of the commonest local features, and this can reduce their invasion ability. Hence, by inspecting the marginality of an invasive population we are also adding new information about its potential ability to continue the invasion process.

Ecological niche modelling is often used to anticipate the potential geographic extent of an invasive process. In particular, ensemble forecast predictions (Araújo and New 2007) have been frequently used in invasive ecology, as they represent a consensus approach that offers more robust predictions for the potential and realized distribution of species than techniques that rely on a single algorithm. Reciprocal distribution modelling (RDM) emerged as a powerful tool by considering both the invasive and native ranges in distribution models (Medley 2010), otherwise, the potential range of a species may be seriously underestimated (Václavík and Meentemeyer 2012). Niche conservation is indicated if both the native model accurately predicts non-native distributions, and non-native models accurately predict the native distribution (Medley 2010). Alternatively, a niche shift is suspected whenever reciprocal models poorly predict one another, although that situation can also be caused by a narrower niche in the invasive range. Indeed, this RDM approach has previously revealed niche shifts for the spotted knapweed and fire ant invasions in the United States (Broennimann et al*.* 2007; Fitzpatrick et al*.* 2007).

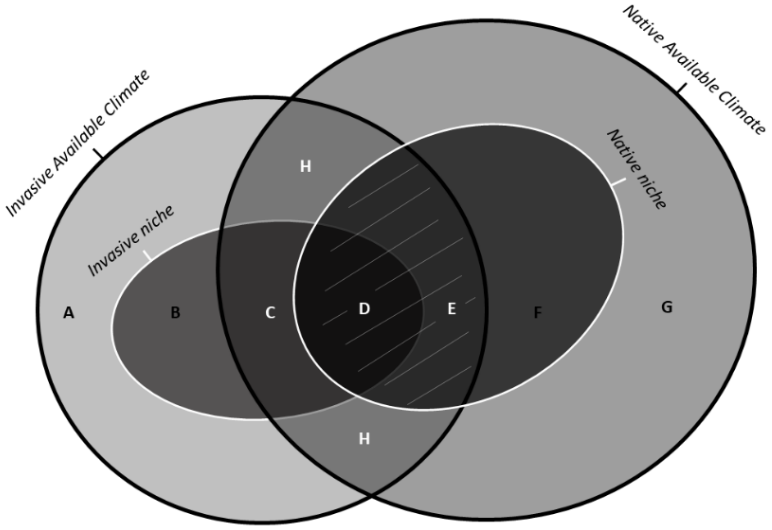

Fig. 1.1 - Schematic representation of the indices of niche change (unfilling, stability, and expansion), adapted from Guisan *et al.* (2014). Unbroken black lines show the density of available environments in the native range (on the right) and in the invasive range (on the left). The unbroken white lines on the left and on the right show the invasive and the native niches, respectively. The area with white uppercase letters shows the most frequent environments common to both ranges (i.e., analogue environments). The uppercase letters represent: (A) Available conditions in the invaded range but outside of the invasive niche and non-analogue to the native range (B) Novel conditions, i.e., conditions inside of the invasive niche but non-analogue to the native range. (C) Niche expansion, that is, conditions inside the invaded niche but outside the native one, due to ecological or evolutionary change in the invaded range. (D) Niche stability, that is, conditions occuppied in both native and invaded range. (E) Unfilling, that is, conditions inside of the native niche but outside the invaded niche, possibly due to recent introduction combined with ongoing dispersal of the invasive species, which should at term fill these conditions. (F) Conditions inside of the native niche but non-analogue to the invaded range. (G) available conditions in the native range, outside of the native niche and non-analogue to the invaded range. (H) Analogue conditions containing niche unfilling (E), stability (D) and expansion (C). The grey lines covering the regions (D) and (E) represent the Maximum Niche Stability that a species can occupy in the invaded range if there is no occupation of entirely novel conditions.

Many invasive insects are not in equilibrium in their invasive ranges, and have the potential to further expansion (Hill et al. 2017). As invasive insects can respond quickly to novel environments (including biotic interactions) through either phenotypic plasticity, changes in adaptive traits or some combination of both (Urbanski et al. 2012, Gibert et al. 2016), niche shifts may be relatively common within this group. Frequently a single species invades several regions, and this provides an opportunity to study and model its invasion ability, particularly to assess potential niche shifts. One of the most recent examples of a successful invasion in Europe is the yellow-legged hornet, *Vespa velutina nigrithorax* Lepeletier, 1836 (hereafter *V. velutina*). Native to south-east Asia, *V.*

velutina is a predator of honeybees and other beneficial insects (Monceau et al. 2014, Rome et al. 2021, Verdasca et al. 2021a), being considered a serious threat to honey production, pollination services and consequently food security (Monceau et al. 2014; Requier *et al.*, 2019). The mere presence of *V. velutina* near beehives induces stress to honeybees (Leza et al*.* 2019) and decreases their foraging activity, thus reducing pollen and nectar collection, with impacts on winter survival of the colony (Requier et al*.* 2019). Further, as *V. velutina* can attack a broad variety of wild pollinators, it could also pose additional negative effects on crop production as well as on biodiversity (Monceau et al. 2014). Since the accidental introduction in France, in 2004 (in a temperate bioclimatic region: Sayre et al*.* 2020) of a single female fertilized by several males (Arca et al. 2015), *V. velutina* has spread to other European countries, being considered an invasive alien species of concern in the European Union (European Commission 2016). In 2011 a new invasion by *V. velutina* was found in the North of Portugal (Fig. 1.2 – P2) in the region of Viana do Castelo (Grosso-Silva and Maia 2012), near a paper mill, where the species probably arrived in a load of timber from France (Marco Portocarrero, pers. comm.), in a transitional region between a temperate hyper oceanic climate to the North and a Mediterranean climate to the South; the latter conditions with no apparent parallel with any bioclimatic region of the native range (Sayre et al*.* 2020). The new invasive population has been expanding South into central Portugal (Verdasca et al. 2021b), and to the North and East into Galicia (Spain), where it arrived at the end of 2012 (Rodríguez-Flores et al*.* 2019). As *V. velutina* is invasive in at least two different regions in Europe, this is a pertinent model organism to study niche dynamics during invasion processes. Furthermore, as both French and north-west Iberian populations are genetically very similar (Quaresma 2019), and have been present in Europe for very few generations, it is possible to assess the role of plasticity, rather than adaptation, on its ability to invade different climatic niches. Recently Barbet-Massin et al*.* (2018), suggested that the climatic niche of *V. velutina* in France expanded during the past few years, raising the hypothesis of a change in the niche dynamics during the invasion process. However, these authors did not predict some of the already invaded areas in Portugal and Germany. Here, we expand on this previous work to assess niche shifts in a newly invaded region and compare them to the niche shifts seen in the older invasive population, incorporating the impacts of time since invasion on niche metrics.

In a recent work by Hill et al. (2017), the authors suggest that distribution models should not be used in isolation to predict insect invasions or invaded range extents, but instead need to be coupled at least with some analysis of changes in environmental/climatic space. For this reason, and again expanding the work of Barbet-Massin et al*.* (2018), here we compared the realized niche of the two main invasive populations of *V. velutina* in Europe and coupled niche dynamic analysis and RDM with an ensemble forecast to address three main questions: (i) Has *V. velutina* shifted its niche during the invasion process? (ii) Is *V. velutina* occupying the most common environmental conditions of the invasive range? (iii) Is there potential for further expansion? Answering these questions is crucial to understand niche flexibility and its potential for evolution in different regions of the invaded range, to identify areas at risk, and to inform management plans.

Material and Methods

Vespa velutina occurrence data

Distribution data sets consisted of presence data only. We enriched the native dataset used by Barbet-Massin et al*.* (2018) (that only used the subspecies *V. velutina nigrithorax*), by considering the different subspecies (see Perrard et al*.* 2014) present in Asiatic mainland (148 records). With the current fragmented knowledge in Asia, none of the different subspecies is geographically well covered in the native range being very difficult to discriminate the spatial limits of each population. A recent work by Takeuchi et al*.* (2017) showed a clear divergence between the Malaysian and Indonesian populations and all the other Asian mainland (and Taiwanese) populations and colour morphs, while also showing that the haplotypes from these continental and Taiwanese forms do not clearly segregate geographically. Genetic exchange is therefore probably still occurring across all the native mainland populations and the validity of several subspecies and colour morphs is questioned; therefore, the records from the native range used here refer to all the records, regardless of the putative subspecies, except for the Indonesian and Malaysian populations (Takeuchi et al*.* 2017). Although there are considerably less records from the native range available (in comparison with invasive range: see below), they capture the entire environmental envelope where the species thrives, being so far the most complete dataset used to model this species.

For the invasive range we gathered a total of 20855 records from different sources (for details see Supporting Information). The effort was not systematic once it results from citizen reports, although all the records were previously validated by the different entities that hold the data. Since by 2017 the records were not continuously distributed in Europe it was possible to identify two different populations that had not yet come into contact in the North of Spain (data provided to us in 2017 by the Spanish Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente). Hence, it was possible to attribute each location to either the first introduction in France or the second introduction in Portugal. To check for commonalities in the set of factors affecting the distribution of *V. velutina* within Europe, we divided the records in two sets: P1 with data from France and contiguous records from Italy and NE Spain; P2 in the NW of Iberian Peninsula, with records from Portugal and from the contiguous Galicia (Fig. 1.2). We could ascribe a date for 81.9 % of the European data. To analyse the variation of realized niche metrics with time since introduction (see methods below), these data were grouped by year (the remaining 18.1 % of the data were discarded only for this specific analysis). We compared each year with the combination of all the previous years.

To avoid spatial autocorrelation, we reduced the number of occurrence data through the "spatially rarefy occurrence data" tool in SDMtoolbox (Brown 2014) weighed by a principal components analysis (PCA) on climatic data, thus keeping the points with unique environmental information (in a 5km pixel resolution); this resulted in a total of 110 points for Asia and 2582 for Europe.

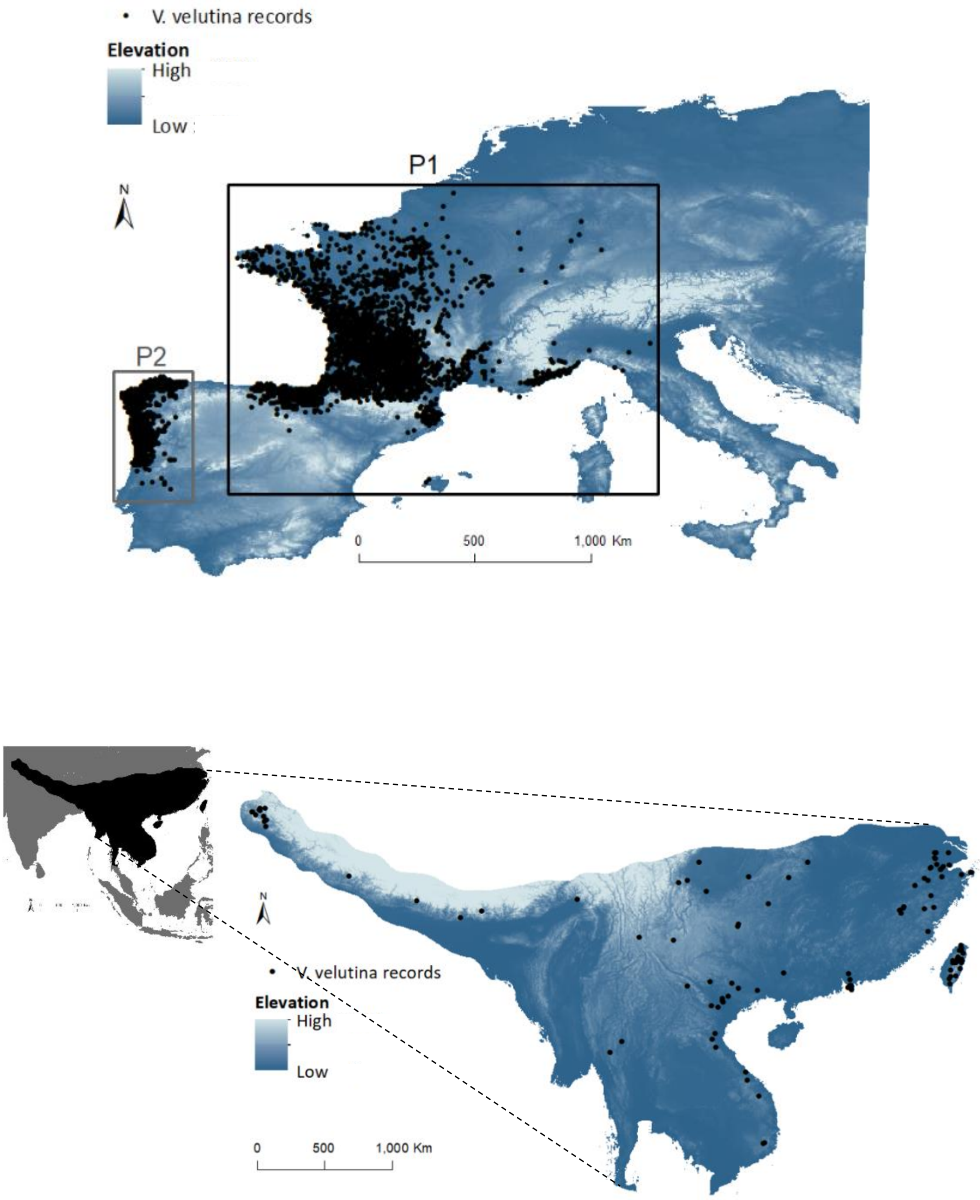

Fig. 1. 2 - Invasive (above) and native (below) ranges of *Vespa velutina*. European study area (with elevation in background) includes the two invasion focuses: P1 (black box containing the population from France and contiguous regions of Italy and Spain) and P2 (grey box containing the population from NW of Iberian Peninsula). Native area in southeast Asia includes south of China, east of India, Bangladesh, Myanmar, Thaylandia, Laos, Cambodia, Vietnam, and Taiwan. Native records include all the different subspecies of *Vespa velutina*, except for Indonesian and Malaysia populations, being the native envelope delimited by the area that encompasses the different closely related subspecies in the native range.

Environmental data

We considered an initial dataset of 22 environmental variables (climatic, topographic and land use: 'see Table S1.1 for details') which potentially affect the ecophysiology of *Vespa velutina* (Villemant et al. 2011; Bessa et al. 2016). We calculated the distance to each land cover type since distance variables are acknowledged to achieve a better performance for species associations to landscape features (Rainho and Palmeirim 2011). As nests are made in trees, but also in the ground, on walls, on slopes with vegetation, we decided to test for a few topographic components (slope included). All the layers were clipped to the same extent and upscaled to a 5 km pixel resolution, to match known *V. velutina* homing ability (Poidatz et al. 2018). Climatic data of the available environmental space and of the occupied niche in both native and invasive ranges were compiled to compare its range of variation (Table S1.2). All calculations regarding the preparation of variables for analysis were carried out in ArcGIS 10.4.1 software (ESRI 2016). To avoid high collinearity, variables that were highly correlated (r ≥ 0.70: (Dormann et al. 2013), examined on both ranges, see Tables S1.3 and S1.4) were excluded from subsequent analysis, keeping the ones with higher acknowledged biological importance. With the remaining twelve variables (Table 1.1), a multivariate environmental similarity surface (MESS) calculated in MaxEnt, spatially identified which areas in the invasive range possess similar environmental characteristics to the native range (Fig. S1.2). The native range was delimited to contain only the regions where the species is acknowledged to live (we took care to not include areas with extreme climates that have no parallel in Europe, like the cold regions of Himalayas or the tropical regions of Malaysia). *Vespa velutina* is still expanding in the invasive range, thus to estimate its potential distribution we used three different non-parametric presence-only algorithms (please see below) using the 'biomod2' package (Thuiller et al. 2014) in R (R Core Team 2019).

Table 1.1 - Variables used in the modelling of the realized niche of *Vespa velutina* (for details of resolution and source of each variable, see Supporting Information - Table S1.1)

Type	Variables
Climatic	BIO1 = Annual Mean Temperature BIO7 = Temperature Annual Range (BIO5-BIO6) BIO10 = Mean Temperature of Warmest Quarter BIO11 = Mean Temperature of Coldest Quarter BIO17 = Precipitation of Driest Quarter BIO19 = Precipitation of Coldest Quarter
Topography	slp = Slope northn = Northeness eastn = Eastness
Land cover	durb = Distance to urban areas dfor = Distance to forest dwat = Distance to water

Has Vespa velutina shifted its niche during the invasion process?

To test this hypothesis, we used the R package ’ecospat’, which can be applied to investigate the niche of invasive species, by using an ordination method to quantify and compare niche shifts between invasive and native ranges (Di Cola et al*.* 2017). We performed two complementary analyses:

(i) We compared the environmental niche space between native and invasive ranges of *V. velutina*. We performed a PCA to summarize the selected environmental variables; this was calculated using environmental values from all the pixels of both the native and the invaded areas, thus maximizing the ecological variance. We then applied a kernel density function of the occurrence data over the PCA space, to estimate the density of the occurrences of the species by cell (Broennimann et al*.* 2012; Di Cola et al*.* 2017; Fig. 1.3). We plotted all datasets (native, P1 and P2) on the environmental space and visually analysed them for niche shifts between native and invasive populations. The niche equivalency (i.e., whether the niche overlap is constant when randomly reallocating the occurrences of both entities among the two ranges) and niche similarity (addressing 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) were tested in ‘ecospat’ (see Broennimann et al*.* (2012) for methodological details).

(ii) We inspected temporal niche dynamics for each focus of invasion (2006-2015 for P1 and 2014-2017 for P2). Based on the three niche metrics (Expansion, Unfilling and Stability) defined by Guisan et al*.* (2014), we decided to rename the metric “Stability” into “Maximum Niche Stability”, to facilitate the interpretation of the results, since the Unfilling metric also corresponds to stability conditions that are not (yet) filled. Niche overlap was then decomposed across time (yearly) in the three metrics of niche (corresponding to the areas E, (D+E) and C; Fig. 1.1). This decomposition provides more information about the drivers of niche dynamics between native and invaded ranges (Petitpierre et al. 2012, Guisan et al. 2014). These calculations only took into account areas with analogous environmental conditions (Qiao et al. 2017) enabling the correction for differences in the availability of environments between study areas and preventing the detection of false niche shifts. We thus avoided the inclusion of environmental conditions that occur in the native but not in the invasive ranges (Petitpierre et al*.* 2012), restricting niche analyses to the intersection between native and invaded ranges in the PCA (areas represented by (C), (D) (E) and (H) on Fig 1). Schoener’s D index (ranging between 0 and 1) was used as a measure of niche overlap (Petitpierre et al*.* 2012). By comparing niche overlap between a given year and all the years prior to that accumulated as one, we then evaluated the dynamics of the realized niche in the invasive range.

Is Vespa velutina occupying the most common environmental conditions of the invasive range?

For each invasive population we performed a theoretical exercise in the R package ‘ecospat’ (Di Cola et al*.* 2017) to inspect for changes in niche metrics when marginal conditions were discarded from the analyses, enabling this way to infer about the use of rare environmental conditions (for details how the niche metrics works please see: Guisan et al*.* (2014)).

We repeated the calculations of the niche metrics, discarding successively an increasing proportion (10 % and 20 %) of the most marginal (i.e., less frequent) available environmental conditions. So, we progressively reduced the “intersect” parameter (quantile of the environmental density used to remove marginal climates), removing up to 20% of the area with analogous conditions between both invasive and native ranges (area (H) in Fig. 1.1). When the “intersect” parameter was set to zero the analysis included all analogue environmental conditions between native and invaded range. By changing the same parameter to 0.1, we excluded 10 % of the marginal climate, being the analysis restricted to 90 % of the overlap between native and invaded environmental space, and so on, until 20 % of the most marginal environmental conditions had been removed. With this we aimed to infer if the two invasive populations of *V. velutina* are occupying the most frequent or rather distinct (i.e., marginal) environmental conditions occurring at both invasive and native ranges. If a low variation in the niche metrics is detected after excluding marginal climates from the analysis, it means that the species is not exploring conditions that are rare in the study area. Conversely, if the exclusion of marginal climates leads to a large variation in the niche metrics, it means that the species is using those uncommon conditions, hence its populations are less prone to further expand.

Is there potential for further expansion?

We used a RDM approach (Medley 2010; Broennimann et al*.* 2012) to evaluate *V. velutina* expansion potential to uninvaded European areas and to identify which regions may be at higher risk of invasion. For this RDM approach, we fitted the species distribution models (SDMs) using data (i) from the native range to fit the first set of SDMs models, (ii) from France and contiguous regions of Italy and Spain invaded range (P1) and (iii) from NW of Iberian Peninsula (P2) invaded ranges. We applied three commonly used non-parametric algorithms: Generalized Boosted Models (GBM; Friedman 2001), Random Forests (RF; Breiman 2001) and Maximum Entropy modelling (Maxent; Phillips et al. 2006) (for details see Supporting Information - A). For each one of the datasets (full, P1 and P2) we fitted 10 model repetitions per algorithm using 75% of the data for modelling training and 25% for model testing, creating the number of pseudo-absences (background data for MaxEnt) as five times the number of presences used. We thus produced 30 spatially explicit predictions (10 model repetitions × 3 algorithms) per dataset. We converted the continuous model suitability scores to binary predictions using the thresholds that maximize the sensitivity and specificity of the models (Liu et al. 2013) and then created a final ensemble model per dataset (full, P1 and P2). For the ensemble models we considered a presence only when more than half of the models predicted a cell as presence (Araújo and New 2007). We used the area under the ROC (Receiving Operating Characteristic) curve (AUC) (Phillips et al. 2006) to evaluate model performance. The AUC is not only threshold independent but also evaluates both the false-positive error rate and the true positive rate in order to obtain a measure for the accuracy of the constructed model (Aguirre-Gutiérrez et al. 2013). We obtained AUC values from each of the models created by the 10 repetitions for each algorithm. Currently the AUC is one of the most used methods for model evaluation (Razgour et al. 2016).

To identify the regions in Europe with environmental conditions similar to the native range, we overlapped the models (from native to invasive and from invasive to invasive) previously produced, highlighting the areas of agreement between environmental conditions of both ranges. High-risk areas were identified as the regions predicted by both native and invasive models, or only by one of the models. Low risk areas were predicted to be unsuitable by both models.

Results

Has Vespa velutina shifted its niche during the invasion process?

We detected evidence of a niche shift in the invasive population from the NW of Iberian Peninsula (P2) towards climatic conditions different from those of the native range (absence of overlap with the native niche; Fig. 1.3a, 1.3 c, Table 1.2). For the French population (P1), the species realized niche is basically nested in environmental conditions similar to the ones used in the native range (Fig. 1.3a, 1.3b). In the NW of Iberian Peninsula, the species thrives under different environmental conditions from those used by the species in France (Fig. 1.3b, 1.3c). Among the environmental variables considered, climatic variables were the ones that better explained the probability of invasion by *V. velutina* (Fig. 1.3d). Niche equivalency was rejected for both populations, indicating that both suffered alteration of their realized niche during the invasion process. However, and for both populations, niche overlap falls within the 95% confidence limits of the null distributions, meaning that niche similarity cannot be rejected. (Fig. S1.3 and Fig. S1.4).

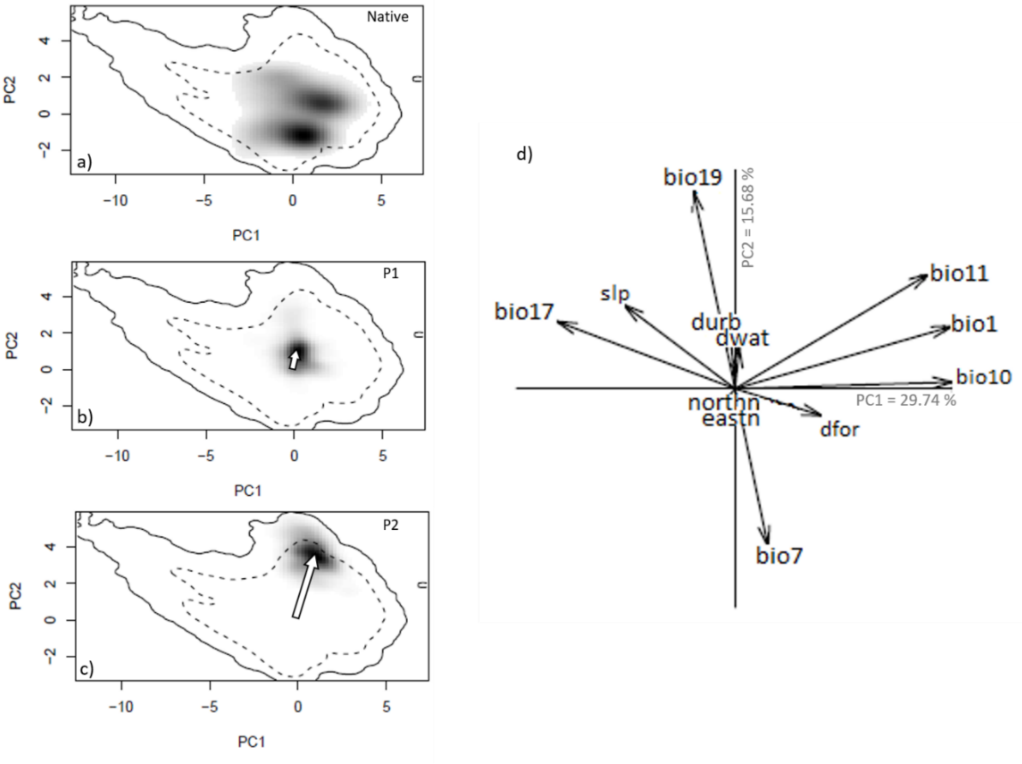

Fig. 1.3 - Niche of *V. velutina* in environmental space of a principal component analysis (PCA-env). The left three panels represent the niche of the species along the two first axes of the PCA in: a) the Asiatic native range; b) in the invaded range of population P1 (from France and contiguous regions of Italy and Spain) and c) in the invaded range of population P2 (from NW of Iberian Peninsula). Grey shading shows the density of the occurrences of the species by cell. The solid and dashed contour lines illustrate, respectively, the kernel density estimates corresponding to 100% and 50% of the available (background) environment. The arrows represent how the centre of the niche has changed between Asian native and European invaded range. (d) Contribution of the environmental variables for all invaded range (Climatic: bio1 - Annual Mean Temperature; bio7 - Temperature Annual Range; bio10 - Mean Temperature of Warmest Quarter; bio11 - Mean Temperature of Coldest Quarter; bio17 - Precipitation of Driest Quarter; bio19 - Precipitation of Coldest Quarter; Land cover: for - Distance to forest; dwat - Distance to water; durb - Distance to urban; Topography: eastn - Eastness; northn - Northness; slp - slope) on the two axes of the PCA and the percentage of variance explained by the two axes.

Analysing the niche dynamics across years following invasion, our results suggest that for P1, in the beginning of the invasion almost 80 % of the environmental conditions similar to the native niche were not occupied (Table 1.2 – Unfilling; corresponding to region (E) in Fig 1), and that this proportion has steadily diminished to ~40 %, after 9 years (Table 1.2). In the NW of Iberian Peninsula (P2), from the very beginning the species expanded into environmental conditions that are apparently not explored in the native range (Table 1.2, corresponding to region (C) in Fig. 1.1). This proportion has been rather stable over the years.

Table 1.2 – Variation of niche metrics (Expansion, Maximum Niche Stability and Unfilling) along the years in the two sets of invasive populations (P1 – from France and contiguous region of Italy and Spain; and P2 – from NW of Iberian Peninsula) and corresponding overlap between native and invaded ranges. The uppercase letters (C), (D) and (E) correspond to the regions presented in Fig. 1.1.

Population	Year	Expansion (C)	Maximum Niche Stability (D) + (E)	Unfilling (E)	Overlap (Schoener's D index)
P1	2006	0.01	0.99	0.78	0.09
	2007	0.01	0.99	0.77	0.10
	2008	0.01	0.99	0.71	0.13
	2009	0.01	0.99	0.65	0.17
	2010	0.02	0.98	0.59	0.20
	2011	0.02	0.98	0.55	0.21
	2012	0.02	0.98	0.50	0.22
	2013	0.02	0.98	0.49	0.24
	2014	0.02	0.98	0.43	0.24
	2015	0.02	0.98	0.38	0.25
P2	2014	1.00	0.00	1.00	0.00
	2015	0.97	0.03	0.97	0.00
	2016	0.97	0.03	0.97	0.00
	2017	0.97	0.03	0.97	0.00

Is Vespa velutina occupying the most common environmental conditions of the invasive ranges?

Reducing in 20% of the environmental space with analogous conditions between both invasive and native ranges (to exclude marginal climates from the analyses) did not lead to a variation in the niche metrics in both populations (Table 1.3). This result suggests that the species is not occupying marginal (i.e., uncommon) environmental conditions in Europe. Instead, the species is occupying the most common conditions available.

Table 1.3 - Effect of marginal environmental conditions on the three niche indices (Expansion, Maximum Niche Stability and Unfilling) calculated by reducing the intersection parameter (from 0% to 50%) and global overlap between native and invaded ranges.

Population	Intersect	Expansion (C)	Maximum Niche Stability (D)+(E)	Unfilling (E)	Overlap (Schoener's D index)
P1	0	0.05	0.95	0.26	0.3
	0.1	0.05	0.95	0.26	
	0.2	0.05	0.95	0.26	
P2	0	0.91	0.09	0.93	0.01
	0.1	0.91	0.09	0.93	
	0.2	0.91	0.09	0.93	

Is there potential for further expansion?

Before modelling procedures, we checked for the existence of analogous conditions between native and invasive ranges. The MESS analysis showed that almost 100% (>99.99%) of the invasive range did not have one or more environmental variables outside the range present in the training data (Fig. S1.2), enabling the predictions for these areas. Regarding the predictive models for the invasive range, AUC values were high (all over 0.93) for the different algorithms, although for the native model the AUC values ranged between 0.73 and 0.91 (Fig. S1.5). The model with highest fit was obtained using RF (higher AUC values than GBM and Maxent). Comparing model sensitivity to small changes in occurrence points and using the model developed for the invasive range, the predicted area for the species in Europe was relatively similar for the different algorithms (mean varying between 551600 km^2 and 781275 km^2), except for Maxent and RF (with larger within-model variance) (see Fig. S1.6a). For the projection of native occurrences into Europe, the sensitivity to small changes in the presence data varied more between algorithms (predicted area varying from 1229625 km^2 to 3978275 km^2), being Maxent the more susceptible regarding model variance (see Fig. S1.6b).

When only the P1 or P2 invasive ranges were considered for the modelling procedure, the full extent of areas already invaded were not detected (Fig. 1.4b, c). However, the native range model overlapped and covered most occurrences in the invasive range (Fig. 1.4a and Fig. 1.5). As the native model identified 99% of the high-risk areas, we considered these as the regions that are not (yet) occupied despite having similar environmental conditions to those used by the species in the native range. Contrarily, the predicted presence of the species in Asia using the model developed for Europe did not accurately predict its native distribution.

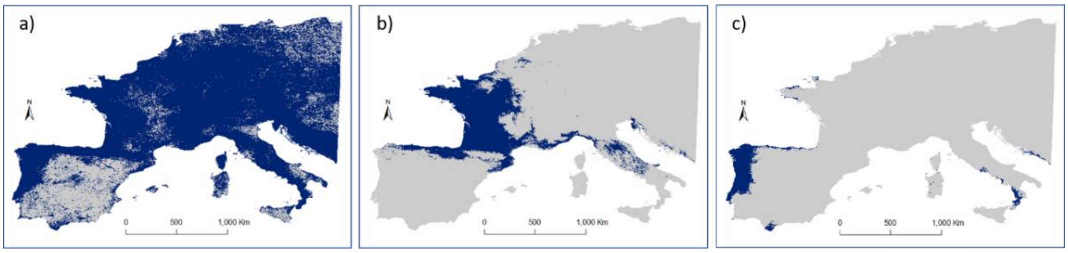

Fig. 1.4 – Predicted distribution (in dark blue) of *Vespa velutina* in the entire Europe region using: a) ensemble model using data collected in the native range (prediction area = 3467700 km^2); b) ensemble model using data from P1 alone (from France and contiguous regions of Italy and Spain; prediction area = 793675 km^2; c) ensemble model based on P2 data alone (from NW of Iberian Peninsula; prediction area = 156675 km^2). Distribution models were calculated in 'biomod2', and then converted into binary predictions using their correspondent thresholds that maximize the sensitivity and specificity of the models. Every cell for which more than half of the models predicted a presence, was considered a presence, the other cells were assigned as absences.

An agreement between native and invasive models occurred for only 15 % of the potential distribution in the invaded range. The remaining areas of concordance between both models that are still unoccupied by the hornet are in the south of Italy, Corsica, Croatia and Montenegro. About 65 % of the area where the species is predicted to occur in Europe was considered at high-risk of being invaded, as it represents similar environmental conditions to those found on *V. velutina's* native range, though this area remains uninvaded (Fig. 1.5). Concerning the presence records, all were located on high-risk areas. At this continental scale, the climatic variables related with precipitation and temperature contributed the most to explain the distribution of *V. velutina* (Fig. S1.7). More specifically, the species is thriving in Europe in regions where the winters are mild and rainy, and the summers are pleasant.

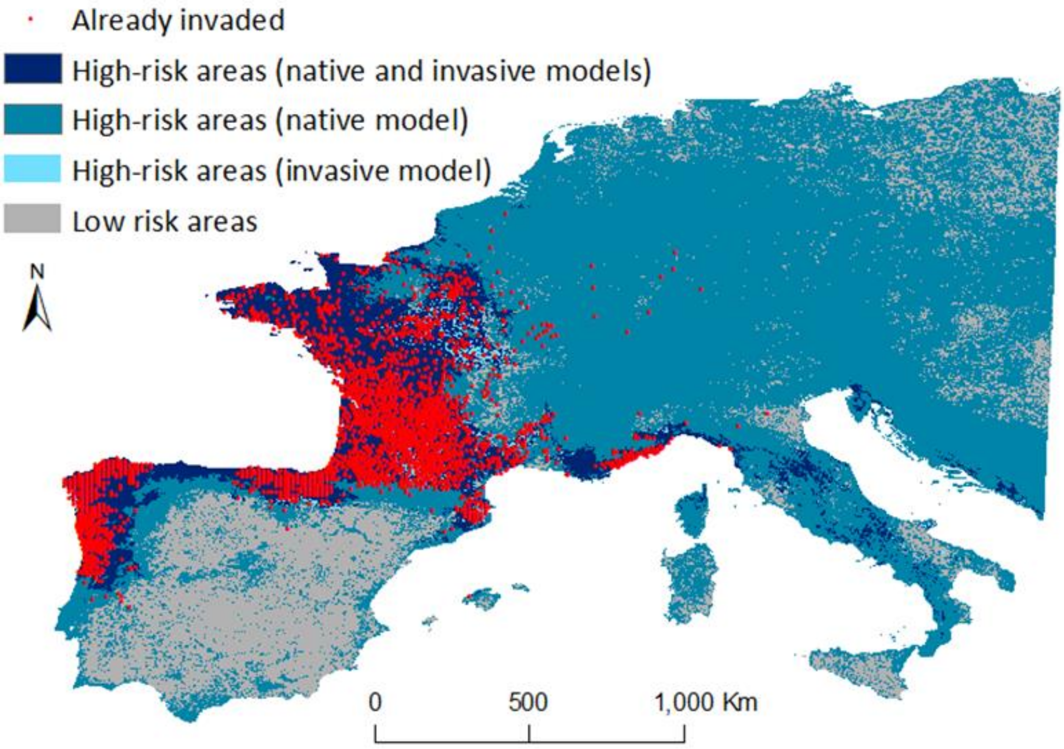

Fig. 1.5 – Risk map highlighting the areas of agreement between the models of the native and invasive ranges of *Vespa velutina*. Distribution models were built in 'biomod2' ('see Methods').

Discussion

Here we showed that the niche of *Vespa velutina* in Europe has probably shifted in the NW of Iberian Peninsula, and that it is essential to consider both native and invasive records in modelling procedures to accurately predict the species' invasive potential. Further, our study revealed that the colonization process by *V. velutina* is still ongoing with a high potential for geographical expansion, as a large extent of Europe with analogue environmental conditions to those of the native niche is still unoccupied.

It has become increasingly important to describe species' niches and understand whether these can change rapidly (niche shifts) or not (niche conservatism) between different geographic areas or time periods (Guisan et al*.* 2014). A genuine shift in the fundamental niche would reflect evolutionary adaptation of a species to novel environmental conditions, whereas occupying new portions of the same fundamental niche does not change the environmental potential of the species (Qiao et al. 2017). Regardless of the clearly different environmental conditions occupied by the two European invasive populations, *V. velutina* is well established in both regions, meaning that the species can successfully invade different environmental envelopes. Differences between environmental conditions in the native range and the P2 invaded range (suggesting niche shift) were particularly clear. However, as the analyses of niche dynamics are necessarily restricted to the environmental space with analogue conditions between native and invaded ranges, we cannot fully assess the use of novel conditions in the invaded range. Still, we can state that, within the same analogue conditions, our estimate of the realized niche of the species in the Iberian Peninsula is different from its equivalent realized niche in its native range, which may help explain its invasiveness. Substantial niche shifts are rare among invaders (Petitpierre et al*.* 2012), and this has been often related with the loss of genetic variability that takes place during all invasion episodes (founder effect: Matute 2013). Furthermore, expansion to novel environmental conditions is frequently the last step in the colonization process as most invasions begin where conditions are similar to those of the native range. In France, the species started to colonize environmental conditions of high stability, and since then has been progressively colonizing the unfilled environmental space. Interestingly, in a very short time (less than ten years) it is possible to assist to significant changes in niche unfilling metric of P1 population. However, it is also possible that other invasive populations (like P2) start from the very beginning of the expansion process to use different environmental conditions from those of the native niche. This reinforces the ability of this species to colonize different environmental envelopes and infer about its invasive potential. For both populations, *V. velutina* experienced measurable changes in environmental niche occupancy but, as would be expected, both invasive niches tend to be more similar to the native niche than would be expected by chance, and niche similarity could not be rejected.

Similar to previous studies (Villemant et al*.* 2011; Bessa et al*.* 2016; Fournier et al*.* 2017; Barbet-Massin et al. 2018), we identified climatic variables as the ones that most contributed to the current distribution of *V. velutina*. Regardless of the adequacy of particular environmental conditions for a species, if they are uncommon on a given area the species can still be limited in its potential to further expand. In our case, by addressing species marginality, we showed that *V. velutina* is using the most common environmental conditions available in Europe. This suggests that the species has all the necessary conditions to continue its expansion process. The maintenance of constant and high values of the metric Maximum Niche Stability for P1 occupied conditions of this population overlap with the core of the species' realized niche in its native range. In contrast, from the very beginning, the population of the Iberian Peninsula thrives on conditions that are not exploited in the native range.

Niche theory tackles a variety of issues, including evolutionary processes, competition and predation dynamics (Hirzel and Lay 2008). Hence, the apparent niche shift of the P2 population must be interpreted with caution. Although the European environmental envelope of the species greatly overlaps with the native one, there are some conditions of the realized niche in the invasive range that are not explored in the native. Therefore, the shift may have resulted from several factors: alleviation of biotic constraints and geographic barriers, adaptation to novel conditions, or under sampling of the species in its native range. Invasive species frequently experience release from biotic interactions and dispersal barriers in their invasion process (Colautti et al*.* 2004). Biotic constraints in the native range (like the presence of parasitoids and pathogens, competition with other hornets or predation) that are not present in the invasive will likely result in the expansion of invasive *V. velutina* to conditions not explored in the native range. Further, if the native dataset is not representative of the full Asian distribution, the entire native environmental envelope might not have been fully captured. This indication of a niche shift may also be simply stochastic (once it depends on the available environmental conditions of the invaded range), or due to a partial filling of the potential native niche in the invaded range, not representing a “true” niche change (Strubbe et al*.* 2013). The most likely scenario is a realized niche shift in P2 population; however, without experimental evidence it is impossible to elucidate the mechanisms driving the observed shift. It is also possible that the species is rapidly evolving and adapting to the novel conditions in the Iberian Peninsula. Although a low level of genetic diversity was detected in the European populations (Arca et al*.* 2015; Quaresma 2019), this does not necessarily cause inbreeding depression and in some cases may lead to local adaptations (Valladares et al*.* 2014). Further studies on the morphology, ecophysiology and plasticity of the European populations will elucidate on their degree of local differentiation.

We detected suitable conditions for *V. velutina* in Europe in areas much larger than those estimated by prior prediction exercises (Ibáñez-Justicia and Loomans 2011, Villemant et al. 2011b, Fournier et al. 2017, Barbet-Massin et al. 2018, 2020, Kim et al. 2021). This is particularly relevant for central and eastern Europe where our models have identified, for the first time, suitable conditions for *V. velutina*. This was expected, as this species is still expanding. As we used a higher amount of available environmental information compared to previous studies, our predictions of expansion potential of *V. velutina* are expected to better reflect the environmental space used by the species (Raes 2012; Carretero and Sillero 2016). In fact, most of the already invaded area in Portugal and the new record of *V. velutina* in Hamburg (Husemann et al*.* 2020) occurred in regions that recent studies identified as unsuitable for the hornet (Fournier et al*.* 2017; Barbet-Massin et al*.* 2018; Barbet-Massin et al. 2020). Barbet-Massin and collaborators (2018) argue that, to predict the hornet short-term potential of expansion, it is better to run species distribution models without accounting for native data. However, such an approach may not capture the entire environmental envelope where the species is able to thrive. In fact, by using only native occurrences, we predicted a large unoccupied extent with suitable conditions for the species in Europe. Another explanation for the narrower expansion area predicted by Barbet-Massin et al. (2018) is that their study is mostly based on the data from France (corresponding to

P1) which used environmental conditions that are relatively similar to those found on the core of the species' native niche. Our study clearly demonstrates that using only part of invasive records has limited predictive power. On the other hand, as the hornet is still expanding and the realized niche is not stable, the invasive model failed to predict most of native occurrences. This may also be an indication that the niche in Europe is currently changing.

The genetic paradox of invasions suggests that the success of invasive species may result from rapid neutral and even adaptative evolutionary changes that do not require high genetic variability (reviewed by Schrieber and Lachmuth 2016). During an invasion the gene pool of the new population is likely much smaller than in the source population (Arca et al*.* 2015). However, Garnas et al*.* (2016) showed that founder effects rarely limit fitness in invasive insects and may in fact be beneficial by purging harmful alleles or increasing additive genetic variance. Indeed, the European invasive process originated from a single founder female in France, was followed by a second colonization process in the north-western Iberian Peninsula, where a second founder effect occurred (Quaresma 2019). However, the different environmental conditions in the Atlantic coastal zone of Iberian Peninsula did not seem to have hampered the invasion (Verdasca et al. 2021b). Apparently, *V. velutina* has a high ability to cope with the challenges presented by the novel European abiotic and biotic features, but the lack of competitors or the enemy release hypothesis (Keane and Crawley 2002) can also justify its success. Little is known about the composition of natural enemies' communities (e.g., predators, parasites) and their potential importance on the regulation of *V. velutina* populations. However, in the Asian tropics and subtropics this hornet faces competition with six other hornet species while in Europe it only competes with *Vespa crabro* (Choi et al. 2012). Identifying such biotic constrains in the native region could be important to predict (and potentially avoid) new invasions, providing essential information for the development of control strategies.

Our results suggest that the invasion process of both invasive populations is likely to continue towards other European countries and that the centre, east and centre-north of Europe are at a high risk of invasion. According to Villemant et al. (2011), other suitable regions for *V. velutina*, located in north and south America and in Australia, may also be vulnerable to invasion if another accidental introduction occurs in these continents. The fact that few (9 %) records of the invasive population are in high-risk areas (that were only predicted by one of the models - native or invasive) is not surprising, since the invasion started recently. In 2017 very few occurrences were detected in British Isles (not dealt in this work), but in the end of 2019 new nests were detected, highlighting the need for attention to the potential of *V. velutina* to rapidly colonise the British mainland, if control strategies are not timely applied (Keeling et al*.* 2017). As *V. velutina* gynes can fly over long distances and as jump-dispersal mediated by humans also occurs (Robinet et al. 2017, Verdasca et al. 2021b), the high-risk regions (south of Italy, Corsica, Croatia and Montenegro) should be vigilant. Hence, action plans should be elaborated by these countries, addressing the expected impacts on native biodiversity, crop pollination, beekeeping activities and public health.

Conclusions

Our approach of combining reciprocal distribution models with analyses of niche dynamics enabled us to assess which areas are most sensitive to invasion while also evaluating for potential niche shifts between invasive and native ranges. This approach is useful to forecast the future of the *V. velutina* expansion in western and central Europe and might be used with success for many other invasives.

Acknowledgments

We thank to ICNF - Instituto da Conservação da Natureza e das Florestas (platform SOSvespa: www.sosvespa.pt/web), Bombeiros Voluntários de Viana do Castelo, Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente and INPN - Muséum national d’Histoire naturelle in Paris for having provided the records of *V. velutina* occurrences from both native and invasive range; European Space Agency for making available the ESA CCI land cover data and user tool. We also thank Margarida Santos-Reis for comments in early versions of the manuscript. Fundação para a Ciência e a Tecnologia (FCT Portugal) provided financial support through the project UIDB/00329/2020 granted to cE3c, and the projects UIDB/50017/2020 and UIDP/50017/2020 to CESAM. M.J.V (PD/BD/128351/2017 and COVID/BD/151632/2021), H.R. (DL57/2016/EEC2018/07) and L.G.C. (LISBOA-01-0145-FEDER-028360/EUCLIPO) were funded by FCT Portugal). LGC was also funded by the Brazilian National Council for Scientific and Technological Development (CNPq. Universal 421668/2018-0; PQ 305157/2018-3). J.A.G. was funded by the Natural Environment Research Council (NERC; NE/T011084/1 and 670 NE/S011811/1). The funding sources had no direct involvement in the study design, or in the collection, analysis and interpretation of data.

2

Invasive hornets on the road: motorway-driven dispersal must be considered on management plans of *Vespa velutina*


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The content of this chapter is published as:

Verdasca MJ, Rebelo H, Carvalheiro LG, Rebelo R (2021) Invasive hornets on the road: motorway-driven dispersal must be considered in management plans of *Vespa velutina*. NeoBiota 69: 177-198. https://doi.org/10.3897/neobiota.69.71352 (IF2020 3,684; Q1 Biodiversity Conservation)

Author Contributions

M.J.V., H.R., L.C., and R.R. conceived the study. M.J.V. gathered the data, ran the models and performed the analyses. M.J.V. produced the first draft of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Invasive hornets on the road: motorway-driven dispersal must be considered in management plans of *Vespa velutina*

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Abstract

Understanding the mechanisms that potentiate the dispersion of an invasive species is essential to anticipate its arrival into new regions and to develop adequate management actions to minimize damage to biodiversity and society. One of the most successful invaders in Europe, the yellow-legged hornet (*Vespa velutina nigrithorax*), is dispersing through self-diffusion and jump dispersal. Using information on species occurrence in Portugal from 2013 to 2018, this study aimed to understand the range expansion trajectory of *V. velutina* and to identify the role of climate, landscape and anthropogenic variables on the two mechanisms of spread. We found that in Portugal the invasion is proceeding faster southwards (45 km/year) along the Atlantic coast than eastwards (20 km/year) where the climatic suitability gradient is more compressed, with jump dispersal playing an important role in this difference and in the acceleration of the invasion process. Dispersal by diffusion was best explained by the annual range of temperature and precipitation of the wettest month, with distance to shrub land also having an important role. Additionally, jump dispersal appeared to be facilitated by motorways, hinting at the role of human-mediated dispersal. Indeed, the number of nests that resulted from this dispersive mechanism were significantly closer to motorways than expected by chance. To prevent the dispersal of *V. velutina* into Mediterranean regions, and in addition to a special attention to the advancing front, early monitoring programs should also target a buffer zone on both sides of motorways, and at freight shipping hubs.

Keywords: climatic gradient, diffusion dispersal, human-mediated dispersal, jump dispersal, motorways, *Vespa velutina*

Introduction

Invasive species can have important environmental and socioeconomic impacts. Knowing the dispersal routes of such species is crucial to anticipate their arrival and define adequate management practices in a timely fashion. Invasiveness (a dynamic property of the species) and invasibility (a property of a location that can change with anthropogenic disturbance, seasons, climate change), two key components of biological invasions, are thought to be primarily determined by species' dispersal ability and habitat suitability, respectively (Brooks 2007). Invasiveness is thus mediated by life strategies (Pysek and Richardson 2007), whereas invasibility is related to local conditions at the site, habitat or landscape levels (Vicente et al. 2010). For a risk assessment of the vulnerability of a site to an invasive species it is therefore important to have detailed information on both the species' ecological niche (*i.e.*, static information, such as climatic tolerance) and movement ability, which will regulate if and when suitable areas away from the site where the invasion started will be reached (*i.e.*, dynamic information). Including such information on distribution models of invasive species can help to distinguish suitable habitat that is, or can be potentially occupied, from suitable habitat that is inaccessible (Miller and Holloway 2015).

As different environmental conditions and landscape heterogeneity may accelerate or hamper the invasive process (Hastings et al. 2005), it is also important to identify the patterns of range expansion. Three different trajectories of range expansion versus time can be considered: linear (Andow et al. 1990), biphasic (with an initial shallow slope followed by a steep linear slope), and accelerating with time, quickly reaching the saturation phase (Shigesada et al. 1995). The three expansion patterns occur through either self-mediated dispersal from an initial location (diffusion; Lockwood et al. 2007) or jump dispersal into regions relatively far from the core distribution area without colonizing the regions in between, leading to the establishment of nascent colonies (Suarez et al. 2001), "outposts" hereafter. If outposts establish in environmentally suitable areas the species can continue the expansion process from there, accelerating it. When a species spreads through both processes (natural diffusion and jump dispersal), stratified diffusion occurs (Hengeveld, 1989; Suarez, et al. 2001). In such a process, the initial range expansion occurs mainly by diffusion, but as the area of the founder population expands, new nests created by long-distance events accelerate range expansion in later phases (Shigesada et al. 1995; see also some insect examples in Andow et al. (1990)).

Insects are the dominant group among non-native terrestrial invertebrates in Europe (Roques et al. 2009). Social insects, in particular social Hymenoptera, are likely to become successful invaders due to their excellent dispersal abilities, high reproductive rates in an annual life cycle, broad diets and habitat ranges, colony initiation by a single inseminated queen, and to their close association with human transportation with relatively low probability of detection (Moller 1996, Beggs et al. 2011). These characteristics favor the invasiveness of eusocial insects, giving them a plasticity of responses that allow their survival and establishment in new environments. Impacts of invasive social insects include changes of ecosystem functions, competitive displacement of native species, hybridization with native species, threats to human or animal health through stings or the transmission of pathogens (Schneider et al. 2004, Lester and Beggs 2019). One example of an invasive eusocial insect that is currently spreading in Europe is the yellow-legged hornet, (*Vespa velutina nigrithorax* Lepeletier, 1836), an aggressive predator of honeybees and wild pollinators, that is putting honey and agricultural production at risk (Monceau et al. 2014, Verdasca et al. 2021a). The high dispersal ability of this invasive combined with the lack of true competitors and with the availability of food resources in Europe, has been favoring its expansion in this continent. After the accidental introduction of *V. velutina* in France in 2004, probably by a single female originating from temperate south-eastern China (Arca et al. 2015), the invasion spread to other European countries - Spain, Portugal, Belgium, Italy, Germany, United Kingdom, Netherlands and Luxembourg, being the hornet now considered an invasive alien species of concern in the European Union (European Commission 2016). In 2011, a new invasion was detected in the north of Portugal (Grosso-Silva and Maia 2012) that then expanded southward into the center of Portugal (Carvalho et al. 2020) across a climate gradient between Temperate and Mediterranean bioclimates (Rivas-Martínez et al. 2017, Sayre et al. 2020) and northward into Galicia (Spain) (Rodríguez-Flores et al. 2019). The current European distribution of this species, mostly concentrated along the Atlantic coast, aligns with the climatic preferences predicted by Villemant et al. (2011). As a central place forager, workers of *V. velutina* optimally explore foraging areas 500 m to 800 m from their nests, although the maximum homing ability (the maximal distance an individual is able to travel on its way home) of the hornet is much greater (5000 m; Poidatz et al. 2018).

The spread of *V. velutina* in Europe has been considered a stratified diffusion process, including a mixture of natural diffusion and jump dispersal events (Bertolino et al. 2016, Robinet et al. 2017, Lioy et al. 2019). Jump dispersal may occur by two different processes: self-dispersal of gynes (queens of the next generation), which are able to fly long distances on their own (18 km/day in flight mill experiments - pers. comm., Dr. D. Sauvard, INRA, France to the authors of Robinet et al. 2017), or through human-mediated dispersal (Robinet et al. 2019). Due to the difficulty in disentangling these processes, only occurrence points found more than 78 km (yearly spread rate in France) from their nearest neighbors were considered to result from long distance dispersal events by Robinet et al. (2017). However, Bertolino et al. (2016), working in Northwest Italy, considered a much shorter distance as the limit over which human-mediated dispersal was considered the most likely explanation for jump dispersal events (yearly spread rate in Italy = 18.3 ±3.3 km). Besides the different methodological approaches (including the scale of analysis), the differences between the two studies are attributed by Bertolino *et al.* (2016) to the Italian mountainous territory, when compared with the largely flat north western and central France, while Robinet et. al. (2017) justify the high spread rate in France by the ability of founder females of flying long distances in flight mill experiments.

Precipitation and temperature are thought to be the strongest predictors of the invasive range of *V. velutina* (Villemant et al. 2011b), with land-use also playing an important role at a regional scale (Bessa et al. 2016). This hornet is now colonizing the Mediterranean peninsulas, and therefore being exposed for the first time to a Mediterranean climate. As the species spreads along the transitional temperate-Mediterranean climate regions, it is important to assess the extent of suitable area at a finer resolution, as well as to understand how the invasion process is unfolding and the role of human-mediated dispersal. A recent study highlighted the need to identify the colonization pathways and plan management approaches to halt the spread of *V. velutina* in Portugal (Carvalho et al. 2020). At a regional scale, the identification of a limited number of key variables explaining the dispersal and establishment success of *V. velutina* can facilitate the creation of effective preventive and control measures. In this study, we incorporated land cover and anthropogenic drivers to predict the risk of invasion by *V. velutina* within the transitional temperate-Mediterranean zones and infer colonization pathways. More specifically, we (i) assessed the roles of diffusion and jump dispersal on *V. velutina* expansion into Mediterranean-type climates and (ii) identified which environmental attributes are most influential on the direction and speed of its dispersal.

Material and Methods

Nest occurrence data in Portugal

For this study, we focused on the secondary introduction event of the hornet in Portugal and used all the available Portuguese presence data of *Vespa velutina* (8610 records of nests, from 2013 to 2018). Data was obtained from Bombeiros Voluntários de Viana do Castelo and from the online platform *STOPvespa* (http://stopvespa.icnf.pt/), which is managed by the Instituto da Conservação da Natureza e das Florestas (ICNF) and aggregates all validated Portuguese records of *V. velutina* nests that were previously registered in the platform by citizens. To avoid spatial autocorrelation, we reduced the number of occurrence data points through the spatially rarefy occurrence data tool (pixel size resolution: 300m) in SDMtoolbox (Brown 2014) in ArcGIS 10.4.1 (ESRI 2016); this resulted in a total of 7847 points. To calculate the continuous distribution area of *V. velutina* (2013 to 2018) we drew a 5 km buffer around each nest. The workers of *V. velutina* probably forage less than 1000 m from their nest, and this buffer of 5 km corresponds to the estimated maximum homing ability of the species, since few workers have the ability to perform long trips (Poidatz et al*.* 2018). Moreover, and according to (Lioy et al. 2019), most new nests (>90%) in NW Italy were located within this radius to their nearest source of the previous year. All the contiguous buffers were aggregated to establish each year's continuous distribution; all records outside the continuous area of the previous year were considered expansion nests. From these expansion nests, those located within the new continuous area were considered to result from diffusion dispersal and those found outside this new limit were considered outposts (*i.e.*, an evidence of jump dispersal, through either self-mediated or human-mediated).

Range expansion

To identify the range expansion trajectory, we calculated the annual increment in the continuous area. The number of new outposts per year was counted and their contribution to the overall expansion was estimated by identifying those outposts that could have functioned as a source for other nests. To ascertain a possible origin for each expansion colony and outpost, we compared its distance to the nearest edge of the continuous area and to the nearest outpost of the previous year; all the records for which the difference between both distances was lower than 5 km (corresponding to 1419 records) were discarded, being considered of non-attributable origin.

The yearly expansion resulting from diffusion dispersal along the N-S and W-E axes was estimated by measuring the distance to the south and east between consecutive limits of the continuous distribution area. The number of new nests established exclusively to the south and east from the previous continuous limit was counted and we identified how many of these were outposts. Yearly, for each outpost, its distance to the nearest source of the previous year was measured. To test for an acceleration of both types of expansion, the slopes of the relationships between these distances and year was compared with zero.

Environmental drivers

Assuming that the same variables influencing distribution have the potential to promote its dispersal, we considered three climatic and eight land cover and anthropogenic variables (Table S2.1). Variables in this study were adapted from Bessa et al. (2016), with the following changes: i) we excluded NDVI and isothermality, ii) we used the distance to each specific land cover class instead of its percentage because using distances assures better performance for landscape features (Rainho and Palmeirim 2011), iii) we incorporated the classes “distance to forest” and “index of human influence” and iv) we included predictors related to the distance to linear structures (motorways and railways). To avoid collinearity, we inspected if there were highly correlated variables ($r \geq 0.70$; Dormann *et al.*, 2013) (Table S2.2 a,b,c). For this analysis we used three datasets: i) bound records (the series of points defining a minimum convex polygon that represents the leading edge of the continuous invaded area for each particular year, presumably resulting from dispersal by diffusion), ii) all outposts (resulting from jump dispersal) and iii) > 18 km outposts, representing the subset of outposts located more than 18km (distance travelled in flight mill experiments - pers. comm., Dr. D. Sauvard, INRA, France to the authors of Robinet et al. 2017) from the continuous area of the respective year, for which there is probably a higher contribution of human-mediated dispersal.

Data analyses

To assess which variables influence the dispersal of *Vespa velutina* for each of the three datasets we used generalized linear mixed models (GLMM) with the package ‘lme4’ (Bates et al. 2015) in R (Core Team 2019). We began by running full models with climatic, land cover and anthropogenic drivers simultaneously. As climatic variables are acknowledged to be the main factors influencing the species distribution across varying spatial scales (Pearson and Dawson 2003), we decided to run additional models with land cover and anthropogenic variables only, in an attempt to find other possible predictors at a regional scale. For each dataset, we set the dependent variable as the minimum distance of the records to the continuous area of the previous year (we discarded three records that were located less than 5 km from an outpost established in the previous year, as that could be an offshoot of that outpost). To detect collinearity between explanatory variables we used the Vifstep function in the usdm R package (Naimi et al. 2014) to calculate the variance inflation factor (VIF) and excluded the variables in models with a VIF value greater than the threshold (th=3). A variable “year” was included as a random effect to account for yearly climatic variations that may affect the dispersal of the hornet. We then selected the best model (using the Akaike Information Criterion – AIC) with the dredge R function, and generated average estimates of the effect of each variable using the model.avg R function (models with delta AIC values < 2) from the MuMIn package (Bartón 2009). The results were plotted using the package visreg (Breheny and Burchett 2017).

As we verified that one anthropogenic predictor (distance to motorways; see Results) was influential on hornet jump dispersal we decided to further explore the data. First, we inspected if the outposts’ distance to motorways was random, *i.e.*, we tested whether motorways may be acting as drivers of jump dispersal. To accomplish this, we generated a twin random point for each outpost, located at the same Euclidean distance to the continuous distribution area as the outpost, and compared their distance to motorways with a paired samples Wilcoxon test. Second, for both data sets of outposts we ran another GLMM model, but this time with the distance to the entire road network to inspect the relative importance of each road category in hornet jump dispersal.

To generate a risk map of *V. velutina* dispersal and identify regions most at risk of imminent invasion, we combined information from suitable areas (regions with rainy winters and pleasant summers, mainly located along the Atlantic coast – chapter 1) with the geographical information of the significant dispersal predictors of a model with climate, land cover and anthropogenic variables. These predictors were combined according to their estimates to produce a dispersal map. To define the risk areas around motorways, we analyzed the pattern of the number of outposts as a function of distance to motorways. As the number of new nests established alongside the motorways decreased linearly with distance up to 17 km from the highway (after that there was no apparent relation with distance - see Results), we calculated this relation to estimate the width of the areas that contained 50% and 75% of the outposts.

Results

From 2013 to 2018, the area occupied by *Vespa velutina* in Portugal experienced a 25-fold increase (from 845 km^2 to 20,561.26 km^2) in a linear manner without acceleration or deceleration (Fig. 2.1). Expansion was much faster along the north-south axis (45 km/year) than along the west-east axis (roughly 20 km/year), regardless of taking place in temperate (in 2014 and 2015) or Mediterranean climate regions (since 2016) (Fig. 2.1 and Fig. 2.2).

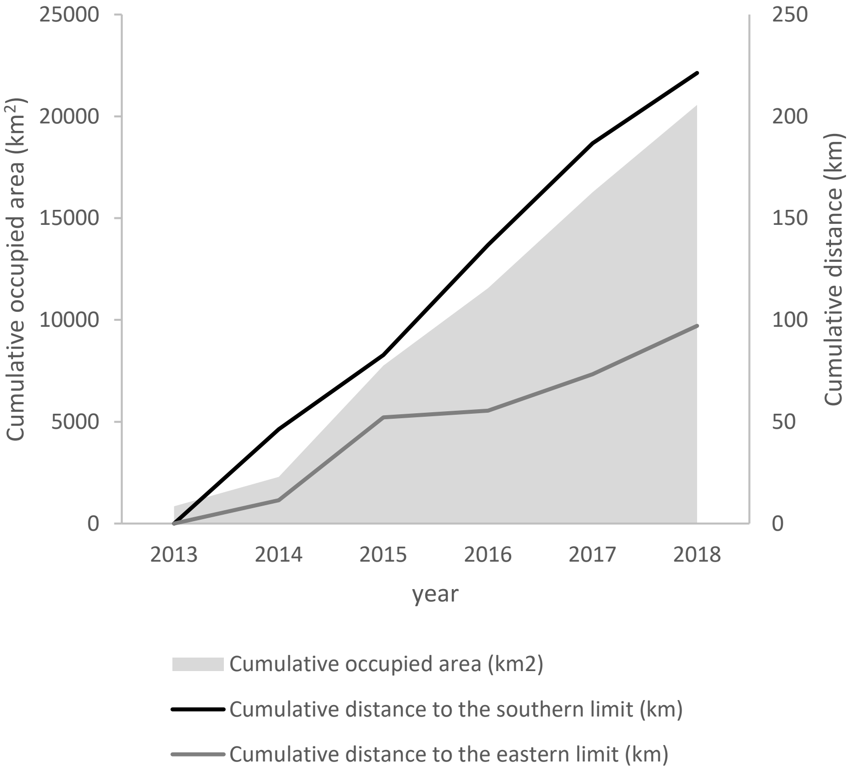

Fig. 2.1 – Annual expansion of *V. velutina*. The picture depicts the two cumulative linear distances (right axis) between the invasion origin and the successive limits of the invasion front to the south (black line) and east (dark grey line). The southern and eastern limits of each year were measured by simply drawing a tangent to the southernmost point and the easternmost point, respectively. The left axis refers to the yearly cumulative invaded area (in km^2) resulting from diffusion dispersal and depicted as a gray area.

Range expansion

The number of outposts varied across the different years from a minimum of 4 in 2016 to a maximum of 46 in 2015. Such outposts had a very high importance for the expansion of the hornet. Indeed, the number of new expansion nests that were located near the outposts established in the previous year was higher than the number of new nests found near the previous continuous limit in all years except 2017 (Table 2.1).

In the first three years (2014-2016), the mean distance of new nests to the nearest outpost was lower than the distance to the continuous area (Fig. S2.1). The reverse scenario occurred in 2017 and 2018, when almost all outposts were established in Mediterranean-climate regions. As the core distribution area expanded to the south and east, some outposts that gave rise to new nests nearby were engulfed into the continuous distribution area (*i.e.*, coalescent colony model; Fig. 2.2).

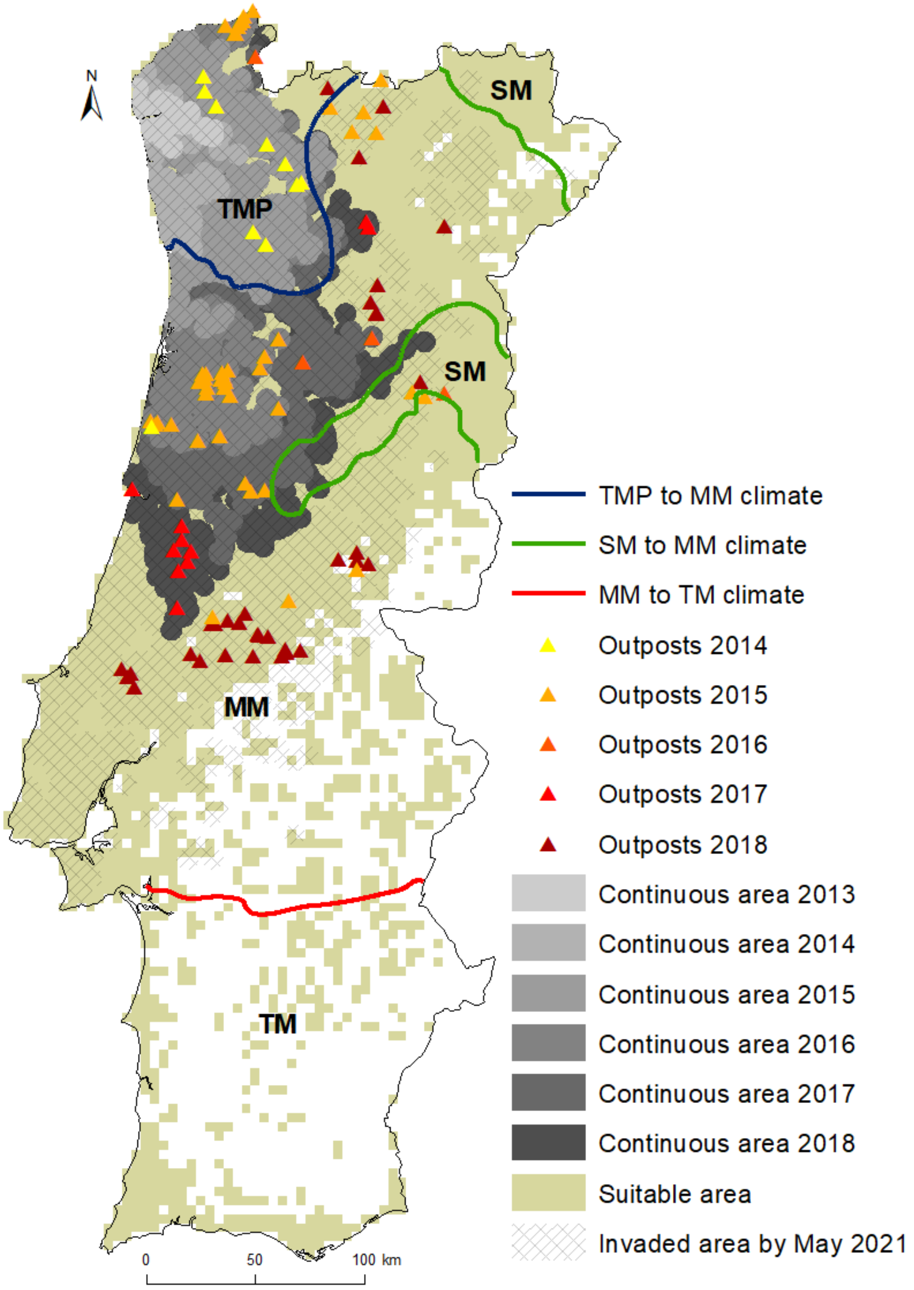

Fig. 2.2 – Invasion pattern of *Vespa velutina* in Portugal between 2013 and 2018 along the climatic gradient (TMP – Temperate climate, SP – Supramediterranean climate, MM – Mesomediterranean climate and TM – Thermomediterranean. The figure depicts the continuous distribution area of *V. velutina* in each year and the location of the outposts (points with the highest dispersal distances; see methods) by year. The suitable area for the species was retrieved from Verdasca et al. (in review; chapter 1). The current invaded area (by May 2021) is also shown.

Outposts established southwards were over 3 times more frequent than those established eastwards (Table 2.2). There was a decrease in the number of successfully established nests since 2016, especially southwards (Table 2.2). The slope of the relationship between time and dispersal distance to the south and east was not significantly different from zero, for both types of dispersal (diffusion or jump dispersal) (Table S2.3). Most outposts (90%) were located more than 18 km from the continuous area of the previous year.

Table 2.1 – The number of new nests and outposts of *V. velutina* (outside the continuous distribution area of the previous year), between 2014 and 2018.

Year	Number of new nests located nearer the continuous area of the previous year	Number of new nests located nearer an outpost of the previous year	Total number of outposts	Number of "> 18 km outposts"
2014	55	83	10	7
2015	418	491	46	46
2016	69	103	4	3
2017	230	163	10	8
2018	165	174	33	29
Total	**937**	**1014**	**103**	**93**

Table 2.2 – Annual number of new occurrences registered exclusively southwards and eastwards.

Year	**Number of new records exclusively southwards**	**Number of new records exclusively eastwards**
2014	128 (+ 1 outpost)	1 (+ 1 outpost)
2015	120 (+ 23 outposts)	330 (+ 6 outposts)
2016	225 (+ 1 outpost)	1 outpost
2017	123 (+ 8 outposts)	19 (+ 2 outposts)
2018	24 (+ 20 outposts)	8 (+ 3 outposts)
TotaL	**620 (+ 53 outposts)**	**359 (+ 13 outposts)**

Environmental drivers

Models with both climatic and land cover variables explained more variability of the dispersal patterns of *Vespa velutina* than models solely with climatic or land cover variables (Tables S2.4, S2.5 and S2.6). A climatic variable - precipitation of the wettest month - was the single variable selected in all the models. Temperature annual range was the only additional climatic variable identified as a driver of diffusion dispersal (Table 2.3, Fig. S2.2; see also Table S2.7 for a model with climatic and land cover variables). In the models with land cover variables only distance to shrub land (plus natural meadows) was identified as influential on diffusion dispersal; however, for jump dispersal, distance to motorways was the only significant predictor (Table 2.3, Fig S2.2). Distance to the entire road network (instead of distance to motorways) had no effect upon either dispersal pattern (Table S2.8). In both datasets (all outposts and the subset located more than 18 km from the continuous area of the previous year), outposts were significantly closer to motorways than expected by chance (all outposts: 103 pairs compared, V = 1820, p-value = 0.0024 (also see Fig. 3.2); >18km outposts: 93 pairs compared, V = 1306, p-value = 0.0004). We found that the number of outposts decreased in a linear function (y = -0.6103x + 10.61) with distance to motorways in a 17 km-width strip along the motorways (Fig. 2.3 – dark grey bars). Further away, there is no apparent relationship with distance. In fact, 50% of the new outposts were located within a 6 km wide buffer zone alongside motorways and 75% up to 12km from these linear structures.

Table 2.3 – Effects of distance to land cover categories and linear features (1) and climate (2) on the dispersal of *V. velutina*". Distance to the previous continuous distribution (as a proxy of dispersion) was used as dependent variable in the GLMM. The results were obtained by averaging model predictions with ΔAIC<2.

	Multivariate model (bound records)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
1. Land cover Model	(Intercept)	21670	7982	8088	2.68	0.007	**
	Distance to shrubs and natural meadows	13.23	4.50	4.61	2.87	0.004	**
	Distance to riparian galleries	-1.14	1.75	1.77	0.64	0.522	
	Distance to motorways	-0.13	0.24	0.24	0.55	0.580	
	Index of Human Influence	-93.37	171.9	173.9	0.54	0.591	
	Distance to urban areas	-1.25	4.48	4.56	0.27	0.784	
	Distance to crops	-0.03	0.43	0.45	0.06	0.951	
	Distance to forest	-0.06	1.73	1.77	0.04	0.972	
	Multivariate model (all outposts)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
	(Intercept)	54507.91	8590.37	8694.48	6.27	< 2e-16	***
	Distance to forest	-30.61	24.87	25.05	1.22	0.222	
	Distance to riparian galleries	4.35	4.24	4.27	1.02	0.308	
	Distance to motorways	-0.93	0.29	0.30	3.14	0.002	**
	Distance to crops	-0.49	2.16	2.18	0.23	0.822	
	Distance to urban areas	0.76	3.55	3.57	0.21	0.831	
	Distance to shrubs and natural meadows	-0.21	1.90	1.92	0.11	0.912	
	Index of Human Influence	-0.28	72.54	73.49	0.00	0.997	
	Multivariate model (> 18 km outposts)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
	(Intercept)	58370	8701	8810	6.626	<2e-16	***
	Distance to forest stands	-16.80	22.77	22.93	0.73	0.464	
	Distance to riparian galleries	2.92	3.94	3.96	0.74	0.462	
	Distance to motorways	-0.72	0.33	0.33	2.17	0.030	*
	Index of Human Influence	-41.95	139.60	140.80	0.30	0.766	
	Distance to urban areas	1.14	4.51	4.56	0.25	0.802	
	Distance to shrubs and natural meadows	-0.46	2.57	2.60	0.18	0.859	
	Distance to crops	-0.09	1.24	1.25	0.08	0.940	
2. Climatic Model	**Multivariate model (bound records)**	**Estimate**	**Std. Error**	**Chisq**	**t value**	**Pr(>Chisq)**	
	(Intercept)	40882.79	18700.1		2.19		
	bio7 - Temperature annual range	188.05	65.92	8.14	2.85	0.004	**
	bio13 - Precipitation of wettest month	-363.64	97.93	13.79	-3.71	0.000	***
	Multivariate model (all outposts)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
	(Intercept)	141041.22	23165.9	23424.59	6.02	<2e-16	***
	bio13 - Precipitation of wettest month	-678.49	100.71	101.99	6.65	<2e-16	***
	bio7 - Temperature annual range	31.22	75.37	76.02	0.41	0.681	
	Multivariate model (> 18 km outposts)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
	(Intercept)	136405.5	26392.8	26729.3	5.10	3E-07	***
	bio13 - Precipitation of wettest month	-630.56	124.99	126.77	4.97	7.00E-07	***
	bio7 - Temperature annual range	30.27	81.27	82.11	0.37	0.712	

The map of dispersal risk produced (based on the model with climatic and landcover variables) evidenced that some isolated suitable areas for *V. velutina* in the south of the country are at risk since they are connected by motorways to other suitable regions (Fig. 2.4).

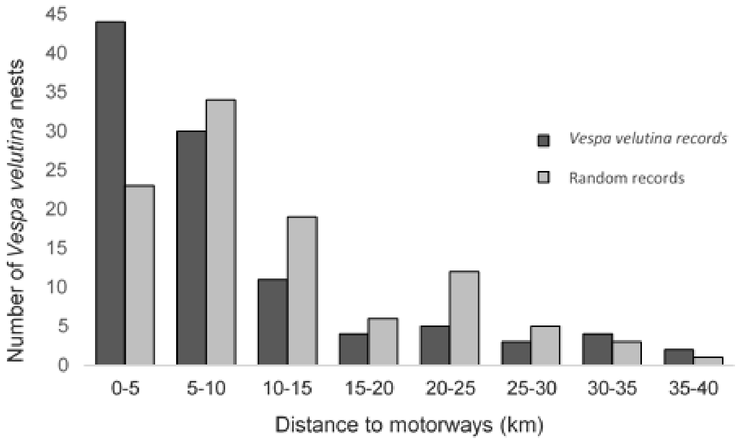

Fig. 2.3 - Distribution *Vespa velutina nigrithorax* outposts (dark gray) and random points (light grey) according to the different classes of distance to motorways (km). The estimated linear function found up to 17 km is “No. nests = - 0.6103 * (Distance to motorways) + 10.61”.

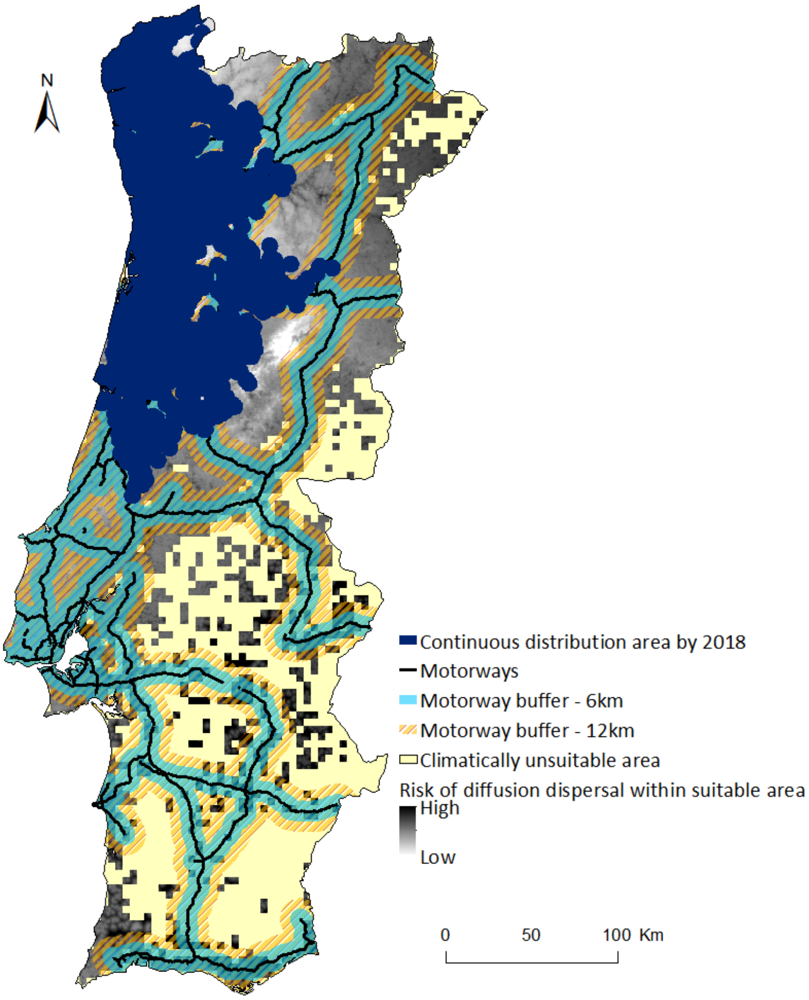

Fig. 2.4 - Risk of dispersal of *Vespa velutina nigrithorax* in Portugal evidencing the buffers alongside motorways where dispersal is likely to be mostly human-mediated. The unsuitable area for the species (Verdasca et al., in review; chapter 1) is depicted in a pale yellow. Almost all the isolated suitable areas located in the south of the country are also at risk as they are connected by motorways to other suitable regions.

Discussion

Major findings

The invasion of *Vespa velutina nigrithorax* is occurring at a slower pace in the northwest of the Iberian Peninsula (spread rate of approximately 45 km/year to the south and 20 km/year to the east) than in other temperate macroclimate regions (*e.g.*, France), but faster than in other Supramediterranean climates (*e.g.*, Italy). In the first few years of the invasion the number of new established nests was much higher near outposts than near the continuous distribution area, an indication that jump dispersal played an important role in the acceleration of the invasion process. Besides climate (namely, precipitation of the wettest month and the annual range of temperature), we found the distance to shrub lands to be influential in the dispersal of *V. velutina*. This finding adds new information to a previous study which also showed that land-use (namely, percentage of agricultural fields) has an important role in the expansion of this species at regional scales (Bessa et al. 2016). We also revealed that one anthropogenic driver (motorways) was important for the jump dispersal events of this flying insect, highlighting the role of these linear infrastructures in accelerating the natural invasion dynamics of *V. velutina* and the need to reinforce early monitoring programs in a 6 km wide buffer around motorways.

Range Expansion

From the initial propagule found in the north of Portugal (near the coast), self-mediated dispersal has been occurring faster towards the south than towards the east. The western Iberian Peninsula encompasses different bioclimatic belts (Mesotemperate, Supramediterranean, Mesomediterranean and Thermomediterranean, Rivas-Martínez et al., 2017) that spread more along the North-South axis near the Atlantic coast than along the West-East axis. In Portugal, *Vespa velutina* is therefore faced with two transition zones, differing in extent, from temperate to Mediterranean climates, as its expansion is predominantly occurring along the Atlantic coast. To better predict the risk of invasion in the short run, it is important to disentangle the spread rate across different climatic gradients. For instance, the average rate of *V. velutina* expansion here identified (45 km/year to south and 20km/year to east) is different from the one recently estimated for Portugal (37.4 ± 13.2 km/year, but considering all the directions, and therefore an intermediate value) (Carvalho *et al.* 2020). We believe that it is important to refine the estimates, as the spread rate of this invasive hornet is clearly not uniform across Portugal, and this same invasion process may occur in other temperate-Mediterranean transition zones. We acknowledge that since our records are reported by citizens, a bias in *V. velutina* detection may be occurring due to more identifications in areas with higher population density (i.e., along the coast). However, this is a very mediatic species in Portugal, and most people are aware of this and its impacts on beekeeping, agriculture, and public health. Despite the lower population density in the eastern part of the country, there are still some important cities, numerous small villages and, even more important, more beekeepers in these rural regions. Given that we used the outermost records for each year (and not the density of records) for most of our estimates, we think that the major patterns detected, such as the differential expansion along the North-South and West-East axes, are barely affected by differences in human density. We did not find a difference in the distance of establishment of new nests between the two directions; however, there was a substantial difference in the number of new nests, as those established southwards were twice the number of those established eastwards. The climatic transitions are more abrupt towards the east, where the hornet is now facing Mediterranean climatic conditions (*i.e.*, drier, higher range of temperatures), which may explain why the species has more difficulty in establishing new nests in this direction. This may be the reason for the decrease in the numbers of established nests since 2016 and supports the importance of climate for the expansion rate of this hornet. In fact, the expansion area is not increasing exponentially as would be expected if diffusion was occurring equally across all directions. In France, the species spread rapidly toward the northeast and not so much to the south (Robinet et al. 2019). In Portugal, the rate of expansion was lower in 2018, potentially due to the major and uncontrolled wildfires that occurred in 2017 precisely over the distribution limit of *V. velutina* in that year. Indeed, by December 2019, the spread rate towards the south was again near 50km/year (see http://stopvespa.icnf.pt/ by ICNF; also check the invaded area in Portugal by May 2021 in Fig. 2.2). Extrapolating to other temperate-Mediterranean transition zones, such as the Italian and Balkan peninsulas, the rate of expansion and invasion pattern may be similar.

Roles of diffusion and jump dispersal

As in other countries, *Vespa velutina* in Portugal is dispersing by both diffusion and jump dispersal. This same pattern was noticed in France (Robinet et al. 2017) and Italy (Bertolino et al. 2016, Lioy et al. 2019), as well as for other social Hymenoptera (like the Argentine ant; Suarez et al. 2001). The frequency and distance of jump-dispersal events are thought to be stochastic, and therefore difficult to predict. For species that spread through stratified diffusion, the distance and rate at which new foci are created through jump dispersal may be more important than the rate of spread through diffusion from established foci (Suarez et al. 2001). Here, the successful long-distance dispersal events played an important role in the expansion of *V. velutina* in almost every year after its establishment, as the number of new nests was higher near outposts than the boundary of the continuous distribution area. These results support a coalescent colony growth model, similar to prior studies that found outposts to accelerate range expansion (Shigesada et al. 1995). Previous studies from Italy (Bertolino et al. 2016, Lioy et al. 2019) found the dispersal of *V. velutina* to be hindered by high mountain ranges (above 700m), and therefore argued that this may be one of the main reasons for the low spread rate in Italy (18 km/year: Bertolino et al. 2016) compared to France (78 km/year: Robinet et al. 2017). Spread rates similar to those in Italy were registered in Korea (10-20 km/ year), although the low spread rate there may be due to competition with six other hornet species (Choi et al. 2012a). Nonetheless, the constant spread rate observed so far in Portugal may begin to decrease southwards when the species reaches Mediterranean climates. Although all outposts were located within the suitable area for the species, *V. velutina* is not (yet) in geographical equilibrium, since, according to our former work, there are still suitable areas to the south and east (Verdasca et al., un review; chapter 1). This apparent limit on the establishment of outposts corroborates our estimates about the adequate areas for the species; however, as the species reaches its estimated limits, it is important to assess how robust they are, as the colonization of adjacent areas, or even the adaptation to novel environmental conditions is possible. If these limits hold, this means that jump dispersal will be the only dispersal mechanism allowing the species to reach the isolated suitable areas in the south of the country.

Influential environmental attributes on the direction and speed of Vespa velutina dispersal

The dispersion of *Vespa velutina* is affected by precipitation and temperature gradients, a result that is similar to those of other studies that modelled the hornet's bioclimatic niche (Villemant et al*.* 2011; Verdasca et al., in review; chapter 1). Besides precipitation of the wettest month and the annual range of temperature, distance to vegetated, but treeless landscapes (covered by shrubs and natural meadows) seem to favor diffusion dispersal. As shrub lands provide a wide variety of nesting sites and food resources for wild pollinators (Chaplin-Kramer et al. 2011), hornets will probably need to fly over longer distances until such pollinator suitable habitats can be reached. In regions that are climatically suitable, the presence of shrubs may thus reduce hornet dispersal. However, shrub land cover is probably not related to the large difference between the eastward and southward rate of expansion, as this habitat is regularly found across the suitable area for the species in Portugal.

Precipitation in the wettest month, and motorways, were the only factors identified as drivers of jump dispersal, but the role of motorways in the dispersal of the hornet was only detected when the climatic predictors were not included in the models. This is in line with the scale dependencies outlined by Pearson and Dawson (2003) - different processes are more important at different scales i.e., at a continental scale, climate can be considered the dominant factor, whilst at more local scales factors including topography and land-cover type become increasingly important. Further down the hierarchy, if conditions at higher levels are satisfied, factors including biotic interactions and microclimate may become significant (for details on hierarchical modeling framework see Pearson and Dawson (2003)).

Motorways facilitate jump dispersal

The fact that motorways were important predictors of outposts is an indication that they may have resulted from human-mediated dispersal. Yet, as motorways are heavily used by people, a potential bias in the detection of nests near these human infrastructures may have occurred. However, most motorways pass through remote places with low population density, and people cannot stop their cars over vast extensions. Therefore, it is unlikely that nest reports come from people using the motorways. Jump dispersal events were predicted by motorways, but not by all roads, railways, or the index of human influence, a variable highly correlated with human population density (e.g., cities). This is an indication that the establishment of outposts is probably mediated through the movement of vehicles and goods, such as wood products and bark or man-made goods (e.g., ceramic pottery associated with garden trade), which in Portugal occurs mostly through the motorways. These products provide suitable refuges for hibernating inseminated *V. velutina* queens (Marris et al. 2011); indeed this was the most probable route of incursion of *V. velutina* in Europe, on pots imported from coastal China, near Shanghai. However, it is also plausible that a dispersing gyne may simply land on a car that then travels a good distance away and starts a nest there. As *V. velutina* gynes can fly over long distances and generate stochastic patterns of spread similar to those resulting from human-mediated dispersal (Robinet et al. 2017), it is probable that some of the records may have originated from self-mediated dispersal. However, the overall detected effect of motorways regardless of the distance group considered, together with the decreasing trend in nest abundance as one proceeds away from the motorways, are difficult to explain through self-dispersal alone.

The association of nest establishment with motorways was only found for outposts (50% and 75% of them established within a 6 km and 12 km wide buffer zone alongside motorways, respectively), and not for records that originated from diffusion dispersal. Our findings corroborate a previous study in Italy (Porporato et al. 2014) where a high number of the observations and captures of *V. velutina* in bait-traps were recorded near highways, emphasizing that freight traffic can contribute to the transport of this species far from the invasion front. The fact that Bessa and collaborators (2016) did not find any relation with the road network could be due to the very restricted region that was used in their study (roughly 10% of the area that we used here). In France, Robinet et al. (2017) used human population density as a proxy for trade to test jump dispersal, not taking into account the road network, and concluded that the rapid spread of the hornet may not be necessarily mediated by humans. So, it is possible that long-distance dispersal events that occurred in France may also have contributed to unintentional introductions via motorways.

Despite it being extremely difficult to provide evidence for early introductions, other social insects have also probably been transported accidentally by humans over long distances since the establishment of long-distance trade routes (Bertelsmeier 2021). For example, New Zealand had no social wasp species prior to human colonization, but over the last century has been invaded by several species of social wasps (Lester and Beggs 2019). Indeed, *Vespula germanica* and *Vespula vulgaris*, both native from Eurasia, have become widespread throughout the New Zealand causing major impacts to native biodiversity (Lester et al. 2014). In Argentina, where *V. germanica* is also invasive, the observed stratified geographical expansion pattern (which frequently exceeds 30 km per year, although faster to south) does not match the observed queen dispersal abilities (only a few hundred meters naturally to find nest sites), suggesting that human-aided transport of hibernating queens is the central driver of the current distribution of these wasps in the country (Masciocchi and Corley 2012). At more local scales, the anthropogenic influence on the spread of invasive insects was also demonstrated. For example, the distance to railroad tracks influenced the spread of the invasive termite species *Reticulitermes flavipes* (Perdereau et al. 2019).

Implications for the management of Vespa velutina invasion process

Identifying pathways that facilitate the dispersal of invasive species is essential for informing efforts to contain invasions (Suarez et. al. 2001). To be successful, every invasive species control program must consider the probability of detecting the species and the cost of the process. In this work, we showed that 50% of the presumed new nests resulting from human-mediated long-distance dispersal established within a 6km wide buffer along motorways. To raise this proportion to 75 %, the buffer must be increased to 12 km, which represents an almost 70% increase in the area to be surveyed. Based on results here, effective measures to contain *V. velutina* invasions should include early monitoring programs in a buffer of 5 km (the maximum homing ability that few hornet workers can reach: Poidatz et al*.* 2018) around the continuous distribution area of the previous year, and 6 km (ideally 12 km) around motorways. If the climatic conditions are met, the vicinity of the main roads is susceptible to be colonized faster through human-mediated transport. Even in highly fragmented habitats, the main roads can connect isolated suitable areas. For instance, in the regions at risk in southern Portugal, the area to be surveyed can be limited only to climatically favourable regions that are reachable by highway. This is particularly relevant in southern Portugal where the isolated fragments of suitable landscape are economically very important for beekeeping activities. The early detection and control of nascent populations in these areas may be a good way to manage its spread, rather than focusing efforts on established invasion fronts. Local outreach activities, especially those targeted to transportation companies, should also be prioritized to prevent the European motorway network from becoming an invasion route for the hornet to new countries. However, different types of cargo do not carry the same risk of being infested (as different species may differ in their commodity associations). Therefore, focused biosecurity policies for *V. velutina*, are needed, particularly targeted to the interception of wooden products’ transportation and man-made goods associated with garden trade, due to the potential of these commodities to shelter hibernating queens. It is also important to promote control actions on ports of species entry, namely harbours along the coast.

Acknowledgements

We thank Instituto da Conservação da Natureza e das Florestas (ICNF) and Bombeiros Voluntários de Viana do Castelo, for having provided the records of *V. velutina* occurrences in Portugal. We thank José Pedro Granadeiro and Pedro Segurado for their insights on data analysis. Fundação para a Ciência e a Tecnologia (FCT Portugal) provided financial support through the project UIDB/00329/2020 granted to cE3c. MJV (PD/BD/128351/2017 and COVID/BD/151632/2021), HR (DL57/2016/EEC2018/07) and LGC (LISBOA-01-0145-FEDER-028360/EUCLIPO) were funded by FCT Portugal. LGC was also funded by the Brazilian National Council for Scientific and Technological Development (CNPq. Universal 421668/2018-0; PQ 305157/2018-3).

3

A metabarcoding tool to detect predation of the honeybee *Apis mellifera* and other wild insects by the invasive *Vespa velutina*


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The content of this chapter is published as:

Verdasca MJ., Godinho R., Rocha RG, Portocarrero M., Carvalheiro L., Rebelo R., Rebelo H. (2021). A metabarcoding tool to detect predation of the honeybee *Apis mellifera* and other wild insects by the invasive *Vespa velutina*. J Pest Sci. https://doi.org/10.1007/s10340-021-01401-3 (IF2020 5,918; Q1 Entomology)

Author Contributions

MJV, RG, LGC, RR & HR conceived the study. MP collected samples. RG & RGR performed the metabarcoding work. MJV analysed data and wrote the manuscript. All authors contributed to the final version.

A metabarcoding tool to detect predation of the honeybee *Apis mellifera* and other wild insects by the invasive *Vespa velutina*

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Abstract

The invasive *Vespa velutina nigrithorax* has been widely referred as an effective predator of honeybees. Despite the potential risk to pollination services provision and honey production, there is no accurate quantification and assessment of its real consequences for honeybees. To date, the identification of the honeybee and other insects in the diet of *V. velutina* has been investigated by direct observation of adult foraging or examination of food pellets. To overcome these limitations, in this study we used a DNA metabarcoding approach to evaluate the usefulness of different types of samples (jaws and stomachs collected from workers and larval faecal pellets taken from the hornet comb) to investigate the predation of *V. velutina* upon honeybees, and potentially on other insects. Honeybee DNA was identified in all types of samples, but larval faecal pellets retrieved the higher number of reads of honeybee DNA and the largest diversity at all taxonomic levels. Over all samples we could identify 4 orders, 9 families, 6 genera and 1 species of prey. We estimate that collecting 6 workers is sufficient to identify honeybee predation by a colony using worker's jaws. Stomachs were the least useful sample type to detect honeybee DNA. The presence of honeybee DNA in all analysed colonies irrespective of collection site, and the variety of insect orders detected in the diet support current concerns over the acknowledged negative impact of *V. velutina* on managed honeybees and its potential threat to pollination services provision.

Keywords: *Apis mellifera*, diet analysis, DNA metabarcoding, invasive species, taxon-specific primers, *Vespa velutina*

Introduction

Invasive alien species are considered one of the major causes for the current global biodiversity crisis, negatively affecting native species and habitats, and the provision of ecosystem services, with important economic impacts (IPBES 2019). Due to broad diets, competitive abilities, high reproductive rates, and ability to establish and spread into new regions, invasive social Hymenoptera have the potential to cause important impacts on native ecosystems (Moller 1996). The invasive yellow-legged hornet (*Vespa velutina nigrithorax* Lepeletier, 1836), an effective predator of honeybees (*Apis mellifera*) and wild pollinators, can affect beekeeping activities and crop production (Monceau et al. 2014, Requier et al. 2019). The presence of *V. velutina* near the hives decreases the foraging activity of honeybees, thus reducing pollen and nectar collection, with the consequent impact on winter survival of the colony (Monceau et al. 2018, Requier et al. 2019). However, there is yet no accurate quantification and assessment of the real consequences to beekeeping.

Adults of *V. velutina* feed on carbohydrates (sugary substances like flower nectars and fruits), which are the main source of energy for flight, but prey seasonally on insects as a source of protein to feed their carnivorous larvae (interestingly, in coastal areas *V. velutina* is attracted to seafood products (fishes, mussels or oysters) that provide a protein-rich alternative food source favouring colony development (Monceau and Thiery 2017)). Adult foragers can accidentally consume some protein while processing prey into food pellets (or meatballs) that they carry back to the nest, but do not consume it directly. The demand for protein by the larvae increases as colony grows, especially from July to November when the pressure upon honeybee colonies becomes more frequent (Monceau et al. 2012). In the nest a worker–larvae trophallaxis occurs, i.e., reciprocal exchange of food between adults and larvae. This process where larvae reward the workers via an oral secretion (a highly energetic solution containing sugars, protein and free amino acids), creates a dependency on the worker and ensures that the larvae are regularly fed (Matsuura and Yamane 1990).

To date, identification of *V. velutina* preys has been undertaken essentially via direct observation of wasps foraging in the wild, near beehives or via examination of body fragments of prey taken to the nest by the workers (Perrard et al. 2009, Villemant et al. 2011a). As hornets tend to chop honeybees into one of their tagmata, adult honeybee body parts are usually easily identifiable. Nevertheless, such direct observations do not allow to identify predation levels of immature or soft bodied insects (i.e., eggs, larvae). Direct observation is also a time-consuming method, generally limited to higher taxonomic level assignment (i.e., Order, Family), not allowing species-level identification of most prey. Thus, inferences on prey selection, dietary variation, and economic impacts of predation are severely constrained by these methods (Clare et al. 2011).

The development of DNA metabarcoding is revolutionizing the characterization of species dietary breadth, including of insect predators (Moran et al. 2019). DNA metabarcoding methodology relies on high throughput sequencing generating DNA sequence reads from the whole content of a sample with mixed origins. Such molecular methods are being increasingly used to identify prey

DNA in predators' faeces (Cheng & Lin, 2016; Kamenova et al., 2018; Long, Kurta, & Clemans, 2013; Moran et al., 2019). Major advantages of using DNA metabarcoding in diet analysis include, but are not limited to, its ability to identify prey items that do not leave remains, like soft bodied species, its speed, and ease-of-use bioinformatics analysis (Egeter et al. 2015). Naturally, the detection of prey DNA in a sample is dependent of a range of interacting factors related to the environment, the predator–prey system and the sample quality (Kamenova et al. 2018). Another methodological limitation of DNA metabarcoding is the concomitant amplification of prey and predator DNA, which may compromise the detection success of scarce and degraded prey DNA (Piñol et al. 2013). However, this can be overcome or greatly mitigated through the exclusion of the predator DNA from the analysis by combining the use of PCR primers targeting potential prey items with predator-specific blocking primers hampering or decreasing the amplification of predator DNA (Vestheim and Jarman 2008).

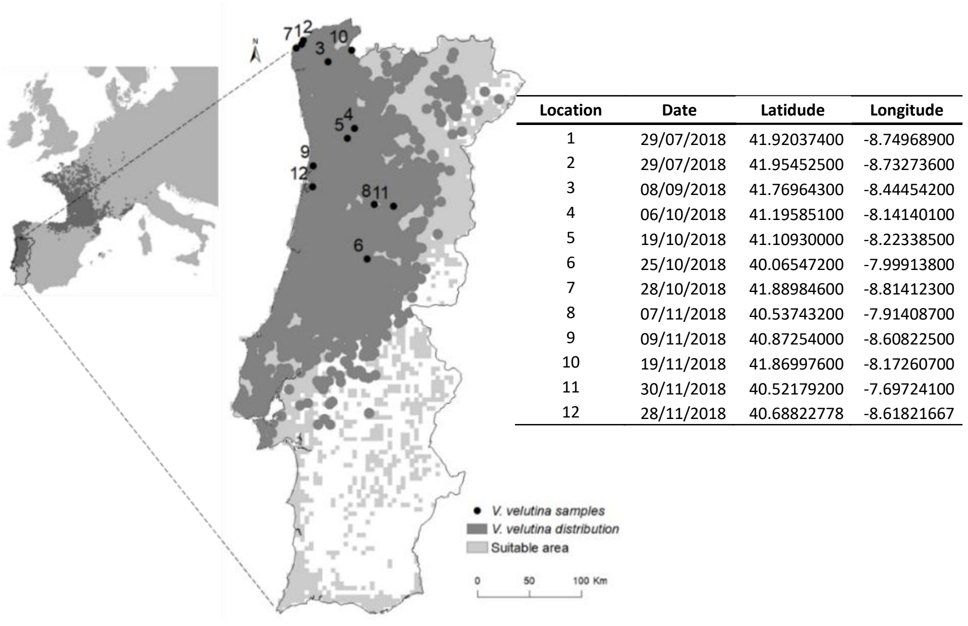
The DNA analysis of the faeces produced by *V. velutina* larvae and accumulated at the bottom of brood cells during its entire larval life, may be a promising way to detect and identify their prey species. This gut content (faecal pellets) is eliminated at once by an individual when moulting from larval to pupal instars, and contrarily to what occurs in honeybees, these pellets are not removed by workers after adult emergence (Rome et al. 2015). Hence, faeces also constitute a historical record of the larval diet throughout its development, enabling to assess the seasonal variation of prey (Lefort et al. 2020). However, the collection of these faecal pellets is particularly problematic as most nests are constructed high within the tree canopy. Furthermore, the nests that produce samples easy to collect are usually retrieved on the course of control operations. These nests are chemically treated, making it necessary to unhook them to avoid environmental contamination (Turchi and Derijard 2018). To ensure complete inactivity, nests can only be removed two weeks after being treated, which implies a second visit to the site and a delay in collecting the faecal samples. Capturing workers of *V. velutina* is much easier than having access to the nests, and therefore the possible detection of honeybee DNA in adult wasp tissues, like jaws (which directly contact with the prey) or stomachs (which probably contain some accidentally consumed protein while processing prey), would make the task of identifying the hornet’s prey much easier. However, by contrast to larval faeces, jaws and stomach samples only provide a snapshot of the diet of *V. velutina* at a given point in time. Given the hornet biology, it is expected that higher amounts of honeybee DNA are found in faecal pellets, followed by forager jaws and lastly in forager crops.

The main aim of this study is to develop a new molecular method to enable the evaluation of the usefulness of different types of samples in the study of predation by *V. velutina* upon honeybees. Following a metabarcoding comparative approach, three different types of samples (jaws and stomachs from workers and faeces from the larvae) were sequenced to identify and quantify the presence of honeybees in the hornet’s diet. We used a specific honeybee primer and designed a specific *V. velutina* blocking primer to test which type of sample has the greatest potential to detect DNA of honeybee. Although we focused on the detection of honeybees, due to the high negative impact of the hornet on both natural and economic services provided by these pollinators, we also identified other arthropod prey. Our results will allow the optimization of protocols for broader diet studies of *V. velutina* and other Hymenoptera.

Material and Methods

Sample collection

Between July and November 2018, twelve nests of *Vespa velutina nigrithorax* distributed along its area of occurrence in Portugal (Fig. 3.1), were opened and dissected *in situ* after having been poisoned during control procedures. Time elapsed between nest treatment and collection varied from 1 day to 4 weeks (for five nests there is no information about treatment procedure and time).



Location	Date	Latidude	Longitude
1	29/07/2018	41.92037400	-8.74968900
2	29/07/2018	41.95452500	-8.73273600
3	08/09/2018	41.76964300	-8.44454200
4	06/10/2018	41.19585100	-8.14140100
5	19/10/2018	41.10930000	-8.22338500
6	25/10/2018	40.06547200	-7.99913800
7	28/10/2018	41.88984600	-8.81412300
8	07/11/2018	40.53743200	-7.91408700
9	09/11/2018	40.87254000	-8.60822500
10	19/11/2018	41.86997600	-8.17260700
11	30/11/2018	40.52179200	-7.69724100
12	28/11/2018	40.68822778	-8.61821667

Fig. 3.1 – Distribution of the collected samples in the area invaded by *Vespa velutina* in Portugal. The distribution area in Portugal (by May 2021) is represented by a buffer of 5 km around each recorded nest (corresponding to the potential foraging area and to the estimated homing ability of the species (Poidatz *et al.*, 2018). The suitable area for the species in Portugal (5 km pixel resolution) and the distribution of *V. velutina* in Europe (on the left) were retrieved from Verdasca et al. (in review; chapter 1). The list of samples with the respective date of collection and geographic coordinates is also shown.

One pellet of larval faeces was collected from each nest at the bottom of one brood cell (as representative of the colony) with sterile forceps and stored in absolute ethanol. Further, one adult worker was also collected from each nest and kept in absolute ethanol for posterior dissection. In the laboratory, the jaws (i.e., mandibles) and stomachs (i.e., midgut, however as the separation is not precise the crop inclusion cannot be excluded) were dissected under a magnifier to be further used for DNA extraction. A total of 12 faecal pellets, 10 jaws and 10 stomachs were considered for the subsequent analysis (2 jaws and 2 stomachs were discarded due to poor conditions of the samples).

Primer design and optimization

To amplify the DNA of honeybee and block the amplification of DNA of *Vespa velutina*, we used available sequences of honeybee (JN293747) and *V. velutina* (NC035146) to design a forward specific honeybee primer (ApisF; Table 3.1) and a reverse hornet-blocking primer (Vespa_blockR; Table 1) for the cytochrome oxidase subunit I (COI). These primers were used together with the general arthropod primers (ZBJ-ArtF1c and ZBJ-ArtR2c) commonly used in metabarcoding studies (Zeale et al. 2011). Illumina adapter overhangs were appended to each primer sequence for compatibility with Illumina index and sequencing adapters (Table 3.1).

Table 3.1. Primer sequences designed and/or used for amplifying a fragment of COI during the first step PCR of library preparation. Underlined sequences are adapter overhangs (McInnes et al. 2017).

Name	**Sequence 5' to 3'**	**Source**
ZBJ-ArtF1c	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGATATTGGAACWTTATATTTTATTTTTGG	Zeale *et al.*, 2011
ZBJ-ArtR2c	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG WACTAATCAATTWCCAAATCCTCC	Zeale *et al.*, 2011
ApisF	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTATTCTWGCWTTATGATCTGG	This study
Vespa_blockR	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTCAGTTACCGAATCCTCCG	This study

The ability of the forward honeybee primer to amplify honeybee DNA was tested by polymerase chain reaction (PCR) using approximately 10 ng of honeybee DNA, 0.4 µM of each primer (ApisF and ZBJ-ArtR2c) and 5 µl of the Multiplex PCR Kit (QIAGEN) in a total volume of 10 µl. The thermal cycling profile used included an initial step of 95°C for 15min, followed by 38 cycles of 95°C for 30s, 56°C for 30s, and 72°C for 30s; with a final extension of 60°C for 10min. PCR products were sequenced using the forward primer and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer´s protocol and sequencing products were separated on a 3130xl Genetic Analyzer (Applied Biosystems).

To test the efficiency of amplification we amplified individually DNA of three potential hornet preys (honeybee, domestic fly *Musca domestica* and Pieris butterfly *Pieris* sp.) using the two forward primers (for general arthropods and honeybee) in the reaction. PCRs were performed using approximately 10 ng of DNA, 0.2 µM of primers ZBJ-ArtF1c and ApisF, 0.4 µM of primer ZBJ-ArtR2c, and 5 µl of the Multiplex PCR Kit (QIAGEN). For these amplifications we used touchdown PCRs with thermal cycles consisting of an initial step of 95°C for 15min; 5 cycles of 95°C for 30s, 56°C - 0.5°C/cycle for 30s, and 72°C for 30s, followed by 32 cycles of 95°C for 30s, 54°C for 30s, and 72°C for 30s, and a final extension of 60°C for 10min. PCR products were Sanger sequenced as described above.

The hornet-blocking primer, used to block DNA amplification of the predator, was designed to overlap with the 3’-end of the reverse arthropod general primer and was modified with a C3 spacer at the 3’-end (Vestheim et al. 2011) (Table 3.1). The specificity of the blocking primer was first tested by blasting its sequence against the GenBank Nucleotide database, in which we observed 100% coverage and identity with *V. velutina* and no other arthropod. To further assess the specificity of the hornet-blocking primer, we pooled equimolar DNA concentration of hornet and one of the same three potential preys and amplified using final volumes of 10 µl containing approximately 10 ng of DNA, 0.2 µM of primers ZBJ-ArtF1c and ApisF, 0.4 µM of primers ZBJ-ArtR2c and Vespa_blockR, and 5 µl of the Multiplex PCR Kit (QIAGEN). For these amplifications we used the same touchdown PCR and Sanger sequence described above. Finally, we tested different concentrations of blocking primer, namely 0.5x, 1x, 2x and 5x. The same touchdown PCR and Sanger sequence described above were used.

DNA extraction, library construction and high-throughput sequencing

DNA was isolated from both tissues (jaws and stomachs from workers) and from larvae faecal pellets using the E.Z.N.A. Tissue DNA Kit (Omega BIO-TEK) according to the manufacturer’s instructions. Blank extractions were performed to monitor possible contaminations. All samples that were in conditions to be analyzed (10 of jaws, 10 of stomachs and 12 of faecal pellets) were used to construct dual-indexed libraries. All samples were amplified in triplicate, totalling 96 DNA libraries. For the first step PCR, the COI region (210 bp) was amplified using the same conditions and the same touchdown thermal profile optimized in the previous steps, including the hornet-blocking primer. A template-free PCR was included throughout the process to control for potential DNA contamination. PCR success was checked for each sample in agarose gels and were purified using reversible immobilization (SPRI) paramagnetic beads (Agencourt AMPure XP, Beckman Coulter, CA, USA) with 0.8x of beads per microliter of PCR product.

Each amplicon from the first step PCR was individually identified on the second step PCR using dual-index barcodes. Second step PCRs were performed in a volume of 25 µl containing 12.5 µl of KAPA HiFi HotStart ReadyMixPCR Kit (Kapa Biosystems, MA, USA), 2.5 µl of 1 µM of each primer and 2.5 µl of 1:10 dilution of first step PCR product. The thermal cycling conditions were 95 °C for 3min, followed by 10 cycles of 95 °C for 30s, 55 °C for 30s, 72 °C for 30s, and a final extension of 72 °C for 5min. PCR products were cleaned using the same procedure described for first step amplicons. All libraries were normalized at 10nM and then pooled in an equimolar mixture. Size distribution of the pool was analysed on a TapeStation 2200 using the HS D1000 kit (Agilent Technologies, CA, USA). Pool concentration was checked by quantitative PCR on a BIORAD C1000 Real Time Thermo cycler using KAPA Library Quantification kit (Kapa Biosystems, MA, USA). Amplicon libraries were sequenced on a MiSeq platform (Illumina) using 30% of a MiSeq Reagent Nano Kit v2 (500 cycles; 2 x 250 bp).

Bioinformatic analysis

Illumina software BASESPACE was used to demultiplex reads from MiSeq. Following Egeter et al. (2019), OBITools (Boyer et al. 2016) was firstly used for: i) aligning paired-end reads; ii) removing alignments with a score <40; iii) removing reads without both primers; iv) clustering reads into Operational Taxonomical Units (OTUs) if 100% identical; v) filtering OTUs with the target size of 135–165 bp; and vi) removing singletons. Then, MEGABLAST (Zhang et al. 2000) was used for BLASTing OTUs against the GenBank Nucleotide database. Finally, MEGAN (Huson et al., 2016) was used for assigning BLAST results to taxa, using the default parameters, apart from the following: minScore=100.0, topPercent=5.0, minSupportPercent=0.0, and minSupport=1.

Data analysis

After bioinformatics analyses, we obtained the final number of reads with taxonomic identification (species, genera, families, orders) per sample (see Supplementary Material – Chapter 3). As low levels of template DNA are a cause of PCR stochasticity in metabarcoding studies, the entire target diversity in the sample might not be amplified in all PCR replicates (Alberdi et al. 2018). Hence, by doing three PCR replicates we improve the probability of detecting different species (Alberdi et al. 2018). To maximize the inclusion of sequences of actual prey items and exclude low-level background noise (secondary predation, contamination, sequencing errors), a threshold was applied to the total number of reads of each replicate (Deagle et al., 2019). A 1% threshold may be suitable for many situations (Deagle et al., 2019), but when diets are extremely diverse with potentially large recovery biases, then a much lower threshold may be justified by increasing the importance of rare diet items (e.g., 0.1% in Deagle *et al.*, 2019 or 0.01% in Alberdi *et al.*, 2018). Here we used the threshold of 0.02% of total number of reads and considered as present only the taxa with the number of reads higher than this threshold that were cumulatively present in at least two replicates. By doing this we discarded plausible errors, preventing the inclusion of possible chimeras in subsequent analysis (Alberdi et al. 2018). The location of each sample was cross-checked with the known distribution of the identified prey taxon (Hjorth-Andersen 2002, Baldock et al. 2018).

The frequency of occurrence (FO, i.e., percentage of samples in which a given taxon was detected) was then calculated. For this calculation, we included in Order, Family and Genus all taxa that are assigned to each hierarchical group, i.e., the FO of the Order considers the detection of families, genera and species within that Order, and the same for Family and Genus. The number of honeybee reads were counted and compared through Mann-Whitney U test between the two pairs of independent samples (faecal pellets/jaws and faecal pellets/stomachs). The same comparison between jaws and stomach of the same worker was done through a paired T-test. We repeated the same comparison procedure described above, but this time using the total number of reads and the number of identified taxa (both including honeybees) in each sample. Being faecal pellets the most informative sample (see results) and to investigate the sequencing effort for honeybee in these samples, we generated a read rarefaction curve to determine if and when the curve plateaued. Given that predation of honeybees did occur on all sampled colonies (see results), it was possible to use the frequency of honeybee detection in the workers' tissues to estimate the number of samples needed to test for honeybee predation.

Results

Primer specificity

A total of four primers were used in this study covering a ~160 bp fragment of the COI region. The primer pair ApisF and ZBJ-ArtR2c amplified honeybee samples, while the primer pair ZBJ-ArtF1c and ZBJ-ArtR2c amplified other potential hornet preys, including those tested in this study (domestic fly and Pieris butterfly). When combined, these three primers amplified both honeybee and other potential hornet preys tested in this study. When combining both forward primers ApisF and ZBJ-ArtF1c, the reverse primer ZBJ-ArtR2c and the blocking primer Vespa_blockR, no amplification of *V. velutina* was obtained, while amplifying honeybee and other potential hornet preys.

Data quality and coverage

Final read numbers (108979 after applying the threshold of 0.02% to discard taxa with low number of reads) were, on average, of 1265 reads/replicate. As for the sample type, faecal pellets recovered the highest read number, on average 1709 reads/replicate, which represents almost twice the average read numbers in jaw samples (933 reads/replicate) and stomachs (786 reads/replicate). Keeping the taxa that were present in at least two replicates, we assigned OTUs to 4 orders, 9 families, 6 genera and 1 species of prey (Table 3.2), all occurring in Portugal.

Table 3.2. List of prey observed in the whole dataset arranged according to taxonomic level, and the frequency of occurrence (FO) per sample type. The FO was calculated considering the presences of the different taxa within each Order, Family and Genus.

Orders	Families	Genus	Species	FO - faecal fellets	FO - jaws	FO - stomachs
Diptera				0.67	0.08	0.08
	Calliphoridae			0.17	—	—
	Culicidae			0.08	—	—
	Muscidae			0.25	—	—
		Musca		0.08	—	—
		Neomyia		0.08	—	—
	Tachinidae			0.17	—	—
	Tephritidae			0.08	—	—
		Ceratitis		0.08	—	—
Hymenoptera				1.00	1.00	0.80
	Apidae			1.00	0.70	0.40
		Apis		1.00	0.70	0.40
			*Apis mellifera*	1.00	0.70	0.40
	Vespidae			0.17	0.60	0.90
		Vespa		—	0.50	0.90
		Vespula		0.17	—	—
Lepidoptera				0.33	—	—
	Noctuidae			0.08	—	—
Orthoptera				0.08	—	—
	Acrididae			0.08	—	—

Honeybee detection in different samples

Honeybee reads represented almost 75% (79143) of the total number of reads of our study (Table 3.3). As for sample type, from the total number of reads present in faecal pellets (60518 reads), in jaws (27546 reads) and stomachs (20915 reads), 84% (50549 reads), 74% (20458 reads) and 39% (8136 reads) respectively, correspond to honeybee (Table 3.3). Irrespective of collection site, honeybee was found in every single replicate of every faecal pellet. Indeed, within faecal pellets samples, rarefaction curves of honeybee reads reached a plateau at 400 reads per sample (Fig. S3.1). Honeybee DNA was also found in workers from all colonies with a single exception (location 10; see Table 3.3). For jaws and stomachs (that correspond to recent predation events), the percentage of presence of the honeybee in each sample was 70% and 40%, respectively, and the level of concordance in prey detection between both samples from the same worker was 37.5%.

Table 3.3. Number of reads per sample type (faecal pellets, jaws, and stomachs) in each location. The comparison of the number of reads of honeybee and its percentage (in brackets) in the respective sample type vs other prey, is also shown.

Local	Sample	Total reads	Nr. Reads honeybee	Nr. Reads other prey
		8032	**5902 (73%)**	**2130 (27%)**
1	Faecal pellets	2338	2082 (89%)	256 (11%)
	Jaws	2760	2006 (73%)	754 (27%)
	Stomachs	2934	1814 (62%)	1120 (38%)
		5385	**4532 (84%)**	**853 (16%)**
2	Faecal pellets	1020	1020 (100%)	0 (0%)
	Jaws	3687	3512 (95%)	175 (5%)
	Stomachs	678	0 (0%)	678 (100%)
		9533	**6302 (66%)**	**3231 (34%)**
3	Faecal pellets	3577	3447 (96%)	130 (4%)
	Jaws	4428	2855 (64%)	1573 (36%)
	Stomachs	1528	0 (0%)	1528 (100%)
		10528	**7291 (69%)**	**3237 (31%)**
4	Faecal pellets	9781	7135 (73%)	2646 (27%)
	Jaws	747	156 (21%)	591 (79%)
	Stomachs	0	0 (0%)	0 (0%)
		19037	**18051 (95%)**	**986 (5%)**
5	Faecal pellets	13435	13054 (97%)	381 (3%)
	Jaws	3562	3539 (99%)	23 (1%)
	Stomachs	2040	1458 (71%)	582 (29%)
		7246	**5809 (80%)**	**1437 (20%)**
6	Faecal pellets	6060	5565 (92%)	495 (8%)
	Jaws	244	244 (100%)	0 (0%)
	Stomachs	942	0 (0%)	942 (100%)
		3705	**3311 (89%)**	**394 (11%)**
7	Faecal pellets	3705	3311 (89%)	394 (11%)
		5957	**5362 (90%)**	**595 (10%)**
8	Faecal pellets	5957	5362 (90%)	595 (10%)
		11395	**8569 (75%)**	**2826 (25%)**
9	Faecal pellets	5478	4217 (77%)	1261 (23%)
	Jaws	4121	4045 (98%)	76 (2%)
	Stomachs	1796	307 (17%)	1489 (83%)
		4006	**583 (15%)**	**3423 (85%)**
10	Faecal pellets	583	583 (100%)	0 (0%)
	Jaws	952	0 (0%)	952 (100%)
	Stomachs	2471	0 (0%)	2471 (100%)
		6815	**2841 (42%)**	**3974 (58%)**
11	Faecal pellets	3394	2597 (77%)	797 (23%)
	Jaws	567	0 (0%)	567 (100%)
	Stomachs	2854	244 (9%)	2610 (91%)
		17340	**10590 (61%)**	**6750 (39%)**
12	Faecal pellets	5190	2176 (42%)	3014 (58%)
	Jaws	6478	4101 (63%)	2377 (37%)
	Stomachs	5672	4313 (76%)	1359 (24%)

Total nr. of reads	108979	79143 (73%)	29836 (27%)
Total nr. of reads in Faecal pellets	60518	50549 (84%)	9969 (16%)
Total nr. of reads in Jaws	27546	20458 (74%)	7088 (26%)
Total nr. of reads in Stomachs	20915	8136 (39%)	12779 (61%)

By assessing the differences between samples regarding the number of reads of honeybees, we found that faecal pellets and stomachs differ significantly from each other (Table 3.4). Moreover, we also found differences between jaws and stomachs of the same worker (Table 3.4). The estimation of the number of samples needed to test for honeybee predation showed that at least six jaws (from different workers) are needed to guarantee less than 0.1% probability of a false negative, while for stomachs, 13 samples are needed.

Table 3.4. Comparison of the different samples through Mann-Whitney U (for independent samples) and a paired T-test (for samples of the same individual: jaws and stomachs) regarding the number of reads of honeybee.

Comparison of the number of honeybee's reads between samples				
Test	**samples**	**w**	**p value**	
Mann-Whitney U	jaws and faecal pellets	85	0.106	
	stomachs and faecal pellets	108	0.002	**
Paired T-test	jaws and stomachs	**t**	**p value**	
		2.35	0.040	*

For seven locations we knew the time elapsed between nest treatment with biocides and sample collection and observed that the DNA of honeybee remained at least 28 days, the maximum known period, in faecal pellets. Time of DNA decay is more difficult to infer for jaws and stomachs once most of applied biocides do not suddenly kill the entire colony. Instead, their action remains for a few days, enabling the hornets to still leave the nest to capture preys, while the colony gradually dies.

Other prey

Although this study was focused on the detection of honeybees in the diet of *Vespa velutina*, it was possible to identify other prey. Besides Hymenoptera, other three orders were identified (Diptera, Lepidoptera and Orthoptera: see Table 3.2). With the single exception of the genus *Vespa*, all the other taxa were found in faecal pellets. In contrast, orders Lepidoptera, Orthoptera and the different families of Diptera were not identified in jaws and stomachs. The total number of reads obtained in each sample (including honeybee and all the other identified prey) was only significantly different between faecal pellets and stomachs (Table 3.5A). Moreover, the number of different taxa retrieved differed between faecal pellets and stomachs or jaws (Table 3.5B).

Table 3.5. Comparison of the total number of reads and of different taxa (both including honeybee) found in each sample (faecal pellets, jaws and stomach), through Mann-Whitney U test (for independent samples) and T-test (for samples of the same individual: jaws and stomachs).

A - Comparison of the total number of reads between samples			
Test	samples	w	p value
Mann-Whitney U	jaws and faecal pellets	84	0.12
	stomachs and faecal pellets	95	0.02 *
Paired T-test	jaws and stomachs	t	p value
		1.15	0.28
B - Comparison of the number of different taxa between samples			
Test	samples	w	p value
Mann-Whitney U	jaws and faecal pellets	93	0.03 *
	stomachs and faecal pellets	96.5	0.02 *
Paired T-test	jaws and stomachs	t	p value
		0.57	0.58

Discussion

The metabarcoding approach developed in this work overcomes one of the major limitations of traditional methods to identify prey items based on morphological features by enabling the detection of immature and/or soft body insects in the diet of *Vespa velutina nigrithorax.* Using this approach, we showed that honeybee DNA can be detected either from larvae faecal pellets or from jaws or stomachs of adult workers, and that 100% of the larvae faecal pellets examined exhibited honeybee DNA. We also showed through our approach that faecal pellets enabled the detection of several orders, families, and genus of other arthropod preys, although accurate assessments of diet composition would need additional efforts because our method was aimed at honeybee. Overall, these findings corroborate previous studies based on direct observation of predation events or on examination of food pellets and provide additional information of prey at a lower taxonomic level.

We identified the presence of honeybees in all analysed colonies irrespective of its collection site. This finding supports current concerns over the acknowledged negative economic impact of *V. velutina* on beekeeping activities (Monceau et al. 2018) and on the provision of crop pollination, for which honeybees play a major role (Garibaldi *et. al*., 2013). However, and despite the significant predation over honeybees, beekeeping seems to be mostly impacted by the mere presence of the hornet hoovering in front of beehives. Such behaviour triggers an increase of oxidative stress in honeybee workers (Leza et al. 2019) and disrupts the foraging activity of the bee colonies, specially threatening isolated or poorly populated and unhealthy beehives (Requier et al. 2019). Monitoring recent predation events may be carried out using the workers. We found honeybee DNA more frequently in jaws than in stomachs of workers, providing evidence that jaws are more suitable to detect recent predation of honeybees. This was an expectable result since adult foragers do not consume insect prey directly; they make a meatball with the nutritious body parts of prey and carry it back to nest to feed the larvae. Thus, only accidentally some honeybee body parts can be consumed (and found in the stomach of the workers) when such food pellets are prepared. Moreover, the simplicity of capturing adult workers as opposed to the time-consuming and laborious work of collecting larval faeces, makes the use of jaws a valuable approach to detect honeybee DNA.

As for our DNA amplification strategy, we designed a specific honeybee forward primer that was multiplexed in the same reaction with general arthropod primers. We acknowledge that this might have increased the amplification rate of honeybee DNA, increasing its relative frequency of occurrence in comparison with other arthropods. Indeed, this was the main goal of this work, although we were also able to identify other prey belonging to six different genus, nine families and four orders of insects. Additionally, our strategy of low sequencing depth was validated by the rarefaction analysis showing that as few as 400 reads would have been enough to detect honeybee DNA in 100% of the faecal pellets examined. This supports that low sequencing depth, and the use of honeybee specific primers is sufficient to identify our target species. However, while this strategy is suitable for studies that specifically focus on honeybee predation, it might need further optimization regarding sequencing coverage and primer selection in studies aiming to assess hornet diet to assure even and accurate rates of amplification. This would be an essential future step to assess the impact of this invasive species on wild native insects and evaluate the potential for negative impacts on ecosystem functioning (pollination of wild plants) and provision of ecosystem services (crop pollination).

Being *V. velutina* an opportunistic forager, its prey diversity varies as a function of the available resources (Rome et al. 2021). Indeed, when fisheries, sea food, are available, they neglect insect preys (Monceau and Thiery 2017). Previous studies based on direct observation, and certainly biased towards organisms easily recognized morphologically, in detriment of soft bodied organisms, suggest that in urban, agricultural or forest areas the hornet preys essentially on flying social Hymenoptera and Diptera (Perrard et al. 2009, Villemant et al. 2011a). Notably, and despite the already discussed limitations of our work, we did not find any genus of solitary bees or bumblebees above the threshold of 0.02% of the total number of reads. A low detectability of these taxa was also found by Rome et al. (2021). The opportunistic nature of *V. velutina* and its predominant association with urban and agricultural areas (Choi et al. 2012a, Rome et al. 2015) in detriment of natural areas (Fournier, Barbet-Massin, Rome, & Courchamp, 2017), make us believe that the predation impact on these main pollinators should be low. However, and despite the large body mass of bumblebees, they are known to change foraging behaviour (e.g., decrease of the time spent in flower visiting) when chased and killed by *V. velutina*, which disrupts their pollination services (Rojas-Nossa and Calviño-Cancela 2020). Therefore, it is possible that in regions where honeybees are less abundant, other preys are more consumed. Long-term monitoring studies focused on the impact of *V. velutina* on wild bees and associated pollinator services are needed.

In this work dipterans were the second most predated group. The most frequent families detected in this Order were the necrophagous Calliphoridae, Muscidae, and the parasitoid Tachinidae; these groups include species that can act as pollinators, and also important parasitoid species that regulate the population of several arthropods (Stireman et al. 2006). Furthermore, the detection of Lepidoptera in 33 % of the faecal samples suggests an important and until now undetected role of this group in the hornet's diet, comparing to its lower detectability in previous studies: (Villemant *et al.* (2011) and Rome *et al*. (2021). This higher detection of Lepidoptera in our study may result from the unsuitability of methods relying on direct observation of foragers in the wild, or on the identification of food pellets carried by adult wasps, to detect immature or soft bodied insect prey, like eggs and larvae. However, it may also be related to the lifetime record of consumed prey depicted in faecal pellets. Lepidoptera of the family Noctuidae include some well-known crop pests (Hobson et al. 2018), which we speculate could uncover a functional role for hornets (Lester et al. 2013).

Although the amplification of DNA of *V. velutina* was hampered by the hornet-blocking primer, we still detected DNA of the genus *Vespa* with high prevalence in jaws and stomachs (50% and 90% FO respectively), but not in faecal pellets. Considering that both jaws and stomachs are expected to contain high amount of hornet DNA, we believe that *Vespa* sp. reads represent DNA of the predator that was not identified to species level, as sequencing errors are frequent in high-throughput sequencing approaches. However, identification of other prey items of the same genus (e.g., *Vespa crabro*) cannot be excluded. In addition, by using a blocking primer for *V. velutina*, we hampered the amplification of DNA of this species, precluding the detection of scavenging or cannibalism, which are acknowledged behaviours of these eusocial insects (Matsuura and Yamane 1990). Hence, this important detail must be considered when applying this method in studies aiming at the full characterization of the wasp's diet.

Prey DNA detection is acknowledged to continuously decrease over time (Kamenova et al. 2018). As in our study the time between nest poisoning and sample collection varies from one day to four weeks, it is possible that some genetic material had already been lost. This probable DNA decay associated with our small sample size and low sequencing coverage strongly suggests that the representativeness of preys here detected is just a part of the total amount of prey, raising the need to more exhaustive diet studies, as performed in the recent work by Rome et al. (2021). Furthermore, our samples were previously exposed to biocides, which may also contribute to loss of DNA, with the consequent decrease in the number of detected prey taxa. Due to our reduced sample size, we were precluded to further explore this issue, but future research should also focus on how the detection of prey DNA varies with time since the destruction of the hornet colony. It is not known the durability of prey DNA on the hornet mandibles but, in what concerns to larval faecal pellets, our results show that it is possible to detect DNA of honeybee almost one month after the nests have been treated.

Final Considerations

The methodological approach developed in this study provides a good monitoring approach of honeybee predation by the invasive hornet, and a starting point for future studies that aim to characterize the diet of *Vespa velutina nigrithorax*. Larvae faecal pellets showed to be the most informative DNA source to detect the presence of honeybee (a single PCR replicate was shown to be enough to produce a high number of reads). Metabarcoding methods enable the detection of soft bodied organisms (e.g., any type of insect larvae, aphids, bee or butterfly eggs), which is a major achievement when compared to methods based on direct observation, which only enable the identification of adult forms. The possibility to identify predation upon honeybees using only the jaws of six workers of the same colony was also a major result of this work; by doing this, it is possible to substantially reduce the time spent in collecting samples and the costs associated with the methodology. Interestingly, and besides honeybees, we found other insect prey, which reinforces the growing evidence that *Vespa velutina* may pose a threat to the provision of ecosystem services (crop pollination, regulation of arthropod populations, structuring of ecological communities). Further, complementary approaches either through barcoding analysis of food pellets (Rome et al. 2021), or of both worker jaws and faecal pellets (this work) could be conducted to provide a more comprehensive description of the diet of the invasive *V. velutina*.

Acknowledgments

We would like to thank *Nativa* association and to Graça Oliveira, for the help provided in sample collection. We are grateful to Diana Castro for laboratory assistance, and to Bastian Egeter and Pedro Silva for the helpful suggestions in the bioinformatics pipeline. Fundação para a Ciência e a Tecnologia (FCT Portugal) provided financial support through the project UIDB/00329/2020 granted to cE3c. M.J.V (PD/BD/128351/2017), R.G. and H.R. (contract under DL/2016), R.G.R (CEECIND/01087/2018) and L.G.C. (LISBOA-01-0145-FEDER-028360/EUCLIPO) were funded by Fundação para a Ciência e a Tecnologia (FCT). L.G.C. was also funded by the Brazilian National Council for Scientific and Technological Development (CNPq. Universal 421668/2018-0; PQ 305157/2018-3). Laboratory work was supported by PTDC/BIA-ECO/31731/2017. The funding sources had no direct involvement in the study design, or in the collection, analysis, and interpretation of data.

4

Beekeepers' perceptions of the impact of the yellow-legged hornet (*Vespa velutina nigrithorax*) on beekeeping activity in Portugal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The content of this chapter is published as:

Verdasca MJ, Carvalheiro L, Rebelo H, Rebelo R. Avaliação da perceção do impacto da vespa-asiática (*Vespa velutina nigrithorax*) na atividade apícola em Portugal (2021). Lucanus – Revista de Ambiente e Sociedade, Volume V, Páginas 84-103. http://www.lucanus.cm-lousada.pt/2022/02/15/vespa-asiatica-em-portugal/

Author Contributions

M.J.V., H.R., L.C., and R.R. conceived the study. M.J.V. gathered the data and performed the analyses. M.J.V. produced the first draft of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Beekeepers' perceptions of the impact of the yellow-legged hornet (*Vespa velutina nigrithorax*) on beekeeping activity in Portugal

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Abstract

The yellow-legged hornet (*Vespa velutina nigrithorax*), native to Southeast Asia and present in Europe since 2004, where it is considered invasive, has been widely referred as an effective predator of the honeybee (*Apis mellifera*) and other wild pollinators. Despite the potential risk for honey production and the provision of pollination services, until now there is no accurate assessment of the impacts on beekeeping. In this work, and through an online questionnaire launched in 2018 to Portuguese beekeepers in the north and centre of the country, it was our aim to make a first assessment of the perception of beekeepers on the impact of *V. velutina* on beekeeping activity and on the control strategies for the species that are currently being implemented in Portugal. We found that the awareness actions carried out so far have been profitable, as most of the beekeepers that answered to the questionnaire are already informed about *V. velutina* and its impacts. Beekeepers who carry out their activity in municipalities where the official number of reported nests is higher were those who perceived the highest impacts of this species. This is a good indicator that survey-based studies are credible and can be used as a tool to support decision makers. Beekeepers reported that there is still a lack of knowledge about which techniques are most effective to combat *V. velutina* and called for a closer relationship with the scientific community through outreach activities. Disappointment with the performance of the local authorities in fighting, and especially in preventing the expansion of *V. velutina*, was also demonstrated. Finally, they also considered that the financial support received so far is scarce and referred as urgent the implementation of a mandatory national strategy that regulates *V. velutina* control actions equally for all municipalities. Globally, given the awareness about *V. velutina* demonstrated by Portuguese beekeepers, their commitment in communicating the nests found to the local authorities or in their registration on the STOPvespa online platform of the Instituto para a Conservação da Natureza e das Florestas (ICNF) can be critical to future monitoring and control of the spread of *V. velutina* nationwide.

Keywords: *Apis mellifera*, beekeepers, control measures, inquiries, invasive species, *Vespa velutina*

Introduction

Biological invasions are considered one of the greatest threats to the preservation of biodiversity and ecosystem services (and to the socio-economic benefits associated with them), due to the changes they cause in the balance of the biosphere on a global scale (Vicente et al. 2018). An example of a successful invasive species is the yellow-legged hornet (*Vespa velutina nigrithorax*). Originally from China, this species was first found in Europe in 2004 in the region of Bordeaux, France. Since then it has been expanding to different European countries such as Portugal, Spain, Italy, Belgium, Luxembourg, Netherlands Switzerland, Germany and England (Fig. 4.1), and is currently considered by the European Union as an invasive species that requires monitoring and control (European Commission 2016).

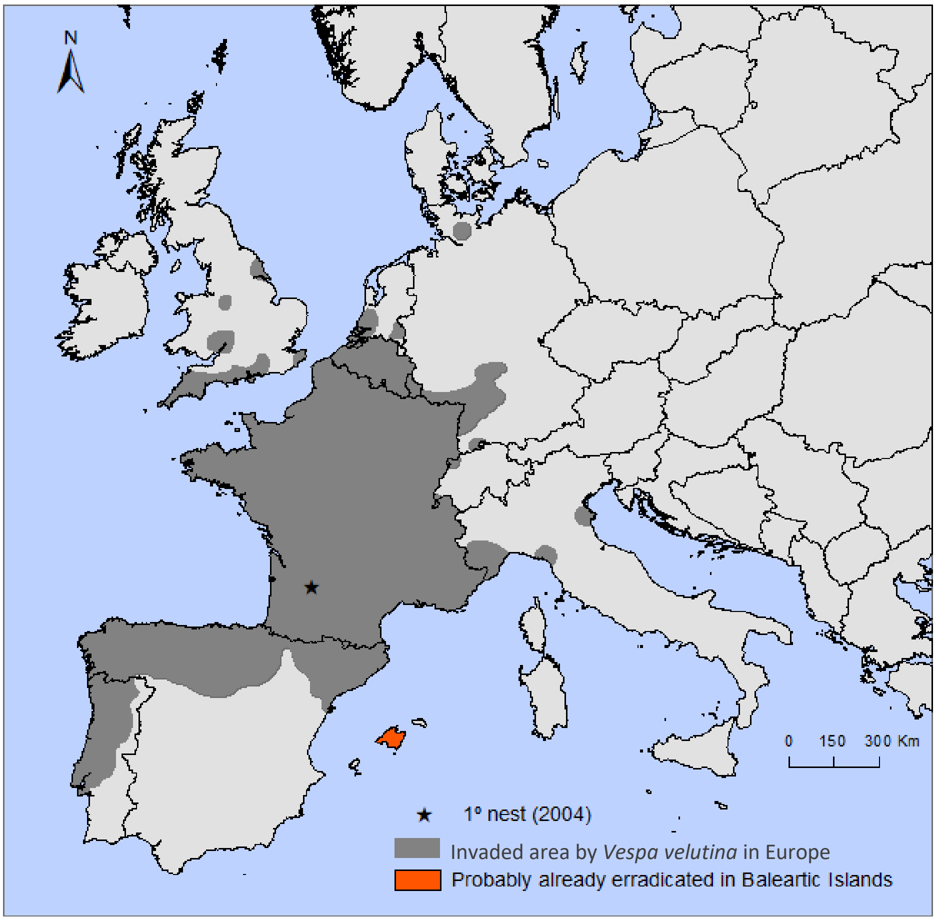

Fig. 4.1 – Distribution of *Vespa velutina* in Europe in 2021. Map adapted from (Rome and Villemant 2021: MNHN-INPN - consulted 6/9/2021) and from Rojas-Nossa et al. (2021). In the Balearic Islands (in orange) the species has probably been eradicated (Leza et al. 2021). The first reported nest in Europe in 2004 near Bordeaux, France, is marked with a star.

In Portugal, the species was first seen in the region of Viana do Castelo in 2011 (Grosso-Silva and Maia 2012). Due to the favourable climatic conditions found along the Portuguese Atlantic coast, *V. velutina* has been dispersing more rapidly southwards than eastwards, and ten years later it was already established in the Lisbon metropolitan area. Nests of *V. velutina* were primarily reported on treetops (Villemant et al. 2006), but recent studies reveal that many can be found in shrubs, vegetated slopes, on the ground, and in artificial structures such as roofs, balconies, and sheds (Monceau and Thiery 2017, Carvalho et al. 2020). For its establishment, suitable climatic conditions are needed to guarantee the survival of the colonies, as well as habitat providing shelter (from rain, wind), and the necessary food resources (Bessa et al. 2016). In a recent work by Carvalho *et al.* (2020), the authors observed that in the last years in Portugal there has been a decrease in the distance between the nests, suggesting that the density of the species is increasing and that this trend may continue in the coming years if control measures are not intensified.

Vespa velutina is a generalist and opportunistic species, and its diet depends on locally available resources (Rome et al. 2021). Adults feed on sugary substances, such as flower and fruit nectars, which provide them the energy needed for flight. However, they prey on honeybee and other wild insects (Beggs et al. 2011, Rome et al. 2021, Verdasca et al. 2021a), using them as a source of protein to feed their carnivorous larvae. In the nest, the larvae reward the workers by regurgitating a highly energetic solution that is fed to the workers, a phenomenon known as trophalaxis (Matsuura and Yamane 1990).

In Portugal, attacks on honeybee hives by *V. velutina* are frequent, especially from July to the end of the year. On the other hand, the mere presence of *V. velutina* near the entrance of the apiaries causes oxidative stress in bees (Leza et al. 2019) and prevents them from leaving the hive to forage, putting at risk the more isolated and weakened hives (Requier et al. 2019). Thus, in addition to the direct impact on beekeeping and honey production, the provision of ecosystem services, namely the pollination of agricultural crops, may also be threatened.

The impacts of *V. velutina* on beekeeping have not been adequately measured so far (Espinosa et al. 2019), but many European beekeepers are already aware of the problem, which has motivated the rapid development of new control methods in recent years (Turchi and Derijard 2018). In 2014, ICNF – Institute for the Conservation of Nature and Forests, launched an online platform for registering occurrences of *V. velutina* by citizens, where it is possible to associate photographs that prove the detection of both nests and adult hornets. Records are then validated or discarded if in presence of similar species. In 2015 an action plan for the surveillance and control of *V. velutina* in Portugal was launched (updated in 2018: DGAV et al. 2018) which aims to propose actions (implementation of a surveillance system, destruction of nests and public awareness), reduce the impacts caused by *V. velutina*, eradicate new outbreaks of invasion and prevent the spread of the species. Also in 2018, a Manual of Good Practices (Marques et al. 2018) was produced, with the aim of providing guidelines for the destruction of nests.

Questionnaire-based research is a common approach to assess the awareness of biological invasions (Novoa et al. 2017). Poor coordination between managers and stakeholders in France is thought to be one of the important reasons for the species continuing spread (Monceau et al. 2014), and so the present study, based on inquiries, aims to assess the perception of Portuguese beekeepers of *V. velutina* and of its impacts, and their opinion on the control and mitigation strategies that are being applied in Portugal. We also tested if the perception of the impacts of *V. velutina* on the beekeeping activity is related with the dedication to the beekeeping activities and with the number of nests officially reported in the area, as well as those seen by the beekeeper. The opinions and responses of beekeepers highlighted in this work may be important for a future review of the Action Plan for *V. velutina* in Portugal.

Material and Methods

Survey

In 2018, a questionnaire was posed to beekeepers in the northern and central regions of Portugal, to collect information on the impacts of *Vespa velutina* on their beekeeping activity between 2015 and 2017 (see Supplementary Material – Chapter 4). In a preliminary phase, a pre-test was carried out. Several CCDRs (Coordination Comissions for Regional Development), City Councils and Beekeeping Associations were contacted for help on the dissemination of the questionnaire. This was divided into 3 sections: (i) characterization of the beekeeper; (ii) perception and impact of the invasive; (iii) business characterization. In the first section we intended to know the region in which the activity takes place, as well as the number of beehives, the age and experience of the beekeeper, the time devoted to beekeeping and which procedures are followed in beehive management. In the second section we aimed to assess whether beekeepers were already aware of *V. velutina*, quantify the number of nests sighted and removed by each beekeeper in the previous three years, an estimation (by the beekeeper) of the proportion of its bees that were lost, and the loss of honey production over the years. They were also asked to evaluate the success of the prevention and control methods used to fight *V. velutina* and promoted by the competent authorities. Open-ended questions were also made to understand the opinion of beekeepers about the techniques used to fight *V. velutina* and which they think are the most effective, the kind of information/support they received about the hornet and by whom they were contacted, the kind of initiatives already developed to face the problem and if they had suggestions for improvement. In the third section we asked if the beekeeping activity was a family business or not, and the average amount (kg) of honey produced in each of the 3 years between 2015 and 2017. We asked for the contact of those interested in knowing the results of the survey.

Presence of Vespa velutina

Records of *Vespa velutina* were obtained from the Firemen Department of Viana do Castelo and from the online platform STOPvespa (http://stopvespa.icnf.pt/), managed by ICNF - Institute for Nature and Forest Conservation, which includes all validated Portuguese records of *V. velutina* nests. The occurrences are confirmed and validated by ICNF, and different entities or local technicians are contacted to remove the nests. The data collection effort is not systematic, as it results from citizen reports. For the period between 2015 and 2017, a total of 4238 nests were registered in the municipalities of the northern and central regions of the country for which beekeepers reported beekeeping activity in the same period.

Data analysis

A descriptive analysis of the responses to the surveys was carried out. To test whether beekeepers' perception of the impacts of *Vespa velutina* is based on actual hornet abundance (number of nests), we used a generalized linear model (GLM) with negative binomial distribution and logit link function. For this purpose, we only used the information provided by beekeepers operating in a single municipality within the invaded area. We used the percentage of honeybee loss reported by beekeepers due to *V. velutina* attacks as the dependent variable. As independent variables we used the number of tasks performed in the maintenance of bee colonies, the number of hives, the number of nests sighted by the beekeeper and the number of nests officially reported for each municipality, the latter being a value most likely unknown by beekeepers to date of completion of the survey. The correlation between independent variables was tested previously to the other analyses (Dormann et al. 2013).

Results

We obtained 259 responses from beekeepers who carry out their beekeeping activity in 116 different municipalities (Fig. 4.2), which corresponds to 2% of the total number of registered beekeepers in Portugal and 3% of beekeepers operating in the north and centre of the country. Most respondents to the online survey have higher education (43%) or secondary education (36%), with 45% being between 50 and 75 years old and 40% between 35 and 50 years old. Regarding beekeeping experience, 39% work with bees for at least 5 to 15 years, 31% started its activity less than 5 years ago and 21% over 20 years ago. Only 25% of respondents say theirs is a family business. Eighty percent of beekeepers have less than 5 apiaries and only 14% are considered professionals for having more than 150 hives. Regarding non-professional beekeepers, 25% have fewer than 10 hives, 49% between 10 and 50 hives and 26% between 50 and 150 hives. Only 8% of respondents said that their beekeeping activity is a full-time job. Of all respondents, 90% reported being a member of a beekeeping association.

Fig. 4.2 – Portuguese municipalities where the respondent beekeepers to the survey develop their beekeeping activity. The background (light grey) depicts the area with suitable climatic conditions for the prevalence of *Vespa velutina* in Portugal (Verdasca et al., in review; chapter 1), i.e., areas with mild winter temperature and pleasant summers.

Most beekeepers (98%) said they were aware of the problem of *Vespa velutina*, and 75% said that their hives had already been attacked by the invasive. In fact, 25% of respondents reported that more than 30% of their time devoted to beekeeping is spent fighting the hornet. Of the total number of nests seen by beekeepers over the 3 years (1877 nests), 17% were removed by themselves and 45% by the competent authorities (for the remaining nests there is no information, most probably were not removed).

From 2015 to 2017, the number of nests sighted by beekeepers substantially increased. The percentage of hives attacked by *V. velutina*, the estimation of honeybee losses (in %) and the estimated loss of honey production (Kg) also depicted a growing trend (Table 4.1).

Table 4.1 - Trends of some indicators on the impact of *V. velutina* on the beekeeping activity.

	2015	**2016**	**2017**
Nr. *V. velutina* nests seen by beekeepers	354	522	1001
Beehives attacked by *V. velutina* (%)	22	33	60
Percentage of honeybee loss due to *V. velutina* (%)	11	16	30
Honey production per hive (kg)	13.8	12.7	10.9

Note: except for the number of nests, the mean value is presented.

Regarding the type of information that beekeepers would like to receive about *V. velutina* (Table 4.2), only 3% reported being fully informed about this topic. A relevant percentage claims for more information on how to protect their honeybees (27%), which type of traps should be used and in which season (25%) and also mention the lack of public awareness sessions about *V. velutina* (22%). Some beekeepers specified that they are acting at their own risk and ask for a binding National Plan that regulates the implementation of control measures in a similar way between the different municipalities. They also mentioned the need for larger involvement of the respective beekeeping associations, higher financial support for trapping procedures and the importance of having information from researchers regarding the advances in this specific field of science. At the end, 70% of beekeepers reported being interested in knowing the results of this study.

Table 4.2 - Synthesis of answers on the question "What kind of information would you like to receive about the *V. velutina* and its impact on beekeeping?"

What kind of information would you like to receive about the *V. velutina* and its impact on beekeeping?	**% beekeepers**
I am already informed and do not intend to receive any further information	3
Know how to distinguish and identify a nest of *V. velutina*	6
Know how to distinguish *V. velutina* from other wasps	4
Know how to act in case of observation of a *V. velutina* nest	10
Know how to protect my honeybees	27
Know which type of traps should be used and in which season	25
Trainings and workshops on the topic	22
Others	4

Regarding prevention and control measures, almost 50% of beekeepers mentioned the development of joint strategies with other colleagues, namely searching for nests, trapping and sharing knowledge. However, 27% of the beekeepers affiliated with beekeeping associations reported not having received any information about *V. velutina* from its respective association (until the date of the survey – 2018). Most beekeepers were clearly dissatisfied with the work done by the competent authorities in fighting and preventing the expansion of *V. velutina* (Fig. 4.3).

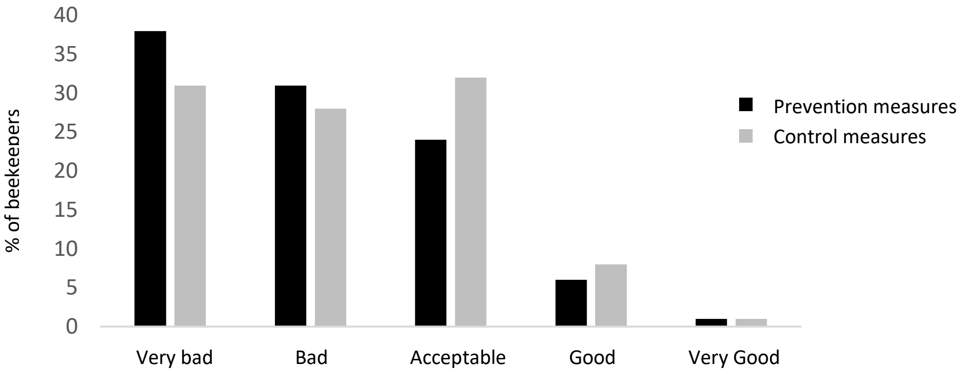

Fig. 4.3 – Beekeeper's classification regarding the performance of the competent authorities in preventing and fighting *Vespa velutina*.

The control measures used by beekeepers are diverse (Fig. 4.4), being the most common the placement of traps near the apiaries to catch the foundresses in spring, the workers in the summer to reduce the pressure on the apiaries, and the future queens in autumn. Fifteen percent of beekeepers reported hornet capture (e.g., using rackets), while 12% reported the use of poisoned baits, that are carried to the nest by the workers to feed the larvae. The latter are thus quickly poisoned, which blocks their development and finally leads to the death of the colony. Alternatively, there are also beekeepers who pick up workers, sprinkle them with insecticide powder and release them back so that when they reach the nest, they can contaminate the entire colony. Most beekeepers (61%) rated the different control techniques as having low effectiveness, with only 23% of respondents reporting the control procedures as effective, noticing however that only if used together.

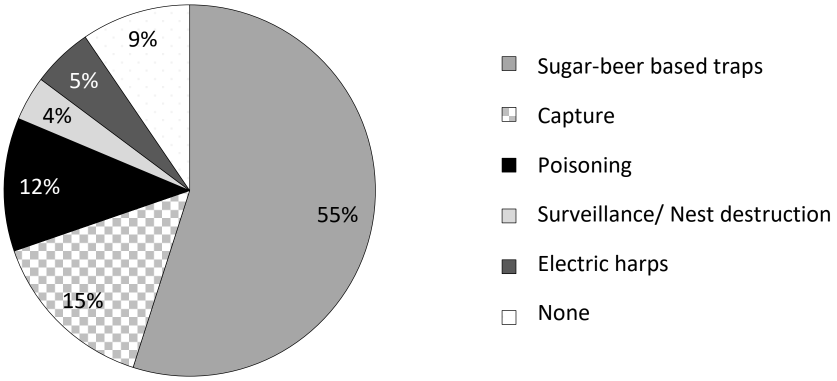

Fig. 4.4 – Techniques used by Portuguese beekeepers to control *Vespa velutina*.

The multivariate model showed that the negative impact of *V. velutina* in the number of bees was particularly mentioned by beekeepers who had seen a high number of nests and by those that develop their activity in the most affected municipalities by *V. velutina* (where the official number of nests reported was higher) (Table 4.3). The number of nests officially reported at the municipality level has no relationship with the number of nests seen by beekeepers, thus there is independence from a variable that respondents do not control (Table S4.1). Still, the beekeepers who have their activity in the most affected municipalities were the ones who reported the highest impacts of this invasive (Fig. S4.1). Beekeepers with a high number of hives had a lower perception of the reduction in the number of their honeybees due to the presence of *V. velutina* (Table 4.3). Finally, beekeepers that performed more tasks with their hives reported less wasp impact, although this relationship was not statistically significant (Table 4.3).

Table 4.3 - Effect of dedication to the beekeeping activity and the number of nests sighted by each respondent and officially reported, in the estimated impact of *V. velutina*.

Multivariate model (% honeybee loss)	Estimate	Std. error	t test	p value	
(Intercept)	2.719	0.140	19.4	< 2e-16	**
No. of tasks performed with beehives	-0.047	0.044	-1.0	0.296	
No. of beehives	-0.004	0.002	-2.1	0.035	*
Official no. of nests in beekeeper municipality 2015-2017	0.002	0.001	2.4	0.020	*
No. of nests seen by beekeeper	0.028	0.008	3.4	0.001	**

Note: Parameters and p values were estimated using a Generalized Linear Model (GLM) with negative binomial distribution and logit link function

Discussion

With this study we aimed to assess the perception by Portuguese beekeepers about the impact of *Vespa velutina nigrithorax* on their activity. The number of respondents was relatively small considering the universe of beekeepers in Portugal, essentially representing the fringe of beekeepers with internet access, high education and membership of beekeeping associations. Among the respondents, we found that the vast majority was already informed about the problems associated with *V. velutina*. This knowledge reinforces that the awareness actions carried out so far by beekeeping associations, research centres, local authorities and other entities of public administration (DGAV, ICNF, INIAV) are bearing fruit. However, the lack of information about the best techniques to fight this invasive and the need of a close relation with the scientific community was also recognized. The inquired beekeepers also mentioned that preventive measure and control procedures have not been effective. They highlighted the urgent need of a binding national strategy that equally regulates control actions between all municipalities.

Most respondents say that their hives have already been attacked by *V. velutina* with a consequent honeybee loss, being this perception positively related both to the number of nests officially reported and to those seen by the beekeeper. Beekeepers are most likely unaware of the official number of occurrences of *V. velutina* in their municipality. However, respondents who keep hives in areas with more nest occurrences reported a higher negative impact. This is a good indicator that beekeepers are correctly assessing these invasive impacts and that these types of survey-based studies are credible and can be used as a decision tool. In this work, it was evidenced that the most professionalized beekeepers, i.e., those who have more hives, and perform more tasks aiming the welfare of their hives (although in this last variable the trend was not considered statistically significant), were the ones who least report the impact of *V. velutina*.

The fact that almost 30% of beekeepers affiliated with associations have mentioned not having been contacted by their beekeeping association reveals that despite the effort made so far, there is still room to improve the information provided to their members. A recent study in Italy (Carisio et al. 2020) revealed that beekeepers use the internet to look for information about *V. velutina*, so the authors suggest that associations and the scientific community should use this tool to promote outreach actions with different stakeholders, thus reducing the costs of organizing awareness campaigns compared to traditional face-to-face meetings. The inquired beekeepers have a high level of education and most likely access to the internet, since 70% provided their e-mail address for this study; this means that the use of digital tools may also be implemented in a more generalized and comprehensive way to facilitate the communication between associations and beekeepers. In this way, the gaps here identified can be minimized.

The beekeeper's awareness about *V. velutina* and its impacts on biodiversity, economy and public health, as well as the explicit desire to learn about the new scientific advances, demonstrates their willingness to contribute for the control and management of this invader. According to data from the Gabinete de Planeamento Políticas e Administração Geral, (2019), there are around 11,883 registered beekeepers in Portugal (corresponding to a universe of approximately 42 thousand apiaries and 768 thousand hives), of which 90% are non-professionals (keeping less than 150 hives). So, their involvement in nationwide early detection and early warning activities (through the communication of all observed nests to the competent authorities and their registration on the STOPvespa platform) could be critical to the monitoring and control of the hornet expansion.

We found that 20% of the nests detected by beekeepers were destroyed by themselves, often with no proper equipment, putting their health at risk due to the danger of attack by the hornets in response to nest disturbance (Choi et al. 2021). Sometimes, and due to unawareness, even using chemical products not approved for this purpose, with the consequent environmental contamination and of local food webs (Turchi and Derijard 2018). The impacts of *V. velutina* control measures on the environment have not yet been investigated; however, the first case of predation of *V. velutina* nests by the European buzzard (*Pernis apivorus*) was recently reported in Spain (Macià et al. 2019) as well as the presence of neocotinoids in this bird of prey (Byholm et al. 2018). These facts highlight the need to deepen the knowledge about bioaccumulation along the food web and the importance of removing insecticide-treated nests from nature (Geiger et al. 2010, Beggs et al. 2011), as recommended in the current Portuguese Good Practices Manual, where it is already (since 2018) possible to verify which products are accepted for nest inactivation (Marques et al. 2018). Trapping continues to be the strategy most used by Portuguese beekeepers; however, they recognize that they are not effective on their own. This result corroborates the findings of a previous survey to beekeepers under the scope of the project GESVESPA, in which 90% of respondents confirmed the use of traps in an unscheduled and non-selective way across the year (GESVESPA 2017). A comparative study of the effectiveness of different types of traps (artisanal and commercial) for the capture of *V. velutina* concluded that regardless of traps, the selectivity for the hornet was always reduced (INIAV 2019). Other European studies have shown precisely the same (Goldarazena et al., 2015; Rojas-Nossa et al., 2018: <1% of the total catches are *V. velutina*; Lioy et al., 2020), which leads to many other wild pollinators being inadvertently captured, with the consequent impact on native biodiversity. The indiscriminate use of traps (regardless of higher or lower density of *V. velutina*) and disregard of the current scientific knowledge may be leading to dramatic impacts on several species of the local entomofauna (Requier et al. 2020). Although poisoned baits have some recognized effectiveness in eliminating *V. velutina* colonies, they can cause serious environmental problems (Turchi and Derijard 2018). Hence, these topics should deserve urgent attention from future work to understand the real impact of these procedures on the environment. Overall and similarly to the work by Requier et al. (2020), here we highlighted the need to improve communication between science and action in the early stages of management plans to improve the sustainability of practices.

The reported honey production was in line with the average productivity of non-professional beekeepers (which represent 86% of our total respondents). Although the number of beekeepers grew exponentially between 2013 and 2016, and stabilized from 2017 onwards (Gabinete de Planeamento Políticas e Administração Geral 2019), the amount of honey per hive reported by beekeepers shows a decreasing trend (Table 4.1). This trend may be related to external factors not related with *V. velutina*. For example, the dramatic fires that hit the region covered by this study in 2017 may have reduced the food resources available for bees and consequently honey production. However, the impact of *V. velutina* cannot be ruled out. Most beekeepers maintain a reduced number of beehives, which increases the difficulty in keeping their hives healthy to better resist *V. velutina* attacks. So, the increasing trend in the abundance and density of this invasive in Portugal may also have contributed to a decrease in honey production.

The less positive rating in relation to the performance of local authorities in fighting, and particularly in the prevention of the expansion of *V. velutina*, is essentially due, according to the respondents, to the low celerity and efficiency with which nests are sometimes destroyed (many of them are removed late and during the day, which can lead to the re-emergence of new colonies), and the lack of specialized teams to prospect for nests in the field (and not acting only after the nests are officially reported). Comparing these results with those of the GESVESPA project presented in December 2017, in the previous study the degree of satisfaction with the performance of the competent authorities was slightly higher. The difference may be due to the small universe of previous respondents (only 30 beekeepers), and because with the ongoing expansion of *V. velutina*, the dissatisfaction with the effectiveness of control actions may now be more evident. These responses highlight the fragility of the alert system and the need for the existing Action Plan for *V. velutina* to be binding and applied equally across all municipalities. The control of this species can only be effective if all agents involved, from public administration entities, municipalities, associations, beekeepers, researchers, and operational technicians agree on the methods to be followed. In 2019, the Portuguese government provided financial assistance to municipalities to support nest destruction campaigns; however, the growing trend of *V. velutina* in Portugal revealed that this financial support is insufficient, especially in the north and centre of the country.

Final Considerations and Future Perspectives

The impact of *Vespa velutina* nigrithorax on beekeeping activity here identified is in line with recent scientific evidence (Leza et al. 2019, Requier et al. 2019, Verdasca et al. 2021a) that confirm the species as an additional stressor for honeybees. In this way, appropriate measures to support the beekeeping sector can be established and the costs resulting from control procedures should be evaluated. Despite some advances, so far, a selective trap for *V. velutina* has not been successfully developed. Being this technique the most widely used in Portugal and Europe among beekeepers, future research on this topic is urgent to mitigate the impact of this control measure on native biodiversity. As *V. velutina* is a voracious predator of the honeybee and other wild pollinators (Rome et al. 2021, Verdasca et al. 2021a), it is also important to know what other species are being impacted and how this is affecting the provision of ecosystem services, namely the pollination of agricultural crops.

This study reflected the urgent need to adjust the current Action Plan to fight *V. velutina* to the issues raised by beekeepers, with mandatory and effective transversal strategies for all municipalities. The results of this work also point to the need for a greater connection between the beekeeping sector and the advances of the scientific community. As Portuguese beekeepers are aware about the problem of *V. velutina* and its associated impacts, their involvement in actions to control the species and early monitoring is crucial to minimize the impacts of this invasive. This way, and with collaboration between public administration entities, local authorities, beekeepers, scientists, associations, and other agents involved in the control of the species, it may be possible to slow down the spread of *V. velutina* in Portugal.

Acknowledgements

We thank the Instituto da Conservação da Natureza e das Florestas (ICNF – Eng. Paulo Carmo) and Bombeiros Voluntários de Viana do Castelo, for having provided the records of *V. velutina* occurrences in Portugal. We thank the more than 250 beekeepers who made available their time to answer the survey and all the beekeeping associations, municipalities and Rural Coordination and Development Committees that helped its dissemination. Fundação para a Ciência e a Tecnologia (FCT Portugal) provided financial support through the project UIDB/00329/2020 granted to cE3c. MJV (PD/BD/128351/2017 and COVID/BD/151632/2021), HR (DL57/2016/EEC2018/07) and LGC (LISBOA-01-0145-FEDER-028360/EUCLIPO) were funded by FCT Portugal. LGC was also funded by the Brazilian National Council for Scientific and Technological Development (CNPq. Universal 421668/2018-0; PQ 305157/2018-3).

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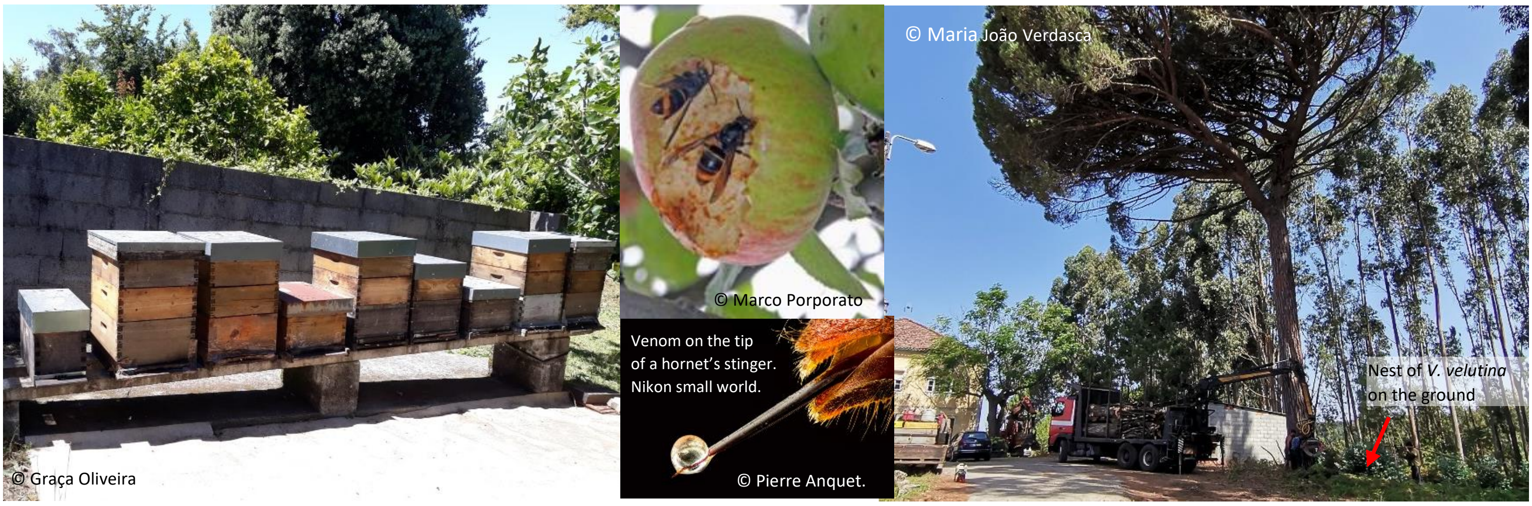
Dynamics of socioeconomic and ecological impacts of invasive species throughout the process of population expansion



The content of this chapter is submitted as:

Verdasca MJ, Rebelo R, Rebelo H, Carvalheiro LG. Dynamics of socioeconomic and ecological impacts of invasive species throughout the process of population expansion (submitted).

Author Contributions

M.J.V., H.R., L.C., and R.R. conceived the study. M.J.V. gathered the data and performed the analyses. M.J.V. produced the first draft of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Dynamics of socioeconomic and ecological impacts of invasive species throughout the process of population expansion

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Abstract

As the density of an invasive species increases, ecological impacts hitherto unknown are expected to arise. Having good predictions of how invader impacts change along the invasion process is critical to better prioritize management actions that may reduce the severity of impacts. Using the invasive *Vespa velutina nigrithorax* as an example, here we assess how the density of this invasive affects the susceptibility to impacts on native biodiversity, human activities, and public health. We gathered data on *V. velutina* occurrence from 2014-2019 from the central region of Portugal and evaluated potential threats to agriculture, wild pollinators, beekeeping, and human health. We found that *V. velutina* starts the invasion process in urban and agricultural areas, and only when these habitats are already colonized with a high nest density the species proceeds to occupy forest patches. This suggests that at an initial stage of invasion the major risks are to urban populations (risk of people being stung) and rural populations (farmers and beekeepers). In Portugal, most of the pollinator dependent crops occupy areas with favourable climatic conditions to this invasive species; so, risks to crop productivity due to the potential decline of managed and wild pollinator populations should be considered in management plans. On the other hand, *V. velutina* only constitutes a risk for humans engaged in forestry activities (e.g., loggers) when it occurs in high densities. We also found that increased cover of suitable areas to wild pollinators and beehive density favour this invasive establishment. Overall, our results show that management plans aiming to reduce the socioeconomic and ecological impacts of the invasion of *V. velutina* should prioritize control actions according to the density and establishment location of this invasive species.

Keywords: Biological invasions, Density–impact relation, Ecological impacts, Invasive species, Species density, *Vespa velutina*

Introduction

To predict the impacts of biological invasions it is critical to understand how the increasing abundance of an invasive species along the invasion process affects local biodiversity, human activities, and public health. After the introduction of a species into a new environment, and early stages of invasion, when impacts are relatively minor, sudden changes are usually observed in the dynamics of successful invaders (Crooks 2005). Population density undergoes an explosive increase, and the rate of expansion accelerates, possibly due to lack of enemies (predators, parasites, and diseases - enemy release hypothesis), competitive advantage over native species (e.g., due to natural or anthropogenic disturbance or lack of defence mechanisms from potential prey/resources), facilitation caused by the presence of other exotic species (invasion meltdown hypothesis: Green *et al.*, 2011). At this stage they come to occupy large areas, sometimes even in higher densities that in the native range (Brzeziński *et al.*, 2019), inflicting substantial damage to native biodiversity and ecosystems and frequently causing considerable socioeconomic harm (Worner and Gevrey 2006). Yet, for most invasive species it is not clear on which habitats, environments, societal groups, are impacts more likely to be felt first.

Frameworks for assessing invasive impacts typically rely on assumed relationships between invader abundance and impact. For instance, Parker et al. (1999) proposed that an invader's impacts are a linear function of its total range, abundance, and per capita effect, with no density-dependent relationship between abundance and per capita effect. However, it is now clear that density-impact relationships vary among species, particularly regarding the threshold density at which significant impacts emerge (Yokomizo *et al*., 2009). Therefore, later impact frameworks explicitly hypothesized density-dependent relationships, with impacts increasing or decreasing nonlinearly with invader abundance (Thiele et al. 2010). Indeed, depending on niche requirements of the invasive, certain habitats may be impacted earlier than others. Therefore, clearly identifying habitats and socioeconomic sectors that are more likely to be impacted at different stages of the invasion process can help to define more targeted and efficient management actions. Here we use the yellow-legged hornet, *Vespa velutina* Lepeletier, 1836, as a case study and try to identify at which stage of the invasion process different habitats and societal sectors are more likely to be affected.

Vespa velutina nigrithorax, native from south-eastern Asia, was introduced in France in 2004, being now present in several European countries (Monceau et al. 2014). Notably *V. velutina* affects managed honeybee colonies by predation on foragers, thus reducing pollen and nectar collection, with impacts on winter survival of the colony (CABI, 2018; Monceau and Thiery, 2018; Requier et al., 2018) and on honey production (Monceau et al. 2014, Requier et al. 2019). Besides honeybees, a broad variety of wild pollinators are also part of the diet of *V. velutina* (Rome et al. 2021, Verdasca et al. 2021a), which may impact the provision of pollination services to pollinator dependent crops. This invasive is also putting at risk human health and other economic activities, due to its close association with humans (Choi et al. 2012a) and because for people who work in rural areas (beekeepers, farmers, loggers) interactions with nests will eventually occur and people will be stung, which may lead to anaphylactic shock and death of allergic individuals (Choi et al. 2012b). The sites selected for nest attachment (treetops, shrubs, roofs, other human infrastructures and on the ground: Monceau and Thiery, 2017; Carvalho *et al.*, 2020) are closely related to its foraging daily routine. Indeed, *V. velutina* is a central place forager, which means that workers forage around their nest (in a radius of *ca.* 500 meters, Poidatz *et al.*, 2018) and then come back to their home base (Monceau and Thiery 2017).

In Europe, *V. velutina* expanded preferentially along the coastlines of the countries it invaded due to favourable climatic conditions (Villemant et al. 2011b, Rodríguez-Flores et al. 2019, Verdasca et al. 2021b) and abundant food resources (Monceau and Thiery 2017). However, and despite differential spread rates (higher along the Atlantic coast and lower to inland areas) this species has also been able to invade many more continental areas than initially expected, with motorways playing an important role in jump dispersal (Verdasca et al. 2021b). A local-scale study on the hornet's nest distribution conducted in southwest France by Monceau and Thiery (2017) evidenced short distances between nests (less than 700m), suggesting that a large overlap occurs between adjacent colonies, which is incompatible with high intraspecific territoriality. These authors also showed that between 2007 and 2014, and despite some fluctuations in nest density in urban areas, the number of nests peaked in 2014, when their density reached 12.26 nest/km^2 (it was of 0.47 nest/km^2 in 2007).

In this work we aim to evaluate how socioeconomic and ecological risks posed by the invasion of *V. velutina* change throughout the invasion process, considering the potential impacts on native biodiversity, human activities (like beekeeping and agriculture), and human health. The information here provided will be important to support future studies aiming to identify the specific threshold density at which significant impacts of *V. velutina* emerge, in a way that an optimal management strategy can be developed for the species.

Methods

Vespa velutina occurrence data

Presence data of *Vespa velutina* for the central region of Portugal (a total of 13112 nests between 2014-2019, Fig. 5.1) was obtained from Instituto da Conservação da Natureza e das Florestas (ICNF - the public administration entity that validates the occurrences reported by citizens on the online platform *STOPvespa: http://stopvespa.icnf.pt/*). This region, which has progressively been invaded by *Vespa velutina*, is organized in a mosaic of similarly small intricated parishes (the smallest administrative subdivision of Portuguese territory) and mostly composed by forest stands of coniferous, eucalypts and mixed forest (>40% of the total area: Table S5.1). With consistent records for all the time considered, it was possible to document the filling up of this area and inspect fluctuations in nest densities across the different years. The detectability of nests may change with the habitat, and a bias may occur towards the detection of more nests in urban than in rural areas. However, agricultural and forestry activities are very intensive in the study area, implying frequent human intervention. Nest density per parish was used as dependent variable in subsequent analyses (see below).

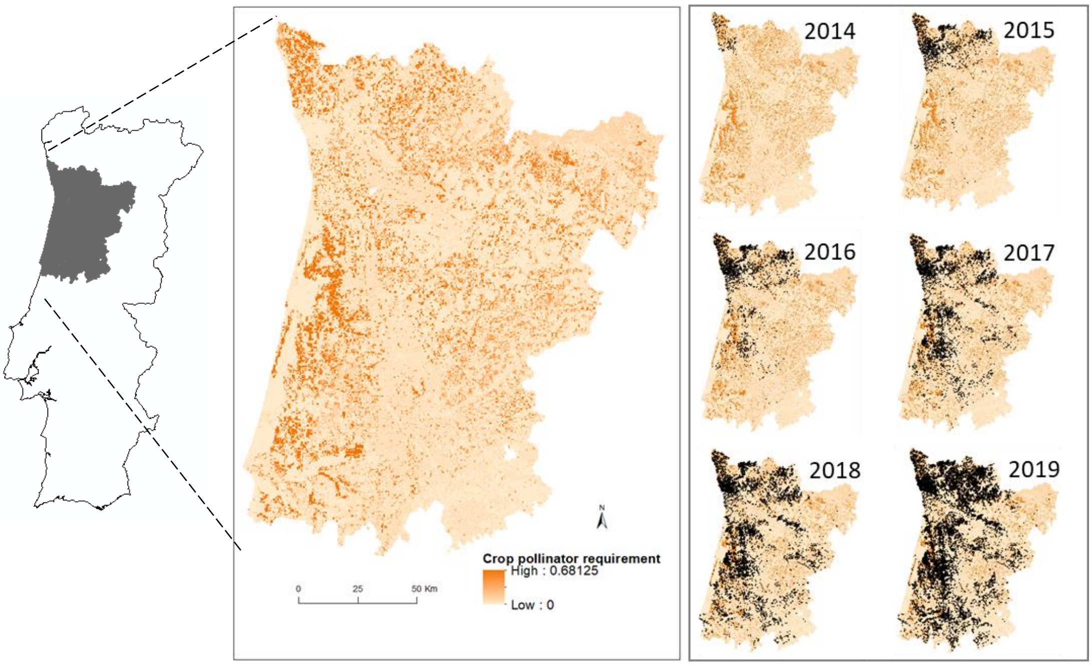


Fig. 5.1– Study area in the centre of Portugal, comprising the districts of Porto, Viseu, Aveiro and Coimbra. The gradient in orange represents the crop pollinator requirement addressed in this paper (crops with high demand for pollinators occur more frequently near the coast). For details on map production see below the *Crop Pollinator requirement* section of the Methods. Cumulative *Vespa velutina* nest locations (in black) from 2014 to 2019 are shown on the right.

Potential impacts:

- ***Proxy for crop pollination susceptibility***

To estimate the crop pollinator requirements of each landscape we combined the information of pollinator dependence of different crop species - a fixed value throughout the period considered in this study (for details see: Klein *et al.*, 2007; Gallai and Vaissière, 2009; Wolowski *et al.*, 2019) with information on the main land uses present in each parish (IGP: COS2007N5, 2010; pixel resolution of 300m). The percentage of the parish area occupied by each crop category was multiplied by its respective pollinator dependence (for details see the review by Klein *et al.* (2007). For each class of dependence we attributed an intermediate value: i) essential (value attributed was 95%): production reduction by ≥ 90%; ii) great (65%): great production increase/ animal pollinators are strongly needed (40 to 90% reduction); iii) modest (25%): modest production increase/ animal pollinators are clearly beneficial (10 - <40% reduction); iv) low (5%): little production increase/ some evidence suggests that animal pollinators are beneficial (>0 - <10%

reduction); v) no increase (0%): no production increase with animal-mediated pollination. The mean value of the percentage of crop pollinator dependence was then calculated for the different parishes. The overlay of the maps of crop pollinator dependence with the ones representing the increasing abundance of *Vespa velutina* in the study area over the years, enabled identifying the risky areas for agriculture, i.e., those areas where the demand of pollinators is high, and the abundance of the invasive hornet was high or was becoming higher.

- ***Proxy for wild pollinators susceptibility***

As *Vespa velutina* is an effective predator of wild pollinators, its impacts to biodiversity are likely greater in areas where densities of wild pollinators (bees, flies, and others) are greater. To identify regions where density of wild pollinators is likely greater, we used the concept of ‘habitat suitability to insect pollinators. Following the method used for other European regions (Vogiatzakis *et al.*, 2014), we classified each land cover class according to its potential to maintain a high diversity of wild pollinators, using expert opinion. More specifically, land cover quality scores were attributed to each class by nine experts on pollinator distribution and ecology (four of them focused only on bees) with a vast experience with wild pollinators and good knowledge on Portuguese landscape and habitats (see list in Acknowledgments). Experts were given the full descriptions of all land-cover classes according to *Carta de Uso e Ocupação do Solo de Portugal para 2007* from IGP (COS2007N5 2010) and were asked to rate them from 0 (lowest) to 5 (highest) overall suitability for pollinators. Responses for every land-cover category were averaged by parish (a fixed value of habitat suitability across the different years) and mapped into the GIS.

- ***Proxy for beekeeping susceptibility***

We used the number of beehives by parish from 2014 to 2019 to inspect for potential impacts on beekeeping activity. This yearly information was obtained from DGAV (Direcção Geral de Alimentação e Veterinária). The density of beehives by parish was calculated and mapped into a GIS.

- ***Proxy for human health susceptibility (2 types of impact)***

Tree cover (retrieved from Copernicus) was used to inspect for impacts on humans working on forestry operations, as most of forest area in the study region is private and managed for timber and fibre production by their owners. Human population density in each parish (obtained from the European Environment Agency, 2020) was used to infer the overall probability of interactions between humans and hornets. We assumed a fixed value for tree cover and for human population density for the time considered in this study.

Data analysis

We used Generalized Linear models (GLM) to inspect for relations between the increasing hornet density and the different proxies of socioeconomic and ecological susceptibility (see description above). The yearly density of *Vespa velutina* nests (number of colonies by km^2) in each parish was used as dependent variable and each of the proxies as independent variable. Before running the models we tested the collinearity between the six independent variables (one by each potential susceptibility, plus the year) and none of them was correlated (all with r<0.7: Dormann *et al.*, 2013). All the interactions between variables were tested and, using the dredge R function from the MuMIn package (Bartón 2009), the best models (with delta values of Akaike Information Criterion (AIC) < 2) were selected. The final model was obtained by keeping all the variables that were consistently chosen by the selected models. The results were plotted using the package visreg (Breheny and Burchett 2017). All the analyses were performed in R 3.6.1 (Team 2019).

Results

Nest density per parish of *Vespa velutina* in invaded parishes fluctuated between 2014-2018, and in 2019 a substantial increase was observed, reaching the maximum of 4.2 nest/ km^2 (Fig. 5.2 and Table S5.2). Nests were mostly observed in urban and agricultural areas (Fig. 5.3 and Table S5.1).

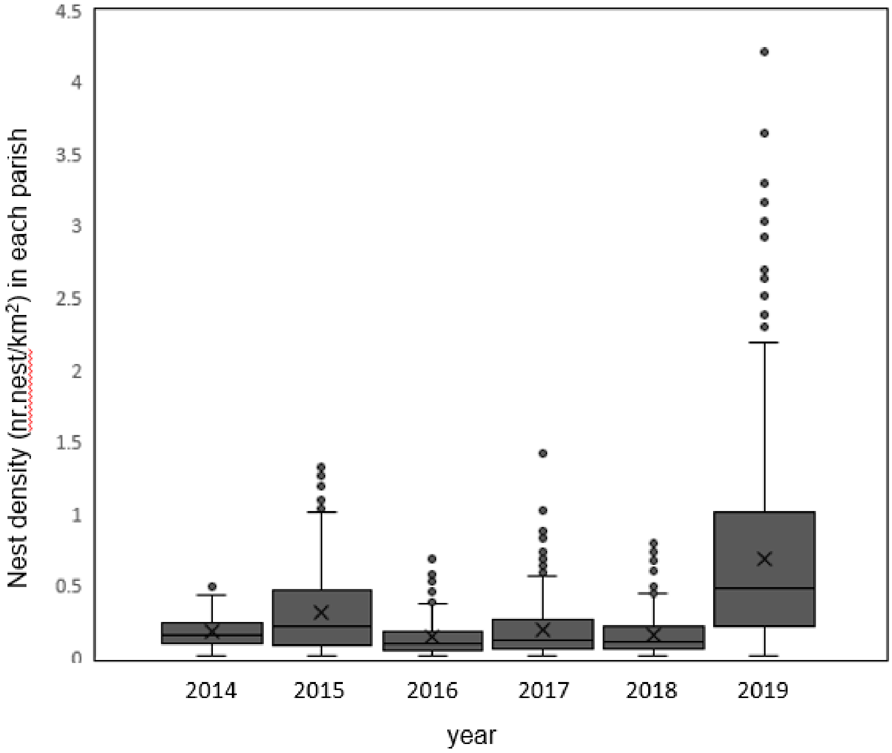


Fig. 5.2 – Variation of nest density (nr of nests/km^2 per parish) between 2014 and 2019

Crop pollination mediated by insects

The classification of the study area according to its crop pollinator requirement varied between 0% (e.g., *Pinus* Forest, built-up urban areas) to 95% (e.g., in fresh fruit orchards, temporary irrigated crop). The agricultural areas with high insect pollinator requirement, specially “temporary irrigated crops”, “fruit crops” and “nut crops”, occupy a small percentage of the study area (10%) being mainly located near the coast (also where the human population density is higher), precisely in areas where *Vespa velutina* has been preferentially settling (Fig. 5.1).

The positive relation between nest density and cover by high pollinator requiring crops was steeper in rural parishes, where the number of inhabitants is lower than 100 per km^2 (Table S5.4 and Fig. 5.3 – left panel), than in urban parishes (Table S5.4 and Fig. 5.3 – right panel).

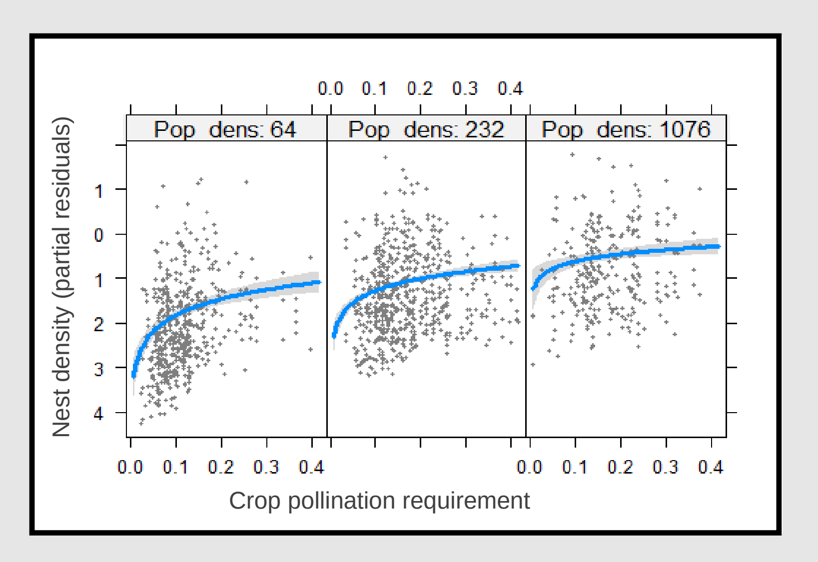


Fig. 5.3 – Relation of *Vespa velutina* nest density with local crop pollinator requirement under different levels of human population density (A – low population density; B - medium population density, C - high population density). Dots represent the partial residuals of the effect of crop pollinator requirement on the nest density. The graphs were built based on the most parsimonious GLM model (for details on model selection see Tables S5.4 and S5.5).

Wild pollinators

For most land use classes there was a general agreement between experts on their value for pollinators (Table S5.3). The classes that received the highest diversity of classifications were those under the general classification of “water”, namely natural water courses, coastal lagoons, ponds and inland lakes. High quality classes corresponded to “shrubs and natural meadows” (with 4.04 points on a 0 to 5 scale), “deciduous forest” (with 3.8 points) and “Mediterranean forest” (3.71 points), which correspond at 20% of the study area (Table S5.1). Low quality classes for pollinators were water, wetlands and constructed urban areas.

Suitability to pollinators is highest in the NE region of the study area (Fig. 5.4) and is positively correlated with the hornet nest density (GLM; P<0.022, Table S5.4). This relation was quite strong and not dependent on either human population density or year (no interactions).

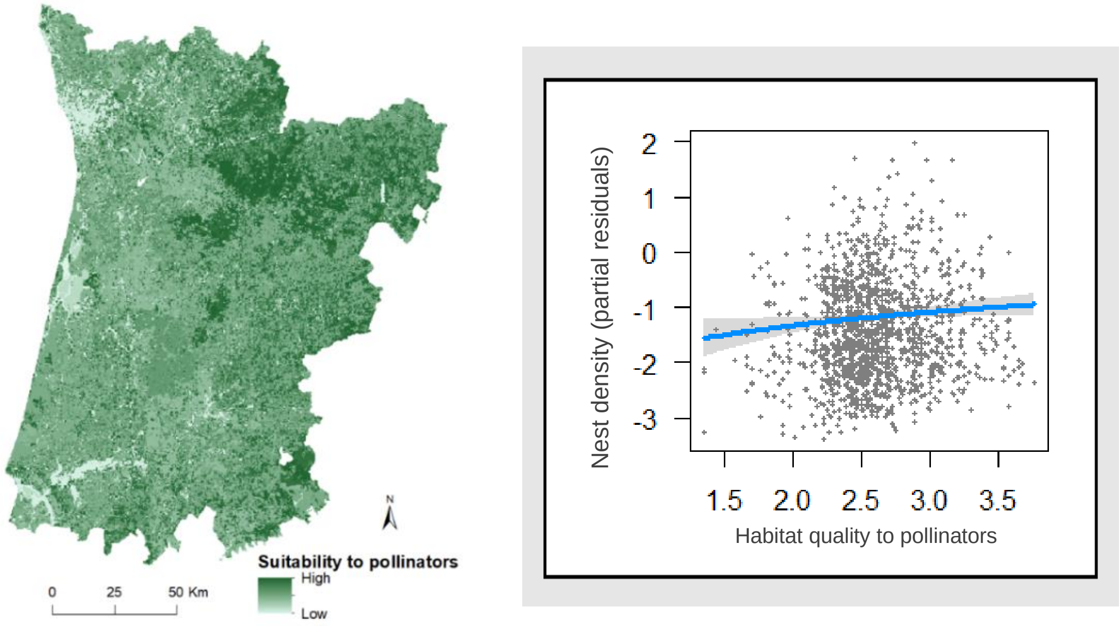


Fig. 5.4 – Habitat quality for pollinators across the study area (left). Dark green areas correspond to the most suitable areas for pollinators according to the expert opinion addressed in this paper (for details on map production see the Methods section). Relation of *Vespa velutina* nest density with habitat quality for pollinators (right). Dots represent the partial residuals of the effect of habitat quality for pollinators on the nest density. The graphs were built based on the most parsimonious GLM model (for details on model selection see Tables S5.4 and S5.5). The plot was obtained with the visreg R package.

Beekeeping

The susceptibility of beekeeping to the hornet varied according to human population density - a positive association between hornets and beehives was only detectable in rural areas in the first years of invasion (Fig. 5.5 – year 2014: left panel). As the years went by, no tendency of the hornet to settle in areas with more hives was observed, particularly in rural areas (left panel) corresponding to the most important honey-production regions.

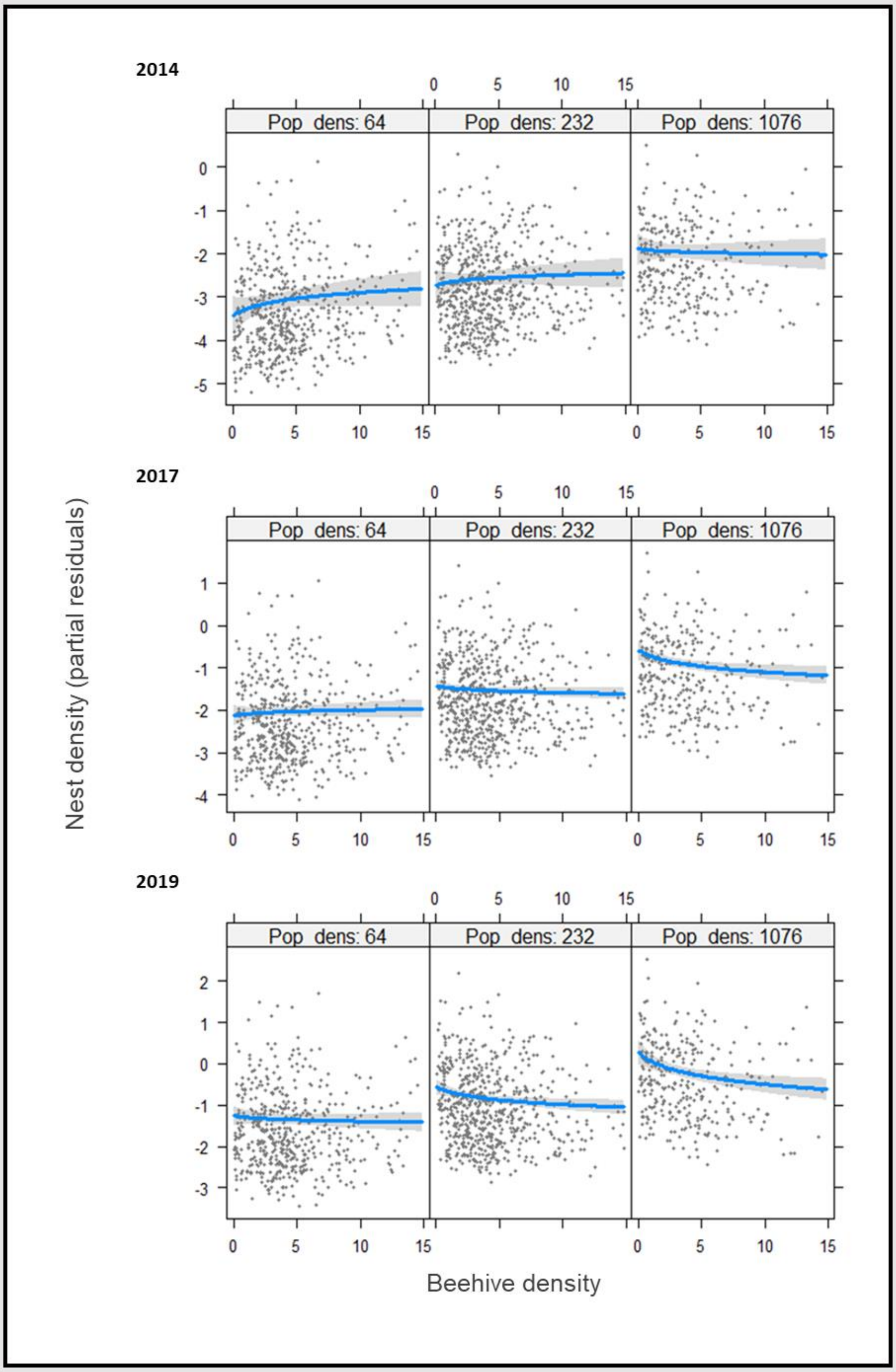


Fig. 5.5 – Relation of *Vespa velutina* nest density with beehive density under different levels of human population density (A – low population density; B- medium population density, C- high population density). Dots represent the partial residuals of the effect of crop pollinator requirement on the nest density. The graphs were built based on the most parsimonious GLM model (for details on model selection see Tables S5.4 and S5.5). The plot was obtained with the visreg R package.

Human Health (Forest loggers | *General human-hornet interactions)*

Vespa velutina established first in areas with low tree cover; as the years went by, this tendency was reverted, specially in 2019 (Fig. 5.6A), which constitutes a new risk for loggers, residual when the density of the hornet is low. On the other hand, the density of nests clearly increased with human population density in a model without year interaction effects (Table S5.4 and Fig. 6.5B).

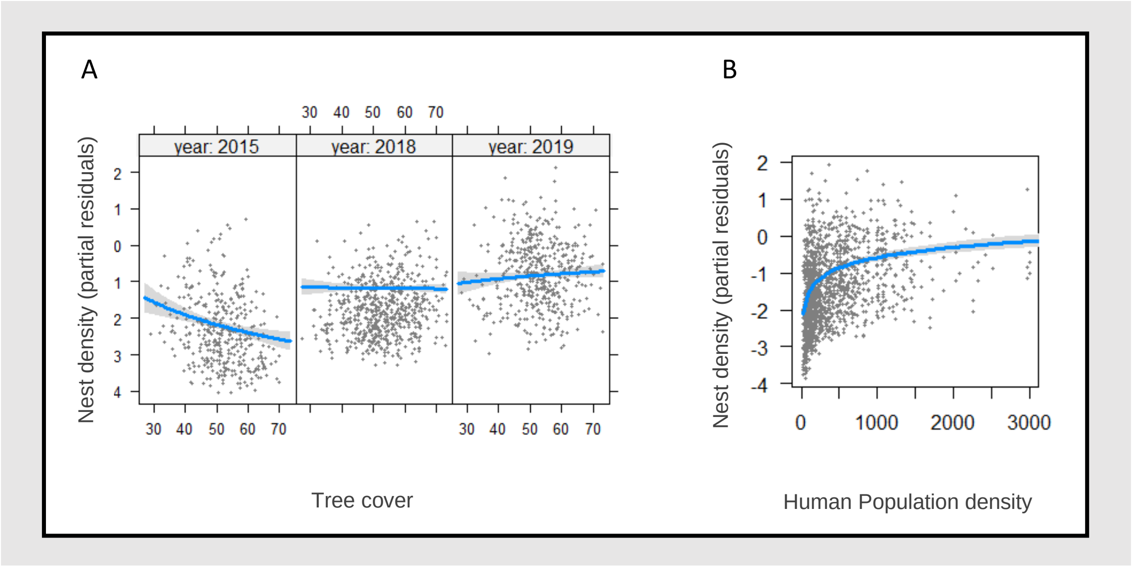


Fig. 5.6 – Relation of *Vespa velutina* nest density with tree cover under different years (on the left) and with Human population density (on the right). Dots represent the partial residuals of each effect on the nest density. The graphs were built based on the most parsimonious GLM model (for details on model selection see Tables S5.4 and S5.5). The plot was obtained with the visreg R package.

Discussion

Our study evidenced that impacts of *Vespa velutina nigrithorax* will probably occur throughout all the different stages of the invasion process and can change according to its density. During the initial invasion stages *V. velutina* associates more with urban and agricultural areas, increasing the risk of having people stung since the very beginning of the invasion process. In those preferred habitats the impacts will probably be high along the entire process, with the consequent increase of the socio-economic risk with time, as the hornet density increases. Moreover, the first establishments may be promoted by the presence of apiaries in rural areas. In our study area we also identified agricultural regions with higher dependency on insect pollination services along the Atlantic coast. Hence, crop production in these areas may be threatened due to the presence of *V. velutina*, even at low densities. On later invasion stages, forest areas begin to be invaded, unravelling an additional risk to the forestry economic sector. In fact, we found that at high densities this invasive will build its nests in forests until then unoccupied, posing a new risk for loggers during vegetation clearing activities or logging operations.

Pollinator habitat suitability - Expert Opinion

The suitability of a habitat for pollinators depends on the availability of floral and nesting/reproduction resources and hence on the characteristics of the landscape (Kremen et al. 2007). Despite the difficulties in defining habitat quality (Johnson 2007), a general agreement on the experts' classification of the different habitats to pollinators was achieved in this study. This same method was already used by Vogiatzakis *et al.* (2014) and Aguirre Gutiérrez *et al.* (2015), also with a general concordance among experts. In our case, water habitats were the ones with higher variation in habitat classification. This can be directly related to the broad description of land-cover classes or, most plausibly, because four experts focused their opinion only in bees while the remaining five considered all pollinators group, which include some insects that live near water or pollinate pond plants in standing waters.

Nest density and habitat associations

Annual fluctuations in nest densities are frequent in Hymenoptera, and can be attributed to four main causes - weather, loss of queens, intraspecific usurpation, or control actions (Martin 1992). Population dynamics and densities of *Vespa velutina* populations in many European countries are not well studied and contrasting demographic processes probably occur in different regions. Since our records came from citizen projects, we are aware that a bias on nest reports could have happened with more identifications in areas with high population density. However, this is a very mediatic species in Portugal, and even in the countryside there are some important cities and villages where people are also informed of this invasive and its impacts on beekeeping, agriculture, and public health. Moreover, important economic activities, namely those linked to agriculture, logging and beekeeping, take place in the countryside and involve many workers (farmers, loggers, beekeepers) who also report nests. Hence, we believe that the susceptibilities to *V. velutina* impact here identified are barely affected by differences in human density. We identified that after the first four years, the species boosted its density in the study region on the fifth year - 2019. The maximum nest density observed (4.2 nest/ km^2) for this region in Portugal was lower than the one measured in a local scale study in the south-west of France (12.26 nest/ km^2: Monceau and Thiery, 2017) or even at an urban local scale in Portugal (5.4 ± 3.3 nest/ km^2: Carvalho *et al.*, 2020), meaning that the carrying capacity has probably not yet been reached.

The expanding distribution of *V. velutina* nests in both semi-urban and agricultural areas indicates that the socio-economic risks they pose is increasing with time (Rodríguez-Flores et al. 2019). The large majority of our records occurred in urban and agricultural areas, being both types of habitat mainly located along the coast of our study area (where the climatic conditions are more favourable for the hornet: Bessa *et al.*, 2016; de Medeiros, Hernández-Lambraño and Sánchez Agudo, 2018; Rodríguez-Flores *et al.*, 2019; Verdasca et al. 2021b). The close relation of the species with anthropized environments may be explained by presence of human structures such as roofs, balconies, etc, that provide shelter for the species (Carvalho et al. 2020), especially during the primary nesting season from February to May. Regarding agricultural areas, in the centre/north of Portugal these are generally embedded within a complex and intricated mosaic of rural and urban landscapes (Moreira, Rego and Ferreira, 2001), which provide suitable foraging conditions for the workers, due to the presence of arthropod crop pests and insect pollinators. The establishment of the species in these urban and rural areas is similar to that found in other studies. For example in France, nest occurrences were basically observed in urbanized (48.5%) and agricultural (42.25%) areas, with only less than 10% of occurrences in natural areas (Rome *et al.*, 2015). In Portugal, Bessa *et al.* (2016) working on a small region in the north of the country found that the percentage of crops had a positive and significant effect on *V. velutina* presence. Interestingly, the crops that were classified as having high crop pollinator requirement (like temporary irrigated crops or fruit crops) although dispersed all over the study area, are mainly concentrated in the west swath of the region, precisely where *V. velutina* occurs in high numbers. Hence these pollinator dependent crops can be at a higher risk if the density of the hornet continues to increase. Soon, or perhaps even now, a decrease in the production of these crops is possible, solely due the expected increase in the predation of pollinators. Additionally, rural areas encompass croplands with high demand for insect pollination, hence local agricultural workers may be more prone to suffer attack by the hornet.

Despite shrublands and natural meadows have few natural or anthropogenic features that can support nest establishment, the high prevalence of wild pollinators in these areas seem to favour the establishment of *V. velutina* nests nearby. This invasive is a generalist and opportunistic predator, capturing honeybees and other wild insects (Rome et al. 2021, Verdasca et al. 2021a). Since adult Vespidae feed on flower nectar from various plants to obtain energy for flight, they can also act as pollinators. However, because they generally lack abundant body hairs and do not feed on pollen, they are considered (with few exceptions) less efficient pollinators than their bee relatives (Hooks and Espíndola 2020). Additionally, it is possible that *V. velutina* may compete with native solitary bees or other pollinators. In fact, changes in flower visitation rates of wild pollinators due to the presence of *V. velutina* were already observed (Rojas-Nossa and Calviño-Cancela 2020). However, the predominant association of *V. velutina* with urban and agricultural areas make us suspect that the predation impact on main pollinators (e.g., bumblebees) should be low. Concerning the possible competitive impacts on native Vespidae, a recent study concluded that these are probably avoiding competition pressure by *V. velutina*, and therefore the presence of the hornet has not been leading to an evident species replacement (Carisio et al. 2020). However, in Korea, Choi, Martin and Lee (2012), highlighted the occurrence of competitive displacement of native *Vespa* species by the invasive *V. velutina*. Hence, soon native Vespidae present in Europe might suffer from predation and competition for food sources or nesting sites.

In Galicia (Spain), intensively reforested areas with *Eucalyptus globulus* and *Pinus pinaster* shelter most of the secondary nests, containing thousands of workers, at high heights (>15m) from June to the end of the season (Rodríguez-Flores et al. 2019). The dominant forest plantations in the centre of Portugal are also composed by these two species of trees. Interestingly, here we showed that when *V. velutina* is present at very low densities (as it happened in the first years of establishment in our study area) the species did not associate with the monoculture plantations or mixed forests of eucalyptus and conifers in this specific Portuguese area, where probably there is less availability of food resources. However, when in high densities, the hornet began to establish in these less favourable habitats. This is an interesting result since to the best of our knowledge it has not been shown before for *V. velutina*. Hence, this invasive can constitute a risk for public health, particularly for loggers that can accidentally disturb the nests during logging or vegetation clearing activities and be attacked. If control procedures can reduce the density of the hornet, these potential risks may become residual. Given that *V. velutina* secondary nests maintain brood temperatures above the surrounding environment, sensitive forward looking infrared (FLIR) cameras, like the ones used by Al-doski, Shattri and Helmi-Zulhai (2016) for other insect pests detection, could be attached to unmanned aerial vehicles or deployed to ground teams to improve nest detection. These techniques have a high economic cost, and therefore quantifying the abundance–impact relationship and identifying effect thresholds will be crucial to enable an efficient management of the invasive and avoid over-investment in control measures (Thiele et al. 2010, Sofaer et al. 2018).

A positive association of the density of nests of *V. velutina* with honeybee hives only occurred at the early stage of the invasive process in rural areas when the hornet was not (yet) spread all over the region. Beehives probably promote the first establishment of *V. velutina*, corroborating its potential impact for beekeeping activities, but when the hornet becomes widespread this relation is no longer evident. As *Vespa velutina* avoids forest when at low densities; we suggest that if beekeepers move their beehives to clearings inside forest patches (but near to food resources for honeybees), the predation upon the apiaries may be reduced. Moreover, if these clearings with beehives can be located not far from the small intricated patches of fruit crops and temporary irrigated crops (highly dependent on pollinators and potentially acting as a supplementary food source for the hornet), it may also be a good strategy to reduce the predation pressure on honeybee colonies. However, these interactions between beehives, hornets, wild pollinators and forests are unknown, and these assumptions need to be properly tested before their incorporation into management plans.

Despite the potential danger of *V. velutina* to public health due to possible serious allergic reactions after multiple stings, this species is not considered more aggressive than the European hornet, *Vespa crabro* (de Haro et al. 2010). However, with increasing densities of nests of this invasive in urban areas, where the species is well adapted (Choi et al. 2012a), humans become more likely to accidentally disturb nests. Therefore, local outreach activities targeted to general public (not only to beekeepers, municipalities or control technicians), either in rural or urban areas, should be reinforced.

Concluding remarks

Understanding the relationship between invasive species density and ecological impact is a pressing topic in ecology, with implications for environmental management and policy (Kornis et al. 2014). Since different population densities pose different ecological risks, it is important to continuously monitor this invasion to establish effective management plans. The apparent lack of intraspecific competition, as a potential mechanism for population regulation (Monceau and Thiery 2017), and the high populations densities if this eusocial insect may favour its invasiveness. Hence, with large suitable areas for *Vespa velutina* in Europe (Verdasca et al., chapter 1), there is no indication that the rapid spread of *V. velutina* will lessen if control strategies do not improve and are not reinforced (Barbet-Massin et al. 2020). A recent study about beekeepers' perception of the impact of *V. velutina* on their activity revealed that almost 40% of the nests reported by beekeepers are not removed (Verdasca et al., *in press*; chapter 4). However, the mitigation of the associated ecological impacts is only possible with effective control procedures aiming at destroying all or a given percentage of detected nests, rather than only those potentially harmful to human (nests close to human habitations) or beekeeping activities (nests close to beehives). Otherwise, a small reduction in nest density due to control procedures, especially in urban areas, can have negligible effects and attract back to the cities the gynes of *V. velutina* that are born on the countryside.

With the growing densities of *V. velutina*, here we identified that human health susceptibility to the hornet may increase in urban areas, even when the hornet occurs at low densities. On the other hand, this species only constitutes a potential risk for loggers when occurring in high densities. Regarding the potential threat to agriculture, insect pollination dependent crops located in suitable areas for *V. velutina* may also be at a higher risk, independently of the invasive density. Moreover, suitable inland areas for wild pollinators (like shrublands and natural meadows) seem to have influence in promoting species establishment, raising the need to evaluate its impact on wild pollinators. Finally, a positive relation was found between the density of beehives and the density of *V. velutina* in rural areas, corroborating the current concerns over the acknowledged negative impact of this invasive on managed honeybees. The novel information of this work will be important to better select the locations to apply effective control measures, especially because the indiscriminate use of traps (regardless of higher or lower density of the *V. velutina*) is probably a greater threat to insect biodiversity than *V. velutina* predation.

Acknowledgments

We thank to ICNF - Instituto da Conservação da Natureza e das Florestas (platform SOSvespa: www.sosvespa.pt/web), for having provided the records of *V. velutina*. We also thank DGAV – Direcção Geral de Alimentação e Veterinária for having provided the data related with beekeeping activity in Portugal. We are grateful to the following experts (in alphabetical order), who took time to participate in the assessment exercise: Andreia Penado, Eduardo Marabuto, Ernestino Maravalhas, José Manuel Grosso-Silva, Luísa Gigante Carvalheiro, Mário Boieiro, Orianne Rollin, Patrícia Garcia Pereira and Thomas Wood. Fundação para a Ciência e a Tecnologia (FCT Portugal) provided financial support through the project UIDB/00329/2020 granted to cE3c. M.J.V (PD/BD/128351/2017 and COVID/BD/151632/2021), H.R. (DL57/2016/EEC2018/07) and L.G.C. (LISBOA-01-0145-FEDER-028360/EUCLIPO) were funded by FCT Portugal). LGC was also funded by the Brazilian National Council for Scientific and Technological Development (CNPq. Universal 421668/2018-0; PQ 305157/2018-3). The funding sources had no direct involvement in the study design, or in the collection, analysis, and interpretation of data.

III

Epilogue

General Discussion

The rising numbers of invasive species have ever-growing impacts on biodiversity, ecosystem functioning, economic activities, and human health. Increasing attention is now being given to the different human dimensions of invasions, to ways of slowing or preventing new invasions and to the mitigation of the negative effects of current invasions (Richardson 2011). This was also the purpose of this work, focused on invasive *Vespa velutina* and its socio-ecological impacts. By providing new information about the invasion dynamics and pathways of dispersion of *V. velutina nigrithorax*, about its diet and impacts on human activities, my results can contribute for future management plans. Most of the chapters also produced new fundamental knowledge on the species and its invasion process in Europe and will probably contribute to the refinement of methodological approaches for its future study, as well as of other invasive Hymenoptera.

Niche dynamics and potential for further distribution

Understanding the adaptation of invasive species to new environments is fundamental to derive robust generalities in invasion biology. Evolutionary shifts that change traits and enhance invasibility are one form of adaptation that affects the outcome of a potential invasion (Hill et al. 2016). Adaptation to novel environmental conditions is also critical to range expansions and population persistence in suboptimal habitats. In this thesis I showed that in Iberian Peninsula *Vespa velutina nigrithorax* thrives on conditions that are not exploited in the native range and that are also different from the ones used by the species in France. Furthermore, I identified a large extent of Europe with analogue environmental conditions to those of the native niche that is not yet occupied, proposing as highly plausible the continuation of the expansion process towards other countries. Regions identified in this work as high-risk regions, as south of Italy, Corsica, Croatia, and Montenegro must be vigilant and implement preventive action plans.

The occupation of novel environmental conditions in an invasive range is not exclusive to *V. velutina* (Broennimann et al. 2007, Beaumont et al. 2009). Failure to take this into account when projecting distributions can result in underestimation of potential invasive ranges with clear implications for invasive species management (Beaumont et al. 2009). Like previous works (Broennimann and Guisan 2008, Jiménez-Valverde et al. 2011, González-Moreno et al. 2014), this thesis showed that modelling a species niche requires information of its occurrences on both native and invasive ranges. However, as invasive species are usually not at equilibrium with climate during the invasive process, care must be taken when exploring the potential for their spread in invaded regions. For instance, I clearly showed (chapter 1) that modelling species distributions based on a subset of location records will underestimate the area at risk of being invaded.

Social Hymenoptera are efficient invaders in several continents. *V. velutina* is currently invading Europe, but according to a previous work by Villemant *et al.* (2011), other parts of the world, such as the east and west coast of north America, south America, south Africa and eastern Australia, were identified as having high climatic suitability for the species and therefore as potentially vulnerable to an accidental introduction through international trade (like what happened in France). Interestingly, the worldwide predicted potential distribution for this invasive matches the distribution of another invasive social wasp, the German yellow jacket, *Vespula germanica* (Villemant *et al.*, 2011). This species is widely distributed in Eurasia and was, as several other invasive wasps, unintentionally but successfully introduced in many regions of the world where it became invasive (Beggs et al. 2011). The invasion success of such species likely relates to the generalist foraging behaviour that the social organization of *Vespula* confers (Beggs et al. 2011). In 2019 another Asian native species, the giant Asian hornet (*Vespa mandarinia*) reached north America at two different locations, British Columbia in Canada and Washington State in the United States of America, causing social alarm about its potential spread (Wilson et al. 2020). Like *V. velutina*, *V. mandarinia* is also known to prey on several native and economically important species, such as honeybees and also thrives on temperate climates (Alaniz et al. 2020). The similar environmental conditions exploited by both species makes it possible that if an accidental introduction of the giant Asian hornet occurs in Europe, the species may become invasive. The approach used in this thesis (chapter 1) to compare the niche dynamics of *V. velutina*, in different regions can also be applied to inspect for potential niche shifts in other invasive social Hymenoptera.

In the native range, biotic constraints probably exclude *V. velutina* from part of the climatic envelope within which it could otherwise grow and reproduce. Indeed, like many other invasive species, *V. velutina* is not associated with economic losses in its native range, where it is likely that biotic factors (e.g., predation and competition) control its populations. However, such factors seem to be absent in Europe. Until now little emphasis has been given to the incorporation of biotic interactions (e.g., presence of hornet predators, co-occurrence of competitors like *V. crabro*, density of honeybee hives, distribution/abundance of prey, parasites, pathogens) into *V. velutina* distribution models at large spatial scales and to their potential importance on the regulation of its population dynamics in both native and invasive ranges; such biotic interactions at invaded areas should be addressed and coupled with the distribution models. Although modelling at large spatial scales allows understanding the potential distribution of a species in the long term, its usefulness for the decision of preventive or control activities is limited, as these activities are developed locally, and often on a short-term basis. In a recent review by Srivastava, Lafond and Griess (2019), the authors show that SDMs with strong underlying biological assumptions will have better predictive powers and produce invasive species risk maps that will be more likely to forecast precise estimates of invasion risk. Therefore, these same authors propose hybrid models that include fitting models with existing ecological knowledge, address uncertainty and biotic interactions and link species dispersal traits with projections of species distributions. This type of modelling approach will become much more frequent in the future and holds promise for possible predictions of the effects of biocontrollers such as predators, prey abundance, competitors, or species-specific parasites.

Human mediated transport as accelerator of Vespa velutina nigrithorax invasion

Predicting the expansion of *Vespa velutina nigrithorax* and identifying the pathways that facilitate its introduction and dispersal (especially human-mediated pathways that can aid long-distance spread and potentiate its impact), are important for informing efforts to contain invasion. We highlighted the role of motorways in accelerating the invasion dynamics of this invasive (chapter 2), showing that the establishment of distant nascent colonies is probably mediated through transportation of commercial goods along motorways. By providing a potential model of spread of *V. velutina* in which the motorway network was shown to contribute significantly, this work contributes with a tool that may help policy makers and local managers to prioritize the implementation of early detection/eradication efforts and anticipate the resulting ecological and social consequences. Because large regions in Europe have similar environmental conditions to the hornet's native niche but until now are not yet occupied (chapter 1), the European motorway network may already be or become the invasion route of the hornet. The role of motorways was only possible to identify when the dispersion models were run with land cover and anthropogenic variables separately from the models with climatic variables, highlighting the major role of climate at large spatial scales, while landscape and anthropogenic variables are more relevant at a regional scale, which is in line with Pearson and Dawson (2003). So far, none of the implemented action plans in Portugal, Spain, France, Italy, and UK, refers the need to increase searching for nests around motorways or to reinforce outreach activities targeted to transportation companies. This new information can be incorporated into biosecurity efforts and management of introduction pathways. For instance, biosecurity efforts in New Zealand have been successful in reducing the establishment rate of non-native insects (Browne et al. 2018).

Understanding the mechanisms of invasion and associated pathways is critical for management, including monitoring, interception, and policies to restrict trade (Hulme 2006). However, the problem of invasive species is as much an economic problem as an ecological problem, and the costs of these actions need to be balanced against the gains, measured in terms of avoided damage (Perrings et al. 2010). Estimates of the economic costs of preventing or mitigating invasive species impacts on biodiversity and ecosystem services are scarce, but are only likely to increase in the future: The global map of expenditures with invasive species shows that societies have been mostly paying for the post-introduction management of invasive species impacts, with very little reported investment in the prevention of biological invasions (Zenni et al. 2021). Recently Barbet-Massin, Salles and Courchamp (2020) estimated that *V. velutina*'s nest destruction costed €23 million between 2006 and 2015 in France with increasing yearly costs as the species spreads, potentially reaching €11.9 million in France, €9.0 million in Italy and €8.6 million in the United Kingdom (if the species fills its current climatically suitable distribution). For Portugal there are yet no estimates of the costs of preventive and control measures to mitigate *V. velutina* impacts.

Increasing volumes of global trade have led to calls for efficient solutions to transport goods which presented new challenges for the interception of insect invasions (Hill et al. 2016). From 2010 to 2018, there have been close to 50 interceptions of *Vespa* (hornets) and *Vespula* (yellow jackets) at US ports of entry (Smith-Pardo et al. 2020). The *Vespa* species intercepted include *V. bellicosa*, *V. crabro*, *V. orientalis*, *V. mandarinia*, and *V. tropica*. One of the interceptions of significance was an entire nest of *V. mandarinia* containing live brood and pupae that was sent via express courier from Asia (Smith-Pardo et al. 2020). In fact, such accidental introductions resulting from human activities are nowadays considered the major cause of biological invasions. *Vespa velutina* can be transported over different regions in wood products and bark, and in other man-made goods (e.g., ceramic pottery associated with garden trade), both providing suitable harbourages for hibernating inseminated *V. velutina* queens (Marris et al. 2011). Indeed, this was the most probable route of incursion of *V. velutina* in Europe, on pots imported from a coastal region of China, near Shanghai (Arca et al. 2015). The absence of borders in the European Union makes it difficult to verify long-distance freight, however it is possible if it is targeted for the interception of wooden products transportation and man-made goods associated with garden trade, due to the potential of these commodities to shelter hibernating queens. These targeted biosecurity policies jointly with local outreach activities, especially those directed to transportation companies, should be prioritized to prevent the European motorway network from becoming an invasion route for *V. velutina* to new countries. Moreover, it is also important to promote control actions on ports of entry, namely harbours along the coast.

Diet characterization

The abundance of honeybees in Europe has probably enhanced the spread of *Vespa velutina nigrithorax* (Perrard et al. 2009, Monceau et al. 2013)**.** Honeybee is probably the hornet's main prey in Europe because, in contrast with the Asian species *Apis cerana* (Ken et al. 2005), *Apis mellifera* has no effective defensive behaviour against this new predator (Monceau et al. 2013). In the third chapter of this thesis, I corroborated previous studies based on direct observation of predation events or on examination of food pellets, which give support to the acknowledged negative impact of *V. velutina* on managed honeybees. Indeed, our samples were collected with no specific prerequisite of being located near beehives, but nevertheless we found honeybee DNA in all examined colonies. Moreover, the variety of insect orders and families detected in the hornet diet supports current concerns over the potential threat to the provision of pollination services.

In chapter 3, a specific primer for honeybees was used to test a new protocol through an innovative metabarcoding approach that enabled us to infer the most informative type of sample to assess the predation on honeybees by *V. velutina*. Larval faecal pellets retrieved the higher number of honeybee reads, followed by jaws and stomachs (which were the less informative sample). The approach used in this work can be used with further optimization, and different primers (targeted to the identification of other species besides honeybees) to inspect the broad diet of *V. velutina*. However, as this framework uses a blocking primer to *V. velutina* (to prevent the detection of its own DNA), caution must be taken since it also precludes the detection of scavenging or cannibalism, which are acknowledged behaviours of these eusocial insects (Matsuura and Yamane 1990). Moreover, this method can also be applied to infer the diet of other Hymenoptera, since it enables the detection of soft bodied insects, namely the larval phases, being particularly relevant in the case of invasive species. The results of chapter 3 of this thesis were possible because larvae faecal pellets (i.e., meconia, the gut content eliminated at once by an individual when moulting from larval to pupal instars: Rome *et al.*, 2015) are not removed by workers when cleaning cells after adult emergence (Rome et al. 2015). Consequently, the number of meconia at the bottom of each cell indicates how many individuals were successively reared to pupal instar in the cell before nest collection (Archer 2008). The study of these gut contents will enable to inspect the seasonal variation of prey. Indeed, this was confirmed in a recent study focused on two species of introduced paper wasps (*Polistes chinensis*, and *Polistes humilis*) in New Zealand, where the authors used generalist degenerated PCR primers to amplify a wide prey spectrum without a priori selection of particular taxa (Lefort et al. 2020). These authors detected several orders of insects including agricultural pests and native species on paper wasps' faecal pellets and confirmed that the consumed prey communities were significantly different between inner and outer nest samples, suggesting a seasonal variation in prey availability and/or a diversification of the wasps' diet as the colony grows. However, as brood cells can be re-used multiple times by subsequent generations, this temporal analysis requires precise knowledge of the nest construction process. Overall, and comparing to traditional DNA recovery methods, the framework presented in chapter 3 and the work by Lefort *et al.* (2020) reinforce the potential of these innovative metabarcoding approaches on providing a temporal overview of the diet of other nest-building Hymenoptera colonies based on a single sampling event.

Impacts of Vespa velutina

Some of the most negative ecological impacts of invasive alien species are related to competition with native species, which can lead to the invasive species occupying a dominant position in their new environment or even to the replacement of the native residents (Vicente et al. 2010). Since nest site selection by *Vespa velutina* foundresses partially overlaps with its European counterpart *Vespa crabro* (Monceau *et al.*, 2015), some level of competitive displacement of the native *V. crabro* by the invasive *V. velutina* may be occurring. However, a recent study by Carisio *et al.*, (2020) analysed the impacts of *V. velutina* presence on *V. crabro*, *Vespula vulgaris* and *Vespula germanica* in Italy and concluded that native Vespidae are probably avoiding or minimising competition pressure, and therefore the presence of *V. velutina* has not been leading to an evident replacement of such species. In fact, Villemant *et al.*, (2011) noted that the low level of interspecific competition with other *Vespa* species (basically *V. crabro*) in Europe could have contributed to the success of *V. velutina* in France. Additionally, and since adult workers feed on flower nectars, it is possible that competition is also occurring with some native solitary bees or other pollinators, thereby lowering pollinator visitation and fruit production. It is possible that this invasive is shifting the functioning of native pollination systems through networks of trophic and competitive interactions (Rojas-Nossa & Calviño-Cancela, 2020). Long-term monitoring studies focused on the impact of *V. velutina* on wild bees and associated pollinator services are clearly needed: being this invasive predominantly associated with urban and agricultural areas (Choi, Martin and Lee, 2012; Rome et al., 2015) in detriment of natural areas (Fournier, Barbet-Massin, Rome, & Courchamp, 2017), the predation impact on the main wild pollinators could potentially be low. Nevertheless, attention is needed since *V. velutina* is an opportunistic forager (Rome et al. 2021) and it is possible that in regions where honeybees are less abundant, other preys are more consumed.

The economic impacts of *V. velutina* on the beekeeping sector have been poorly addressed so far. This is partly because it is difficult to disentangle the causes of colony losses, which may be due to weather, insecticides, diseases, parasites, etc. A recent study (Ferreira-Golpe et al. 2018) found that Spanish beekeepers used 20% of the value of their production to fight *V. velutina*. Despite not being able to show a clear relationship between the presence of the hornet and a decrease in honey production, they verified a relationship between strong attacks and a higher incidence of production losses. Those authors estimated that the average value of production per hive dropped about 15% from 2015 to 2016 without any variation in the sale prices. According to the inquiry to Portuguese beekeepers (chapter 4), a decreasing trend in honey production from 2015 to 2017 is also reported. This trend may be related to external factors not related to *V. velutina* (e.g., the dramatic fires of 2017 may have reduced the food resources for bees and consequently honey production), but the increase in abundance and density of this invasive in Portugal may also have contributed to the drop in honey production. The analysis of the questionnaire revealed, with no surprise, that most beekeepers were already informed about *V. velutina* and its impacts and wanted to be involved in the control of this invasive. Hence, their involvement in activities of rapid detection and early warning on a national scale can be essential to monitor the expansion of *V. velutina*. However, they still claim for a closer relationship with the scientific community and for extra advice about the most effective strategies to fight *V. velutina*. In addition to the problems evidenced for the beekeeping activity, agriculture is probably also being impacted but until now its quantification has not been done. From this thesis (chapter 5), I found that cultures with high demand for pollinators, namely temporary irrigated crops or fruit crops located on suitable areas, may be more susceptible to *V. velutina*. Therefore, it is expected than in a near future economic losses on crop production may become more evident.

The impacts on public health cannot be discarded. The association of *V. velutina* to urban environments (Choi, Martin and Lee, 2012 and this thesis - chapter 5) makes contact with humans inevitable. Indeed, increasing numbers of people stung by this hornet have been reported in Portugal (more than 500 in the last three years: Marco Portocarrero from Nativa Association, personal communication), sometimes with severe allergic reactions. Since nests are established in a large variety of natural and human structures (top of the trees, walls, soil, roofs, balconies, sheds) (Carvalho et al. 2020), people can accidentally disturb nests more frequently near their houses, when harvesting fruit or during vegetation clearing activities. As its density increases, the hornet is already starting to colonize forestry areas; hence there is a new risk for loggers. *Vespa velutina* is a mediatic species, but most people does not know how to act after being stung. Therefore, outreach activities about this invasive should provide information about the different sting reactions. Learn to recognize an allergic reaction to the sting and how to act is extremely important and ultimately can help to save lives.

Future research

The impact of *Vespa velutina* on beekeeping activity and on local entomofauna highlights the importance to focus future attention on local scale studies. The future identification of risky areas for beekeeping can serve as a tool to support decisions by beekeepers (namely about the placement of apiaries and traps), to better manage and minimize the impact of *V. velutina* on the regional beekeeping sector and on local biodiversity. Moreover, the specificity of traps is very low (see introduction), which raises the need to more applied scientific research to overcome this trap limitation.

Broad diet studies are also needed to identify all the spectrum of *V. velutina* prey across climatic and landscape gradients. So far little is known about which native species are being predated and what consequences this has on the pollination of agricultural crops with the consequent loss in the final production. It is also possible that this invasive is predating on some crop pests, which can uncover a different functional role. Moreover, it is also not known whether there are some landscape features or climatic conditions that promote or hinder the predation of specific prey. Addressing these questions would be an essential step to assess the impact of this invasive species on wild native insects and evaluate the potential for negative impacts on ecosystem functioning (pollination of wild plants) and provision of ecosystem services (crop pollination).

The impacts of control measures in the environment have not been addressed thus far. Most detected nests in Europe are inactivated by permethrin injection, requiring nest unhook after injection to avoid contamination of the environment and of local food webs (Turchi and Derijard 2018). A research group from Laboratório Ibérico Internacional de Nanotecnologia (INL) in Portugal, has been developing a selective method of nest treatment, in which the hornets carry a bait (a small biocide-injected sphere of the size of the bee's thorax) to the nest, resulting in its elimination. Despite the promising results, further research is needed to guarantee its selectivity. As insecticides have negative effects on biodiversity (Geiger et al. 2010), and in predators of *V. velutina* (e.g. *Pernis apivorus*: Byholm *et al.*, 2018), it is crucial to eliminate the colonies treated with insecticides from the natural environment (Beggs et al. 2011) and follow the accepted recommendations for *V. velutina* nest destruction (Marques et al. 2018). As such, these themes should deserve urgent attention to understand the real impact of these procedures on the environment and local food webs.

Besides the detrimental impacts on biodiversity, human health, and ecosystem services, the economic impacts of *V. velutina* also need to be considered and quantified; and that information is lacking for Portugal and for most of the countries where *V. velutina* is invasive. While invasive species prevention might not necessarily be cheaper than control and impact mitigation efforts, in many cases it can help diminish the costly environmental, agricultural, and health impacts (Zenni et al. 2021). Reducing the damage costs of *V. velutina* likely requires spending more money and effort undertaking prevention, early detection, and rapid response. Such actions should be done by intercepting and preventing the establishment of new colonies (by nest destruction and eradicating localized outbreaks), as well as outreach activities directed to general public, beekeepers, farmers, pest control technicians and transportation operatives.

In a very recent work, a promising technology to detect the presence of *V. velutina* nest used sensitive thermal images advices (Lioy et al. 2021). Despite some limitations evidenced by the authors, their results show the applicability of thermography in detecting *V. velutina* nests before the beginning of the reproductive phase, and consequently its potential in control strategies. In Portugal, researchers from University of Trás-os-Montes are also conducting new experiments that consist in attaching a micro transmitter to the hornet dorsal side and follow the flight of *V. velutina* back to the nest. This method will help to detect nests earlier (and evaluate the best options to destroy it) and to study the dynamics of *V. velutina* in the area surrounding the nest. Further research to improve the technique with reduced costs will allow its application by the different municipalities across the invaded area in Portugal and other European countries.

In this work I showed that *V. velutina nigrithorax* has a huge potential for further expansion in Europe, since there are large available areas with suitable conditions for the species (i.e., rainy winters and pleasant summers). Despite the ability of this invasive to adapt to different environmental conditions, it is important to understand, at different scales, to what extent these suitable areas will be modified under the current climate change trends. It is possible that with increasing temperatures and reduction of precipitation, a potential reduction in the suitable area for *V. velutina* may occur in Portugal. However, climate change has a direct impact on other processes such as land use changes and increased occurrence of fires, which in turn can have additional effects on biodiversity. Hence, to manage climate change impacts on invasive species is fundamental to assess the vulnerability of a territory and its communities, through the implementation of programs dedicated to monitoring, early detection and support for its management and control (Gonçalves et al. 2021). In Portugal a monitoring network for active surveillance of *V. velutina* has been implemented, first through the project Gesvespa and more recently through Atlantic Positive project, both conducted by INIAV and other national and international teams. These projects also include beekeepers' associations, city councils and protected areas as partners, which are monitoring the presence of *V. Velutina* across the country in sentinel stations, and its arrival in non-invaded regions. The continuous monitoring on these stations in the future will certainly help to better control the species and quickly act when in the presence of new outbreaks.

In Asia, where various *Vespa* species are native, the medicinal and culinary motivations have caused a recent rise in efforts to develop a sustainable hornet production method (Van Itterbeeck et al. 2021). The traditional use of insects as food continues to be widespread in tropical and subtropical countries and to provide significant nutritional, economic and ecological benefits for rural communities (DeFoliart 1999). Indeed, *Vespa* hornets are used as food in multiple Asian countries like China, Japan, Laos, Malasya, Indonesia, Korea, Myanmar and Thailandia (Van Itterbeeck et al. 2021). However, in European countries, a bias against insect as food persist as a result of different cultural conditionings (DeFoliart 1999). Despite of the long history of the utilization of social wasps as food and pharmaceutical bioresources - see some examples in Jeong *et al.* (2020), systematic investigations on the value of social wasps as a food resource are scarce. Bee and wasp brood (Apidae and Vespidae) are among the insects that are most notable for their flavour and quality, not only among indigenous populations but among Europeans and other Westerners who have tasted them (DeFoliart 1999). A recent study by Jeong *et al.* (2020), evidenced the potential of *V. velutina* as a food resource. These authors used nutritional and heavy metal analyses of the larvae to reveal their balanced and rich nutritional value and safety as a food resource. Sometimes the effective control of alien species occurs when their utilization as a potential resource is discovered; research on the potential use of *V. velutina* as a bioresource in Europe is also necessary.

Final considerations

Given the increasing number of insect invaders worldwide, overlapping with new global environmental issues including climate change (Huang et al. 2011) and the rapid transformation of habitats, the challenges associated with prevention, eradication and management of insect invasions are increasing in scope and complexity (Roderick and Navajas 2015). *Vespa velutina nigrithorax* is a major invasive species in terms of its abundance, ability to spread and invade different environmental envelopes from those existing in its native range, and its ecological and economic impacts. Engaged in the control of *V. velutina*, the international working group "*Velutina Task Force*", yearly debates and gathers knowledge about the recent scientific outcomes on the biology, ecology, impacts and management of this invasive. So far, local authorities of different countries have implemented action plans for the destruction of the nests with little success (Turchi and Derijard 2018). However, in the Balearic Islands a successful management of this invasive was achieved (Leza et al. 2021). For that, an early detection strategy and the involvement of citizens in the detection of nests were crucial.

Despite the existence of a national action plan to fight *V. velutina*, a binding national strategy is lacking to equaly regulate the control procedures among municipalities. To hamper this invasive process, it is necessary that early control measures can be implemented, particularly by preventing the initial establishment of the species. For that, efforts of monitoring and early detection must be prioritized on a radius of 5 km from the continuous distribution area, and on a buffer of 6 km around motorways. The control of the species can only be effective if all agents involved (government, public administration, municipalities, associations, beekeepers, researchers, and operational technicians) agree on the best approach to be followed. And here, researchers need to be more proactive in disseminating the results of their studies to bridge the gap between the various stakeholders. These efforts associated with local outreach activities may be a good way to manage the spread and anticipate resulting ecological and social consequences of this hornet.

By coupling climate modelling with niche analysis, diet molecular approaches and computational methods, this thesis provided novel insights into the dynamics of invasion of *Vespa velutina nigrithorax* and will certainly contribute for future management plans.

IV

Supplementary Material and References

A

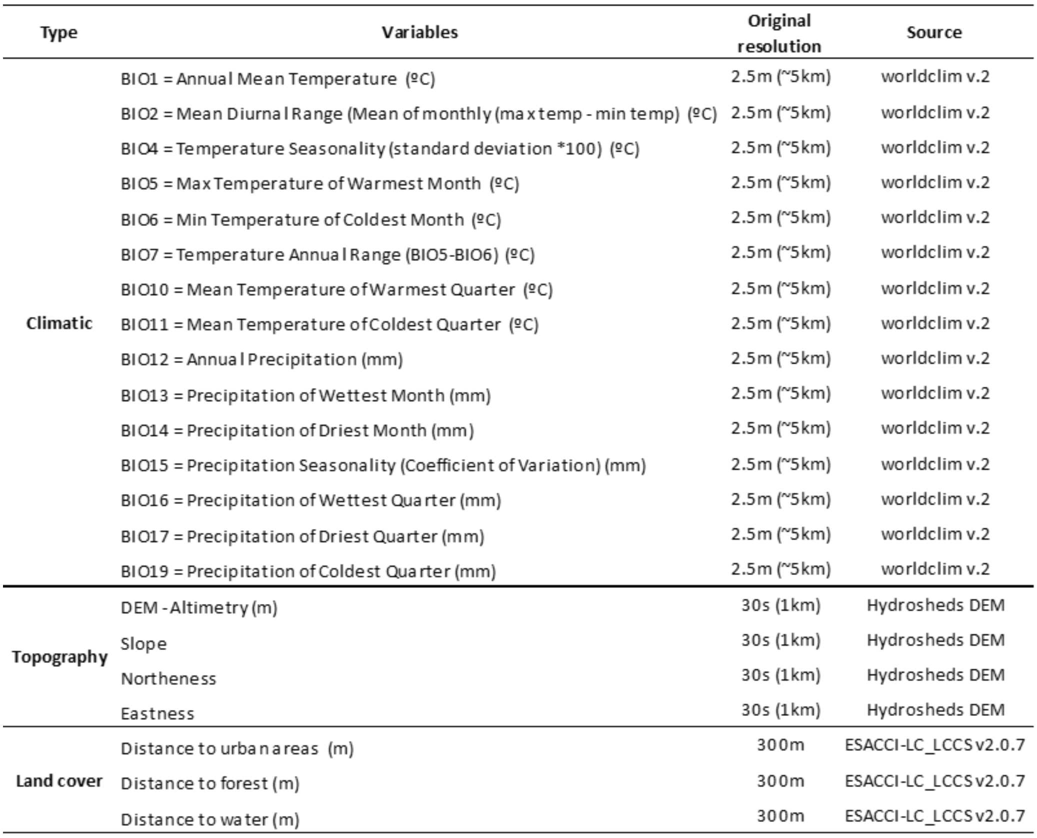
Supplementary Material - Chapter 1

Sources of Information

- ICNF - Instituto da Conservação da Natureza e das Florestas (Portuguese records);
- Bombeiros Voluntários de Viana do Castelo (Portuguese records);
- Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente (Spanish records provided on a 1 km x 1 km grid - we used the latitude and longitude of the centroid of each presence);
- INPN - Muséum National d'Histoire Naturelle in Paris (native and invasive French records);
- GBIF – Global Biodiversity Information Facility (native and invasive records with a geographical uncertainty below the resolution of our pixel size - 5km). Available at: www.gbif.org
- Life STOP vespa (italian records): Data available on the online website of the project at: https://www.vespavelutina.eu/it-it/ (Accessed: 1 September 2018).
- Data available in *Vespa velutina* in Europe - Google Maps 2017. Available at: https://www.google.com/maps/d/u/0/viewer?mid=1jRfoi4oF6GmiGRgbXuD71Qpbw8s&hl=en_US&ll=47.89184612561176%2C10.028028255145273&z=6 (Accessed: 1 September 2018).

Tables

Table S1.1 – Environmental variables with potential to affect the ecophysiology of *Vespa velutina*. The source where each variable was obtained, and its original resolution is indicated in the table.



Type	Variables	Original resolution	Source
Climatic	BIO1 = Annual Mean Temperature (ºC)	2.5m (~5km)	worldclim v.2
	BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp) (ºC)	2.5m (~5km)	worldclim v.2
	BIO4 = Temperature Seasonality (standard deviation *100) (ºC)	2.5m (~5km)	worldclim v.2
	BIO5 = Max Temperature of Warmest Month (ºC)	2.5m (~5km)	worldclim v.2
	BIO6 = Min Temperature of Coldest Month (ºC)	2.5m (~5km)	worldclim v.2
	BIO7 = Temperature Annual Range (BIO5-BIO6) (ºC)	2.5m (~5km)	worldclim v.2
	BIO10 = Mean Temperature of Warmest Quarter (ºC)	2.5m (~5km)	worldclim v.2
	BIO11 = Mean Temperature of Coldest Quarter (ºC)	2.5m (~5km)	worldclim v.2
	BIO12 = Annual Precipitation (mm)	2.5m (~5km)	worldclim v.2
	BIO13 = Precipitation of Wettest Month (mm)	2.5m (~5km)	worldclim v.2
	BIO14 = Precipitation of Driest Month (mm)	2.5m (~5km)	worldclim v.2
	BIO15 = Precipitation Seasonality (Coefficient of Variation) (mm)	2.5m (~5km)	worldclim v.2
	BIO16 = Precipitation of Wettest Quarter (mm)	2.5m (~5km)	worldclim v.2
	BIO17 = Precipitation of Driest Quarter (mm)	2.5m (~5km)	worldclim v.2
	BIO19 = Precipitation of Coldest Quarter (mm)	2.5m (~5km)	worldclim v.2
Topography	DEM - Altimetry (m)	30s (1km)	Hydrosheds DEM
	Slope	30s (1km)	Hydrosheds DEM
	Northeness	30s (1km)	Hydrosheds DEM
	Eastness	30s (1km)	Hydrosheds DEM
Land cover	Distance to urban areas (m)	300m	ESACCI-LC_LCCS v2.0.7
	Distance to forest (m)	300m	ESACCI-LC_LCCS v2.0.7
	Distance to water (m)	300m	ESACCI-LC_LCCS v2.0.7

Note: Climatic data were obtained from WORLDCLIM at 2.5-arcmin resolution (Hijmans et al. 2005), HydroSHEDS topographic data (Lehner et al. 2006) were obtained at 30 s resolution and land-cover data were extracted from European Space Agency (2017) at a resolution of 300 m.

Table S1.2 – Ranges of the selected climatic variables used for modelling of the ecological niche of *Vespa velutina* in native and invasive distribution areas. The range of climatic variables used for each invasive population P1 (population from France and contiguous records) and P2 (population from NW of Iberian Peninsula) is also shown.

Variables	**Non-native Available Climate**		**Native Available Climate**		**Invaded niche**				**Native niche**	
	Min	**Max**	**Min**	**Max**	**Min P1**	**Max P1**	**Min P2**	**Max P2**	**Min**	**Max**
Bio1= Annual Mean Temperature (ºC)	-8.7	18.6	-13.5	28.9	3.5	15.9	10.1	16.7	4.4	25.3
Bio7 = Temperature Annual Range (BIO5-BIO6) (ºC)	12.3	33.5	9.1	41.9	15.7	30.0	14.9	26.2	14.6	35.6
Bio10 = Mean Temperature of Warmest Quarter (ºC)	-2.5	26.8	-7.0	32.4	10.8	22.9	15.7	24.1	13.0	29.4
Bio11 = Mean Temperature of Coldest Quarter (ºC)	-14.3	13.3	-22.7	27.5	-2.8	9.6	3.6	10.7	-8.8	21.7
Bio17 = Precipitation of Driest Quarter (mm)	10	562	0	773	6.5	294	46	161	16	493
Bio19 = Precipitation of Coldest Quarter (mm)	63	713	0	3228	104	425	246	644	21	495

Table S1.3 - Correlation matrix of the environmental variables that have the potential to affect the ecophysiology of *Vespa velutina* in Europe. Correlated variables (r≥0.70) are shown in grey.

Variables	**bio1**	**bio2**	**bio4**	**bio5**	**bio6**	**bio7**	**bio10**	**bio11**	**bio12**	**bio13**	**bio14**	**bio15**	**bio16**	**bio17**	**bio19**	**dem**	**dfor**	**durb**	**dwat**	**northn**	**eastn**	**slope**
bio1	1.00																					
bio2	-0.04	1.00																				
bio4	-0.33	0.66	1.00																			
bio5	0.54	0.69	0.54	1.00																		
bio6	0.77	-0.58	-0.81	-0.07	1.00																	
bio7	-0.19	0.86	0.93	0.70	-0.76	1.00																
bio10	0.82	0.34	0.26	0.89	0.31	0.36	1.00															
bio11	0.88	-0.37	-0.73	0.13	0.96	-0.60	0.47	1.00														
bio12	0.31	-0.25	-0.68	-0.21	0.55	-0.53	-0.09	0.57	1.00													
bio13	0.44	-0.36	-0.73	-0.16	0.68	-0.59	0.03	0.69	0.95	1.00												
bio14	-0.60	0.26	0.36	-0.23	-0.60	0.27	-0.42	-0.64	-0.22	-0.46	1.00											
bio15	0.57	-0.42	-0.70	-0.04	0.77	-0.57	0.19	0.78	0.69	0.86	-0.81	1.00										
bio16	0.43	-0.38	-0.77	-0.19	0.70	-0.62	0.00	0.70	0.95	0.99	-0.47	0.87	1.00									
bio17	-0.45	0.32	0.32	-0.11	-0.51	0.29	-0.27	-0.49	-0.05	-0.31	0.95	-0.71	-0.33	1.00								
bio19	0.42	-0.31	-0.76	-0.17	0.68	-0.60	-0.02	0.68	0.95	0.96	-0.44	0.83	0.98	-0.31	1.00							
dem	-0.44	0.04	0.10	-0.16	-0.34	0.14	-0.35	-0.32	0.20	0.15	0.01	0.07	0.13	0.07	0.08	1.00						
dtrees	0.01	0.03	0.16	0.07	-0.07	0.09	0.09	-0.09	-0.31	-0.29	0.06	-0.21	-0.28	0.00	-0.26	-0.25	1.00					
durb	-0.22	0.24	0.13	0.04	-0.26	0.21	-0.13	-0.20	0.11	0.04	0.16	-0.09	0.02	0.22	0.02	0.45	-0.18	1.00				
dwat	-0.11	0.07	0.11	0.04	-0.13	0.12	-0.04	-0.12	0.02	0.00	0.02	0.00	0.00	0.06	-0.01	0.23	-0.08	0.21	1.00			
northn	0.01	0.00	-0.03	-0.02	0.01	-0.02	-0.01	0.01	-0.01	-0.01	0.03	-0.02	-0.01	0.02	-0.01	-0.12	0.05	-0.05	-0.04	1.00		
eastn	-0.02	-0.01	0.05	0.02	-0.03	0.03	0.02	-0.03	-0.05	-0.04	-0.01	-0.02	-0.05	-0.01	-0.06	0.09	-0.03	0.03	0.01	0.02	1.00	
slope	-0.11	-0.12	-0.11	-0.12	-0.01	-0.07	-0.15	0.01	0.34	0.33	-0.17	0.27	0.31	-0.07	0.26	0.60	-0.27	0.27	0.12	-0.01	0.04	1.00

Table S1.4 - Correlation matrix of the environmental variables that have the potential to affect the ecophysiology of *Vespa velutina* in Asia. Correlated variables (r≥0.70) are shown in grey.

Variable	bio1	bio2	bio4	bio5	bio6	bio7	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio19	dem	dfor	durb	dwat	northn	eastn	slope
bio1	1.00																					
bio2	-0.16	1.00																				
bio4	-0.44	-0.05	1.00																			
bio5	0.67	-0.04	0.34	1.00																		
bio6	0.91	-0.25	-0.73	0.33	1.00																	
bio7	-0.42	0.22	0.95	0.38	-0.75	1.00																
bio10	0.76	-0.22	0.25	0.97	0.45	0.24	1.00															
bio11	0.91	-0.10	-0.77	0.32	0.98	-0.74	0.42	1.00														
bio12	0.20	-0.43	-0.64	-0.38	0.49	-0.75	-0.25	0.44	1.00													
bio13	0.26	-0.24	-0.68	-0.34	0.51	-0.74	-0.22	0.49	0.87	1.00												
bio14	0.01	-0.56	-0.04	-0.08	0.14	-0.19	0.00	0.05	0.54	0.14	1.00											
bio15	0.29	0.28	-0.47	-0.09	0.34	-0.39	-0.05	0.41	0.08	0.50	-0.66	1.00										
bio16	0.26	-0.25	-0.70	-0.36	0.52	-0.76	-0.23	0.50	0.90	0.99	0.17	0.48	1.00									
bio17	0.01	-0.54	-0.06	-0.08	0.14	-0.20	-0.01	0.05	0.56	0.15	0.99	-0.67	0.19	1.00								
bio19	-0.13	-0.42	0.07	-0.11	-0.02	-0.06	-0.08	-0.11	0.45	0.06	0.89	-0.72	0.10	0.91	1.00							
dem	-0.71	0.40	-0.18	-0.84	-0.47	-0.12	-0.90	-0.42	0.02	0.03	-0.14	0.08	0.04	-0.13	-0.01	1.00						
dtrees	0.10	-0.16	0.31	0.34	-0.03	0.27	0.35	-0.06	-0.26	-0.24	-0.06	-0.07	-0.25	-0.09	-0.09	-0.37	1.00					
durban	-0.11	0.16	-0.46	-0.47	0.10	-0.42	-0.46	0.13	0.32	0.34	-0.04	0.22	0.35	-0.01	0.00	0.45	-0.36	1.00				
dwat	-0.20	0.27	-0.23	-0.35	-0.08	-0.17	-0.40	-0.04	0.11	0.22	-0.16	0.18	0.21	-0.15	-0.11	0.44	-0.19	0.46	1.00			
northn	0.09	0.22	-0.07	0.07	0.07	-0.01	0.04	0.09	0.01	0.09	-0.09	0.13	0.07	-0.07	-0.06	-0.09	-0.02	0.06	0.03	1.00		
eastn	0.03	-0.04	0.11	0.11	-0.03	0.10	0.12	-0.03	-0.18	-0.18	-0.03	-0.10	-0.20	-0.05	-0.10	-0.02	0.06	0.05	-0.20	0.00	1.00	
slope	-0.28	0.15	-0.30	-0.54	-0.09	-0.29	-0.54	-0.07	0.21	0.32	-0.06	0.25	0.31	-0.05	-0.06	0.47	-0.40	0.34	0.24	0.02	-0.11	1.00

Figures

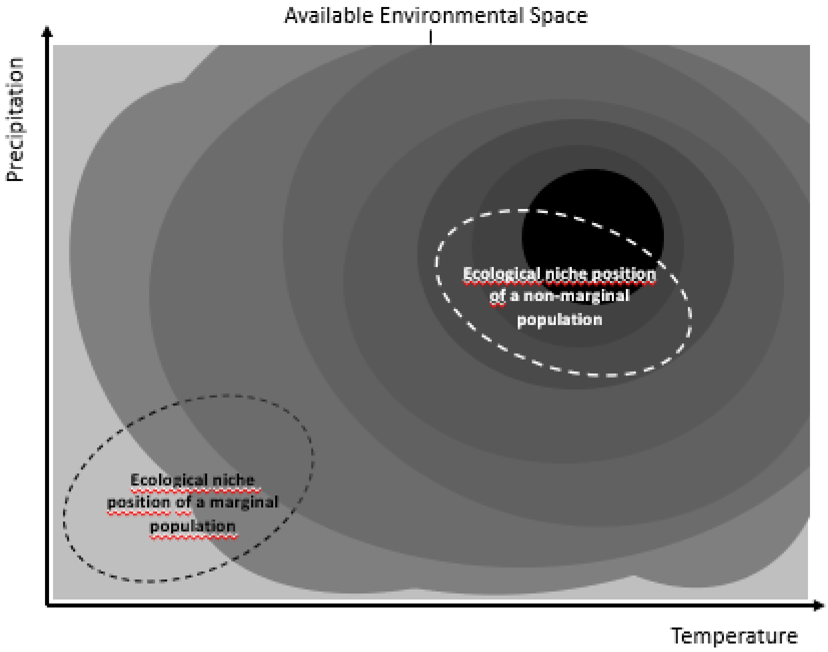


Fig. S1.1 - Schematic representation of the species' marginality concept. The large square represents the total available environmental space. The growing intensity of the colour is proportional to the frequency of the combined environmental conditions in a geographical area, from the less frequent or marginal environmental conditions (light grey) to the most common conditions (black). The dashed lines, black and white, represents the ecological niche position of a marginal and non-marginal population, respectively.

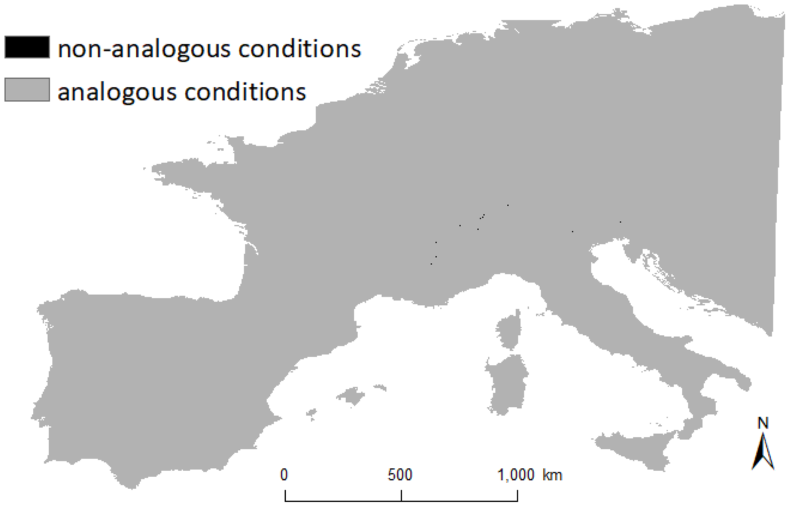


Fig. S1.2 - Comparison of the environmental similarity of variables to the environmental data used for training the model. Multivariate Environmental Similarity Surfaces (MESS) shows areas in grey, representing the regions with environmental similarity to the native range and areas in black (very small: 0.008% of the Europe area) having one or more environmental variables outside the range present in the training data, so predictions in those areas should be treated with strong caution.

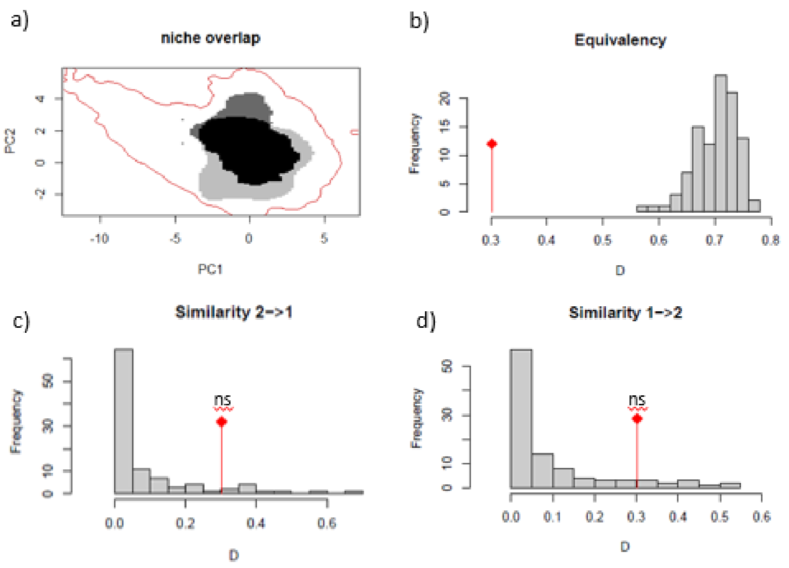


Figure S1.3 – Similarity and equivalence test between native and invasive distribution of P1. Visual representation of niche overlap between native and invasive ranges where the solid contour line illustrates the kernel density estimates corresponding to 100% of the available (background) environment (a); Histograms (b-c-d) show the observed niche overlap D between the two ranges (bars with a diamond) and simulated niche overlaps (grey bars) on which tests of niche equivalency (b), niche similarity of Europe-P1 to Asia (c), and niche similarity of Asia to Europe-P1 (d) are calculated from 100 iterations. The significance of the tests is shown (ns, non-significant).

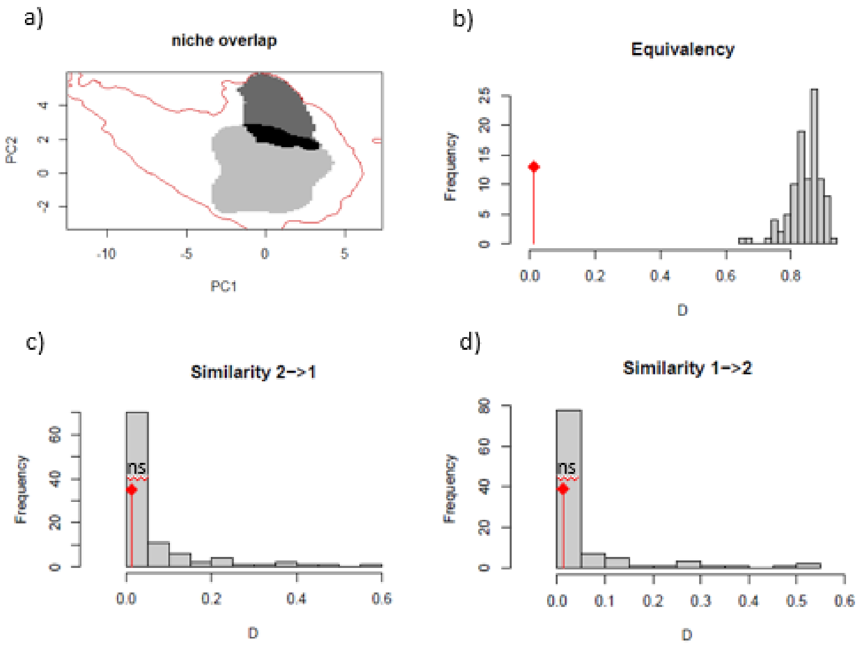


Figure S1.4 – Similarity and equivalence test between native and invasive distribution of P2. Visual representation of niche overlap between native and invasive ranges where the solid contour line illustrates the kernel density estimates corresponding to 100% of the available (background) environment (a); Histograms (b-c-d) show the observed niche overlap D between the two ranges (bars with a diamond) and simulated niche overlaps (grey bars) on which tests of niche equivalency (b), niche similarity of Europe-P2 to Asia (c), and niche similarity of Asia to Europe-P2 (d) are calculated from 100 iterations. The significance of the tests is shown (ns, non-significant).

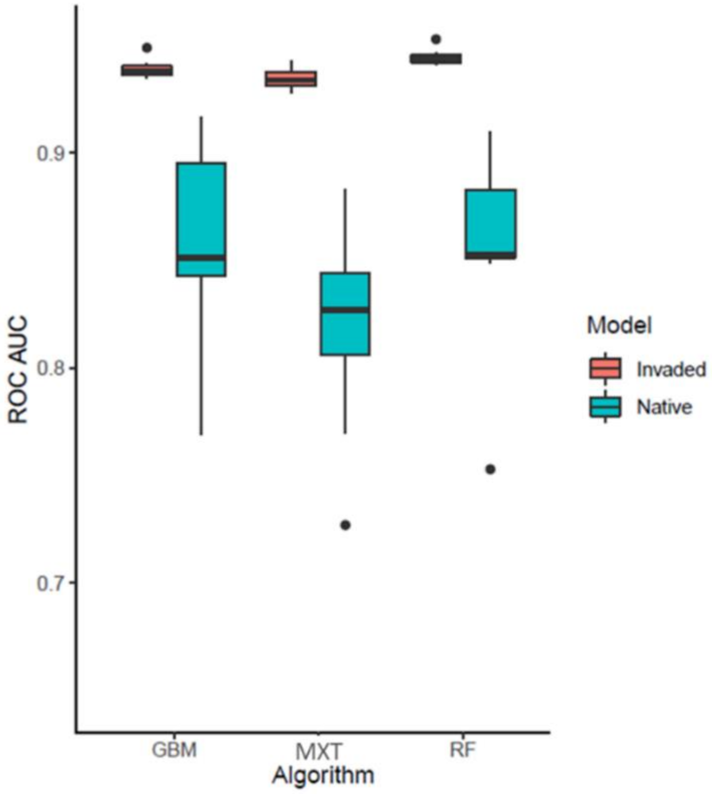


Fig. S1.5 – Variation of the Area Under the Curve (AUC) across the different modelling techniques: Generalized Boosted Models (GBM), MaxEnt (MXT) and Random Forest (RF).

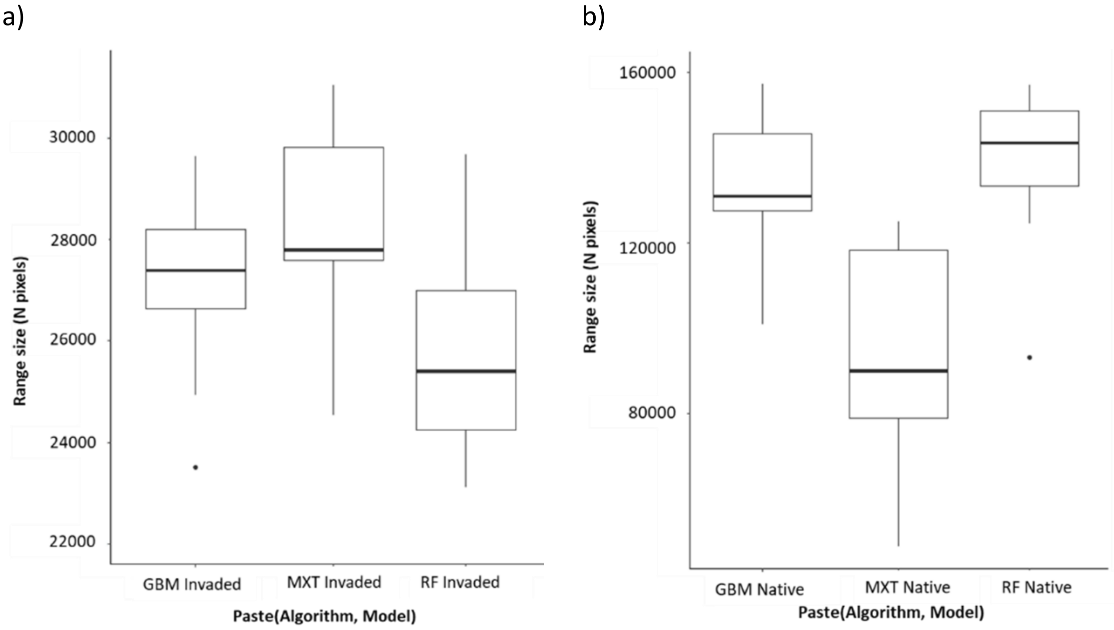


Fig. S1.6 – Variation of the predicted European range size with the different modelling techniques using the invasive (a) and native (b) models. Generalized Boosted Models (GBM), MaxEnt (MXT) and Random Forest (RF).

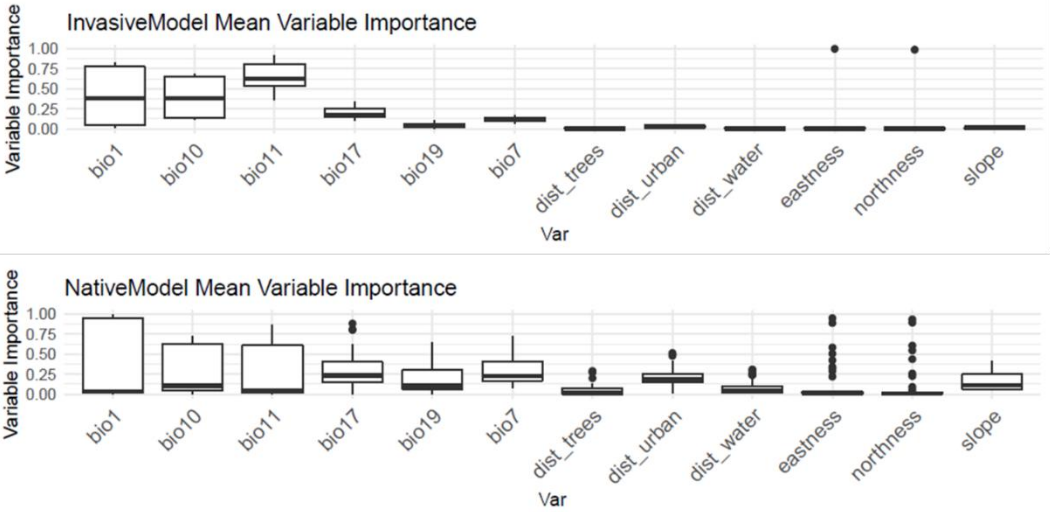


Fig. S7 – Variation of variable importance according to invasive and native models.

Methodological details of modelling techniques

The GBM models were constructed by fitting 2500 trees and 3 cross-validations to identify the number of trees that produced most accurate predictions. For RF models 500 trees were used as the building criterion with a node size of 5. MaxEnt was run with linear, quadratic, product, threshold and hinge features.

To evaluate the importance of variables for determining the suitable environment biomod2 applies a randomisation procedure randomly shuffling the values of the focus environmental variable and fitting a model. Then the model predictions of the ‘random’ and original model are compared, and their correlation value is obtained. Then the variable importance equals 1 – correlation, so that unimportant variables have values as low as 0 (not important) and important variables have high values of up to 1 (highly important). We extracted the resulting variable importance values for all models created from biomod2.

B

Supplementary Material - Chapter 2

Table S2.1– Climate, land cover and anthropogenic variables with potential to affect the behaviour and establishment of *Vespa velutina*. The source where each variable was obtained, and its original resolution is indicated in the table.

Type	**Original classes**	**Acronym**	**Original resolution**	**Source**
Climate	Annual Mean Temperature (ºC)	bio1	30s (~1km)	Worldclim
	Temperature annual range (ºC)	Bio 7	30s (~1km)	Worldclim
	Precipitation of the wettest month (mm)	bio 13	30s (~1km)	Worldclim
Land Cover	Distance to urban areas (m)	durb	25m	COS2005N5
	Distance to forest (m)	dfor	25m	COS2005N5
	Distance to riparian galeries (m)	dgal	25m	ECRINS; COS2005N5
	Distance to crops (m)	dcrops	25m	COS2005N5
	Distance to shrub and natural meadows (m)	dshr	25m	COS2005N5
Anthropogenic	Distance to motorways (m)	dmo	25m	Open Street Map
	Distance to railways (m)	drail	300m	Forest GIS
	Human Influence Index	hi	30s (1km)	CIENSIN

Note: Climatic variables were obtained from Worldclim (Hijmans *et al.*, 2005: 30 s resolution). Land-cover variables were obtained from IGP (COS2007N5 2010) and European Environment Agency (EEA-ECRINS, 2012) and the respective distance to each class was calculated in ArcGis (ESRI). Anthropogenic variables were obtained from Wildlife Conservation Society - WCS and Center for International Earth Science Information Network - CIESIN (2005), Forest GIS, 2019 and OpenStreetMap - ODbL MapCruzin 2019, being the distances to each linear structure also calculated. All layers were clipped to the same extent and scaled to 300 m pixel resolution.

Table S2.2 (a, b, c) - Correlation matrix of the climatic, land cover and anthropogenic drivers that have the potential to affect the behaviour and establishment of *Vespa velutina* in Europe: a) bound records; b) outposts; c) outposts >18km. One of each pair of correlated variables (r≥0.70) was excluded from subsequent analysis.

a)

Variables	bio1	bio7	bio13	dfor	dgal	dmo	drail	dro	dshrubs	durb	dcrops	hi
bio1 - Annual Mean Temperature	1.00	-0.44	-0.72	0.01	0.22	-0.44	-0.40	-0.42	0.34	-0.43	-0.06	0.31
bio7 - Temperature Annual Range	-0.44	1.00	0.18	0.00	-0.35	0.17	0.33	0.23	-0.05	0.20	-0.15	-0.41
bio13 - Precipitation of wettest month	-0.72	0.18	1.00	-0.02	-0.23	0.33	0.11	0.24	-0.32	0.29	-0.11	0.04
dfor - Distance to Forest	0.01	0.00	-0.02	1.00	0.39	-0.24	-0.25	-0.19	0.29	0.06	-0.10	0.14
dgal - Distance to riparian galleries	0.22	-0.35	-0.23	0.39	1.00	-0.20	-0.16	-0.23	0.19	-0.19	0.10	0.04
dmo - Distance to motorways	-0.44	0.17	0.33	-0.24	-0.20	1.00	0.77	0.57	-0.36	0.48	0.07	-0.40
drail - Distance to railways	-0.40	0.33	0.11	-0.25	-0.16	0.77	1.00	0.49	-0.20	0.30	0.10	-0.59
dro - Distance to road network	-0.42	0.23	0.24	-0.19	-0.23	0.57	0.49	1.00	-0.16	0.17	-0.04	-0.38
dshrubs - Distance to shrubs and natural meadows	0.34	-0.05	-0.32	0.29	0.19	-0.36	-0.20	-0.16	1.00	-0.12	-0.10	-0.04
durb - Distance to urban areas	-0.43	0.20	0.29	0.06	-0.19	0.48	0.30	0.17	-0.12	1.00	0.22	-0.23
dcrops - Distance to crops	-0.06	-0.15	-0.11	-0.10	0.10	0.07	0.10	-0.04	-0.10	0.22	1.00	-0.04
hi - Index of Human Influence	0.31	-0.41	0.04	0.14	0.04	-0.40	-0.59	-0.38	-0.04	-0.23	-0.04	1.00

b)

Variables	bio1	bio7	bio13	dfor	dgal	dmo	drail	dro	dshrubs	durb	dcrops	hi
bio1 - Annual Mean Temperature	1.00	-0.29	-0.82	0.05	-0.07	-0.39	-0.40	-0.01	0.02	-0.16	-0.15	0.24
bio7 - Temperature Annual Range	-0.29	1.00	0.11	0.09	0.06	0.09	0.03	0.06	-0.13	0.31	0.14	-0.15
bio13 - Precipitation of wettest month	-0.82	0.11	1.00	-0.11	-0.14	0.57	0.41	0.22	0.07	0.00	0.04	-0.22
dfor - Distance to Forest	0.05	0.09	-0.11	1.00	0.15	-0.09	-0.16	-0.18	-0.10	-0.01	-0.17	0.41
dgal - Distance to riparian galleries	-0.07	0.06	-0.14	0.15	1.00	-0.07	0.00	-0.09	-0.11	-0.11	0.15	0.09
dmo - Distance to motorways	-0.39	0.09	0.57	-0.09	-0.07	1.00	0.77	0.64	0.05	-0.06	-0.15	-0.24
drail - Distance to railways	-0.40	0.03	0.41	-0.16	0.00	0.77	1.00	0.45	0.04	0.00	-0.05	-0.47
dro - Distance to road network	-0.01	0.06	0.22	-0.18	-0.09	0.64	0.45	1.00	0.18	0.04	-0.12	-0.29
dshrubs - Distance to shrubs and natural meadows	0.02	-0.13	0.07	-0.10	-0.11	0.05	0.04	0.18	1.00	0.02	0.24	-0.08
durb - Distance to urban areas	-0.16	0.31	0.00	-0.01	-0.11	-0.06	0.00	0.04	0.02	1.00	0.01	-0.23
dcrops - Distance to crops	-0.15	0.14	0.04	-0.17	0.15	-0.15	-0.05	-0.12	0.24	0.01	1.00	-0.06
hi - Index of Human Influence	0.24	-0.15	-0.22	0.41	0.09	-0.24	-0.47	-0.29	-0.08	-0.23	-0.06	1.00

c)

Variables	bio1	bio7	bio13	dfor	dgal	dmo	drail	dro	dshrubs	durb	dcrops	hi
bio1 - Annual Mean Temperature	1.00	-0.25	-0.81	0.10	-0.09	-0.34	-0.32	-0.05	0.00	-0.20	-0.17	0.21
bio7 - Temperature Annual Range	-0.25	1.00	0.10	0.01	0.04	0.07	-0.02	0.10	-0.11	0.31	0.22	-0.18
bio13 - Precipitation of wettest month	-0.81	0.10	1.00	-0.16	-0.12	0.55	0.31	0.30	0.10	0.05	0.02	-0.17
dfor - Distance to Forest	0.10	0.01	-0.16	1.00	0.14	-0.14	-0.26	-0.17	-0.09	-0.03	-0.16	0.45
dgal - Distance to riparian galleries	-0.09	0.04	-0.12	0.14	1.00	-0.09	-0.01	-0.11	-0.12	-0.13	0.21	0.07
dmo - Distance to motorways	-0.34	0.07	0.55	-0.14	-0.09	1.00	0.73	0.74	0.08	-0.02	-0.10	-0.20
drail - Distance to railways	-0.32	-0.02	0.31	-0.26	-0.01	0.73	1.00	0.61	0.06	0.09	-0.01	-0.46
dro - Distance to road network	-0.05	0.10	0.30	-0.17	-0.11	0.74	0.61	1.00	0.17	0.03	-0.11	-0.32
dshrubs - Distance to shrubs and natural meadows	0.00	-0.11	0.10	-0.09	-0.12	0.08	0.06	0.17	1.00	0.03	0.26	-0.08
durb - Distance to urban areas	-0.20	0.31	0.05	-0.03	-0.13	-0.02	0.09	0.03	0.03	1.00	0.02	-0.28
dcrops - Distance to crops	-0.17	0.22	0.02	-0.16	0.21	-0.10	-0.01	-0.11	0.26	0.02	1.00	-0.07
hi - Index of Human Influence	0.21	-0.18	-0.17	0.45	0.07	-0.20	-0.46	-0.32	-0.08	-0.28	-0.07	1.00

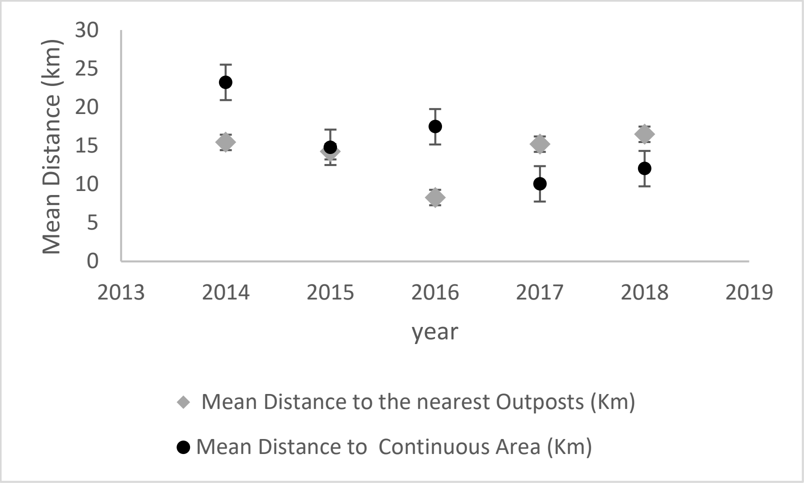


Fig. S2.1 - Variation of the mean distance of the new records within a given year to the nearest potential source: continuous area or outpost. Error bars depict standard errors.

Table S2.3 - Relation between dispersion distance (to south and east) vs time either for self mediated or jump dispersal by testing through t-test the significance of the slope of the regression line when compared to zero (H0: the slope of the regression line is 0).

Curve	**Equation**	**R^2**	**p value**
Outposts South Distance to the nearest source	y = 0.4748x - 924.73	0,0015	0,78
Outposts East Distance to the nearest source	y = -2.725x + 5522.2	0,1166	0,25
Distance to south between the limits of consecutive years	y = -x +2060.3	0,0344	0,77
Distance to east between the limits of consecutive years	y = 0.19x - 363.62	0,0005	0,97

Table S2.4 – Set of best models with climatic and land variables according to the different datasets (1. bound records; 2. all outpost and 3. outpost 18 km). Only the models with ΔAIC<2 are shown. Nomenclatures: bio 7 – temperature annual range; bio 13 – precipitation of the wettest month; dfor – distance to forests; dmo – distance to motorways; dcrops – distance to crops; dgal - distance to riparian galleries; dshrubs – distance to shrubs; hi – index of human influence.

1. Climatic + Land cover model selection table - bound records dataset

	(Intrc)	bio13	bio7	dcrps	dfor	dgal	dmo	dshrb	durb	hi	df	AIC	delta	weight
68	23430	-258.60	167.30					10.05			6	1153.6	0	0.055
324	35110	-277.80	152.40					9.09		-162	7	1155	1.39	0.028
84	29110	-261.70	148.70			-1.34		10.69			7	1155	1.45	0.027
196	19960	-240.70	173.90					10.04	-5.19		7	1155.2	1.59	0.025
100	20720	-232.70	167.50				-0.15	9.57			7	1155.2	1.64	0.024
76	23370	-258.50	168.00		-1.057			10.19			7	1155.6	1.98	0.02
72	23820	-258.60	166.20	-0.17				10.04			7	1155.6	1.99	0.02

2. Climatic + Land cover model selection table - all outposts dataset

	(Intrc)	bio13	bio7	dcrps	dfor	dgal	dmo	dshrb	durb	hi	df	AIC	delta	weight
10	149200	-667.3			-26.52						5	2310.8	0	0.03
258	160400	-706								-291.1	5	2310.9	0.07	0.03
2	147100	-671.7									4	2310.9	0.11	0.02
138	147800	-670.8			-26				8.04		6	2311.6	0.78	0.02
130	145800	-675.2							8.30		5	2311.6	0.84	0.02
26	144700	-651.2			-29.13	3.22					6	2311.9	1.07	0.02
12	130000	-688.5	98.12		-27.34						6	2311.9	1.12	0.02
74	151000	-668.4			-27.34			-4.84			6	2311.9	1.14	0.02
322	162800	-708.9						-5.01		-305.1	6	2312	1.15	0.01
266	157700	-692.2			-18.73					-200.7	6	2312	1.2	0.01
4	129500	-691.3	90.19								5	2312.2	1.38	0.01
274	156900	-694.1				2.71				-300.4	6	2312.2	1.4	0.01
66	148700	-672.8						-4.44			5	2312.2	1.4	0.01
386	157500	-703.8							6.15	-250.3	6	2312.2	1.4	0.01
154	142400	-652.5			-28.98	3.78			9.15		7	2312.3	1.5	0.01
18	143600	-659.9				2.44					5	2312.4	1.58	0.01
260	145400	-719.7	72.55							-273.5	6	2312.4	1.6	0.01
34	152100	-714.7					0.1752				5	2312.6	1.82	0.01
42	152600	-698.3			-25.74		0.126				6	2312.7	1.85	0.01
202	149600	-672			-26.83			-4.99	8.21		7	2312.7	1.85	0.01
290	163300	-734.1					0.1189			-281.5	6	2312.7	1.94	0.01
14	149700	-667.9		-0.62	-26.88						6	2312.8	1.98	0.01

3. Climatic + Land cover model selection table - Outpost 18km dataset

	(Intrc)	bio13	bio7	dcrps	dfor	dgal	dmo	dshrb	durb	hi	df	AIC	delta	weight
258	157200	-661.5								-324	5	2087.5	0	0.03
2	142600	-625.9									4	2087.8	0.28	0.03
10	146400	-633.5			-27.27						5	2087.9	0.34	0.02
130	142500	-639.5							9.03		5	2088.5	0.96	0.02
322	160000	-666.4						-5.43		-338.8	6	2088.5	1.01	0.02
138	146100	-646			-26.29				8.55		6	2088.7	1.14	0.02
274	152600	-642.1				2.89				-334.9	6	2088.8	1.31	0.02
266	155700	-656.9			-17.54					-238.1	6	2088.9	1.35	0.01
386	155000	-665.6							6.25	-277.1	6	2088.9	1.4	0.01
74	148600	-637			-28.15			-5.20			6	2088.9	1.44	0.01
26	140900	-610.4			-29.88	3.32					6	2089	1.45	0.01
66	144600	-628.9						-4.82			5	2089	1.52	0.01
4	123800	-640	91.74								5	2089.2	1.7	0.01
18	138100	-607.5				2.57					5	2089.3	1.75	0.01
260	144000	-668.2	59.25							-303.6	6	2089.3	1.76	0.01
12	127800	-647.2	89.95		-27.11						6	2089.3	1.77	0.01
290	161700	-701.8					0.15			-314.9	6	2089.3	1.83	0.01
154	139800	-621			-29.17	3.81			9.54		7	2089.5	1.96	0.01
34	149200	-681.5					0.21				5	2089.5	1.97	0.01
262	156600	-660.2		0.53						-321.9	6	2089.5	1.99	0.01

Table 2.5 – Set of best models with climatic variables according to the different datasets (1. bound records; 2. all outpost and 3. outpost 18 km) Only the models with ΔAIC<2 are shown. Nomenclatures: bio 7 – temperature annual range; bio 13 – precipitation of the wettest month.

1. Climatic model selection table - bound records dataset

	(Intrc)	bio13	bio7	df	AIC	delta	weight
4	40880	-363.6	188	5	1156.90	0	0.91

2. Climatic model selection table - all outposts dataset

	(Intrc)	bio13	bio7	df	AIC	delta	weight
2	147100	-671.7		4	2310.9	0	0.65
4	129500	-691.3	90.19	5	2312.20	1.27	0.35

3. Climatic model selection table - Outpost 18km Dataset

	(Intrc)	bio13	bio7	df	AIC	delta	weight
2	142600	-625.9		4	2087.8	0	0.67
4	123800	-640	91.74	5	2089.2	1.42	0.33

Table S2.6 – Set of best models with land variables according to the different datasets (1. bound records; 2. all outpost and 3. outpost 18 km). Only the models with ΔAIC<2 are shown. Nomenclatures: dfor – distance to forests; dmo – distance to motorways; dcrops – distance to crops; dgal - distance to riparian galleries; dshrubs – distance to shrubs; hi – index of human influence.

1. Land cover model selection table - bound records dataset

	(Intrc)	dcrps	dfor	dgal	dmo	dshrb	durb	hi	df	AIC	delta	weight
17	15790					13.80			4	1158.6	0	0.06
21	18320			-2.57		14.97			5	1158.7	0.11	0.05
89	31440				-0.43	10.37		-336.9	6	1159.1	0.46	0.05
29	22280			-2.89	-0.31	13.12			6	1159.1	0.51	0.04
93	32830			-2.58	-0.46	11.56		-288.6	7	1159.2	0.6	0.04
25	18710				-0.24	12.22			5	1159.6	1.02	0.03
81	21690					13.47		-189.5	5	1159.7	1.05	0.03
53	19970			-2.85		14.66	-7.73		6	1159.9	1.28	0.03
49	16790					13.48	-5.61		5	1160.2	1.56	0.03
85	22160			-2.28		14.69		-133.6	6	1160.3	1.66	0.02
18	16400	-0.57				13.70			5	1160.5	1.91	0.02
19	15920		-1.32			14.00			5	1160.6	1.97	0.02
##	25460					12.79	-9.01	-256.7	6	1160.6	1.99	0.02

2. Land cover model selection table - all outposts dataset

	(Intrc)	dcrps	dfor	dgal	dmo	dshrb	durb	hi	df	AIC	delta	weight
15	54000		-39.71	6.05	-0.93				6	2335.7	0	0.08
11	58030		-34.88		-0.96				5	2336.2	0.55	0.06
9	54180				-0.93				4	2337	1.27	0.04
16	56810	-4.24	-43.07	6.77	-0.97				7	2337	1.27	0.04
47	51970		-39.63	6.48	-0.91		6.83		7	2337	1.35	0.04
13	50370			5.04	-0.90				5	2337.2	1.52	0.04
31	54920		-39.94	5.85	-0.93	-2.43			7	2337.5	1.84	0.03

3. Land cover model selection table - Outposts 18 km Dataset

	(Intrc)	dcrps	dfor	dgal	dmo	dshrb	durb	hi	df	AIC	delta	weight
15	56880		-34.65	5.75	-0.72				6	2102.7	0	0.05
9	57600				-0.71				4	2102.8	0.12	0.05
11	60880		-30.05		-0.76				5	2102.9	0.25	0.05
13	53760			4.91	-0.67				5	2103.2	0.49	0.04
73	65220				-0.78			-249.1	5	2103.7	1.05	0.03
77	61840			5.21	-0.73			-271.8	6	2103.9	1.2	0.03
47	55010		-34.20	6.13	-0.70		6.43		7	2104.1	1.45	0.03
79	60420		-29.30	5.77	-0.74			-135.2	7	2104.4	1.73	0.02
41	56370				-0.71		5.17		5	2104.5	1.77	0.02
25	58660				-0.71	-3.41			5	2104.5	1.81	0.02
31	57880		-34.83	5.52	-0.71	-2.55			7	2104.5	1.83	0.02
16	58110	-2.02	-36.36	6.13	-0.73				7	2104.5	1.86	0.02
27	62120		-30.60		-0.76	-3.78			6	2104.5	1.86	0.02
45	51820			5.32	-0.66		6.81		6	2104.6	1.88	0.02
43	59720		-29.51		-0.76		4.62		6	2104.6	1.96	0.02

Fig. S2.2 – Relation between the dispersion of *Vespa velutina* and significant climatic and land cover variables according to the different datasets: bound records, all outposts and outpost 18km (see Table 2.3). Plots were obtained with the visreg R package.

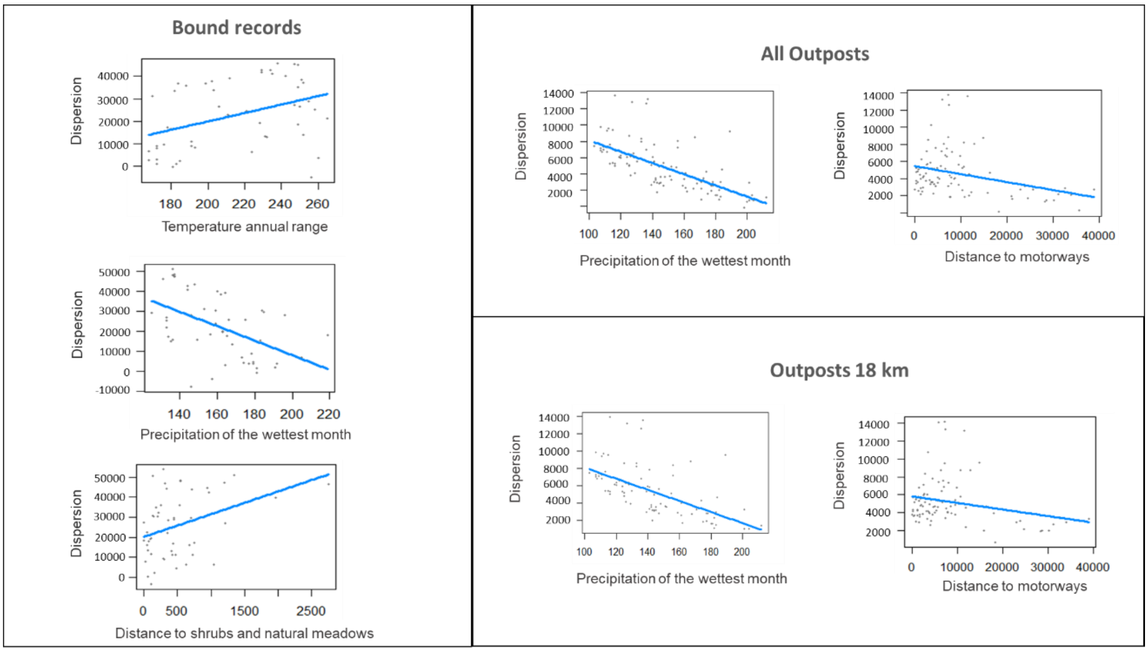


Table S2.7 – Effects of climatic, land cover and anthropogenic variables on the dispersal of *V. velutina*”. Distance to the previous continuous distribution (as a proxy of dispersion) was used as dependent variable in the GLMM. The results were obtained by averaging model predictions with ΔAIC<2.

Multivariate model (bound records)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
(Intercept)	25070	20580	21120	1.187	0.2351	
bio13 - Precipitation of wettest month	-256.3	102.2	105	2.441	0.0147	*
bio7 - Temperature annual range	163.6	64.81	66.56	2.457	0.014	*
Distance to shrubs and natural meadows	9.954	4.194	4.308	2.311	0.0209	*
Index of Human Influence	-22.37	87.41	88.84	0.252	0.8012	
Distance to riparian galleries	-0.1786	0.7877	0.8023	0.223	0.8238	
Distance to urban areas	-0.6487	3.323	3.391	0.191	0.8483	
Distance to motorways	-0.01772	0.09452	0.09647	0.184	0.8543	
Distance to forest	-0.1087	2.324	2.387	0.046	0.9637	
Distance to crops	-0.01713	0.5781	0.5939	0.029	0.977	
Multivariate model (all outposts)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
(Intercept)	149500	21780	22020	6.792	<2e-16	***
bio13 - Precipitation of wettest month	-684.1	104.7	106	6.453	<2e-16	***
Distance to forest	-11.86	18.06	18.16	0.653	0.514	
Index of Human Influence	-86.04	172.9	173.9	0.495	0.621	
Distance to urban areas	1.736	4.762	4.794	0.362	0.717	
Distance to riparian galleries	0.4945	1.757	1.771	0.279	0.78	
bio7 - Temperature annual range	10.79	46.86	47.24	0.228	0.819	
Distance to shrubs and natural meadows	-0.7866	2.754	2.775	0.283	0.777	
Distance to motorways	0.01336	0.1079	0.109	0.123	0.902	
Distance to crops	-0.01868	0.7344	0.7438	0.025	0.98	
Multivariate model (>18km outposts)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
(Intercept)	1.47E+05	2.49E+04	2.53E+04	5.834	<2e-16	***
bio13 - Precipitation of wettest month	-6.46E+02	1.29E+02	1.30E+02	4.957	7.00E-07	***
Index of Human Influence	-1.25E+02	2.06E+02	2.07E+02	0.603	0.546	
Distance to forest	-9.11E+00	1.72E+01	1.74E+01	0.525	0.6	
Distance to urban areas	1.61E+00	4.79E+00	4.83E+00	0.332	0.74	
Distance to shrubs and natural meadows	-7.58E-01	2.78E+00	2.80E+00	0.271	0.786	
Distance to riparian galleries	5.26E-01	1.86E+00	1.88E+00	0.281	0.779	
bio7 - Temperature annual range	9.47E+00	4.89E+01	4.94E+01	0.192	0.848	
Distance to motorways	1.29E-02	1.08E-01	1.10E-01	0.118	0.906	
Distance to crops	1.85E-02	8.68E-01	8.80E-01	0.021	0.983	

Table S2.8 - Effects of the different land cover and anthropogenic predictors on the dispersion of *V. velutina*, considering the predictor distance to the entire road network, instead of distance to motorways in bound records and both sets of outposts. Distance to the previous continuous distribution (as a proxy of dispersion) was used as dependent variable in the GLMM. The presented results were obtained by averaging model predictions with ΔAIC<2.

Multivariate model (bound records)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
(Intercept)	14400	4551	4652	3.096	0.001964	**
Distance to road network	0.6752	0.5598	0.5688	1.187	0.235211	
Distance to shrubs and natural meadows	14.95	4.181	4.292	3.483	0.000496	***
Distance to riparian galleries	-0.7513	1.479	1.498	0.501	0.616105	
Distance to urban areas	-1.881	5.264	5.341	0.352	0.724676	
Distance to crops	-0.0299	0.5218	0.5353	0.056	0.95546	
Index of Human Influence	-2.722	52.69	54.07	0.05	0.959852	
Distance to forest	0.04941	2.138	2.196	0.023	0.982043	
Multivariate model (all outposts)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
(Intercept)	44580	8695	8785	5.074	4.00E-07	***
Distance to forest	-28.12	25.56	25.75	1.092	0.275	
Distance to riparian galleries	5.266	4.547	4.581	1.149	0.25	
Distance to urban areas	2.446	6.153	6.196	0.395	0.693	
Distance to road network	-0.145	0.4167	0.4195	0.346	0.73	
Index of Human Influence	25.9	116.2	117.1	0.221	0.825	
Distance to shrubs and natural meadows	-0.1495	1.627	1.646	0.091	0.928	
Distance to crops	-0.09848	1.283	1.298	0.076	0.94	
Multivariate model (> 18 km outposts)	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**	
(Intercept)	52778.442	8491.2861	8585.3193	6.148	<2e-16	***
Distance to riparian galleries	3.2727	4.1678	4.197	0.78	0.436	
Distance to forest	-9.1976	18.6108	18.7305	0.491	0.623	
Distance to road network	-0.2759	0.5535	0.5573	0.495	0.621	
Distance to urban areas	0.8934	3.9733	4.0111	0.223	0.824	
Index of Human Influence	-35.498	130.1473	131.2565	0.27	0.787	
Distance to shrubs and natural meadows	-0.2286	1.9015	1.9237	0.119	0.905	
Distance to crops	0.1175	1.5016	1.5209	0.077	0.938	

C

Supplementary Material - Chapter 3

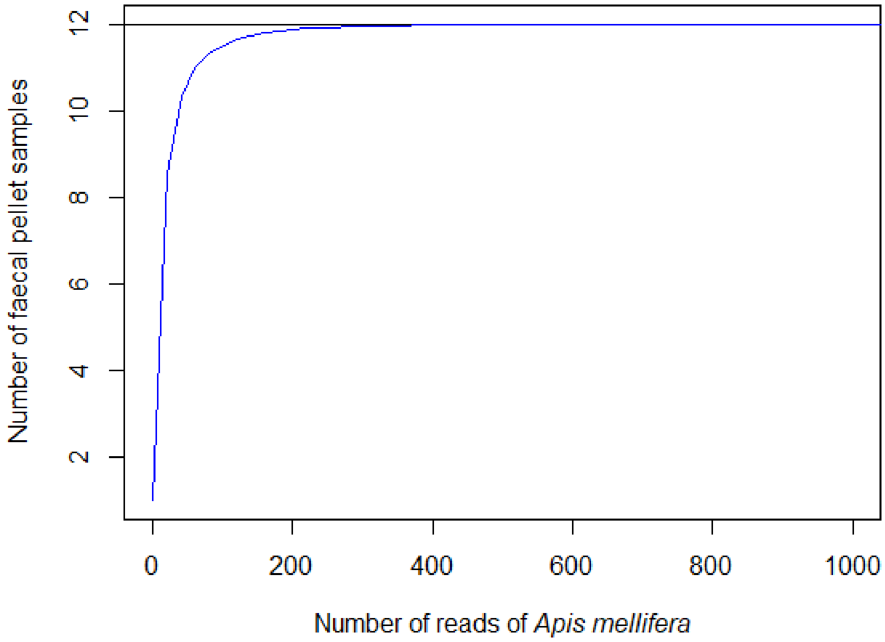


Fig. S3.1 - Rarefied honeybee accumulation curve by read count in faecal pellet samples in the COI dataset. Read curve was plotted using the total number of reads per faecal pellet sample in each location (a total of 50549 reads). Curve is only shown for 1000 reads, for readability purposes.

D

Supplementary Material - Chapter 4

Inquérito sobre o impacto da vespa-asiática (*Vespa velutina nigrithorax*) na atividade apícola

Este estudo a realizar juntos dos apicultores da região norte e centro do país, pretende recolher informações sobre o impacto socioeconómico da vespa asiática na atividade apícola em Portugal.

Este instrumento metodológico enquadra-se numa investigação no âmbito de um capítulo de uma tese de doutoramento ao abrigo do programa doutoral BIODIV do CE3C – Centre for Ecology, Evolution and Environmental Changes da Universidade de Lisboa, a fim de que seja possível complementar a respetiva tese.

Todas as informações recolhidas são estritamente confidenciais e não terão qualquer tipo de exploração comercial. Por favor responda com sinceridade pois não há respostas corretas ou incorretas. A sua opinião é muito importante. Obrigado pela colaboração.

Caso seja do seu interesse, no final será convidado a participar num *workshop* onde não só lhe serão facultados os resultados finais deste estudo, como também serão discutidas as perspetivas futuras relativamente ao impacto socioeconómico da vespa asiática em Portugal.

Caracterização do apicultor

1. **Qual a freguesia e o concelho onde desenvolve a sua atividade apícola?**

Distrito ________________ Concelho ________________ Freguesia __________

2. **Qual o tempo que dedica à atividade apícola?**

Tempo inteiro ☐ Tempo parcial ☐

Se respondeu **Parcial**, por favor indique com um **X** a opção que melhor se adequa:

Superior a 50% ☐ Entre 40% a 50 % ☐ Entre 10 a 30 % ☐ Inferior a 10 % ☐

3. **Qual a sua escolaridade?**

☐ Ensino Básico (antiga 4ª classe)
☐ Ensino Preparatório
☐ Ensino Secundário
☐ Curso Profissional
☐ Formação Superior (licenciatura, mestrado, doutoramento)
☐ Outro. Qual? ____________________________

4. Qual o número de apiários, colmeias, núcleos e colónias que possui?

Apiários ☐ Colmeias ☐ Núcleos ☐ Colónias ☐

5. Com que regularidade visita as suas colmeias?

☐ Todos os dias
☐ Entre duas a três vezes por semana
☐ Uma vez por semana
☐ Duas vezes por mês
☐ Uma vez por mês
☐ Outro. Qual? ____

6. Relativamente a alguns procedimentos relativos à manutenção das colmeias, assinale com um X, o(s) que costuma realizar.

☐ Limpeza da zona envolvente aos apiários
☐ Substituição de quadros e ceras
☐ Renovação das rainhas, se necessário
☐ Alimentação artificial das abelhas
☐ Verificação do estado e evolução da criação
☐ Aplicação de preventivos de doenças
☐ Prevenção de eventuais enxameações
☐ Vigilância do excesso de temperatura sobre as colmeias
☐ Vigilância das colmeias para inspecionar possíveis doenças

☐ Eliminação das colmeias doentes
☐ Desinfestação do material
☐ Utilização de estrado sanitário
☐ Utilização de recolectores de polén
☐ Preparação das colmeias para o Inverno
☐ Outro. Qual? ____

7. **Nos últimos 3 anos as suas abelhas foram afetadas por algum tipo de doença de declaração obrigatória?**

Sim ☐ Não ☐

Se sim indique qual/ quais e o respetivo grau de infestação (baixo, moderado ou grande):

☐ Varrose → Grau de infestação: ________
☐ Nosemose → Grau de infestação: ________
☐ Ascoferiose → Grau de infestação: ________
☐ Outra. Indique: _____________ → Grau de infestação: _______

Perceção e impacto do problema

8. **Está informado sobre o problema da predação das abelhas do mel por parte da vespa asiática? Assinale com um X a opção escolhida.**

Sim ☐ Não ☐

Neste caso, entenda-se **predação** quando a vespa asiática mata e se alimenta de abelhas do mel.

9. **Quantos ninhos de vespa asiática avistou nos últimos anos?**

2015: _______________ 2016: ________________ 2017: ______________

10. **Da quantidade de ninhos avistados quantos foram destruídos por si e quantos pelas autoridades competentes?**

Por si: ______________ pelas autoridades competentes: __________________

11. **Já teve colmeias que foram alvo de ataque por parte da vespa asiática?**

Sim ☐ Não ☐

Se respondeu Não, passe para a questão n.º 15

12. Que percentagem das suas colmeias foi atacada pela vespa asiática nos últimos anos?

2015: ________________ 2016: _________________ 2017: _______________

13. Que percentagem do número total de abelhas perdeu devido ao ataque da vespa asiática nos últimos anos?

2015: ________________ 2016: _________________ 2017: _______________

14. Nos últimos 3 anos, a presença da vespa asiática teve impacto na sua produção de mel?

Sim ☐ Não ☐

Se respondeu sim, indique a percentagem de Kg de mel por colmeia que estima ter perdido, devido à vespa asiática.

2015: ________________ 2016: _________________ 2017: _______________

15. Qual a percentagem do seu tempo de trabalho na apicultura que dedica ao combate à vespa?

0% - 10% ☐ 11% - 20% ☐ 21% - 30% ☐ >30% ☐

16. Que métodos usa no combate à vespa? Considera que os mesmos são eficazes para controlar o impacto da vespa asiática na apicultura?

____

____

____

17. Quais as armadilhas que considera mais eficazes para combater a vespa asiática?

____

____

18. Já procurou desenvolver uma estratégia conjunta com outros apicultores no que respeita ao combate à vespa asiática?

Sim ☐ Não ☐

Se respondeu sim, especifique o tipo de iniciativa:

____

19. Já foi contactado por alguma entidade competente para que recebesse informação sobre a vespa asiática?

Sim ☐ Não ☐

Se respondeu sim, indique:

- A entidade que o contactou: __________________________
- Que tipo de informação recebeu: ______________________________________ ____

20. No geral, como classifica a atuação das entidades competentes na prevenção à vespa asiática na sua região? Assinale com um X a opção escolhida.

Muito mau ☐ Mau ☐ Razoável ☐ Bom ☐ Muito bom ☐

21. No geral, como classifica a atuação das entidades competentes no combate à vespa asiática na sua região? Assinale com um X a opção escolhida.

Muito mau ☐ Mau ☐ Razoável ☐ Bom ☐ Muito bom ☐

22. É membro/sócio de alguma associação de apicultores?

Sim ☐ Não ☐

Se respondeu sim, indique:

- O nome da associação a que pertence: ____
- Se tem recebido apoio da sua associação relativamente à prevenção e combate à vespa asiática: ____

23. Que tipo de informação gostava de receber sobre a vespa asiática e o seu impacto na apicultura. Assinale a(s) resposta(s) que melhor se adequa(m).

☐ Já estou informado e não pretendo receber mais informação.
☐ Saber distinguir e identificar um ninho de vespa asiática
☐ Como distinguir as vespas asiáticas das outras vespas
☐ Saber como agir e que entidade contactar em caso de observação de um ninho
☐ Saber como posso proteger as minhas abelhas
☐ Saber que armadilhas colocar e qual a melhor altura
☐ Formações e workshops sobre o tema
☐ Outro. Qual: ____

24. O que sugere para melhorar a eficácia das ações de combate à vespa asiática?

____

____

Caracterização Socio-económica

25. Qual a sua idade?

☐ < 35 anos ☐ 35-49 anos ☐ 50-75 anos ☐ >75 anos

26. A sua atividade ligada à apicultura é um negócio que já vem de família?

Sim ☐ Não ☐

27. Indique qual a média de Kg de mel por colmeia que produziu nas épocas de colheita de 2015, 2016 e 2017?

2015: _______________ 2016: __________________ 2017_________________

28. Gostaria de ser contactado para estar presente num workshop final de apresentação dos resultados deste estudo?

Sim ☐ Não ☐

Se respondeu sim, indique o seu e-mail e/ou contacto telefónico:

E-mail:
____

Contacto telefónico:
____

Muito obrigada pela sua participação!!

Tabela S4.1 – Correlation matrix of the variables used in the multivariate analysis on the perception of the impact of *Vespa velutina* on beekeeping activity. None of the variables (r≥0.70) was excluded from the analysis.

	VARIAVÉIS	**1**	**2**	**3**	**4**
1	Number of tasks performed in the maintenance of bee colonies	1.00			
2	No. of beehives	0.00	1.00		
3	No. of nests reported in the beekeeper's municipality between 2015-2017	0.25	-0.02	1.00	
4	No. of nests seen by the beekeeper	0.23	0.16	0.25	1.00

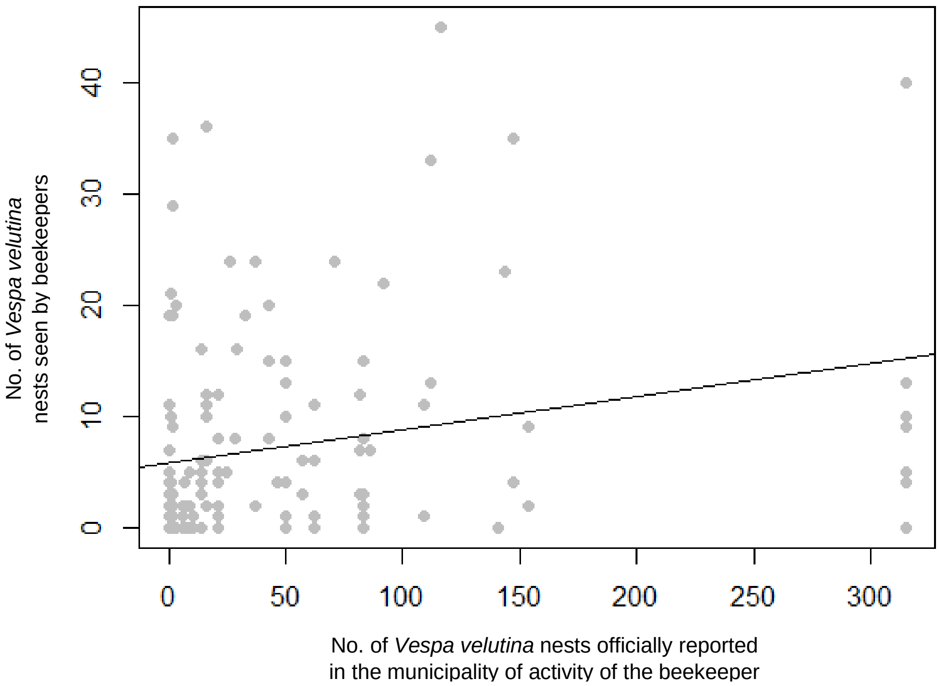


Fig. S4.1 – Relation between the number of nests officially reported in the municipality of activity of the beekeeper and the number of nests seen by beekeepers (two independent and uncorrelated variables). The black line indicates the positive trend of the relationship.

E

Supplementary Material - Chapter 5

Table S5.1 – Reclassified land cover areas with the respective area of occupancy within the study area.

Land cover classes	Area (km2)	Nr. Nests
Agro_forestry areas	12.22	13
Complex_cultivation_patterns	1685.51	1601
Coniferous Forest	3646.65	665
Constructed Urban areas	1235.21	2031
Deciduos Forest	1162.56	621
Discovered areas with sparce vegetation	999.32	166
Eucaliptos plantations	2856.79	954
Fruit crops	130.86	59
Green Urban areas	1025.65	1449
Invasive tree forests	124	38
Mediterranean forest	1750.18	259
Mixed Forest	3529.92	1764
Montado	6.87	0
Nut crops	72.55	2
Olive crops	225.44	80
Pastures	24.66	7
Rice plantations	177.71	28
Shrubs and natural meadows	1912.48	307
Temporary irrigated crops	2223.24	2386
Temporary rainfed crops	457.71	265
Vineyards	730.09	304
Water (mostly riparian galleries)	251.13	76
Wetlands	187.38	19

Table S5.2 – Variation of nest density of *V. velutina* between 2014 and 2019. The minimum, maximum and mean values are shown.

year	Max. Density	Min. Density	Mean	St. Dev.
2014	0.50	0.01	0.18	0.11
2015	1.32	0.01	0.31	0.28
2016	0.71	0.01	0.15	0.14
2017	1.42	0.01	0.20	0.19
2018	0.80	0.01	0.15	0.13
2019	4.21	0.01	0.69	0.65

Table S5.3 - Summary of land-cover suitability for pollinators in the Portugal according to nine experts' opinion (four of them focused their opinion only in relation to bees). The percentage of crop pollinators requirement is also shown for each categorie.

Caterory	**Reclass**	**% Crop Pollinator requirement**	**Suitability to Pollinators***	
			Mean	SE
1.1.1.01.1 Predominantly vertical continuous urban fabric	Constructed urban areas	0.00	0.4	0.5
1.1.1.02.1 Predominantly horizontal continuous urban fabric	Constructed urban areas	0.00	0.9	0.6
1.1.1.03.1 Parking and public areas	Green Urban areas	0.00	1.3	1.0
1.1.2.01.1 Discontinuous urban fabric	Green Urban areas	0.00	1.7	0.8
1.1.2.02.1 Sparse discontinuous urban fabric	Green Urban areas	0.00	2.2	1.0
1.2.1.01.1 Industry	Constructed urban areas	0.00	0.6	0.5
1.2.1.02.1 Commerce	Constructed urban areas	0.00	0.4	0.5
1.2.1.03.1 Agricultural installations	Constructed urban areas	0.00	1.5	1.2
1.2.1.04.1 Public and Private Equipment	Constructed urban areas	0.00	0.6	0.7
1.2.1.05.1 Renewable energy production infrastructures	Constructed urban areas	0.00	1.0	0.9
1.2.1.05.2 Non-renewable energy production infrastructures	Constructed urban areas	0.00	0.8	0.8
1.2.1.06.1 Infrastructure for abstraction, treatment and supply of drinking water	Constructed urban areas	0.00	0.7	0.9
1.2.1.07.1 Waste and wastewater treatment infrastructures	Constructed urban areas	0.00	0.9	0.8
1.2.2.01.1 Road network and associated spaces	Constructed urban areas	0.00	1.8	0.7
1.2.2.02.1 Railway network and associated spaces	Constructed urban areas	0.00	1.7	0.7
1.2.3.01.1 Sea and river port terminals	Constructed urban areas	0.00	0.4	0.5
1.2.3.02.1 Shipyards and dry docks	Constructed urban areas	0.00	0.6	0.5
1.2.3.03.1 Marinas and fishing docks	Constructed urban areas	0.00	0.8	1.0
1.2.4.01.1 Airports	Constructed urban areas	0.00	1.7	0.9
1.2.4.02.1 Aerodromes	Constructed urban areas	0.00	1.8	0.8
1.3.1.01.1 Open pit mines	Constructed urban areas	0.00	1.3	0.9
1.3.1.02.1 Quarries	Constructed urban areas	0.00	1.8	1.3
1.3.2.01.1 Landfills	Constructed urban areas	0.00	0.9	1.1
1.3.2.02.1 Dumpsters and Scraps	Constructed urban areas	0.00	0.8	1.0
1.3.3.01.1 Areas under construction	Constructed urban areas	0.00	1.1	0.9
1.3.3.02.1 Abandoned Areas in Artificial Territories	Constructed urban areas	0.00	2.2	1.1
1.4.1.01.1 Parks and gardens	Green Urban areas	0.00	3.2	0.8
1.4.1.02.1 Cemeteries	Green Urban areas	0.00	2.6	1.1
1.4.2.01.1 Golf Courses	Green Urban areas	0.00	1.9	0.9
1.4.2.01.2 Other sports facilities	Constructed urban areas	0.00	1.3	1.0
1.4.2.02.1 Campsites	Green Urban areas	0.00	2.4	1.2
1.4.2.02.2 Other leisure equipment	Constructed urban areas	0.00	1.4	1.1
1.4.2.03.1 Cultural facilities and historical areas	Constructed urban areas	0.00	1.3	1.0
2.1.1.01.1 Temporary rainfed crops	Temporary rainfed crops	0.05	2.1	1.2
2.1.1.02.1 Greenhouses and Nurseries	Constructed urban areas	0.05	1.4	0.9
2.1.2.01.1 Temporary irrigated crops	Temporary irrigated crops	0.95	1.9	1.0
2.1.3.01.1 Rice paddies	Rice plantations	0.00	1.1	0.8
2.2.1.01.1 Vineyards	Vineyards	0.05	1.9	0.8
2.2.1.02.1 Vineyards with orchards	Vineyards	0.25	2.8	0.9
2.2.1.03.1 Vineyards with olive groves	Vineyards	0.03	2.6	0.7
2.2.2.01.1 Fresh fruit orchards	Fruit crops	0.95	3.0	0.5
2.2.2.01.2 Almond Orchards	Nut crops	0.65	3.1	0.6
2.2.2.01.3 Chestnut Orchards	Nut crops	0.25	2.9	0.8
2.2.2.01.4 Carob orchards	Nut crops	0.65	2.6	1.1
2.2.2.01.5 Citrus orchards	Fruit crops	0.65	2.7	1.1

2.2.2.01.6 Other orchards	Fruit crops	0.65	2.6	1.0
2.2.2.02.1 Fresh fruit orchards with vines	Fruit crops	0.66	3.0	0.9
2.2.2.02.2 Almond orchard with vineyard	Nut crops	0.46	3.1	0.8
2.2.2.02.3 Chestnut orchard with vineyard	Nut crops	0.19	2.9	0.8
2.2.2.02.4 Carob orchards with vines	Nut crops	0.46	2.7	1.2
2.2.2.02.5 Citrus orchards with vines	Fruit crops	0.46	2.8	1.2
2.2.2.02.6 Other orchards with vines	Fruit crops	0.46	2.7	1.0
2.2.2.03.1 Fresh fruit orchards with olive groves	Fruit crops	0.64	2.8	1.0
2.2.2.03.2 Almond orchard with olive groves	Nut crops	0.44	2.9	1.0
2.2.2.03.3 Chestnut orchard with olive grove	Nut crops	0.17	2.8	1.0
2.2.2.03.4 Carob orchards with olive groves	Nut crops	0.44	2.6	1.2
2.2.2.03.5 Citrus orchards with olive groves	Fruit crops	0.44	2.8	1.2
2.2.2.03.6 Other olive orchards	Fruit crops	0.44	2.7	1.2
2.2.3.01.1 Olive Groves	Olive crops	0.00	2.8	1.2
2.2.3.02.1 Olive groves with vines	Olive crops	0.02	2.7	1.0
2.2.3.03.1 Olive groves with orchard	Olive crops	0.17	2.9	1.1
2.3.1.01.1 Permanent pastures	Pastures	0.00	3.4	1.0
2.4.1.01.1 Temporary rainfed crops associated with vineyards	Temporary rainfed crops	0.05	2.6	1.0
2.4.1.01.2 Temporary rainfed crops associated with orchard	Temporary rainfed crops	0.35	2.8	0.9
2.4.1.01.3 Temporary rainfed crops associated with olive groves	Temporary rainfed crops	0.03	2.6	1.1
2.4.1.02.1 Temporary irrigated crops associated with vineyards	Temporary irrigated crops	0.50	2.3	0.9
2.4.1.02.2 Temporary irrigated crops associated with orchard	Temporary irrigated crops	0.80	2.5	0.7
2.4.1.02.3 Temporary irrigated crops associated with olive groves	Temporary irrigated crops	0.48	2.5	0.7
2.4.1.03.1 Pasture associated with the vineyard	Pastures	0.03	3.1	1.0
2.4.1.03.2 Pastures associated with orchard	Pastures	0.33	3.3	1.0
2.4.1.03.3 Pastures associated with olive groves	Pastures	0.00	3.3	1.0
2.4.2.01.1 Complex cultural and parcel systems	Complex cultivation patterns	0.65	3.5	1.0
2.4.3.01.1 Agriculture with natural and semi-natural spaces	Complex cultivation patterns	0.13	3.9	0.7
2.4.4.01.1 Cork oak AFS with temporary rainfed crops	Montado	0.05	3.5	1.2
2.4.4.01.2 Holm oak AFS with temporary dryland crops	Montado	0.05	3.5	1.2
2.4.4.01.3 AFS from other oaks with temporary rainfed crops	Agro-forestry areas	0.05	3.4	1.2
2.4.4.01.4 AFS of other species with temporary rainfed crops	Agro-forestry areas	0.05	3.4	1.2
2.4.4.01.5 Cork oak AFS with holm oak and temporary rainfed crops	Montado	0.05	3.5	1.3
2.4.4.01.6 AFS from other mixtures with temporary rainfed crops	Agro-forestry areas	0.05	3.3	1.1
2.4.4.02.1 Cork oak AFS with temporary irrigated crops	Montado	0.50	2.9	1.0
2.4.4.02.2 Holm oak AFS with temporary irrigated crops	Montado	0.50	2.9	1.0
2.4.4.02.3 AFS from other oaks with temporary irrigated crops	Agro-forestry areas	0.50	2.8	0.9
2.4.4.02.4 AFS of other species with temporary irrigated crops	Agro-forestry areas	0.50	2.8	0.9
2.4.4.02.5 Cork oak AFS with holm oak and temporary irrigated crops	Montado	0.50	2.9	1.0
2.4.4.02.6 AFS of other mixtures with temporary irrigated crops	Agro-forestry areas	0.50	2.8	0.9

2.4.4.03.1 Cork oak AFS with pastures	Montado	0.03	3.9	0.9
2.4.4.03.2 Holm oak AFS with pastures	Montado	0.03	3.9	0.9
2.4.4.03.3 AFS of other oaks with pastures	Agro-forestry areas	0.03	4.1	0.9
2.4.4.03.4 AFS of other species with pastures	Agro-forestry areas	0.03	3.6	0.7
2.4.4.03.5 Cork oak and holm oak AFS with pastures	Montado	0.03	3.9	0.9
2.4.4.03.6 AFS of other mixtures with pastures	Agro-forestry areas	0.03	3.8	0.8
2.4.4.04.1 Cork oak AFS with permanent crops	Agro-forestry areas	0.12	3.0	1.2
2.4.4.04.2 Holm oak AFS with permanent crops	Agro-forestry areas	0.12	2.8	1.0
2.4.4.04.3 AFS from other oaks with permanent crops	Agro-forestry areas	0.12	3.0	1.0
2.4.4.04.4 AFS of other species with permanent crops	Agro-forestry areas	0.12	2.8	1.0
2.4.4.04.5 Cork oak and holm oak AFS with permanent crops	Agro-forestry areas	0.12	2.8	1.0
2.4.4.04.6 AFS from other mixtures with permanent crops	Agro-forestry areas	0.12	2.8	1.0
3.1.1.01.1 Cork oak forests	Mediterranean forest	0.05	3.6	1.1
3.1.1.01.2 Holm oak forests	Mediterranean forest	0.05	3.5	1.2
3.1.1.01.3 Forests of other oaks	Deciduous Forest	0.05	3.7	1.1
3.1.1.01.4 Chestnut Forests	Deciduous Forest	0.25	3.7	1.0
3.1.1.01.5 Eucalyptus Forests	Eucaliptos plantations	0.05	1.7	0.7
3.1.1.01.6 Forests of invasive species	Invasive tree forests	0.00	1.9	0.7
3.1.1.01.7 Forests of other broadleafs trees	Deciduous Forest	0.05	3.8	0.8
3.1.1.02.1 Cork oak forest with broadleaf trees	Mediterranean forest	0.05	3.9	0.8
3.1.1.02.2 Holm oak forests with broadleaf trees	Mediterranean forest	0.05	3.8	0.8
3.1.1.02.3 Forests of other oaks with broadleaf trees	Deciduous Forest	0.05	3.8	0.8
3.1.1.02.4 Chestnut woods with broadleaf trees	Deciduous Forest	0.19	3.8	0.8
3.1.1.02.5 Eucalyptus forests with broadleaf trees	Mixed Forest	0.05	2.4	0.7
3.1.1.02.6 Forests of invasive species with broadleaf trees	Invasive tree forests	0.02	2.4	0.7
3.1.1.02.7 Forests of other broadleaf trees	Deciduous Forest	0.05	3.6	0.7
3.1.2.01.1 Pine forests	Coniferous forest	0.00	2.3	0.9
3.1.2.01.2 Stone pine forests	Coniferous forest	0.00	2.4	1.0
3.1.2.01.3 Forests of other softwood trees	Coniferous forest	0.00	2.2	0.8
3.1.2.02.1 Pine forests with softwood trees	Coniferous forest	0.00	2.4	0.9
3.1.2.02.2 Stone pine forests with softwood trees	Coniferous forest	0.00	2.5	1.0
3.1.2.02.3 Forests of other softwood trees	Coniferous forest	0.00	2.3	0.9
3.1.3.01.1 Cork oak forests with softwood trees	Mixed Forest	0.03	2.9	0.7
3.1.3.01.2 Holm oak forests with softwood trees	Mixed Forest	0.03	2.8	0.9
3.1.3.01.3 Forests of other oaks with softwood trees	Mixed Forest	0.03	2.9	1.0
3.1.3.01.4 Chestnut forests with softwood trees	Mixed Forest	0.17	2.7	0.9
3.1.3.01.5 Eucalyptus forests with softwood trees	Mixed Forest	0.03	1.9	0.8
3.1.3.01.6 Forests of invasive species with softwood trees	Invasive tree forests	0.00	1.9	0.9
3.1.3.01.7 Forests of other broadleafs trees with softwood trees	Mixed Forest	0.03	2.5	0.8
3.1.3.01.8 Broadleaf mixed forest with softwood trees	Mixed Forest	0.03	2.6	1.0
3.1.3.02.1 Pine forests with broadleaf trees	Mixed Forest	0.02	2.6	0.9

3.1.3.02.2 Stone pine forests with broaleaf trees	Mixed Forest	0.02	2.7	0.8
3.1.3.02.3 Forests of other softwood trees with broadleaf trees	Mixed Forest	0.02	2.7	0.8
3.1.3.02.4 Softwood mixed forests with broadleaf trees	Mixed Forest	0.02	2.8	0.7
3.2.1.01.1 Natural herbaceous vegetation	Shrubs and natural meadows	0.00	4.9	0.3
3.2.2.01.1 Dense shrubs	Mediterranean forest	0.00	3.6	1.2
3.2.2.02.1 Sparce shrubs	Shrubs and natural meadows	0.00	4.4	0.5
3.2.3.01.1 Dense sclerophyte vegetation	Mediterranean forest	0.00	3.9	0.6
3.2.3.02.1 Sparce sclerophyte vegetation	Shrubs and natural meadows	0.00	4.2	1.0
3.2.4.01.1 Open cork oak forests	Montado	0.05	4.1	1.1
3.2.4.01.2 Open holm oak forests	Montado	0.05	4.0	1.1
3.2.4.01.3 Open forests of other oaks	Deciduous Forest	0.05	4.0	0.9
3.2.4.01.4 Open chestnut forests	Deciduous Forest	0.25	3.7	1.1
3.2.4.01.5 Eucalyptus open forests	Eucaliptos plantations	0.05	2.4	0.9
3.2.4.01.6 Open forests of invasive species	Invasive tree forests	0.00	2.4	0.7
3.2.4.01.7 Open forests of other broadleaf trees	Deciduous Forest	0.05	3.8	0.7
3.2.4.02.1 Open cork forests with broadleaf trees	Mixed Forest	0.05	4.1	0.8
3.2.4.02.2 Open holm oak forests with broadleaf trees	Mixed Forest	0.05	4.0	0.9
3.2.4.02.3 Open oak forests with other broadleaf trees	Deciduous Forest	0.05	4.1	0.8
3.2.4.02.4 Open Chestnut Forests with broadleaf trees	Deciduous Forest	0.19	4.0	0.7
3.2.4.02.5 Open eucalyptus forests with broadleaf trees	Mixed Forest	0.05	2.7	0.9
3.2.4.02.6 Open invasive forests with broadleaf trees	Invasive tree forests	0.02	2.6	0.9
3.2.4.02.7 Open forests of other hardwood with broadleaf trees	Deciduous Forest	0.05	3.7	0.7
3.2.4.03.1 Open pine forests	Coniferous forest	0.00	2.6	1.1
3.2.4.03.2 Open stone pine forests	Coniferous forest	0.00	2.6	1.1
3.2.4.03.3 Open forests of other softwood trees	Coniferous forest	0.00	2.3	1.1
3.2.4.04.1 Open pine forests with softwood trees	Coniferous forest	0.00	2.6	1.1
3.2.4.04.2 Open stone pine forests with softwoods	Coniferous forest	0.00	2.6	1.1
3.2.4.04.3 Open softwood forests of other softwood trees	Coniferous forest	0.00	2.4	1.0
3.2.4.05.1 Open cork oak forests with softwood trees	Mixed Forest	0.03	2.8	0.9
3.2.4.05.2 Open holm oak forests with softwood trees	Mixed Forest	0.03	3.1	0.9
3.2.4.05.3 Open forests of other oak with softwood trees	Mixed Forest	0.03	3.2	0.8
3.2.4.05.4 Open chestnut forests with softwood trees	Mixed Forest	0.17	3.1	1.1
3.2.4.05.5 Open eucalyptus forests with softwood trees	Mixed Forest	0.03	1.9	0.6
3.2.4.05.6 Open invasive forests with softwood trees	Invasive tree forests	0.00	2.2	1.0
3.2.4.05.7 Open forests of other broadleaf trees with softwood trees	Mixed Forest	0.03	2.9	1.2
3.2.4.05.8 Open broadleaf forests with softwood trees	Mixed Forest	0.03	3.1	0.9

3.2.4.06.1 Open pinewood forests with broadleaf trees	Mixed Forest	0.02	2.8	0.9
3.2.4.06.2 Open stone pine forests with broadleaf trees	Mixed Forest	0.02	2.9	0.7
3.2.4.06.3 Open forest of other softwood trees with broadleaf trees	Mixed Forest	0.02	3.0	0.7
3.2.4.06.4 Open softwood mixture forest with broadleaf trees	Mixed Forest	0.02	3.2	0.8
3.2.4.07.1 Other woody formations	Mixed Forest	0.00	2.6	0.8
3.2.4.08.1 Shallow cuts	Discovered areas with sparce vegetation	0.00	2.0	0.7
3.2.4.08.2 New plantations	Discovered areas with sparce vegetation	0.00	1.6	0.7
3.2.4.09.1 Forest nurseries	Discovered areas with sparce vegetation	0.00	1.8	0.8
3.2.4.10.1 Fire breaks	Shrubs and natural meadows	0.00	2.6	1.2
3.3.1.01.1 Beaches, dunes and inland sands	Discovered areas with sparce vegetation	0.00	3.1	1.2
3.3.1.02.1 Beaches, dunes and coastal sands	Discovered areas with sparce vegetation	0.00	3.1	1.4
3.3.2.01.1 Bare rock	Discovered areas with sparce vegetation	0.00	0.9	1.1
3.3.3.01.1 Sparcely vegetated areas	Discovered areas with sparce vegetation	0.00	3.1	1.1
3.3.4.01.1 Burnt areas	Discovered areas with sparce vegetation	0.00	1.4	1.1
4.1.1.01.1 Inland marshes	Wetlands	0.00	2.6	1.0
4.1.2.01.1 Peatlands	Wetlands	0.00	2.7	1.2
4.2.1.01.1 Marshlands	Wetlands	0.00	2.1	0.9
4.2.2.01.1 Salt pans	Wetlands	0.00	0.9	1.3
4.2.2.02.1 Coastal aquaculture	Wetlands	0.00	0.4	0.7
4.2.3.01.1 Intertidal zones	Wetlands	0.00	0.3	0.5
5.1.1.01.1 Natural water courses	Water	0.00	1.9	2.3
5.1.1.02.1 Artificial channels	Water	0.00	0.6	0.7
5.1.2.01.1 Artificial inland lakes and lagoons	Water	0.00	1.2	1.3
5.1.2.01.2 Natural inland lakes and lagoons	Water	0.00	1.8	2.1
5.1.2.02.1 Dam reservoirs	Water	0.00	0.8	1.1
5.1.2.03.1 Weir reservoir	Water	0.00	1.1	1.4
5.1.2.03.2 Puddles	Water	0.00	1.1	1.5
5.1.2.03.3 Indoor aquaculture	Water	0.00	0.1	0.3
5.2.1.01.1 Coastal lagoons	Water	0.00	1.3	1.7
5.2.2.01.1 Estuaries	Water	0.00	0.8	1.1
5.2.3.01.1 Ocean	Water	0.00	0.0	0.0

Table S5.4 – Relationship of hornet density with the selected independent variables and its interactions (with population density and year) identified in the best model. Details of the model selection with ΔAIC<2 are presented in Table S5.5.

	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	
(Intercept)	2196.2	875.9	2.5	0.012	*
Crop Pollinator requirement	0.9	0.2	4.2	0.000	***
Suitability to Pollinators	0.6	0.3	2.3	0.022	*
Beehive density	116.0	63.1	1.8	0.066	.
Tree cover	-779.0	224.0	-3.5	0.001	***
Human population density	0.3	0.1	3.0	0.002	**
Year	-1.1	0.4	-2.5	0.012	*
Crop Pollinator requirement: Human population density	-0.1	0.04	-2.6	0.009	**
Beehive density: Human population density	-0.1	0.04	-2.4	0.016	*
Beehive density:year	-0.1	0.03	-1.8	0.067	.
Tree cover: year	0.4	0.1	3.5	0.001	***

Table S5.5 – Set of best models used to evaluate the susceptibility for socioeconomic and ecological caused by *Vespa velutina*. Only the models with ΔAIC<2 are shown. Nomenclatures: CPR – crop pollinator requirement; BD – beehive density; HPD – human population density; SP – suitability to pollinators; TC – tree cover.

	(Intercept)	CPR	BD	HPD	SP	TC	year	CPR:BD	CPR:HPD	CPR:SP	CPR:TC	CPR:year	BD:HPD	BD:SP	BD:TC	BD:year	HPD:SP	HPD:TC	HPD:year	SP:TC	SP:year	TC:year	df	logLik	AIC	delta	weight
1498304	726.64	-114.04	119.84	69.01	-9.08	-563.35	-0.36		-0.09			0.06	-0.15	-0.65		-0.06	0.24		-0.03	2.39		0.28	17	511.63	-989.26	0	0.013
1465536	807.62	-114.12	121.19	67.49	-6.24	-582.95	-0.40		-0.09			0.06	-0.14	-0.76		-0.06			-0.03	2.05		0.29	16	510.53	-989.06	0.20	0.012
1268928	746.54	-118.57	121.29	69.15	1.80	-569.80	-0.37		-0.10			0.06	-0.14	-0.77		-0.06		-0.27	-0.03			0.28	16	510.51	-989.02	0.24	0.011
1473728	914.46	-111.88	122.44	67.05	-7.57	-608.27	-0.45		-0.09			0.06	-0.15	-0.84	-0.33	-0.06			-0.03	2.42		0.30	17	511.43	-988.86	0.41	0.010
1301696	695.71	-119.19	120.52	70.48	0.63	-558.10	-0.35		-0.10			0.06	-0.15	-0.68		-0.06	0.19	-0.29	-0.03			0.28	17	511.20	-988.40	0.86	0.008
1891520	1849.38	-96.98	137.18	0.23	-485.05	-633.64	-0.91		-0.08			0.05	-0.15	-0.68		-0.07	0.26			2.37	0.24	0.31	17	511.13	-988.26	1.00	0.008
1494208	789.10	-111.69	117.20	64.68	-10.04	-570.97	-0.39		-0.09			0.06	-0.10			-0.06	0.30		-0.03	2.33		0.28	16	510.03	-988.07	1.19	0.007
1277120	838.55	-117.46	122.40	68.87	1.88	-592.02	-0.42		-0.10			0.06	-0.15	-0.82	-0.25	-0.06		-0.29	-0.03			0.29	17	511.03	-988.06	1.20	0.007
1466048	763.48	-118.19	122.50	70.01	-6.18	-577.63	-0.38		-0.10		-0.24	0.06	-0.13	-0.73		-0.06			-0.03	2.03		0.29	17	510.91	-987.82	1.44	0.006
1531072	729.56	-115.99	120.51	68.68	-2.92	-564.31	-0.36		-0.09			0.06	-0.14	-0.78		-0.06		-0.16	-0.03	1.21		0.28	17	510.88	-987.76	1.50	0.006
1858752	1898.18	-96.48	136.38	0.45	-445.44	-655.21	-0.94		-0.08			0.05	-0.14	-0.80		-0.07				2.00	0.22	0.32	16	509.86	-987.71	1.55	0.006
1662144	1810.62	-101.08	136.76	1.48	-446.06	-632.65	-0.90		-0.09			0.05	-0.15	-0.81		-0.07		-0.27			0.22	0.31	16	509.86	-987.71	1.55	0.006
1866944	2009.80	-94.63	138.65	0.47	-454.07	-681.28	-0.99		-0.08			0.05	-0.16	-0.88	-0.35	-0.07				2.40	0.22	0.34	17	510.84	-987.69	1.57	0.006
1497280	1241.39	0.92	121.82	52.22	-9.40	-613.43	-0.61		-0.10				-0.15	-0.64		-0.06	0.24		-0.03	2.48		0.30	16	509.83	-987.67	1.59	0.006
1890496	2082.72	0.88	137.27	0.20	-413.44	-661.41	-1.03		-0.09				-0.15	-0.67		-0.07	0.26			2.45	0.20	0.33	16	509.77	-987.54	1.72	0.005
1989824	1089.08	-114.10	130.26	52.51	-188.70	-592.72	-0.54		-0.08			0.06	-0.14	-0.79		-0.06			-0.03	2.03	0.09	0.29	17	510.70	-987.41	1.85	0.005
1793216	1028.17	-118.64	130.73	53.44	-189.80	-576.57	-0.51		-0.09			0.06	-0.14	-0.80		-0.06		-0.27	-0.03		0.09	0.29	17	510.70	-987.41	1.86	0.005
1472704	1425.56	0.93	124.48	50.33	-7.90	-658.58	-0.70		-0.10				-0.15	-0.83	-0.35	-0.06			-0.02	2.51		0.33	16	509.70	-987.41	1.86	0.005
1269440	725.83	-121.55	122.44	70.89	1.78	-568.85	-0.36		-0.10		-0.17	0.06	-0.14	-0.75		-0.06		-0.26	-0.03			0.28	17	510.70	-987.39	1.87	0.005
1694912	1781.53	-102.26	137.75	1.38	-477.74	-618.06	-0.89		-0.09			0.05	-0.16	-0.72		-0.07	0.21	-0.29			0.24	0.31	17	510.69	-987.37	1.89	0.005

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