PhD Thesis

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Enhancing Biological Control of Mealybugs in Mediterranean subtropical crops

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Mealybugs are major agricultural insect pests in subtropical crops worldwide, including citrus and persimmon from Mediterranean basin. Enhancing the biological control of mealybugs can contribute to reducing crop damage and reliance on insecticides for their control, which is essential for sustainable agriculture. To identify and evaluate strategies that can enhance mealybug biological control in Mediterranean subtropical crops, this thesis studies several factors potentially influencing mealybug abundance, including mutualistic ants, parasitoids, climate warming, and habitat context.

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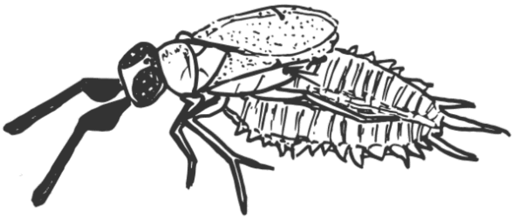

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“*Bugs are not going to inherit the earth.*
They own it now. So we might as well
make peace with the landlord.”

Thomas Eisner

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Summary

Pseudococcids (Hemiptera: Pseudococcidae), commonly known as mealybugs, are sap-sucking hemipteran insects that feed on the phloem of plants. These insects are among the most damaging pests to various subtropical crops. In the Mediterranean basin, several species of invasive mealybugs have established and become key pests in various crops such as citrus and persimmon. These crops cover a large territory and hold high economic importance in the Valencian Community. Mealybugs have been traditionally managed with insecticides. However, their environmental risks have led to the implementation of policies to reduce insecticide use, including the prohibition of several active substances. Therefore, it is necessary to implement alternative management strategies. Among these strategies, biological control is one of the most promising. To identify and evaluate different strategies that can enhance the biological control of mealybugs, in this thesis I studied several factors potentially influencing mealybug abundance in Mediterranean subtropical crops. These factors include mutualistic ants, parasitoids, climate warming, and habitat context.

In the **first chapter**, I described the interactions between Mediterranean native ants and the invasive mealybug *Delottococcus aberiae* in Mediterranean citrus. Here, I demonstrated that the Mediterranean ant *Lasius grandis* and *D. aberiae* have established a mutualistic relationship that can facilitate the invasion of the mealybug. In the **second chapter**, I assessed whether the physical exclusion of *L. grandis* from citrus canopies may enhance the control of *D. aberiae*. I found that ant-exclusion from citrus canopies can reduce *D. aberiae* density and damage, likely because of an increase in the abundance of generalist predators. In the **third chapter**, I identified the complex of mealybugs attacking Mediterranean persimmon and described their seasonal trend. This study revealed that *Pseudococcus longispinus* has become the most abundant mealybug species in the crop and can reach high infestation levels at harvest. Importantly, I found that fruit infestation at harvest can be predicted by early samplings. I also described the phenology of *P. longispinus* and evaluated the potential effect of climate warming on mealybug phenology, predicting that the number of generations and potential damage will increase in the following years. In the **fourth chapter**, I identified the parasitoid complex of *P. longispinus* in persimmon. I found that *P. longispinus* was parasitized by a diverse complex of parasitoids dominated by one encyrtid species, *Anagyrus fusciventris*. This parasitoid can effectively reduce the density of the pest despite the high abundance of hyperparasitoids. Therefore, *A. fusciventris* has a high potential to be used in augmentative biological control programs. I also determined the preferred host size used by *A. fusciventris*, which will be valuable to design an augmentative biological control program. Finally, in the **fifth chapter**, I assessed whether habitat heterogeneity affects the density of mealybugs in citrus and persimmon. First, I found that the proportion of surrounding monoculture increased the abundance of both *D. aberiae* in citrus and *P. longispinus* in persimmon. Furthermore, non-crop habitats, both semi-natural habitats surrounding the crop and inter-row ground cover vegetation, reduced the abundance of *P. longispinus* in persimmon by increasing parasitism. These findings revealed that habitat diversification strategies can enhance mealybug control.

Overall, this thesis emphasizes that mealybugs have become primary pests that can cause high economic losses in several subtropical fruit crops from Mediterranean basin, such as citrus and persimmon, and revealed that this increase in mealybug abundance is caused by multiple factors. Remarkably, this thesis has identified different strategies that can be implemented to enhance the biological control of mealybugs in Mediterranean citrus and persimmon, including the management of mutualistic ants, the augmentative release of parasitoids, and habitat management. The findings of this thesis must be considered within the Integrated Pest Management programs against mealybugs in subtropical crops.

Resumen

Los pseudocóccidos (Hemiptera: Pseudococcidae), comúnmente conocidos como cochinillas harinosas, son insectos hemípteros chupadores de savia que se alimentan del floema de las plantas. Estos insectos están entre las plagas más perjudiciales de varios cultivos subtropicales. En la cuenca mediterránea, diversas especies invasoras de pseudocóccidos se han establecido y convertido en plagas clave de cítricos y caquis, dos cultivos que abarcan un amplio territorio y tienen una gran importancia económica en la Comunidad Valenciana. Convencionalmente, los pseudocóccidos han sido controlados mediante el uso de insecticidas; sin embargo, los riesgos ambientales que conlleva su uso han llevado a la implementación de políticas para reducir su uso, incluida la prohibición de varias materias activas. Por lo tanto, es necesario desarrollar e implementar estrategias de gestión alternativas. Entre estas estrategias, fomentar el control biológico es una de las más prometedoras. Para identificar y evaluar diferentes estrategias que puedan mejorar el control biológico de pseudocóccidos, en esta tesis estudié varios factores que pueden influir en la abundancia de estas plagas en cultivos subtropicales mediterráneos. Estos factores incluyen las hormigas mutualistas, los parasitoides, el cambio climático y el paisaje.

En el **primer capítulo**, describí las interacciones entre las hormigas nativas del Mediterráneo y el pseudocóccido invasor *Delottococcus aberiae* en los cítricos mediterráneos. Demostré que la hormiga mediterránea *Lasius grandis* y *D. aberiae* han establecido una relación mutualista que puede facilitar la invasión del pseudocóccido. En el **segundo capítulo**, evalué si la exclusión física de *L. grandis* de las copas de los cítricos puede mejorar el control de *D. aberiae*. Descubrí que la exclusión de hormigas de las copas de los cítricos puede reducir la densidad y el daño de *D. aberiae*, probablemente debido a un aumento en la abundancia de depredadores generalistas. En el **tercer capítulo**, identifiqué el complejo de pseudocóccidos que ataca al caqui mediterráneo y describí su dinámica estacional. Este estudio reveló que *Pseudococcus longispinus* se ha convertido en la especie de pseudocóccido más abundante en el cultivo y puede alcanzar niveles altos de infestación en la cosecha. Es importante destacar que encontré que la infestación de la fruta en la cosecha puede predecirse mediante muestreos tempranos. También describí la fenología de *P. longispinus* y evalué el efecto potencial del cambio climático en su fenología, prediciendo que el número de generaciones y el daño potencial aumentarán en los próximos años. En el **cuarto capítulo**, identifiqué el complejo de

parasitoides de *P. longispinus* en caqui. Descubrí que *P. longispinus* estuvo parasitado por un complejo diverso de parasitoides dominado por una especie de encírtido, *Anagyrus fusciventris*. Este parasitoide puede reducir eficazmente la densidad de la plaga a pesar de la alta abundancia de hiperparasitoides. Por lo tanto, *A. fusciventris* tiene un alto potencial para ser utilizado en programas de control biológico aumentativo. También determiné el tamaño del hospedador parasitado por *A. fusciventris*, lo cual sería valioso para programar liberaciones de parasitoides. Finalmente, en el **quinto capítulo**, evalué si la heterogeneidad del hábitat afecta la densidad de pseudocóccidos en cítricos y caquis. Por un lado, encontré que la proporción de monocultivo circundante aumentó la densidad tanto de *D. aberiae* en los cítricos como de *P. longispinus* en caqui. Por otro lado, los hábitats no agrícolas, tanto los hábitats seminaturales que rodean el cultivo como la cubierta vegetal entre filas, redujeron la abundancia de *P. longispinus* en caqui al aumentar su parasitismo. Estos hallazgos mostraron que las estrategias de diversificación del hábitat pueden mejorar el control de los pseudocóccidos.

En resumen, esta tesis destaca que los pseudocóccidos se han convertido en plagas clave que pueden causar pérdidas económicas importantes en varios cultivos frutales subtropicales de la cuenca mediterránea, como cítricos y caquis, y reveló que el aumento en la abundancia de pseudocóccidos es causado por múltiples factores. Es destacable que esta tesis ha identificado diferentes estrategias que pueden implementarse para mejorar el control biológico de las cochinillas en cítricos y caquis mediterráneos, incluyendo la gestión de hormigas mutualistas, la liberación aumentativa de parasitoides y la gestión del hábitat. Los hallazgos de esta tesis deben ser considerados dentro de los programas de Gestión Integrada de Plagas contra pseudocóccidos en cultivos subtropicales.

Resum

Els pseudocòccids (Hemiptera: Pseudococcidae), coneguts com cotonets, són insectes hemípters xucladors de saba que s'alimente del floema de les plantes. Aquests insectes estan entre les plagues més perjudicials per a diversos cultius subtropicals. A la conca mediterrània, diverses espècies invasores de cotonets s'han establert i esdevingut plagues clau en diversos cultius, com ara els cítrics i els caquis, que abasten un ampli territori i tenen una gran importància econòmica a la Comunitat Valenciana. Convencionalment, els cotonets s'han controlat mitjançant l'ús insecticides; no obstant això, els riscos ambientals han portat a la implementació de polítiques per reduir el seu ús, incloent la prohibició de diverses matèries actives. Per tant, és necessari implementar estratègies de gestió alternatives. Entre aquestes estratègies, fomentar el control biològic és una de les més prometedores. Per identificar i avaluar diferents estratègies que puguen millorar el control biològic dels cotonets, en esta tesi vaig estudiar diversos factors que poden influir en l'abundància d'estes plagues en cultius subtropicals mediterranis. Aquests factors inclouen les formigues mutualistes, els parasitoids, el canvi climàtic i el paisatge.

Al **primer capítol**, vaig descriure les interaccions entre les formigues natives del Mediterrani i el cotonet invasor *Delottococcus aberiae* en els cítrics mediterranis. Ací vaig demostrar que la formiga mediterrània *Lasius grandis* i *D. aberiae* han establert una relació mutualista que pot facilitar la invasió del pseudocòccid. Al **segon capítol**, vaig avaluar si l'exclusió física de *L. grandis* de les copes dels cítrics pot millorar el control de *D. aberiae*. Vaig descobrir que l'exclusió de formigues de les copes dels cítrics pot reduir la densitat i el danys de *D. aberiae*, probablement a causa d'un augment en l'abundància de depredadors generalistes. Al **tercer capítol**, vaig identificar les espècies de cotonets que ataquen el caqui mediterrani i vaig descriure la seua dinàmica estacional. Aquest estudi va revelar que *Pseudococcus longispinus* s'ha convertit en l'espècie de cotonet més abundant en el cultiu i pot arribar a nivells alts d'infestació en la collita. És important destacar que vaig trobar que la infestació del fruit en la collita pot predir-se mitjançant mostrejos en primavera i estiu. També vaig descriure la fenologia de *P. longispinus* i vaig avaluar l'efecte potencial del canvi climàtic en la fenologia del cotonet, predient que el nombre de generacions i el possible dany augmentaran en els propers anys. Al **quart capítol**, vaig identificar el complex de parasitoids de *P. longispinus* en caqui. Vaig descobrir que *P. longispinus* estava parasitat per un complex divers de parasitoids dominat per una espècie d'encírtid, *Anagyrus fusciventris*. Aquesta espècie pot reduir

eficaçment la densitat de la plaga malgrat l'alta abundància d'hiperparasitoids. Per tant, *A. fusciventris* té un alt potencial per a ser emprat en programes de control biològic augmentatiu. També vaig determinar el tamany de cotonet parasitat per *A. fusciventris*, aquesta característica biològica és clau per dissenyar els programes de control biològic augmentatiu. Finalment, al **cinquè capítol**, vaig avaluar si la heterogeneïtat del hàbitat afecta la densitat de pseudocòcids en cítrics i caquis. Per una banda, vaig trobar que la proporció de monocultiu circumdant va augmentar la densitat tant de *D. aberiae* en els cítrics com de *P. longispinus* en caqui. Per altra banda, els hàbitats no agrícoles, tant els hàbitats semi-naturals que envolten el cultiu com la vegetació de cobertura entre files, van reduir l'abundància de *P. longispinus* en caqui al augmentar el seu parasitisme. Aquests descobriments van a mostrar que les estratègies de diversificació del hàbitat poden millorar el control dels cotonets.

En resum, aquesta tesi destaca que els cotonets s'han convertit en plagues clau que poden causar pèrdues econòmiques importants en diversos cultius de fruites subtropicals a la conca mediterrània, com ara cítrics i caquis, i va revelar que l'augment en l'abundància de cotonets és causat per múltiples factors. És destacable que aquesta tesi ha identificat diferents estratègies que poden ser implementades per millorar el control biològic dels cotonets en cítrics i caquis mediterranis, incloent la gestió de formigues mutualistes, la solta massiva de parasitoids i la gestió de l'hàbitat. Els resultats d'aquesta tesi han de ser considerats dins dels programes de Gestió Integrat de Plagues contra els cotonets en cultius subtropicals.

General Introduction

1. Towards a sustainable agriculture that ensures food security

Ensuring the access to a healthy diet for a growing population is a critical challenge currently faced by humanity (Prosekov & Ivanova, 2018). The scale of this challenge, known as food security, is vast, necessitating action across the entire food system, including improving distribution, reducing waste, and increasing food production (Godfray & Garnett, 2014; Rask & Rask, 2014; Schneider et al., 2011; Willet et al., 2019). The human population has almost tripled in the last 50 years and per capita food consumption has increased 1.5 times across this period (Ritchie et al., 2023; Roser et al., 2023). This surge in demand has met by escalating food production, which has been achieved by expanded crop areas and agricultural intensification (Daily et al., 1998; Evenson & Gollin, 2003; Kopittke et al., 2019). However, the increase in agricultural yield is slowing down because of technological limitations and, remarkably, some key resources used in agriculture are finite, while the human population and per capita consumption are expected to continue increasing (Gilland et al., 2002; Maja & Ayano, 2021; Pimentel & Pimentel, 2006; Tian et al., 2021). Furthermore, crop expansion and agricultural intensification has led to a range of environmental impacts. Indeed, agriculture has become a primary contributor to soil degradation, soil and water pollution, habitat loss, spread of invasive species, and global warming (Alam, 2014; Crist et al., 2017; Dudley & Alexander, 2017; IPCC, 2023; Paini et al., 2016; Tudi et al., 2021). These impacts can negatively affect human health and wildlife conservation, but also the long-term productivity of agricultural systems (Kopittke et al., 2019; Tscharntke et al., 2012). Therefore, it is urgent to implement sustainable agricultural systems that ensure global food security for current and future generations (Godfray & Garnett, 2014; Tian et al., 2021).

2. The challenge of manage agricultural pests in the Anthropocene

Agricultural pests are organisms, including animals, weeds, and pathogens, that cause damage to crops, posing a threat to food production and security. Herbivorous arthropods stand out among these pests causing substantial crop losses, estimated to be around 20% of annual crop production (Bruce et al., 2010; Oerke, 2006; Sharma et al., 2017). Current agricultural systems are particularly vulnerable to arthropod pests because of different reasons. First, modern agriculture is characterized by low diversification and intensive farming, facilitating the establishment and spread of pests in agricultural landscapes (Bernal & Medina, 2018; Tscharntke et al., 2012). Furthermore, globalization is facilitating the geographical expansion of many species that have been translocated from their native range, resulting in an exponential increase in the number of invasive arthropod species (Meurisse et al., 2019; Paini et al., 2016; Seebens et al., 2017). Some of these invasive species can reach pest status in different crops from the invaded areas, causing severe food losses and economic damage (Bradshaw et al., 2016; Paini et al., 2016; Venette & Hutchison, 2021). Invasive terrestrial invertebrates cost the global economy US$ 712.44 billion up to 2020 (Renault et al., 2022), and the number of invasive species is expected to keep increasing in the coming years due to economic development and global trade (Seebens et al., 2017; **Figure 1**). In addition, current and predicted climate scenarios can exacerbate pest incidence in some crops. The increase in global greenhouse gas emissions across the last century has resulted in several environmental changes, including temperature warming (IPCC, 2023). Global warming can facilitate the expansion of important arthropod pests, hence escalating pest damage (Harvey et al. 2023; Robinet & Roques, 2010; Skendžić et al., 2021). Overall, the current global context makes the management of agricultural pests a formidable challenge that cannot be tackled without a multidisciplinary perspective that includes various approaches, including cultural practices, host plant resistance, insecticides, and biological control (Dent & Binks, 2020). Various policies have attempted to ensure that all these approaches are considered for pest management, prioritizing the most sustainable methods, in what is known as Integrated Pest Management (Bottrell, 1979; Stenberg, 2017). However, insecticide spraying continues to be the most used method for control pests (Bernhardt et al., 2017; Zhang, 2018).

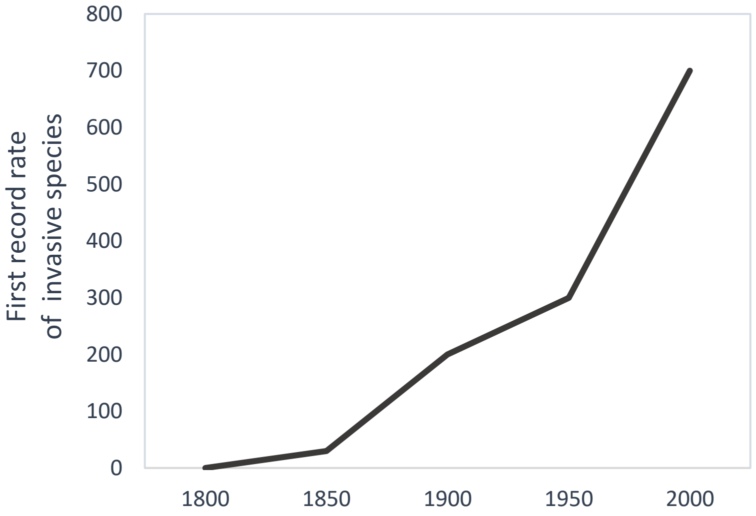

Figure 1. The number of invasive insect species have exponentially increased across the last two centuries. This graph shows the temporal trend of the number of first records of established insect species on a continent per 5 years from 1800 to 2000 (Based on Seebens et al., 2017).

3. Reducing the dependence on insecticides

The chemical development of the 20th century allowed the synthesis of various active substances with a high insecticidal capacity (Casida & Quistad, 1998; Rezende-Teixeira et al., 2022; Sparks, 2013). These products proved effective against major agricultural pests, reducing damage from pests and substantially increasing crop yield (Evenson & Gollin, 2003). This efficacy, together with the low cost of synthesis of several insecticides, made them a popular tool to control pests (Nansen & Ridsdill-Smith 2013; Wilson & Tisdell, 2001;). The global use of pesticides has been increasing in the last 60 years and has been estimated in 3.5 million tons by 2020 (Bernhardt et al. 2017; Sharma et al., 2019; Zhang, 2018; Zhang et al., 2011; **Figure 2**). In Spain, around 75,000 tons of pesticides were used in 2021, of which 13% were insecticides (MAPAMA, 2021a). In fruit crops, insecticides represent a higher percentage. For example, in Spanish citrus, insecticides account for more than 60% of the pesticides used (MAPAMA, 2021b).

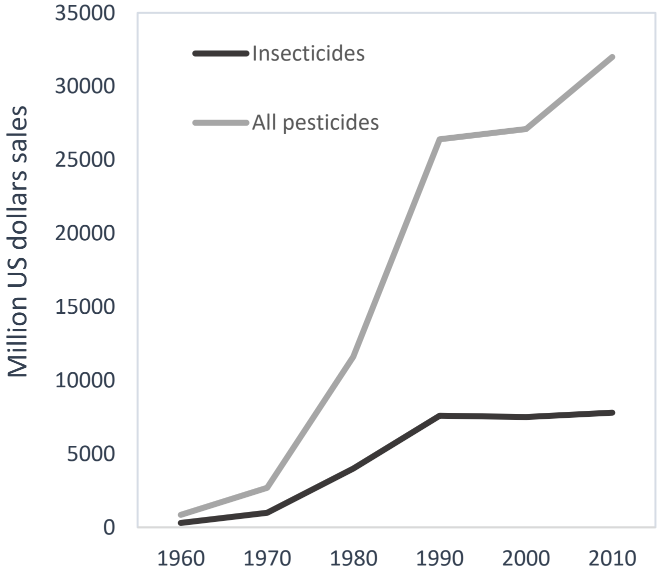

Figure 2. The global pesticide consumption has been increasing across the last 50 years. Despite increasing sales, the proportion of insecticide consumption in overall pesticide consumption has decreased in the last 20 years. The graph shows the changes of pesticides and insecticides consumption worldwide, expressed in million US dollars sales (Based on Zhang et al. 2011).

Although the use of insecticides can be effective in controlling significant pests in the short term, it also presents critical drawbacks. Notably, insecticides usage has been associated with a range of environmental risks, including food, soil, and water contamination, which can pose a threat to human health and wildlife conservation (Ansari et al., 2014; Bourguet & Guillemaud, 2016; Goulson, 2013; Pimentel, 1996; Stehle & Schulz, 2015). Furthermore, various aspects question the success of insecticides as a long-term pest control method. Firstly, pests can develop resistance to insecticides, causing these products to gradually lose their effectiveness (Bass & Jones, 2018; Bielza et al., 2019; Metcalf, 1989;). More than 500 pest insect species have developed resistance to at least one insecticide (Sparks, 2013; Whalon et al., 2008). Secondly, insecticides can affect non-target organisms and its use has been linked to a reduction in the abundance and efficacy of beneficial insects that provide essential ecosystem services, including pollination and pest control (Calvo-Agudo et al., 2022; Desneux et al., 2007; Siviter & Muth, 2020). Paradoxically, the fact that insecticides have a negative impact on organisms that control pests can exacerbate pest damage and lead to an increase in dependence on insecticides.

For the reasons stated above, the reduction in insecticide usage has become a key goal of European and global policies to enhance the sustainability of agricultural systems and ensure access to healthy food while conserving ecosystems. In accordance with this goal, the use of insecticides is being restricted in European Union, with many products being banned in recent years (Lamichhane, 2017; Lamichhane et al., 2016; Lee et al., 2019). These restrictions increase the need to develop strategies that allow pest management with reduced reliance on insecticides. Among these strategies, enhancing biological control stands out as one of the most promising (van Lenteren et al., 2018).

4. Why biological control?

Natural enemies of pests, also known as biological control agents, are predators, parasitoids, nematodes, or microbes that reduce the density and damage caused by pests. Biological control is the exploitation of these living agents to combat pests, directly or indirectly, for human good (Stenberg et al., 2021). The presence of biological control agents is essential for agriculture, since most herbivorous insects do not reach pest status because of the biological control exerted by their natural enemies. The economic value of this ecosystem service has been estimated at 4.5 billion dollars only in United States (Losey & Vaughan, 2006). However, it is believed that the value of biological control is far from its potential because in modern agricultural systems biological control is hindered by different factors, including insecticide usage, lack of efficient natural enemies in pest invaded areas, lack of resources for these natural enemies, and other agronomic and environmental factors (Heimpel & Mills, 2017; Landis et al., 2000; Van Driesche & Bellows, 2012;).

First, the density of a pest is spontaneously regulated by natural biological control carried out by resident natural enemies without human intervention. However, natural biological control is not effective enough for the control of some pests. In such cases, biological control can be enhanced through human intervention using various approaches, including classical biological control, which consists of the importation of natural enemies to the regions invaded by the invasive pest; augmentative biological control, which refers to the release of mass-reared natural enemies; and conservation biological control, which consist of enhance the diversity, abundance and efficacy of resident natural enemies through different practices, such as habitat management or the reduction in the use of insecticides toxic for natural enemies (Heimpel & Mills, 2017; Stenberg et al., 2021; Van Driesche & Bellows, 2012; **Figure 3**). The benefits of implementing these practices far outweigh the associated costs (Bale et al., 2007; Naranjo et al., 2015). Therefore, enhancing biological control has become a key goal in agricultural systems worldwide (van Lenteren et al., 2018). To achieve this goal, a significant amount of research is needed to identify best practices in each region, crop, and pest. This research can reveal global patterns and improve decision-making for stakeholders and policymakers that design pest management programs.

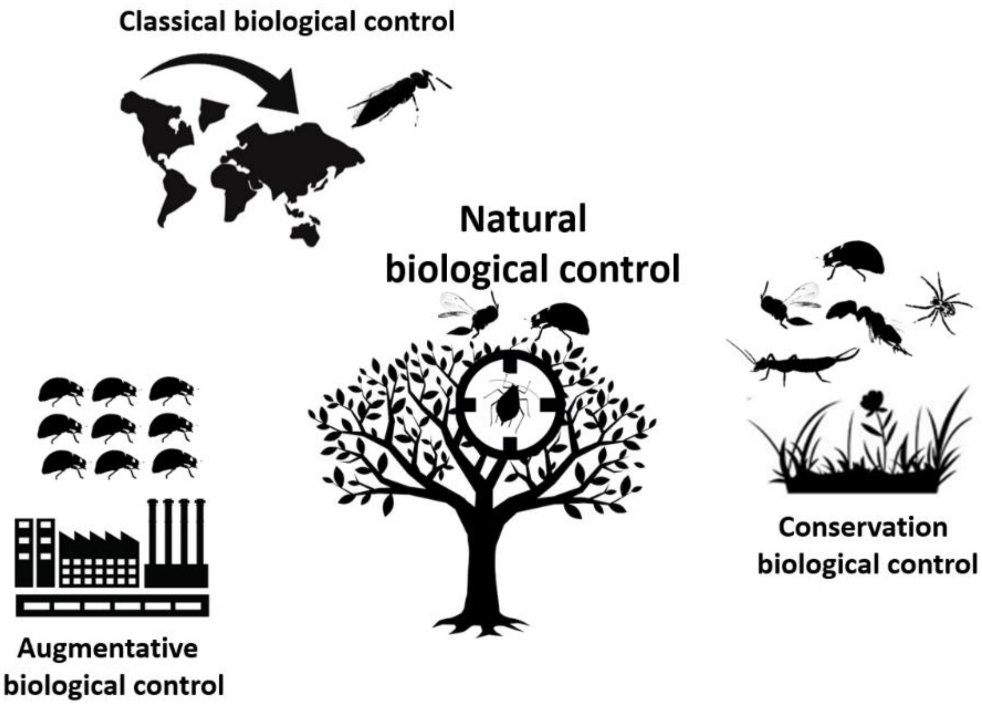

Figure 3. Pest populations are regulated by resident natural enemies without human intervention. Furthermore, biological control can be enhanced through different strategies, including classical biological control, which involves importing natural enemies to pest-invaded regions; augmentative biological control, referring to the release of mass-reared natural enemies; and conservation biological control, which aims to enhance the diversity, abundance, and efficacy of resident natural enemies through habitat and agronomic management.

Multiple examples have demonstrated that adopting biological control strategies is a cornerstone for shifting agricultural models towards more sustainable systems less dependent on chemical insecticides. For example, classical biological control has proven effective in managing devastating exotic pests that could not be satisfactorily controlled with other methods, from the pioneering example of *Icerya purchasi* Maskell (Hemiptera: Monophlebidae) in citrus from California to the cassava mealybug *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae) in extensive regions of Africa (Caltagirone, 1981; Cock et al., 2016). Similarly, augmentative biological control can lead to improved control of primary pests, drastically reducing the reliance on insecticides. For instance, in greenhouse horticultural crops from southern Spain, the notable effectiveness of augmentative biological control in managing key pests such as *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) and *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) led to the cessation of chemical control (Calvo et al., 2009; 2012; Urbaneja et al., 2012). Finally, there are also numerous cases demonstrating that habitat management can enhance biological control and reduce dependence on insecticides (Gurr et al., 2017). For example, diversifying habitats at landscape and orchard levels can reduce pest pressure and insecticide use in vineyards and olives from southern Spain (Paredes et al., 2013; 2021).

5. Mealybugs as major crop pests

Mealybugs (Hemiptera: Pseudococcidae) are a family of sap-sucking hemipteran insects that feed on a wide variety of plant species, potentially causing high damage in agriculture, especially in subtropical and tropical crops (Mani & Shivaraju, 2016; Miller et al., 2002). With near 2000 described species worldwide, mealybugs exhibit remarkable diversity (Ben-Dov, 1994; Kondo et al., 2022). The small size of mealybugs and their cryptic behaviour facilitate their dispersion through global trade, for instance, via imported plant material (Franco et al., 2009; Mani & Shivaraju, 2016; Subramanian et al., 2021). As a result, several mealybug species have been accidentally imported from their native areas to various parts of the world. Some of these mealybug species have become primary agricultural pests in the invaded areas (Miller et al., 2002; Pellizari & Germain, 2010). Some invasive mealybug species are oligophagous and reach pest status in specific crops, such as *P. manihoti*, which causes devastating damage in cassava and pose threats to regions heavily reliant on this staple crop (Herren & Neuenschwander, 1991; Nwanze, 1982;). However, most invasive mealybug species are polyphagous and can affect a wide variety of crops. This is the case of widely distributed species such as *Planococcus citri* (Risso), *Planococcus ficus* (Signoret), *Pseudococcus longispinus* (Targioni-Tozzetii) and *Phenacoccus solenopsis* Tinsley (Miller et al., 2002; Pellizari & Germain, 2010; **Figure 4**).

Figure 4. Invasive mealybug species have become primary agricultural pests worldwide. In this picture, a colony of the long-tailed mealybug *Pseudococcus longispinus*, a polyphagous species that attacks various crops, is established under the sepal of a persimmon fruit.

Mealybugs cause direct damage on crops due to their phloem-feeding habit, that weaken their host plants (Puspitasari et al., 2023; Subramanian et al., 2021). In addition, one major indirect consequence of mealybug infestation is that these insects produce copious amount of honeydew, a sugary waste product that is excreted by mealybugs and other hemipteran insects (Douglas, 2009; Zhou et al., 2013). This honeydew serves as a medium for the growth of other harmful organisms, including other pest insects like honeydew moths and fungi, particularly sooty mold (Fang et al., 2020; Lucchi et al., 2018; Nelson, 2008). The proliferation of sooty mold on plant surfaces leads to reduced photosynthetic capacity of leaves and can stain fruits, diminishing their commercial value (Chomnunti et al., 2014; Romanazzi et al., 2016). Moreover, various mealybug species can exacerbate the damage inflicted on crops. For instance, some species, such as *Dismicoccus* spp. in pineapple or *P. ficus* in vines, act as virus vectors (Dey et al., 2018; Sether et al., 1998; Tsai et al., 2010). Additionally, other mealybug species deform or impede the growth of the organs they infest. For instance, *P. manihoti* induces leaf curling in cassava leaves (Takano et al., 2023), while *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae) cause dwarf and deformations in citrus fruits (Martínez-Blay et al., 2018a; Pérez-Rodríguez et al., 2017). Notably, the economic implications of mealybug infestation extend beyond harvest losses, as many species are considered quarantine organisms (Huang et al., 2014; Song et al., 2023). This classification imposes strict regulations, restrictions, and treatments on the imported fruits to prevent the spread of invasive mealybugs, resulting in significant economic losses for industries involved in fruit exportation. Considering the worldwide spread of invasive mealybugs and their capacity to inflict substantial damages on valuable crops, it becomes imperative to prioritize the comprehension of their biology and ecological dynamics. The development of pest management programs that are both effective and sustainable is paramount to mitigating the socio-economic consequences arising from mealybug infestations.

6. Management of mealybugs

The management of mealybugs presents unique challenges due to various biological and ecological traits of these insects. Firstly, their small size and preference for concealed locations within the plant make difficult to detect and control their geographic expansion through human trade (Franco et al., 2009; Subramanian et al., 2021; Watson & Mifsud, 2017;). This cryptic behaviour also hinders early detection in crops and reduce the efficacy of insecticides and some natural enemies (Berlinger & Gol'berg, 1978; Daane et al., 2002). In addition, the resemblance between different mealybug species, particularly in their early stages, can further delay the identification of cryptic species (Correa et al., 2023; Malausa et al., 2011;). Furthermore, mealybugs are covered by a wax coating that provides them with enhanced resistance to insecticidal treatments and some natural enemies (Ulusoy et al., 2022). Lastly, ecological factors, such as the mutualistic relationship that mealybugs can establish with ants, complicate their control (Anjos et al., 2022; Daane et al., 2007; Feng et al., 2015).

Traditionally, chemical control has been the primary method for controlling mealybug pests (Daane et al. 2012; Franco et al., 2004; Furness, 1977; Mansour et al., 2018; Noureen et al., 2016). However, in recent years, different factors have prompted the adoption of alternative management strategies. First, environmental concerns associated with insecticide use have led to the restriction of many active ingredients commonly used against mealybugs, such as chlorpyrifos and methyl-chlorpyrifos (EFSA, 2019; European Comission, 2020), and the remaining approved insecticides are less effective (Mansour et al., 2018). Additionally, mealybugs can develop resistance to various insecticides (Ahmad & Akhtar 2016; Shankarganesh et al., 2022; Venkatesan et al., 2016). Critically, insecticide applications can lead to an increase in the density of mealybugs. For instance, severe outbreaks of *P. citri* and *P. longispinus* in citrus from California and avocados from Israel were reported following the application of broad-spectrum insecticides (Bartlett, 1957; DeBach & Bartlett, 1951; Furness, 1977; Swirski et al., 1980; Wysoki et al., 1981). This is likely because some widely used insecticides negatively affect mealybug natural enemies (Cloyd & Dickinson, 2006; Mansour et al., 2011; 2023; Mgocheki & Addison, 2009a; Planes et al., 2013;), and may disrupt mealybug biological control. Consequently, alternative control strategies have gained interest as viable alternatives, particularly those based on biological control, as well as other approaches including cultural practices, the use of pheromones, and the management of mutualistic ants (Daane et al., 2012; Franco et al., 2009; Vacas et al., 2019; **Figure 5**).

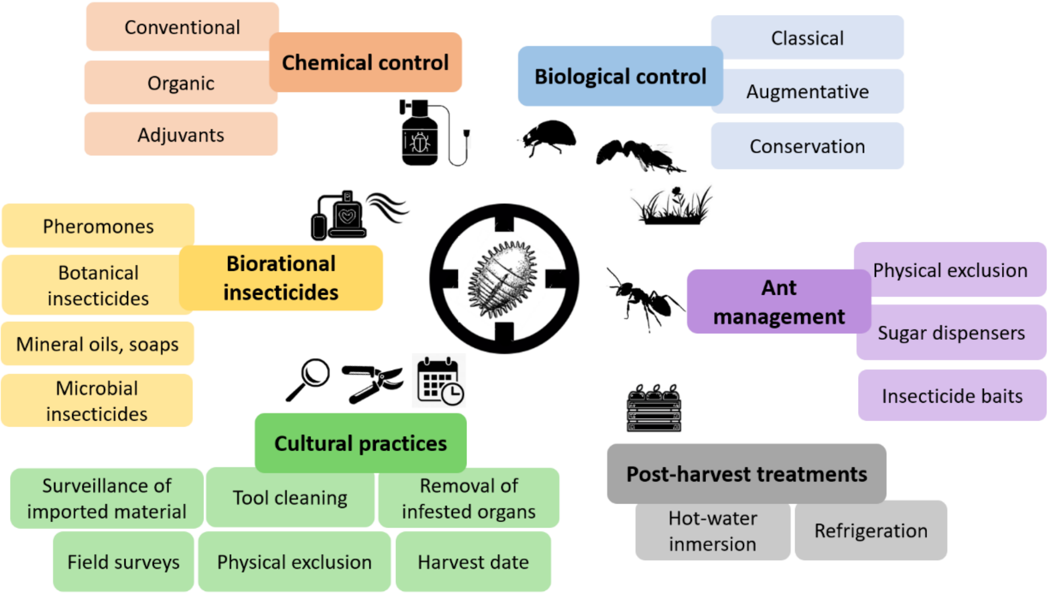

Figure 5. Mealybug pests can be controlled using different tools and approaches. Although chemical control is still the most common approach to deal with mealybug outbreaks, alternative strategies have been developed to control mealybug populations. These include biorational insecticides, cultural practices, biological control, ant management, and post-harvest treatments.

7. Biological control of mealybugs

Mealybugs are attacked by a diverse and extensive complex of natural enemies, including both generalist and oligophagous predators, as well as specific parasitoids (Berlinger, 1977; Charles, 1993; Daane et al., 2012; Rodrigues-Silva et al., 2017; Shylesha & Mani, 2016). While this complex of natural enemies typically regulates mealybug populations within their native range, mealybugs often reach pest status in their exotic range (Charles et al., 2011; Flanders, 1940; Mani & Shivaraju, 2016; Miller et al., 2002). It has been postulated that one of the main factors explaining the success of mealybugs in exotic regions is the absence of specific natural enemies attacking them in the invaded areas. For this reason, classical biological control has been widely employed over the past century to control mealybug pests. This approach involves introducing natural enemies found in the native range of pests, which has yielded favourable results controlling invasive mealybugs in a high number of cases (*e.g.*, Cock et al., 2016; Myrick et al., 2014). Particularly, mealybugs are often well-controlled by specific encyrtid parasitoids (Hymenoptera: Encyrtidae) (Moore, 1988; **Figure 6A**), that are considered the key biological control agents of mealybugs and have been imported to control various mealybug species with notable success (*e.g.*, Cock et al., 2016; Flanders, 1944; Neuenschwander, 2001;). Besides parasitoids, the Australian ladybird *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae), a predatory coccinellid known for its ability to prey on various mealybug species, has been intentionally imported to numerous countries worldwide (Kairo et al., 2013; **Figure 6B**).

Figure 6. The most effective biological control agents of mealybugs are oligophagous predators and specific parasitoids, some of which have been imported in classical biological control programs to enhance the control of invasive mealybugs. On the left (A), the parasitic wasp *Anagyrus aberiae* parasitizing *Delottococcus aberiae* in citrus. On the right (B), the predatory ladybird *Cryptolaemus montrouzieri* preying on *Pseudococcus longispinus* in persimmon.

In addition to the deliberate importation of natural enemies, some natural enemies have also spread spontaneously together with its mealybug hosts (Beltrà et al., 2013b; Culik et al., 2013; Ram & Saini, 2010; Spodek et al., 2018; Viggiani and Battaglia, 1983). Furthermore, native generalist predators, including mites, earwigs, or lacewings, may contribute to the control of mealybugs in invaded areas (Koutsoula et al., 2023; Navasero, 2006; Pérez-Rodríguez et al., 2018; Tapajós et al., 2016). Collectively, mealybugs face a broad array of natural enemies that can reduce their density, but some factors can make biological control ineffective. As mentioned, the lack of natural enemies in invaded areas and the use of insecticides harmful to natural enemies are factors that can facilitate outbreaks of mealybugs. Moreover, additional factors can contribute to mealybugs reaching pest status, including the presence of mutualistic ants, climate factors and habitat context (Daane et al., 2012; Franco et al., 2009; Mani & Shivaraju, 2016).

8. Other factors facilitating mealybug pests

Mutualism with ants

Mealybugs and other phloem-feeder hemipterans have a close association with ants. This is because phloem-feeder hemipterans excrete excess sugars in the form of honeydew. Honeydew serves as a highly valuable carbohydrate source for many organisms, including ants, that frequently exploit it. Ants feed on mealybug honeydew and, often, they exhibit an attendance behaviour towards the mealybugs (Detrain et al. 2010; Holldobler and Wilson 1990; Nelson & Mooney, 2022; **Figure 7**). In return for honeydew, mealybugs can receive various services from ants, including cleaning, transportation, and notable protection against natural enemies (*e.g.*, Herbert & Horn, 2008; Ho & Khoo, 1997; Queiroz & Oliveira, 2001; Xu et al., 2020). This mutualistic relationship with ants, called trophobiosis, can disrupt the biological control of mealybugs and, consequently, increase mealybug density and damage to crops. For instance, *P. solenopsis* in cotton, *P. ficus* in vines, and *P. citri* in citrus, benefit from different species of tending ants (Feng et al., 2015; Mansour et al., 2012; Mgocheki & Addison, 2009b).

Figure 7. Ants feeding on the honeydew excreted by mealybugs often protect them from their natural enemies. In the picture, the Mediterranean ant *Lasius grandis* is stalking the parasitoid *Anagyrus vladimiri* as it attempts to parasitize a colony of the mealybug *Planococcus citri*.

Climate factors

Environmental conditions can lead to an increase in pest abundance. Critically, temperature is the most important variable for insect development and climate changes can led to high impacts on insect dynamics (Harvey et al. 2022). Consequently, the population growth rate of certain pests, including mealybugs, can be pervasively affected (Daane et al. 2012). This is particularly relevant in the current context as global warming is occurring and being exacerbated by global human emissions (IPCC 2023). As a result, agricultural pests may be highly impacted. Many of these pests can expand their geographic range, accelerate their growth rate, increase the number of generations, and ultimately cause higher crop damage (Jactel et al., 2019; Lehmann et al., 2020; Skendžić et al., 2021). For example, it has been predicted that several mealybug species causing significant damage in agriculture, such as *Maconelicoccus hirsutus* (Green), *P. solenopsis*, and *P. ficus*, will increase their invasive potential by increasing their number of generations and expanding their geographic distribution range under the projected climate scenarios (Fand et al., 2014; Jara et al., 2013; Ji et al., 2020). Notably, these effects are expected to be modulated by the effects that climate warming may have on mealybug natural enemies (Daane et al., 2004; Hance et al., 2007).

Habitat context

Agricultural intensification has led to a progressive depletion in the diversity of habitats in agricultural landscapes (Bianchi et al., 2006; Latourneau et al. 2011). Diverse landscapes featuring various crops and patches of natural vegetation have given way to landscapes dominated by a single crop. These monoculture systems can be particularly vulnerable to certain pests for several reasons. First, monoculture systems provide a concentration of resources for herbivorous insects, which may facilitate pest dispersion in landscapes dominated by their host plants (Martinson & Fagan, 2014; O'Rourke & Petersen, 2017). Additionally, non-crop habitats provide essential resources for the natural enemies of pests, and their absence in agricultural landscapes may lead to a reduction in their abundance and effectiveness, compromising biological control (Chaplin-Kramer et al., 2011; Landis et al., 2000; Russell, 1989). Several studies suggest that the incidence of mealybugs is influenced by habitat context. For instance, the presence of semi-natural habitats has been associated to lower abundance of *P. ficus* in vines (Muneret et al. 2018; Shapira et al. 2018).

Study System

1. Study region

Mediterranean agriculture represents a highly interesting model for the study of various insect pests. The historical, economic, and environmental conditions of the Mediterranean basin have positioned it as one of the most important agricultural areas both in terms of production and crop diversity, including cereal crops, vines, olives, horticultural and several fruit crops (FAO, 2023). The Mediterranean basin, moreover, is one of the areas most affected by global issues that threaten agriculture. First, the strategic position of this region within trade routes, along with its environmental conditions and agricultural surface, has made it one of most affected regions by invasive insect pests (Early et al. 2016; Meyerson and Mooney, 2007; Paini et al., 2016). Furthermore, its geographical location between subtropical and temperate climates makes it particularly sensitive to climate change phenomena (IPCC, 2023; Zittis et al., 2019). These factors, coupled with the fact that the Mediterranean climate is present in other important agricultural regions (Di Castri, 1991; **Figure 8**), makes the Mediterranean basin an area of particular interest to be studied. Several invasive pests have become a matter of significant social and political concern within Mediterranean agriculture. Mealybugs stand out among these pests because of its rapid increase and the high crop losses these insects cause in various major crops.

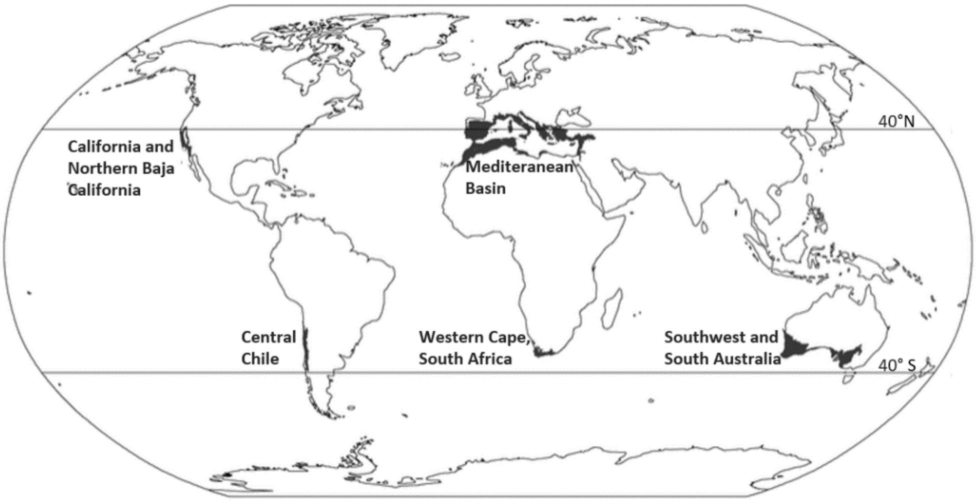

Figure 8. The five mediterranean climate regions of the world: California and Northern Baja California, Central Chile, Mediterranean Basin, Western Cape of South Africa, and Western and South Australia.

2. Mealybugs in Mediterranean subtropical crops

Within Mediterranean agriculture, invasive mealybugs have emerged as a growing concern in several important crops, including citrus, vines, vegetables, and fruit crops. Notably, some species such as *P. citri* in citrus, *P. ficus* in vines and *P. solenopsis* in vegetable crops have been of particular concern throughout the last decades (Cox & Ben-Dov, 1986; Franco et al. 2004; Mansour et al., 2017; Ricupero et al., 2021). Although mealybug pests have been commonly considered as secondary pests, they can become in primary pests under some circumstances. This is occurring in several subtropical crops from western Mediterranean basin, such as citrus and persimmon, where mealybugs are increasing in abundance and currently rank among the most damaging pests (García-Martínez et al., 2017; Pérez-Rodríguez et al., 2017).

3. *Delottococcus aberiae*, a recent invader in Mediterranean citrus

Mediterranean citrus has been traditionally associated with mealybug pests. Until a few years ago, the most abundant mealybug species in Mediterranean citrus was *P. citri* (Cox & Ben-Dov, 1986; Franco et al., 2004). However, in 2009, *Delottococcus aberiae*, native to South Africa, was detected in the core of the citrus-producing area of Spain (Beltrà et al., 2013a; 2015). Probably due to the absence of effective natural enemies against *D. aberiae* in Mediterranean basin, this species became stablished and spread in the following years (Navarro-Llopis et al., 2019; Tena et al., 2017). The geographical expansion of *D. aberiae* caused great alarm because, in addition to the damage caused by the excreted honeydew, *D. aberiae* causes severe deformations and dwarfism in citrus fruit (Beltrà et al., 2013a; Pérez-Rodríguez et al., 2017; **Figure 9**). As a result, the economic damage caused by *D. aberiae* can be much higher than by other mealybug species.

In recent years, considerable research has been conducted to enhance the control of *D. aberiae* in Mediterranean citrus. Firstly, a genetic study was undertaken, revealing its South African origin, which was essential to explore the potential biological control agents in the native area of the mealybug (Beltrà et al., 2015). Additionally, the effectiveness of resident natural enemies has been evaluated. While resident parasitoids fail to parasitize *D. aberiae* (Tena et al., 2016), some predators can contribute to the control of this mealybug species (Pérez-Rodríguez et al., 2018; 2019). However, the efficacy of these resident natural enemies is not sufficient to control *D. aberiae*, necessitating the application of insecticides for its control. The phenology and seasonal trend of *D. aberiae* have been described, providing valuable insights to improve the efficacy of insecticide treatments (Martínez-Blay et al., 2018a; 2018b). Moreover, economic and environmental damage thresholds have been determined, and sampling protocols have been devised to ascertain the need for insecticide application (Pérez-Rodríguez et al., 2017). Recently, the most-used insecticide against *D. aberiae*, methyl-chlorpyrifos, has been banned in the European Union due to its environmental impact (European Comission, 2020), and the remaining approved insecticides are less effective (Mansour et al., 2018). This reduced the available effective tools to control *D. aberiae* for citrus growers. To address this critical situation and reduce dependence on insecticides, several alternatives for *D. aberiae* control have been developed in the last years, including the importation of one of its specific parasitoids, *Anagyrus aberiae* Guerrieri (Hymenoptera: Encyrtidae) (Guerrieri & Cascone, 2018; Soto et al., 2021), and the development of pheromone traps for mass capture (Vacas et al., 2019). Strategies using allowed insecticides have been also evaluated (Vacas et al., 2022). While these methods are expected to enhance *D. aberiae* management in the coming years, additional solutions must be explored to achieve a successful control of *D. aberiae* in Mediterranean citrus. Some methods with high potential to improve the management of this mealybug species could be the management of mutualistic ants and the enhance of biological control through habitat management strategies.


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Figure 9. The invasive mealybug *Delottococcus aberiae*, native from South Africa, was detected in Mediterranean citrus in 2009 and rapidly spread in Valencian citrus. In the image, a mealybug colony feeding on a mandarin fruit. Unlike other mealybugs, *D. aberiae* causes severe deformations in the fruit

4. Emerging mealybug pests in Mediterranean persimmon

Unlike citrus, which is a long-established crop in the Mediterranean region, persimmon is a relatively novel crop in the area that has expanded drastically in this region in the last 20 years. In this period, western Mediterranean basin has become in the second-largest persimmon-producing area in the world (FAO, 2023; MAPAMA, 2022a). In the first years of persimmon expansion, pest pressure was very low; however, since 2015, there has been a significant increase in the density of some pests, including mealybugs (García-Martínez et al., 2019; Tena et al., 2015). Previous studies identified the most abundant mealybug species in Mediterranean persimmon. These species were long-established species in the Mediterranean basin, such as *P. citri*, *P. longispinus*, and *Pseudococcus viburni* (Signoret) (García-Martínez et al., 2017; Prieto, 2016), that are present in the Mediterranean area since 19th century (Pellizzari & Germain, 2010). Notably, the damage caused by mealybugs in Mediterranean persimmon has sharply increased in the last years, reaching worrisome values. For example, in 2021, farmers reported a 20-25% decrease in production due to the damage caused by mealybugs (ASAJA, 2021; **Figure 10**). These insects, therefore, have become in one of the factors causing highest crop losses, and it is urgent to study the factors causing this increase and develop management strategies. Firstly, the geographical distribution, seasonal trend and fruit infestation levels of the different mealybug species is unknown. Likewise, their phenology has not been studied, and sampling strategies and treatment thresholds have not been defined. Moreover, the complex of natural enemies attacking mealybugs in Mediterranean persimmon has not been described nor evaluated. This research must be conducted to develop an Integrated Pest Management program against mealybugs in persimmon that allow farmers to optimize the control of these emerging pests.

Figure 10. Mealybugs have become the most damaging pest for persimmon crop in Spain. These mealybugs can reach high density beneath the sepals of the fruit. In the picture, sooty mold has proliferated on the honeydew excreted by the mealybugs, staining the fruit.

Objectives

This thesis studies the biology and ecology of mealybug pests attacking two important Mediterranean subtropical crops, citrus and persimmon. Basic questions that can improve our understanding of the nature of these mealybug pests are addressed, aiming to identify the main factors explaining the increase in mealybug abundance and damage in these crops. Specifically, this thesis aims to identify and evaluate different strategies that can enhance mealybug biological control, ultimately reducing crop damage. The sense of urgency for local farmers increases the practical implications of our findings. Therefore, it is intended that this thesis provide practical solutions usable by policymakers and stakeholders to improve mealybug control in the short and long term. Ultimately, it is expected that this knowledge will contribute to understanding the mechanisms underlying mealybug outbreaks in other crops and geographic regions, thereby allowing for improvements in the overall management of these major pests.

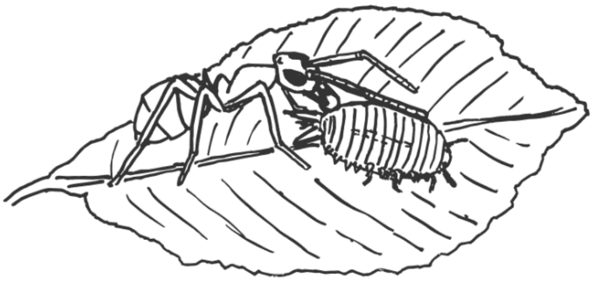
The thesis has been divided into three sections. The first section focuses on the management of the invasive mealybug *D. aberiae* in citrus. This section includes two chapters where I study the relationship between *D. aberiae* and mutualistic ants. In the **first chapter**, entitled **"Native ants facilitate the invasion by the invasive mealybug *Delottococcus aberiae* in Mediterranean citrus"**. I will try to solve the question: Can Mediterranean ants favour the invasion of *D. aberiae* in citrus? To answer it, I pose the following specific questions: Which species of ants attend *D. aberiae* in Mediterranean citrus? What are the levels of ant attendance? Is there a correlation between the density of tending ants and that of *D. aberiae*? Then, in the **second chapter**, entitled **"Exclusion of Mediterranean ant species enhances biological control of the invasive mealybug *Delottococcus aberiae* in citrus"**, I will try to answer: What effect do ants have on the natural enemies of *D. aberiae*? What effect do ants have on the density of *D. aberiae* and the damage it causes?, and finally, Can we reduce *D. aberiae* damage by excluding ants from citrus canopies?

The second section focuses on the management of mealybugs in persimmon. This section includes two chapters. In the **third chapter**, entitled **"Mealybugs in Mediterranean persimmon: fruit infestation, seasonal trend and effect of climate change"**, I address the following questions: Which species of mealybugs currently predominate in Mediterranean persimmon? What are their spatial and temporal dynamics? Can their damage at harvest be predicted by early samplings? Is there a relationship between temperature and mealybug phenology? What consequences might climate change have on their dynamics? Then, in the **fourth chapter**, titled **"Do hyperparasitoids disrupt the biological control of *Pseudococcus longispinus* in persimmon?"**, I study the biological control exerted by resident parasitoids against the most abundant mealybug species in persimmon, *P. longispinus*. Here, I will try to answer: Which species parasitize *P. longispinus* in Mediterranean persimmon? Which mealybug stages do they parasitize? Is hyperparasitism affecting them? How effective are these parasitoids?

Finally, the third section focus on how habitat context at local and landscape scale can affect mealybug species attacking both crops. This section includes the **fifth chapter**, titled **"Habitat heterogeneity reduces abundance of invasive mealybugs in subtropical fruit crops"**. In this chapter, I will assess the effect of habitat heterogeneity on the abundance of mealybugs in Mediterranean citrus and persimmon. To do so, I will address the following specific questions: Are mealybugs affected by inter-rows ground cover vegetation? Are mealybugs affected by surrounding natural habitats and/or monoculture at landscape scale? Are these effects mediated by mealybug natural enemies?, and finally, What habitat management strategies would be recommended to reduce mealybug damage?

Section I

Improving the management of *Delottococcus aberiae* in citrus through the management of ants



Chapter 1

Native ants facilitate the invasion by *Delottococcus aberiae* in Mediterranean citrus

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Abstract

The invasive mealybug *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae) has become a key pest in Mediterranean citrus. This mealybug species excretes honeydew that can be consumed by ants, which may give rise to mutualistic relationships and increase the invasibility of this exotic pest. Here, we studied the interaction between *D. aberiae* and ants in 16 citrus orchards located in the main citrus-growing area of Spain (Valencia) during two consecutive years. Four native ant species were observed feeding on *D. aberiae* honeydew, and *Lasius grandis* Forel (Hymenoptera: Formicidae) represented more than 95% of these ants. *Lasius grandis* was observed attending *D. aberiae* throughout the year and in all the orchard sites where the mealybug was present. Mealybug colony size had a positive effect on both relative and absolute ant-attendance by L. grandis, but these interactions varied across seasons. Moreover, we found a positive correlation between *L. grandis* activity and *D. aberiae* density in all citrus orchards. Our results suggest that the native ant *L. grandis* and the invasive pest *D. aberiae* have established a mutualistic association that promotes the establishment and accelerate the invasion of *D. aberiae* in Spanish citrus orchards. Further research with the aim of evaluating whether the management of this ant species can improve the control of *D. aberiae* in citrus would be recommended.

1. Introduction

Exotic phytophagous insects are a major threat for agriculture because they can increase crop loss and decrease food security (Paini et al., 2016). Climate change and human transport have increased the spread of exotic phytophagous insects that, in the absence of natural enemies, can become invasive pest species (Lövei, 1997; Thomson et al., 2010). This invasion can be accelerated through mutualistic relationships between invasive and native insect species (Traveset & Richardson, 2014; Zhou et al., 2017). The mutualistic relationship between ants and honeydew-excreting hemipterans has been widely studied. Honeydew is the sugar-rich waste product excreted by some hemipteran species (Holldobler & Wilson, 1990; Way, 1963). Many arthropods feed on honeydew because it is the main carbohydrate source in most agroecosystems, when nectar is scarce (Calvo-Agudo et al., 2022; Lundgren, 2009; Tena et al., 2016). Of the complex of arthropods that feed on honeydew, ants are the most efficient due to their ubiquity across terrestrial ecosystems (Holldobler & Wilson, 1990; Way, 1963). Some honeydew-feeding ant species clean, transport, and protect the honeydew-excreting hemipterans in return for the sugar meal, termed trophobiosis. Thus, survival rates of the trophobiont may be increased by the presence of tending ant species. Several studies have suggested that the mutualistic interactions with ants promote the invasibility of exotic hemipterans (Abbot & Green, 2007; Helms & Vinson, 2003; Tena et al., 2013; Wang et al, 2021; Zhou et al., 2014a).

Delottococcus aberiae De Lotto (Hemiptera: Pseudococcidae) is among the most damaging invasive pests in Spanish citrus and it is rapidly spreading in eastern Spain (Navarro-Llopis et al., 2019; Pérez-Rodríguez et al., 2017). This mealybug, native to South Africa, was first detected in 2009 in Valencia (eastern Spain), the main citrus-producing area of Europe and the Mediterranean basin (Beltrà et al., 2015). In spring and summer, *D. aberiae* can feed on leaves and twigs but shows preference for the fruit (Martínez-Blay et al., 2018b) where it causes distortions in oranges, and fruit-size reduction in mandarins (Beltrà et al., 2013a).

Delottococcus aberiae excretes honeydew that might be exploited by ants. In the Mediterranean basin, the dominant ant species in citrus tree canopy is the native *Lasius grandis* Forel (Hymenoptera: Formicidae) (Cerdá et al., 2009; Martínez-Ferrer & Campos-Rivela, 2017; Palacios et al., 1999), which is known to feed on hemipteran honeydew including that of other mealybug species such as *Planococcus citri* (Risso)

(Hemiptera: Pseudococcidae) (Pekas et al., 2011). Other species of ants that tend mealybugs in Mediterranean citrus are *Pheidole pallidula* (Nylander) (Hymenoptera: Formicidae) or, less frequently, the invasive argentine ant *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) (Calabuig et al., 2014; Pekas et al., 2011). In addition, subordinate ants of the genus *Plagiolepis*, that share territory with dominant ants, feed on mealybug honeydew when colonies are unattended by the more dominant species in citrus from Spain and Portugal (Pekas et al., 2011; Zina et al., 2017).

The mutualism between tending ants and mealybugs have been studied for mealybug species commonly found in citrus (Anjos et al., 2021). However, the interaction and potential mutualism between ants and the invasive mealybug *D. aberiae* have neither been studied from its origin nor its invaded range. The aim of the present research was to study the interactions between *D. aberiae* and ant species in Spanish citrus orchards to determine whether these interactions can accelerate the invasion by this mealybug species. For this, 16 citrus orchards were sampled during two consecutive years to: i) identify the ant species that attend *D. aberiae*; ii) determine the level and density-dependence of ant-attendance; and finally, iii) evaluate the correlation between ant activity and *D. aberiae* density. These findings will facilitate the development of suitable management strategies of this new invasive mealybug in Spain.

2. Material and methods

Citrus orchards

Sixteen citrus orchards in the main citrus-producing area of Spain (Valencia), with history of *D. aberiae* infestations, were selected for the present study (Figure 1). Within each orchard, a plot consisting of 40 trees (8 × 5) was established. From each plot, nine alternate trees were sampled, as shown in **Figure 1**. All citrus orchards were between 10 and 15-year-old, drip irrigated, and managed under Integrated Pest Management (IPM) guidelines. For further details refer to the supplementary material (**Table S1**).

Survey protocol

Orchards were sampled in late spring (between June 10 and 20), mid-summer (between July 27 and August 5) and mid-autumn (between October 15 and 26) for two consecutive years (2020 and 2021). The same trees were sampled throughout the study. For each tree and date, 120 leaves (30 per orientation), 40 fruits (10 per orientation) and the surface of the trunk (from the base to a 50 cm height) were surveyed by direct visual observations. All mealybugs and ants observed in each organ were counted and identified to the species level and counted; the mealybug developmental stage (instar) was also reported. When necessary, insects were collected and transported to the laboratory for identification under stereo- (ants and adult mealybugs) or compound- (young nymphs of mealybugs) microscopy. All observations were made between 9 a.m. and 4 p.m.

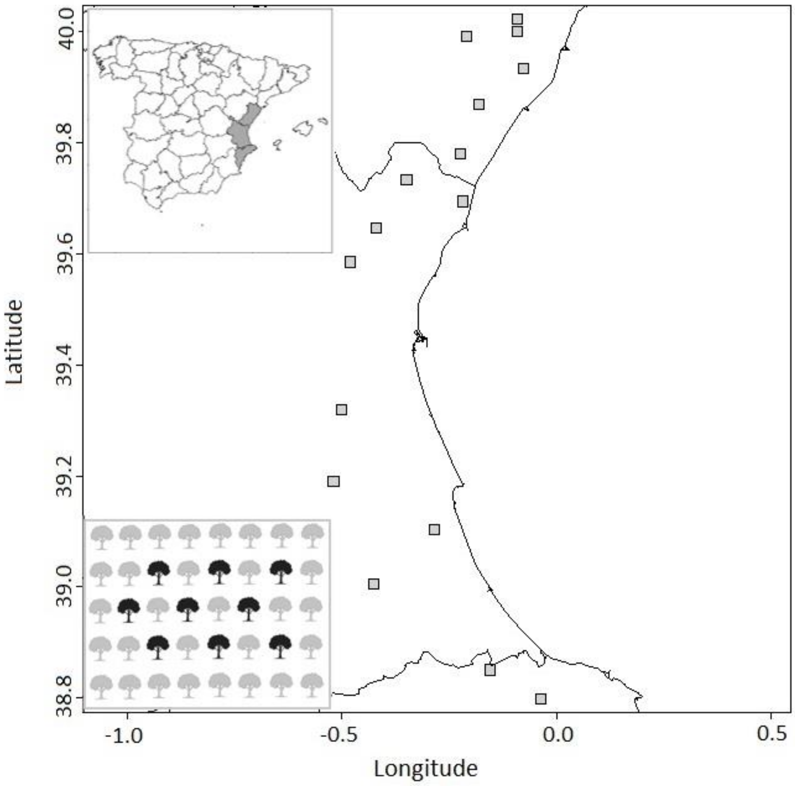

Figure 1. The location of the 16 orchards sampled from the main citrus-producing region in Spain (Valencia). The bottom left box represents the plot of 40 trees selected in each orchard, the trees in black were sampled in spring, summer and autumn 2020 and 2021. Geographical reference system: WGS84.

Mealybug and ant complexes

The mean number of mealybugs per tree (sum of mealybug of each species observed in 120 leaves, 40 fruits, and trunk) and orchard (mean of nine trees) was calculated to describe the complex of mealybugs in each sampling date. Similarly, the mean number of ants per tree (sum of ants of each species observed in 120 leaves, 40 fruits, and trunk) and orchard (mean of nine trees) was calculated to describe the complex of ants in each sampling date. Additionally, the total number of each ant species sampled across each year was presented separately in the trunk, citrus canopy (120 leaves and 40 fruits) and attending colonies of *D. aberiae* in the canopy (**Table 1**).

Ant-attendance

Four measures were used to assess the interactions between the invasive mealybug *D. aberiae* and the native ant *L. grandis*. i) 'Ant-attendance' was calculated as the ratio of *D. aberiae* colonies attended by ants to total *D. aberiae* colonies (*D. aberiae* colonies with at least one ant divided by the total number of *D. aberiae* colonies). For each ant-attended colony, we also calculated ii) absolute, iii) relative, and iv) weighted relative ant-attendance. 'Absolute ant-attendance' is used to measure the preference of ants for a specific honeydew source, and it was calculated as the number of ants per *D. aberiae* colony. 'Relative ant-attendance' was used to obtain a parameter for the intensity of ant-attendance that was independent of colony size (Fischer et al. 2001) and it was calculated as the number of ants divided by the number of mealybugs in each colony. Finally, 'Weighted relative ant-attendance' was calculated as the number of ants divided by the estimated amount of honeydew produced by each colony. This value was calculated because the number of ants foraging sugar sources is theoretically proportional to the volume of "desired" source (Mailleux et al., 2003). Therefore, recruiting ants are supposed to be directly related to the weight of honeydew excreted by each colony (Völkl et al., 1999; Fischer et al., 2005). Each mealybug individual was weighted according to the theoretical weight of honeydew each instar excretes, following the calculation of Zhou et al. (2013) for *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae): each first instar mealybug nymph is weighted to 1 individual, second instar nymphs are weighted as 3 individuals, third instar nymphs = 27, young adult females (without ovisacs) = 42, and adult females with ovisacs = 14. We used this weighted colony size to evaluate the density dependence of the mutualism between *L. grandis* and *D. aberiae*. This was justified because the correlation was higher when individuals were weighted according to the honeydew they produce than when mealybugs were not weighted.

Data analysis

Ant-attendance

To evaluate whether the measures of ant-attendance were affected by season (spring, summer and autumn) and year (2020 and 2021), we used a separate generalized linear model (GLM) for each of the four measures described above and we considered each *D. aberiae* colony as a sampling unit. We assumed a binomial error variance for 'ant-attendance' and Poisson error variance for 'absolute ant-attendance', 'relative ant-attendance' and 'weighted relative ant-attendance'. The assumed error structures were assessed using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If over- or underdispersion was detected, we re-evaluated the significance of the explanatory variables using an F-test after re-scaling the statistical model by a Pearson's χ^2 statistic divided by the residual degrees of freedom (Crawley 2007). Multiple comparisons to assess differences among seasons were based on Tukey's HSD post hoc test.

Effect of D. aberiae colony size on ant-attendance by L. grandis

To evaluate the effect of *D. aberiae* colony size on i) ant-attendance, ii) absolute ant-attendance, and iii) relative ant-attendance by *L. grandis,* we used GLMs for each measure and we considered each *D. aberiae* colony as a sampling unit. To evaluate whether the response of these measures to *D. aberiae* colony size was affected by season (spring, summer and autumn) and year (2020 and 2021), we included them as categorical factors as well as their pairwise interactions with colony size. In these analyses, model reduction was conducted by removing non-significant interactions ($P > 0.05$). We assumed a binomial error variance for 'ant-attendance' and Poisson error variance for 'absolute ant-attendance' and 'relative ant-attendance'. As previously, the assumed error structures were assessed using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If over- or underdispersion was detected, we re-evaluated the significance of the explanatory variables using an F-test after re-scaling the statistical model by a Pearson's χ^2 statistic divided by the residual degrees of freedom.

Relationship between L. grandis activity and D. aberiae density

Finally, a linear function was fitted in order to evaluate the correlation between the activity of *L. grandis* and the density of *D. aberiae* per orchard for both experimental years. The activity of *L. grandis* and the density of *D. aberiae* were calculated by summing up the number of ants and mealybugs, respectively, observed in the three sampling seasons in each tree and year. Then, the mean of the nine sampled trees per orchard was calculated for both numbers. The normality and homoscedasticity of each data set was evaluated by graphical analysis of residuals.

All statistical analyses were carried out with R and RStudio (RStudio Team, 2022), under R version 4.1.2 (R Core Team, 2021). We used the packages "rstatix" (Kassambara, 2021) and "rriskDistributions" (Belgorodski et al., 2017) to evaluate the distribution of data sets.

We used the package "emmeans" (Lenth, 2022) to carry out post hoc tests. We used the packages "ggplot2" (Wickham et al., 2016) and "ggpubr" (Kassambara, 2020) to build the graphs. For the geographical representation of the orchards, we used the package "terra" (Hijmans 2022).

3. Results

Complex of mealybug species

A total of 10,171 mealybugs were observed and identified during the two years (3,852 in 2020 and 6,319 in 2021). Four species of mealybugs were identified: *Delottococcus aberiae*, *Planococcus citri*, *Pseudococcus longispinus* (Targioni-Tozzetti) and *Paracoccus burnerae* (Brain) (**Figure 2**; **Table S2** of supplementary material). *Delottococcus aberiae* was the most abundant species (76.1% in 2020 and 91.6% in 2021) throughout the study and in all the seasons both years. Moreover, it was present in 14 out of the 16 sampled orchards in both years. *Planococcus citri* was the second species in relative abundance (21.1% in 2020 and 4.3% in 2021), it was present in all the seasons both years and it was observed in nine orchards in 2020 and seven orchards in 2021. *Pseudococcus longispinus* (2.8% in 2020 and 1.3% in 2021) was present in all the seasons, but its abundance was very low in summer and autumn. *Pseudococcus longispinus* was present in four orchards both years. *Paracoccus burnerae* (2.7% in 2021) was present in one orchard in 2021 (the southernmost sampled orchard), where it was present in all the seasons, but its abundance was low in summer and autumn.

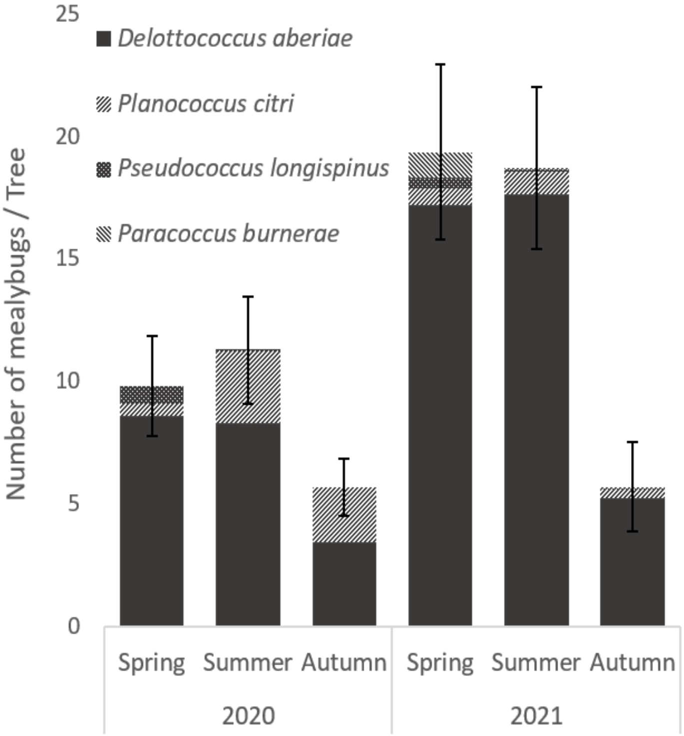

Figure 2. Mealybug complex and abundance from 16 citrus orchards in Valencia during 2020 and 2021. Presented as mean number of mealybugs sampled per tree and orchard (± SE).

Complex of ant species

A total of 15,685 ants were observed and identified during the two years (9,299 in 2020 and 6,386 in 2021). Twelve species of ants were identified: *Lasius grandis* (90.1%), *Pheidole pallidula* (3.6%), *Plagiolepis* sp. (4%) (*Plagiolepis pygmaea* (Latreille) and *Plagiolepis schmitzii* (Forel)), *Formica* sp. (1.3%) (*Formica rufibarbis* Fabricius and *Formica gerardi* Bondroit), *Linepithema humile* (0.8%), *Camponotus* sp. (0.2%) (*Camponotus sylvaticus* (Olivier), *Camponotus foreli* Emery and *Camponotus lateralis* (Olivier)), *Tapinoma nigerrimum* (Nylander) (0.1%), and *Aphaenogaster iberica* Mayr (<0.1%) (**Figure 3**; **Table 1**).

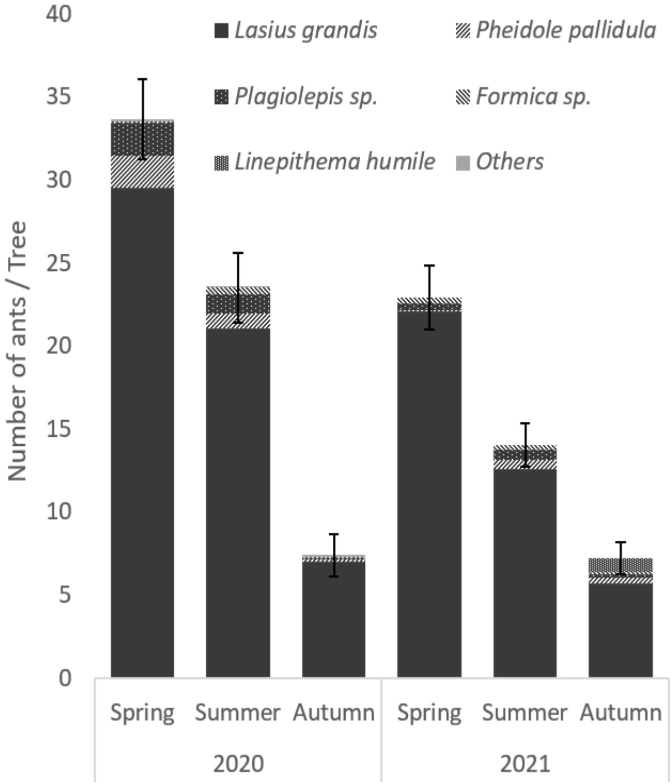

Figure 3. Ant complex and abundance from 16 citrus orchards in Valencia during 2020 and 2021. Presented as mean number of ants sampled per tree and orchard (± SE).

Lasius grandis was the most abundant ant species throughout the study and in all the seasons. Moreover, it was the most abundant species in all the orchards. During the two years of study, the highest activity of *L. grandis* was reported in spring, which gradually decreased until autumn. *Lasius grandis* was observed in the trunk, canopy and associated with *D. aberiae* colonies (**Table 1**). Four ant species were observed attending *D. aberiae* colonies. 95.6% of the ants observed attending *D. aberiae* were *L. grandis*, 2.5% *P. pallidula*, 1% *F. rufibarbis*, and 0.9% *P. pygmaea* (**Table 2**). Therefore, *L. grandis* was the ant with the highest level of interaction with *D. aberiae*. Moreover, 43.1% (575 out of 1335) and 60% (296 out of 434) of the total number of *L. grandis* observed in the canopy sample (120 leaves and 40 fruits) were attending *D. aberiae* colonies in 2020 and in 2021, respectively (**Table 2**).

Table 1. Number of ants sampled from 16 citrus orchards in the main citrus-producing area of Spain (Valencia) during 2020 and 2021. Ants were sampled in the trunk and in the canopy (leaves and fruit). The number of ants observed attending *Delottococcus aberiae* colonies in the canopy was also recorded.

Year	**Sampled unit**	***Lasius grandis***	***Pheidole pallidula***	***Plagiolepis* sp.**	***Formica sp.***	***Linepithema humile***	**Others**	**Total number of ants**
2020	Trunk	6968 88.5%	375 4.8%	448 5.7%	56 0.7%	1 0.01%	26 0.3%	7864
	Canopy	1335 93%	53 3.7%	14 1%	25 1.7%	0	8 0.6%	1435
	D. aberiae colonies	575 92.9%	36 5.8%	2 0.3%	6 1%	0	0	619
2021	Trunk	4497 80.1%	118 19%	145 0.02%	71 0.5%	109 0.4%	4	4944
	Canopy	1333 95.2%	21 3.9%	28	52	8 0.9%	0	1442
	D. aberiae colonies	800 97.6%	0	11 1.3%	9 1.1%	0	0	820

Ant-attendance of *Delottococcus aberiae* colonies by *Lasius grandis*

To evaluate the relation between the invasive mealybug *D. aberiae* and the native ant *L. grandis*, we measured: i) the ant-attendance, ii) the absolute, iii) the relative and iv) the weighted relative ant-attendance in spring, summer and autumn of 2020 and 2021. Throughout the study, 35.4 ± 1 % of *D. aberiae* colonies were attended by *L. grandis*. However, this percentage varied with year ($\chi^2_{2208} = 29.4$, $P < 0.001$), season ($\chi^2_{2206} = 55.1$, $P < 0.001$) and their interaction ($\chi^2_{2204} = 177$, $P < 0.001$). While in 2020 the percentage of mealybug colonies attended by ants was at its maximum in summer, in 2021 maximum ant attendance was observed in autumn and at a lower rate than the previous year (76.6 in 2021 *vs* 43.1% in 2020) (**Table 2**).

The mean number of *L. grandis* ants per *D. aberiae* colony (absolute ant-attendance) was 1.84 ± 0.05. Absolute ant-attendance did not differ between years ($\chi^2_{780} = 0.001$, $P = 0.97$), but it was affected by season ($\chi^2_{778} = 34.1$, $P < 0.001$) and this seasonal effect was different each year (interaction year × season: $\chi^2_{778} = 10.5$, $P = 0.006$). As in the case of ant-attendance, absolute ant-attendance was maximum in the summer of 2020 (2.05 ants per ant-attended colony) and the autumn of 2021 (2.37 ants per ant-attended colony) (**Table 2**).

The mean number of ants per mealybug (relative ant attendance) was 0.72 ± 0.02. There was moderate evidence that relative ant-attendance was higher in 2020 than in 2021($F_{1, 780}$ = 4.15, P = 0.043), it was weakly affected by season ($F_{2, 778}$ = 2.9, P = 0.055) and this seasonal effect was different between year (interaction year × season: $F_{2, 776}$ = 3.08, P = 0.047) (**Table 2**).

The mean number of ants per mealybug individual (weighted size) in ant-attended colonies (weighted relative ant-attendance) was 0.12 ± 0.021 throughout the study. Weighted relative ant-attendance was not affected by year ($F_{1, 780}$ = 3.49, P = 0.062) or season ($F_{2, 778}$ = 0.44, P = 0.64), but it was affected by the interaction between year and season ($F_{2, 776}$ = 4.64, P = 0.01) (**Table 2**).

Table 2. Ant-attendance of the invasive mealybug *Delottococcus aberiae* by the native ant *Lasius grandis* from 16 citrus orchards in the main citrus-producing area of Spain (Valencia) in 2020 and 2021. Presented as total number of mealybug colonies observed, percentage of colonies attended by *L. grandis* and absolute, relative and weighted relative ant-attendance calculated for ant-attended colonies. Different letters in columns denote significant differences between seasons in both years (Tukey test, P <0.05).

Year	Season	*D. aberiae* colonies	Ant-attendance	Absolute ant-attendance	Relative ant-attendance	Weighted relative ant-attendance[a]
2020	Spring	247	0.32 ± 0.018 bc	1.44 ± 0.14 ab	0.7 ± 0.07 ab	0.09 ± 0.02 a
	Summer	274	0.766 ± 0.026 d	2.06 ± 0.1 cd	0.79 ± 0.05 b	0.15 ± 0.02 a
	Autumn	263	0.179 ± 0.024 a	1.55 ± 0.18 abc	0.8 ± 0.1 ab	0.17 ± 0.04 a
		784	**0.429 ± 0.018**	**1.84 ± 0.07**	**0.77 ± 0.03**	**0.14 ± 0.01**
2021	Spring	580	0.291 ± 0.019 b	1.44 ± 0.09 a	0.69 ± 0.05 ab	0.14 ± 0.02 a
	Summer	630	0.292 ± 0.018 b	1.94 ± 0.1 bcd	0.59 ± 0.04 a	0.11 ± 0.02 a
	Autumn	216	0.431 ± 0.034 c	2.37 ± 0.16 d	0.84 ± 0.07 b	0.07 ± 0.02 a
		1426	**0.313 ± 0.012**	**1.84 ± 0.08**	**0.68 ± 0.03**	**0.11 ± 0.01**

[a] Weighted considering the estimated amount of honeydew excreted by mealybugs.

Effect of colony size on ant-attendance

The probability that *D. aberiae* colonies were attended by *L. grandis* increased significantly with the weighted size of *D. aberiae* colonies ($\chi^2_{2208} = 194.4$, $P < 0.001$) and there was a strong interaction with year ($\chi^2_{2204} = 14.2$, $P < 0.001$) and season ($\chi^2_{2202} = 48$, $P < 0.001$) (**Figure 4a**; **Table 3**).When we analysed each season separately, there was strong evidence that colony size has a positive effect on ant-attendance in all the seasons, except spring of 2020 (spring 2020: $\chi^2_{245}= 0.3$, $P = 0.59$; spring 2021: $\chi^2_{578}= 15.8$, $P < 0.001$; summer 2020: $\chi^2_{272}= 17.2$, $P < 0.001$; summer 2021: $\chi^2_{628}= 106.6$, $P < 0.001$; autumn 2020: $\chi^2_{261}= 9.7$, $P = 0.002$; autumn 2021: $\chi^2_{214}= 79.4$, $P < 0.001$).

The number of tending ants per ant-attended mealybug colony (absolute ant-attendance) also increased as the size of *D. aberiae* colony increased ($\chi^2_{780} = 108.1$, $P < 0.001$). Absolute ant-attendance was not affected by the interaction between colony size and year ($\chi^2_{776} = 0.3$, $P = 0.59$), but it was affected by the interaction between colony size and season ($\chi^2_{774} = 12.6$, $P = 0.002$) (**Figure 4b**; **Table 3**). When we analysed each season separately, there was a strong evidence that colony size had a positive effect on absolute ant-attendance in all the seasons (spring 2020: $\chi^2_{77} = 8$, $P = 0.005$; spring 2021: $\chi^2_{167} = 15.4$, $P < 0.001$; summer 2020: $\chi^2_{208} = 23.9$, $P < 0.001$; summer 2021: $\chi^2_{182}= 15$, $P < 0.001$; autumn 2020: $\chi^2_{45} = 6.8$, $P = 0.009$; autumn 2021: $\chi^2_{91} = 31.7$, $P < 0.001$).

The number of tending ants per mealybug (weighted relative ant-attendance) decreased as the size of *D. aberiae* colony increased ($F_{1,\ 780} = 41.1$, $P < 0.001$). Relative ant-attendance was not affected by the interaction between colony size and year ($F_{1,\ 776} = 0.07$, $P = 0.79$) or season ($F_{1,\ 774} = 1.58$, $P = 0.21$) (**Figure 4c**; **Table 3**).

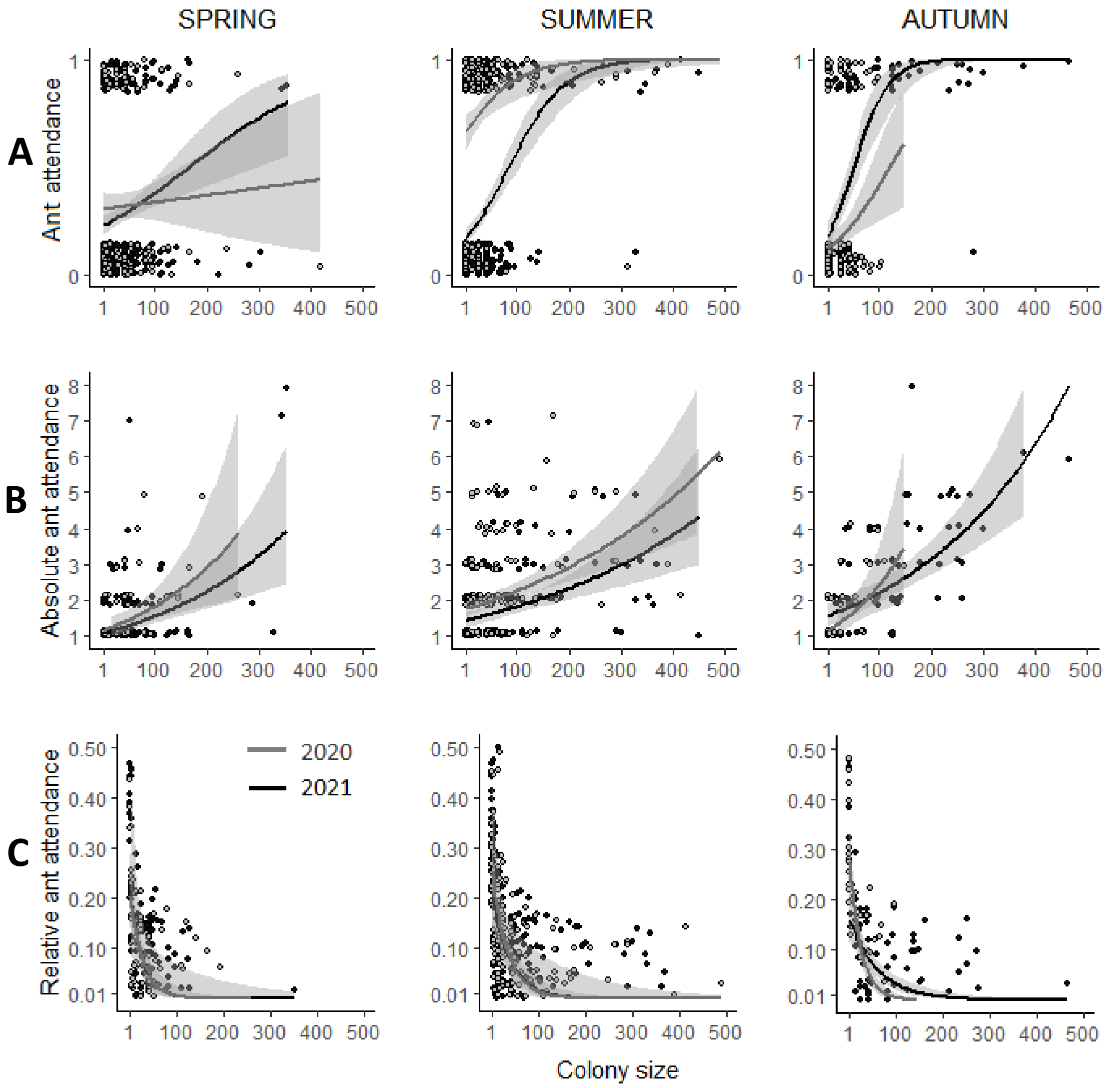

Figure 4. Ratio of *Delottococcus aberiae* colony size to (A) ant-attendance (ant-attended colonies), (B) absolute ant-attendance (number of ants per ant-attended colony), and (C) relative ant-attendance (ants/mealybugs per ant-attended colony), by the Mediterranean ant *Lasius grandis* in spring, summer and autumn of 2020 and 2021. Mealybugs were weighted to account for the estimated amount of honeydew that each instar excretes. Lines represent the generalized linear models (GLMs) based on binomial (ant-attendance) or Poisson distributions (absolute and relative ant-attendance).

Table 3. Effect of *Delottococcus aberiae* colony size on ant-attendance (ratio of ant-attended colonies), absolute ant-attendance (number of ants per ant-attended colony), and relative ant-attendance (ants/mealybugs per ant-attended colony), by the Mediterranean ant *Lasius grandis*. Generalized linear models (GLMs) based on binomial (ant-attendance) or Poisson distributions (absolute and relative ant-attendance). Non-significant interactions were not included in the models.

	Ant-attendance			**Absolute ant-attendance**			**Relative ant-attendance** [a]		
	D. aberiae colonies (2210)			*D. aberiae* atended colonies (782)			*D. aberiae* atended colonies (782)		
Variable	Parameter stimate	*z*	*P*	Parameter stimate	*z*	*P*	Parameter stimate	*z*	*P*
Intercept	-1.41	-6.98	<0.001	0.34	3.87	<0.001	0.09	0.81	0.42
Colony size*	0.027	7.07	<0.001	0.004	7.55	<0.001	-0.005	-5.66	<0.001
Year									
2020	-0.68	-2.73	0.006	-0.07	-1.95	0.23	-0.042	0.48	0.63
2021	0.0	-	-	0.0	-	-	0.0	-	-
Season									
Spring	0.2	0.88	0.38	-0.21	-1.95	0.052	-0.27	-2.21	0.027
Summer	-0.18	-0.77	0.44	0.16	1.67	0.096	-0.24	-2.12	0.034
Autumn	0.0	-	-	0.0	-	-	0.0	-	-
Colony size × Year									
2020	-0.006	-2.41	0.016						
2021	0.0	-	-						
Colony size × Season									
Spring	-0.019	-4.9	<0.001	-0.00019	-0.21	0.83			
Summer	-0.007	-1.66	0.098	-0.0018	-2.9	0.004			
Autumn	0.0	-	-	0.0	-	-			
Year × Season									
Spring	1.08	3.78	<0.001						
Summer	3.01	10.32	<0.001						
Autumn	0.0	-	-						

[a] Mealybugs were weighted according to the estimated amount of honeydew that each instar excretes.

Relationship between *L. grandis* activity and *D. aberiae* density

A positive correlation between *L. grandis* activity and *D. aberiae* density was detected throughout 2020 {Number of *D. aberiae* = (2.47 × (Number of *L. grandis*) – 3.2} ; r = 0.68; $F_{1,\,14} = 8.22$, $P = 0.0037$) and 2021 {Number of *D. aberiae* = (3.04 × (Number of *L. grandis*) + 10.2} ; r = 0.76; $F_{1,\,14} = 13.4$, $P < 0.001$) (**Figure 5**).

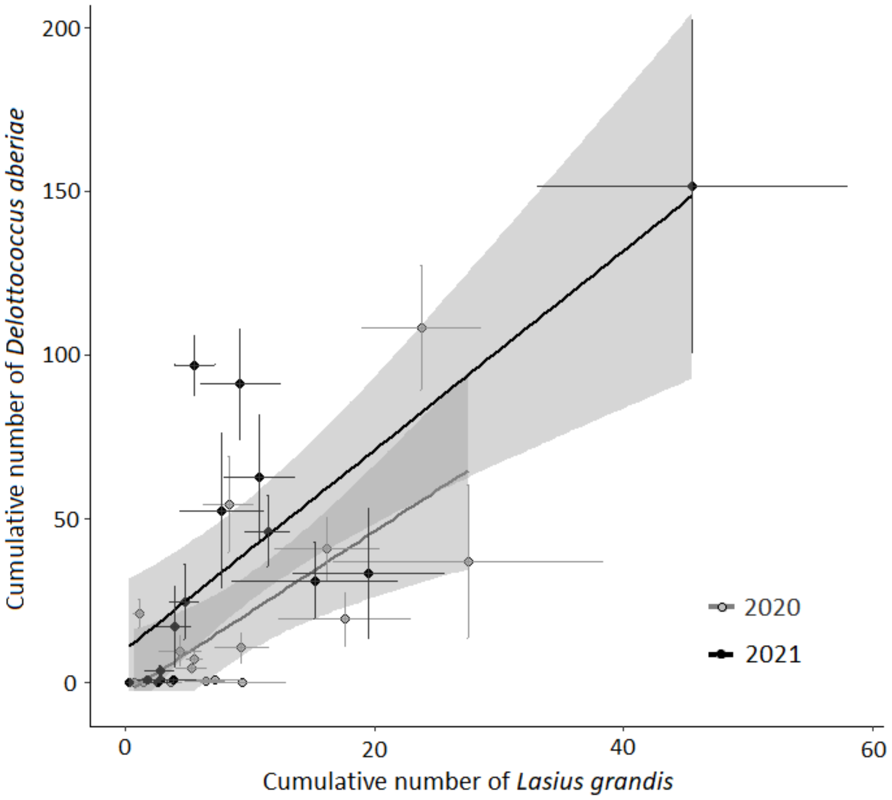

Figure 5. Density of the invasive mealybug, *Delottococcus aberiae*, to activity of the native ant *Lasius grandis* in 16 citrus orchards from the main citrus-producing area of Spain (Valencia) in 2020 and 2021. Each point represents the mean (± SE) number of *D. aberiae* and the mean (± SE) number of *L. grandis* sampled in each citrus orchard throughout 2020 and 2021 separately. Tree was the sampling unit for each orchard and there were nine trees per orchard. Lines represent the linear function for each year.

4. Discussion

Our results demonstrate that the invasive mealybug *D. aberiae* is frequently attended by native ants in Spanish citrus. The dominant ant species in the orchards, the native species *L. grandis,* has established a close association with this mealybug from South Africa. *Lasius grandis* attended *D. aberiae* throughout the year and in all the orchards where the mealybug was present. Our results suggest that this new mutualistic association may contribute to the establishment and expansion of this pest in the Mediterranean Basin. This is because the invasibility of honeydew-excreting hemipterans is usually determined by the mutualism with ants (Abbott & Green, 2007; Helms & Vinson, 2003; Tena et al., 2013; Zhou et al., 2014a).

Between 20% and 80% of *D. aberiae* colonies were attended by ants during each season of the two-year study. This level of ant-attendance is similar to that reported for *P. citri*, which is long-established in this citrus-producing area and its mutualistic relationship with ants has been broadly evidenced (Pérez-Rodríguez et al., 2021). Another study, conducted by Pekas et al. (2011), observed up to 100% of *P. citri* colonies attended by the dominant Mediterranean ants *L. grandis* and *P. pallidula* (Pekas et al., 2011), however, this may have been due to the low number of mealybug colonies in the orchard. Pekas et al. (2011) also observed variable activity of *L. grandis* in Spanish citrus throughout the day in the canopies, with maximum activity at 10 p.m. Therefore, ant-attendance may be underrepresented as we measured attendance between 9 a.m. and 4 p.m., when the number of *L. grandis* in the canopy is lower (Pekas et al., 2011).

Four ant species, *L. grandis*, *P. pallidula*, *F. rufibarbis*, and *P. pygmaea*, were observed feeding on *D. aberiae* honeydew and all them were native species from Mediterranean Basin. *Lasius grandis* was the most widely distributed ant species and it was the most abundant ant in all study orchards. This ant species represented 90% of the total number of ants observed on citrus trees and more than 95% of the ants consuming *D. aberiae* honeydew. The prevalence of *L. grandis* in citrus from eastern Spain have been widely reported (Alvis & García-Marí, 2006; Cerdá et al., 2009; Martínez-Ferrer & Campos-Rivela, 2017; Palacios et al., 1999; Plata & Tena, 2022; Vanaclocha et al., 2005). *Lasius grandis* is a dominant and aggressive ant, which nests in the base of citrus trees and does not allow other dominant ant species to forage in the same tree (Pekas et al., 2011). *Lasius grandis* workers are active from March to November and, despite reaching maximum activity over night, are present in the canopy during the whole day (Pekas et al., 2011). *Lasius grandis* has a marked preference for sugar resources and is associated with honeydew-excreting insects in citrus, such as soft scales, whiteflies, aphids and mealybugs (Paris & Espadaler, 2009; Pekas et al., 2011; Zina et al., 2017). *Pheidole pallidula* was present in 13 out of the 16 sampled orchards, but at very low densities. This species is also dominant (Cerdá et al., 1998) and does not overlap with *L. grandis* in citrus trees (Pekas et al., 2011). *Pheidole pallidula* is also associated with honeydew and frequently attends mealybugs (Pekas et al., 2011) and other hemipterans (Zina et al., 2017). However, *P. pallidula* was not abundant in orchards where *D. aberiae* was present and the percentage of colonies attended by *P. pallidula* was low. *Formica rufibarbis* was sporadically observed feeding on *D. aberiae* honeydew. This species scouts individually for resources that are not being used by more dominant ant species. *Plagiolepis pygmaea* were rarely observed feeding on honeydew excreted by *D. aberiae.* Ants belonging the genus *Plagiolepis* are also subordinate and can forage on the same trees as dominant ant species (Pekas et al., 2011). We observed *P. pygmaea* feeding on *D. aberiae* honeydew but only when unattended by dominant ant species, as has been observed for other honeydew-excreting hemipterans such as aphids or scale insects (Zina et al., 2017; Pekas et al. 2011). Finally, the invasive ant *L. humile*, which is abundant in the citrus orchards of southern Portugal, was very scarce and was not observed attending *D. aberiae*, despite commonly reported attending *P. citri* in Portugal (Zina et al., 2017; 2020).

This work reveals a positive correlation between the activity of the native ant *L. grandis* and the density of the invasive mealybug *D. aberiae* in Spanish citrus canopies, with similar responses in both study years. This is likely due to the mutualism that these species have established in citrus, as has occurred with other invasive honeydew-excreting hemipterans such as aphids, whiteflies and other mealybug species (Anjos et al., 2021). The increase of honeydew-excreting hemipterans is generally attributed to several services that ants provide them, in particular defence against natural enemies. Aggressive ant species such as *L. grandis* can provide better protection to mutualists than less aggressive ant species (Buckley & Gullan, 1991; Itioka & Inoue, 1999). Thus, the presence of *L. grandis* may limit the biological control of *D. aberiae.* This ant species protects other mealybug species against encyrtid parasitoids, an important group of natural enemies of mealybugs (Campos et al., 2006; Mansour et al., 2012; Mouratidis et al., 2021). The parasitoid *Anagyrus aberiae* Guerrieri (Hymenoptera: Encyrtidae) has been recently imported from South Africa (Soto et al., 2021) as native parasitoids cannot successfully parasitise *D. aberiae* (Tena et al., 2017). However, no *D. aberiae* sampled during our study were parasitised, including unattended colonies. This suggests that *L. grandis* may be limiting the effectiveness of *D. aberiae* predators and conferring an advantage to *L. grandis* attended colonies. Among mealybug predators in the Mediterranean Basin, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinelidae) has been observed feeding on *D. aberiae* (Pérez-Rodríguez et al., 2019), however, its predatory potential has shown to decrease in the presence of ants such as *T. nigerrimum* (Mansour et al., 2012), and other generalist predators that also feed on mealybugs, such as lacewings, are less abundant in the presence of *L. grandis* (Calabuig et al., 2015; Morris et al., 1998). Finally, it is worth mentioning that *L. grandis* might provide other services to *D. aberiae*, such as transport (Ho & Khoo, 1997), sanitizing (Nielsen et al., 2010; Queiroz & Oliveira, 2001;) or the stimulation of reproduction and feeding (Morales, 2000; Xu et al., 2021), that lead to an increase of the mealybug density.

Our field observations describe the mutualistic relation between *D. aberiae* and *L. grandis* throughout the year. In general, larger colonies of *D. aberiae* were more likely to be attended by *L. grandis*, but this effect was weaker in summer (June), when *L. grandis* activity was at its maximum. This might occur because mealybug colonies provide ants with persistent reward (honeydew), especially after spring when colonies have individuals of different instars and the aphid critical and citrus bloom period is over (Mockford et al., 2022). Then, *L. grandis* protection would allow attended mealybug colonies to grow at a greater rate that unattended ones. In ant-attended colonies, we found a positive relation between the colony size, weighted with the production of honeydew, and the number of attending ants (absolute ant-attendance). Although there was an interaction between colony size and season, absolute ant-attendance increased sharply with colony size across the seasons and in both years. This suggests that *L. grandis* follows a predictable seasonal pattern exploiting the detected *D. aberiae* colonies. Relative ant-attendance was also similar throughout the year and, as occurs with other ant-attended hemipterans, decreased with hemipteran density, which could result in a lower ant protection for larger colonies (Breton & Addicott, 1992; Itioka & Inoue, 1996; Harmon et al., 2007). To the best of our knowledge, this is the first time that the mutualistic relationship between mealybugs and ants is described across the seasons in agroecosystems. Further research should evaluate ant-attendance in spring, when ants start to search for food and mealybugs establish the first colonies.

The mutualism between ants and honeydew-excreting hemipterans requires that both partners recognize each other, which is essential to establish trophobiosis (Lang & Menzel, 2011; Xu & Chen, 2021). Exaptations are known to facilitate the ant-hemiptera recognition between invasive ants and native honeydew-excreting species (Mondor & Addicott, 2007). In our case, where the ant is the native species and the honeydew producer the invasive, several exaptations of the invasive mealybug might have contributed to this recognition. For example, *D. aberiae* might share some semiochemicals with other mealybug species long-established in Mediterranean citrus such as *P. citri* and, therefore, *L. grandis* might have recognized *D. aberiae* as a trophobiont partner. Alternatevely, *L. grandis* might have learnt to associate the cuticular hydrocarbons of *D. aberiae* to profitable sugar sources, as demonstrated by Hertaeg et al. (2021) for *Lasius niger* L. (Hymenoptera: Formicidae). The high ant-attendance observed during two consecutive years demonstrates that *D. aberiae* excretes honeydew suitable for *L. grandis*, suggesting that this mealybug is adapted to be attended by other ant species in its area of origin, which is supported by recent field observations in South Africa (Urbaneja-Bernat personal observations 2022). From the other side, *L. grandis* fulfils several requirements that can also be considered exaptations promoting mutualisms with exotic honeydew-excreting hemipterans. *Lasius grandis* have high sugar needs and it performs an aggressive behavior when exploiting sugar sources. Moreover, this ant species is capable of mass recruitment. These conditions make *L. grandis* an ideal protector of honeydew-excreting hemipterans (Buckley & Gullan, 1991; Itioka & Inoue, 1999; Ness et al., 2010).

Twelve years after the first detection, *D. aberiae* and *L. grandis* have established a mutualism that can have a wide range of cascading effects on other citrus-inhabiting species. For example, it could favour the presence of other pests that do not excrete honeydew because their natural enemies are disturbed by the presence of ants, such as the Californian red scale *Aonidiella aurantii* (Maskell) in Spanish citrus (Pekas et al., 2010). On the other hand, the preference of ants for one honeydew-excreting species can negatively affect other less-preferred hemipterans that would be unattended, such as shown for different aphid species (Fischer et al., 2001). Therefore, the high abundance of *D. aberiae* and the mutualism with *L. grandis* could prevent the establishment of exotic hemipterans that excrete honeydew of lower quality or more ephemeral, such as the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), that vectors the disease huanglongbing (Tena et al., 2013). In citrus from southern California, *D. citri* has established a mutualistic relationship with the invasive ant *L. humile* (McCalla et al., 2023; Milosavljević et al., 2021). This vector has not been detected yet in Spain but is present in Israel. Likewise, the competition for honeydew can determine the displacement of native ant species by other invasive ones (Zhou et al., 2014b). Thus, the mutualism between native ants and honeydew-excreting insects can prevent the colonization of invasive ant species. The high competitiveness of *L. grandis* in exploiting honeydew could partially explain why the invasive ant *L. humile* does not colonize citrus areas where *L. grandis* prevails such as eastern Spain or some areas in Portugal (Zina et al., 2020).

The literature demonstrates that resident honeydew-excreting hemipterans can be a key factor in the establishment of invasive ants, and many studies have also shown that two invasive species can mutually benefit colonizing new ecosystems. However, few studies have explained how native ants can enhance the establishment and spread of a recent honeydew-excreting species arrival. This paper shows that native ant species should be considered as potential drivers in the establishment of exotic honeydew-excreting species. Further research should assess whether the management of ants can improve the control of *D. aberiae* in citrus.

Data accessibility

Data from this study are available at the IVIA Digital Repository:
https://doi.org/10.58582/redivia.8585

Supplementary material

Table S1. Agronomic characteristics of the 16 citrus orchards from the main citrus-producing area of Spain (Valencia) sampled in 2020 and 2021. All orchards were between 10 and 15 years old, were drip irrigated and farmers followed the IPM guidelines (http://gipcitricos.ivia.es/).

Orchard	Longitude (WGS84 coordinates)	Latidude (WGS84 coordinates)	Altitude (m a.s.l)	Citrus variety	Orchard size (ha)	Green cover	Surrounding landscape
1	-0.09	40.02	121	Clemenules	1.7	Low	Citrus
2	-0.09	40.00	90	Clemenules	2.05	None	Citrus
3	-0.21	39.99	151	Clemenules	0.71	Low	Citrus
4	-0.08	39.93	30	Hernandina	0.56	None	Citrus
5	-0.18	39.87	41	Clemenules	0.49	None	Citrus
6	-0.22	39.78	56	Fortune	0.7	None	Citrus/Forest
7	-0.35	39.73	160	Clemenpons	2.9	Medium-Low	Citrus/Forest/Gardens
8	-0.22	39.69	6	Clemenules	0.37	None	Citrus
9	-0.42	39.65	189	Marisol	1.35	None	Citrus/Forest/Gardens
10	-0.48	39.59	110	Satsuma	0.46	None	Citrus/Gardens
11	-0.50	39.32	142	Navelina	2	Low	Citrus/Persimmon
12	-0.52	39.19	30	Navelina	0.43	None	Persimmon
13	-0.29	39.10	75	Hernandina	0.81	Medium-Low	Citrus/Forest
14	-0.43	39.01	142	Okitsu	2.57	None	Citrus/Forest
15	-0.16	38.85	121	Navelina	0.29	None	Citrus/Forest
16	-0.04	38.80	85	Orogrande	0.33	None	Citrus

Table S2. Total number and percentage of mealybugs sampled in 16 citrus orchards from the main citrus producing area of Europe (eastern Spain) in 2020 and 2021.

Year	Season	*Delottococcus aberiae*	*Planococcus citri*	*Pseudococcus longispinus*	*Paracoccus burnerae*	Total mealybugs
2020	Spring	1240 87.8%	68 4.8%	105 7.4%	0	2562
	Summer	1193 73.6%	426 26.3%	2 0.1%	0	101
	Autumn	498 60.9%	320 39.1%	0	0	44
		2931 **76.1%**	**814** **21.1%**	**107** **2.8%**	**0**	**3852**
2021	Spring	2479 88.9%	93 3.3%	67 2.4%	148 5.3%	4197
	Summer	2559 94.4%	132 4.9%	11 0.4%	10 0.4%	333
	Autumn	753 91.8%	49 6%	7 0.9%	11 1.3%	268
		5791 **91.6%**	**274** **4.3%**	**85** **1.3%**	**169** **2.7%**	**6319**

Chapter 2

Exclusion of Mediterranean ant species enhances biological control of the invasive mealybug *Delottococcus aberiae* in citrus

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Abstract

Delottococcus aberiae is an invasive mealybug that produces severe damage in Spanish citrus. This mealybug has established a mutualistic relationship with native Mediterranean ant species that may limit biological control of this pest. Herein, we evaluated the effect of tending ants on the biological control of *D. aberiae*. To do this, we compared: i) the density of *D. aberiae*, ii) the density of its natural enemies and iii) the damage produced by the mealybug in trees with (control) and without ants (ants excluded with sticky barriers) in two citrus orchards across two consecutive years. *Lasius grandis* was the most abundant ant species in both orchards and represented more than 95% of the ants tending *D. aberiae* in control trees. Spiders and lacewings were the most abundant predators observed in mealybug colonies, and the exclusion of mutualistic ants increased their abundance. Moreover, in control trees, ant activity throughout the year was negatively correlated with the relative abundance of predators (number of predators per mealybug). No parasitoid were recovered during field experiments. Ant exclusion reduced the density of *D. aberiae* and the ratio of damaged fruit at harvest across years and orchards. This work corroborates that *D. aberiae* benefits from its mutualistic relationship with *L. grandis* likely because the presence of ants reduced the abundance of generalist predators. This mutualism can be disrupted using physical barriers in the trunk. Further research should assess other methods of ant control that are more economic and feasible for citrus producers.

1. Introduction

The invasive mealybug *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae) is among the most damaging citrus pests in Spain. This mealybug species is native to Southern Africa and arrived Spain in 2009 Since then, it has spread throughout the main citrus producing area of Spain (Beltrà et al., 2015; Plata et al., 2024a). In Mediterranean citrus orchards, *D. aberiae* completes up to seven generations (Martínez-Blay et al., 2018a). Between February and July, it migrates from the canopy to the soil via the trunk (Martínez-Blay et al., 2018b). Unlike other mealybugs species occurring in citrus, *D. aberiae* causes distortion and size reduction of fruit that can reduce yields by up to 80% (Pérez-Rodríguez et al., 2017). Fruit damage is produced in late spring, when *D. aberiae* feeds on the immature fruit. The damage produced by *D. aberiae* has progressively increased due to the lack of effective natural enemies and the ban of effective insecticides against this mealybug (Pérez-Rodríguez et al., 2017; 2019; Tena et al., 2017). Therefore, it is necessary to develop alternative methods to reduce population densities of *D. aberiae* and associated economic losses.

As with other hemipterans, *D. aberiae* excretes honeydew, a sugar-rich product that is commonly consumed by tending ant species (Hölldobler & Wilson, 1990; Way, 1963). In exchange for honeydew, ants protect the honeydew-producing hemipterans from natural enemies. This mutualism between tending ants and mealybugs has been largely studied and it reduce the efficacy of biological control agents in citrus (Anjos et al., 2021; DeBach et al., 1951; Itioka & Inoue, 1996; 1999; Mansour et al., 2012; Mouratidis et al., 2021;). The Mediterranean native tree-foraging ant *Lasius grandis* Forel (Hymenoptera: Formicidae) is closely associated with *D. aberiae* in Valencian citrus orchards.[2] This relationship might increase the abundance and facilitate the spread of *D. aberiae* in Spanish citrus, where the ant *L. grandis* is widely distributed and it is active most of the year (Pekas et al., 2011; Plata et al., 2024a) However, it is still unknown whether excluding this ant species might reduce the abundance of *D. aberiae* due to increased natural enemy activity.

Ant exclusion might increase the number of natural enemies and reduce *D. aberiae* density and damage, as has been observed with *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), a long-established pest mealybug species in Spanish citrus (Campos et al., 2006; Marras et al., 2008; Pérez-Rodríguez et al., 2021; Villalba et al., 2006). One way to exclude ant access to mealybug colonies is using sticky physical barriers. These barriers might also prevent the movement of mealybugs across the citrus trunk and they may trap some of the adult females that migrate from the canopy to the soil[4], thus increasing *D. aberiae* mortality. On the other hand, the barrier would prevent mealybug from descending to the soil and this might reduce mealybug mortality caused by ground-dwelling predators (Pérez-Rodríguez et al., 2018). Simultaneously, barriers can exclude some predator species that reach the canopy via the trunk (Piñol et al., 2009). The application of insecticides harmful to natural enemies in commercial citrus orchards, coupled with the lack of effective parasitoids against *D. aberiae,* might limit the benefits of ant exclusion (Tena et al., 2017).

D. aberiae is such a destructive invasive pest that it is necessary to design new strategies to manage *D. aberiae* and reduce its spread. To this end, we evaluated the impact of ant exclusion from citrus canopies on the density of *D. aberiae* and its natural enemies, as well as on the percentage of fruit at harvest damaged by the mealybug. To assess these potential effects, a randomize-block design with ant-allowed and ant-excluded treatments was carried out in two commercial citrus orchards across two consecutive years using sticky barriers on trunks to exclude ants in an attempt to promote natural enemy activity against *D. aberiae*. Results from these studies help to better understand the mutualistic relationship that *D. aberiae* has established with the native ant *L. grandis* and the impact of this relationship on the biological control agents and associated pest density suppression.

2. Material and methods

Study area

The experiment was carried out, over two consecutive years, from March 2020 to November 2021, in two Clementine mandarin orchards from Valencian Community (Spain), the main citrus producing area of Europe. The orchards were located in Borriol (40°00'45.5"N 0°05'56.3"W 110 m. a.s.l) and Moncófar (39°48'12.1"N 0°09'58.7"W 15 m. a.s.l) and were 1.4 and 1.1 ha, respectively. Both orchards were selected because they had a medium abundance of *D. aberiae*. Pest management was conventional during the study period and farmers applied insecticides uniformly across orchards regardless of the placement of experimental plots within orchards. One to three insecticide treatments were applied per orchard and year. The active ingredients were clofentezine and abamectin to control spider mites, pyriproxyfen for California red scale *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) and acetamiprid for mealybugs. Paraffin oil was normally added as adjuvant.

Experimental design

Eight replicated blocks were evenly distributed across the two orchards. Each block was divided into paired 4x4 tree plots, one receiving the "ant-allowed" treatment and the other receiving the "ant-excluded" treatment. In ant-excluded plots, sticky barriers were applied to trunks of trees to prevent ants accessing up to the canopy (Pekas et al., 2010). To do this, the tree trunk was wrapped with Parafilm® 10-30 cm above ground. The wrapped zone was coated with Tad All-Weather® adhesive. Sticky barriers were inspected every month and, if necessary, the adhesive was reapplied. Pruning and trimming of the branches and ground vegetation were done when necessary, in order to avoid contact between adjacent trees and between the canopies and the ground. This cultural practice eliminated non-trunk access points for ants to reach mealybug colonies.

Insect sampling

The four central trees of each plot were sampled monthly. Therefore, we sampled 16 trees (four trees × four plots) for each treatment (ant allowed / ant excluded) and orchard. From each tree, 40 leaves (ten leaves per cardinal direction) and 20 fruits (five fruit per cardinal direction) were visually inspected and the number of mealybugs, ants and potential predators were identified and counted. An imaginary cross section of the trunk was drawn at 30 cm above ground and the number of ants crossing (both up and down) were counted for two minutes. Parasitism was also recorded. Mummified mealybugs and mealybugs with parasitism symptoms (*i.e.*, loss of wax and/or black dot) were collected and transported to the laboratory. Once there, mealybugs were individually introduced in a crystal vial sealed with cotton. However, no parasitoids developed and emerged from these potentially parasitized *D. aberiae* individuals. When necessary, insects were collected and transported to the laboratory for identification. Ants were identified to species and predators identification varied among groups from order (*e.g.*, spiders) to species (*e.g.*, coccinellids) (For further details see Supplementary material: Table 1). All observations were made between 9 a.m. and 4 p.m., as ant activity in citrus canopies is stable over this period (Pekas et al., 2011).

Statistical analyses

All statistical analyses described here were conducted using R and Rstudio (R version 4.1.2) (R Core Team 2021; RStudio Team, 2022) at the 0.05 level of significance. The packages "rstatix" (Kassambara, 2021), "rriskDistributions" (Belgorodski et al., 2017), and "ggpubr" (Kassambara, 2020), were used to evaluate the data distribution prior to analyses being performed. The package "ggplot2" was used to construct the graphs (Wickham, 2016). The description of each statistical analysis is provided in each section in detail.

For generalized linear model (GLMs), error structures were assessed using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If over- or underdispersion was detected, a re-evaluation of the significance of the explanatory variables using F-test after re-scaling Pearson's χ^2 statistic divided by the residual degrees of freedom. Significant values are given for the minimal model, while the non-significant values are those obtained before we deleted the variable from the initial model (Crawley, 2007; Mayhew & van Alphen, 1999).

Ant complex and seasonal trend of ant activity

Ant-allowed trees were used to identify the ant complex in each orchard. The ant complex is presented as the number of ants by species sampled throughout each year and orchard. The number of ants observed on the trunk (two minutes visual observations per tree), citrus canopy (40 leaves and 20 fruits per tree) and attending colonies of *D. aberiae* in the canopy were recorded.

To determine the seasonal trend of ants in ant-allowed and ant-excluded trees and evaluate the effectiveness of ant-exclusion, the total number of ants moving up and down the tree trunk during two minutes was calculated and used to determine the mean number of ants per tree and orchard for each sampling date.

Ant attendance on *Delottococcus aberiae* colonies

Ant-attendance on experimental trees was assessed between June and September both years (2020 and 2021), when *D. aberiae* was most abundant in the canopies. To measure ant-attendance, three indices were measured for each tree (Pekas et al., 2011; Plata et al., 2024a; Tena et al., 2013;); i) 'Ant-attendance' was calculated as the ratio of *D. aberiae* colonies attended by ants (*D. aberiae* colonies observed with at least one ant divided by the total number (*i.e.*, colonies with and without attending ants) of *D. aberiae* colonies). For each ant-attended colony, ii) absolute, and iii) relative ant-attendance were also calculated. 'Absolute ant-attendance' is used to measure the preference of ants for a specific honeydew source and it was calculated as the number of ants per *D. aberiae* colony. 'Relative ant-attendance' was used to obtain a parameter for the intensity of ant-attendance that was independent of colony size and it was calculated as the number of ants divided by the number of mealybugs in each colony (Fischer et al., 2001). To evaluate the effect of year (2020 and 2021) and orchard (Borriol and Moncofar) on ant-attendance, a generalized linear model (GLM) for each ant-attendance measure was used. Year, orchard and their interactions were considered as categorical fixed factors and each *D. aberiae* colony as a sampling unit. After checking the distribution of each data set, a binomial error variance for ant-attendance and a Poisson error variance for absolute and relative ant attendance were assumed.

Predator density

Predator density was calculated by summing the total number of potential predators of *D. aberiae* observed in each tree across years. Predators identified during field experiments and their absolute and relative abundance are presented as Supplementary material (Supplementary material, **Table S1**). Additionally, predator density was calculated for the most abundant predator orders. The mean number of predators counted across the 16 sampled trees per orchard and treatment was calculated. To evaluate the effect of the treatment (ant-exclusion) on predator density, GLMs for the total number of predators and for each predator order, with Poisson error variances, were conducted. Treatment, year, orchard and their interactions were considered as categorical fixed factors.

Furthermore, since predator density may be dependent on prey density, relative predator density as the total number of predators divided by the total number of *D. aberiae* was calculated. To evaluate the effect of the treatment (ant-exclusion) on relative predator density, GLM with Poisson error variance was conducted. Treatment, year, orchard and their interaction were considered as categorical fixed factors.

Finally, to further evaluate the effect of ant activity on relative predator density, we carried out an analysis using the total number of ants throughout the year (ant density) in ant allowed-trees. To evaluate the effect of ant density (total number of ants per tree throughout each year) on the relative predator density in ant-allowed trees, a GLM with

Poisson error variance was conducted. As previously described, the effects of treatment, orchard, year, and their interactions were included as categorical fixed factors.

Delottococcus aberiae density and damage

Delottococcus aberiae density was calculated by summing the number *D. aberiae* (nymphs and adults) observed in each tree and sampling date. The mean number of *D. aberiae* across the 16 sampled trees per orchard and treatment was calculated for each sampling date. To evaluate the effect of ant-exclusion on *D. aberiae* density, repeated-measures analyses of variance (ANOVA) were separately used for each year because the sampling dates differed in 2020 and 2021. Data satisfied normality and sphericity.

To evaluate levels of damage caused by *D. aberiae*, 40 fruit (10 fruit per cardinal direction) were observed before harvest (September) and the number of fruit damaged (deformed or size-reduced) was recorded. The ratio of damaged fruit was calculated by dividing the number of damaged fruits by the total number of fruits observed. The mean number of damaged fruit observed on the 16 sampled trees per orchard and treatment was calculated for each year. To evaluate the effect of ant-exclusion on the ratio of damaged fruits at harvest, generalized linear models (GLM) were used. The effects of treatment, orchard, year and their interactions were included as categorical fixed factors and a binomial error variance was assumed.

3. Results

Ant complex

In ant-allowed trees, six ant species were identified during the two-year study. In order of abundance, ant species observed were: *Lasius grandis*, *Plagiolepis pygmaea*, *Formica rufibarbis*, *Pheidole pallidula*, *Camponotus foreli* and *Tapinoma nigerrimum* (**Table 1**). The six species were observed in the orchard of Borriol, while *C. foreli* and *T. nigerrimum* were not observed in Moncófar. The most abundant species, *L. grandis*, represented more than 70% of ants observed on trunks, and accounted for more than 75% in canopies (fruits and leaves) and more than 95% of ants attending *D. aberiae* colonies. Finally, *P. pygmaea* and *F. rufibarbis* were sporadically observed attending *D. aberiae* colonies.

Table 1. Ant species complex in two citrus orchards located in the main citrus producing area of Europe (eastern Spain) sampled between May 2020 and November 2021. Ants were sampled at the base of the trunk and in the canopy.

			Total Number of Ants Counted by Species						
Orchard	**Year**	**Sampled unit**	***Lasius grandis***	***Plagiolepis pygmaea***	***Formica rufibarbis***	***Pheidole pallidula***	***Camponotus foreli***	***Tapinoma nigerrimum***	**Total ants**
Borriol	2020	Trunk	1812 70.7%	691 27%	9 0.4%	7 0.3%	29 1.1%	14 0.5%	2562
		Canopy	76 75.2%	19 18.8%	0	0	6 5.9%	0	101
		Mealybug colonies	41 93.2%	3 6.8%	0	0	0	0	44
	2021	Trunk	3361 80.1%	797 19%	1 0.02%	20 0.5%	18 0.4%	0	4197
		Canopy	317 95.2%	13 3.9%	0	0	3 0.9%	0	333
		Mealybug colonies	261 97.4%	7 2.6%	0	0	0	0	268
Moncófar	2020	Trunk	4882 96.4%	42 0.8%	79 1.6%	60 1.2%	0	0	5063
		Canopy	141 94%	3 2%	6 4%	0	0	0	150
		Mealybug colonies	103 96.2%	2 1.9%	1 0.9%	0	0	0	107
	2021	Trunk	6922 97.3%	90 1.3%	105 1.5%	0	0	0	7117
		Canopy	293 97%	0	9 3%	0	0	0	302
		Mealybug colonies	193 99%	0	2 1%	0	0	0	195

Seasonal trend of ants

In ant-allowed trees, ants ascended to citrus canopies from the beginning of spring (March-April) until the end of fall (November-December) in both years and orchards. Ant activity in canopies peaked in June-July in Borriol and between May and August in Moncófar (**Figure 1**).

Sticky barriers that received regular maintenance completely excluded ants from citrus canopies. As exceptions, however, sticky barriers that were not maintained failed to exclude ants. In 2020 both experimental orchards were not visited over February-May due to COVID-19 and sticky barriers could not be renewed in that period. In May and June 2021, sticky barriers failed in some trees due to the effect of rains, but sticky barriers were renewed and ants were completely excluded until the end of the experiment.

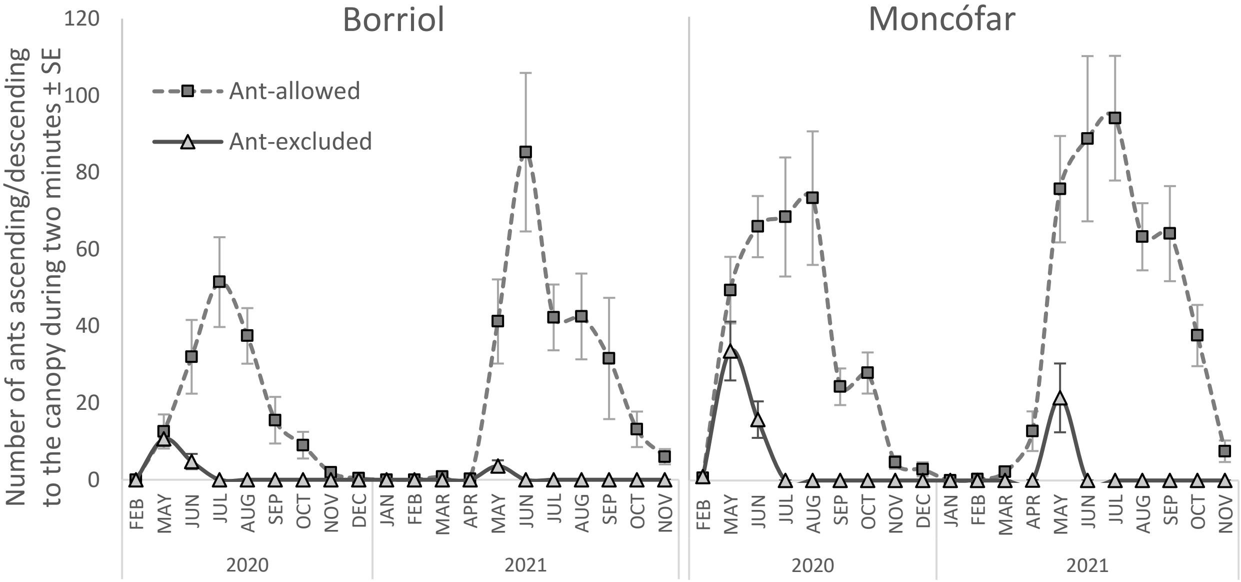


Figure 1. Seasonal trend of ant activity in ant-allowed and ant-excluded trees from two citrus orchards located in the main citrus producing area of Europe (eastern Spain). Represented by the number of ants (Mean ± SE) crossing an imaginary line in the base of the trunk during two minutes (ascending or descending to the canopy).

Ant attendance on *Delottococcus aberiae* colonies

In the ant-excluded trees, no ants were observed attending colonies of *D. aberiae*, while ants frequently attended *D. aberiae* in the ant-allowed trees (Table 2). The percentage of colonies attended by ants in 2020 was 32% and it was moderately higher than observations made in 2021 (25%) (χ^2_{1211} = 4.21, *P* = 0.04). This percentage was similar in both orchards (χ^2_{1210} = 0.2, *P* = 0.65) across years (orchard × year interaction: χ^2_{1209} = 0.02, *P* = 0.89).

The mean number of ants per attended colony, absolute ant-attendance, was moderately higher in 2020 when compared to 2021 ($F_{1,\ 310} = 4.03$, $P = 0.046$) and was higher Borriol when compared to Moncófar ($F_{1,\ 309} = 9.6$, $P = 0.002$) (Table 2). The response in each orchard was constant between years ($F_{1,\ 308} = 0.005$, $P = 0.94$).

The mean number of ants per mealybug in ant-attended colonies, relative ant-attendance, was higher in 2020 than in 2021 ($F_{1,\ 310} = 9.45$, $P = 0.002$), but it did not differ between orchards ($F_{1,\ 309} = 0.42$, $P = 0.52$). The interaction between orchard and year was not significant (orchard × year interaction: $F_{1,\ 308} = 0.51$, $P = 0.48$) (**Table 2**).

Table 2. Ant-attendance of *Delottococcus aberiae* in two citrus orchards located in the main citrus producing area of Europe (eastern Spain) sampled from June to September of 2020 and 2021. Presented as total number of mealybug colonies observed, percentage of colonies attended by ants and absolute and relative ant-attendance calculated for attended colonies.

Orchard	**Year**	***D. aberiae* colonies**	**Ant-attendance***	**Absolute ant-attendance****	**Relative ant-attendance*****
Borriol	2020	54	0.315 ± 0.064	2.29 ± 0.41	0.67 ± 0.07
	2021	542	0.24 ± 0.018	1.8 ± 0.11	0.49 ± 0.06
Moncófar	2020	138	0.319 ± 0.04	1.82 ± 0.21	0.72 ± 0.09
	2021	479	0.253 ± 0.02	1.41 ± 0.07	0.43 0.04

*Ant-attendance: Ratio of mealybug colonies attended by ants
**Absolute ant-attendance: Number of ants per attended mealybug colony
***Relative ant-attendance: Number of ants per mealybug in attended mealybug colonies.

Effect of ant-exclusion on predator density

The total number of potential predators of *D. aberiae* sampled in the canopy was higher in ant-excluded trees than in ant-allowed trees ($F_{1,\ 126} = 18.7$, $P < 0.001$), independently of the orchard (ant-exclusion × orchard interaction: $F_{1,\ 123} = 0.49$, $P = 0.48$) or year (ant-exclusion × year interaction: $F_{1,\ 122} = 0.13$, $P = 0.71$) (**Table 3**). The number of predators was strongly affected by orchard ($F_{1,\ 125} = 27.4$, $P < 0.001$), year ($F_{1,\ 124} = 41.5$, $P < 0.001$) and their interaction ($F_{1,\ 123} = 6.82$, $P = 0.01$). The most abundant groups of predators were spiders (33.5 % in Borriol and 41.5% in Moncófar) and neuropterans (42.5% in Borriol and 50.5% in Moncófar) (**Table 3** and Supplementary material: **Table S1**). Spider density was significantly higher in ant-excluded trees than in ant-allowed trees ($\chi^2_{126} = 176$, $P = 0.011$). This effect was independent of orchard (ant-exclusion × orchard interaction: $\chi^2_{123} = 2.63$, $P = 0.1$) or year (ant-exclusion × year interaction: $\chi^2_{122} = 0.8$, $P = 0.37$) (**Table 3**). Spiders density was strongly affected by orchard ($\chi^2_{125} = 7.15$, $P =$

0.007), year (χ^2_{123} = 20.03, $P < 0.001$) and their interaction (χ^2_{123} = 7.24, $P = 0.009$). Likewise, neuropterans density was higher in ant-excluded trees than in ant-allowed trees ($F_{1,\,126} = 15.1$, $P < 0.001$). This effect was independent of orchard (ant-exclusion × orchard interaction: $F_{1,\,123} = 1.15$, $P = 0.29$) and year (ant-exclusion × year interaction: $F_{1,\,122} = 0.05$, $P = 0.82$) (**Table 3**). Neuropteran density was strongly affected by orchard ($F_{1,\,125} = 8.83$, $P = 0.004$), year ($F_{1,\,124} = 26.2$, $P < 0.001$) and their interaction ($F_{1,\,123} = 4.59$, $P = 0.034$).

Table 3. Mean number (± SE) of predators in citrus canopies of ant-allowed and ant-excluded trees in two citrus orchards from the main citrus producing area of Europe (eastern Spain), between May 2020 and November 2021, observed in 20 fruits and 40 leaves per tree.

		2020		**2021**	
Orchard	**Predator order**	**Ant-allowed**	**Ant-excluded**	**Ant-allowed**	**Ant-excluded**
Borriol	Araneae	1.06 ± 0.17	1.56 ± 0.3	3.13 ± 0.58	3.31 ± 0.45
	Neuroptera	1.13 ± 0.31	2.13 ± 0.4	2.88 ± 0.5	5.38 ± 0.85
	Coleoptera	0.63 ± 0.26	0.5 ± 0.2	0.25 ± 0.11	1.06 ± 0.25
	Hemiptera	0.38 ± 0.2	0.13 ± 0.09	0.31 ± 0.15	2.69 ± 1.84
	Diptera	0	0.06 ± 0.06	0.19 ± 0.14	0.06 ± 0.06
	Total predators	3.25 ± 0.44	4.44 ± 0.65	6.88 ± 0.97	12.5 ± 2.09
Moncófar	Araneae	1.06 ± 0.28	1.88 ± 0.34	1.25 ± 0.3	2.25 ± 0.32
	Neuroptera	1.25 ± 0.32	2 ± 0.35	2 ± 0.39	2.56 ± 0.36
	Coleoptera	0.13 ± 0.09	0.13 ± 0.09	0.56 ± 0.27	0.06 ± 0.06
	Hemiptera	0	0.06 ± 0.06	0	0.06 ± 0.06
	Diptera	0	0	0.13 ± 0.09	0.13 ± 0.13
	Total predators	2.44 ± 0.33	4.06 ± 0.54	3.94 ± 0.69	5.06 ± 0.45

Relative predator density was higher in ant-excluded trees than in ant-allowed trees ($F_{1,\,120} = 57.94$, $P < 0.001$). This effect was independent of orchard (ant-exclusion × orchard interaction: $F_{1,\,117} = 1.36$, $P = 0.25$), but was weakly affected by year (ant-exclusion × year interaction: $F_{1,\,115} = 3.94$, $P = 0.05$) (**Figure 2**). Relative predator density was not affected by orchard ($F_{1,\,119} = 1.89$, $P = 0.17$), but was affected by year ($F_{1,\,118} = 22.58$, $P < 0.001$) and the interaction between orchard and year ($F_{1,\,115} = 37.39$, $P < 0.001$).

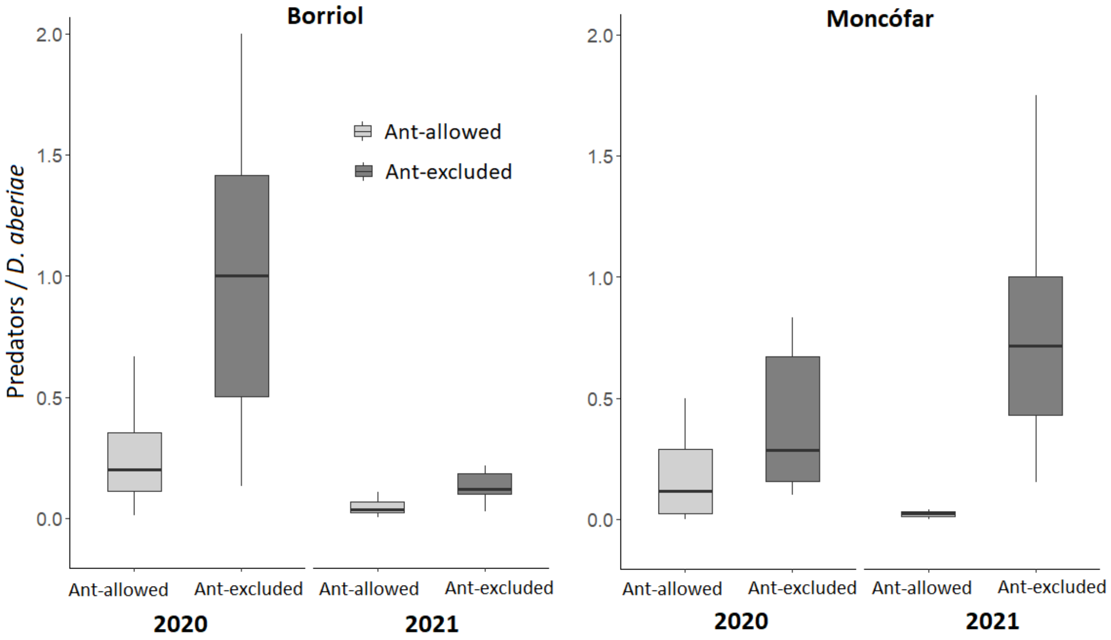


Figure 2. 'Relative predator density' (cumulative number of predators divided by cumulative number of *Delottococcus aberiae*) in ant-allowed and ant-excluded trees in two citrus orchards from the main citrus producing area of Europe (eastern Spain), between May 2020 and November 2021. Each rectangle represents the second and third quartiles and the inner horizontal line indicates the median value. The lower and upper quartiles are shown as vertical lines either side of the rectangle.

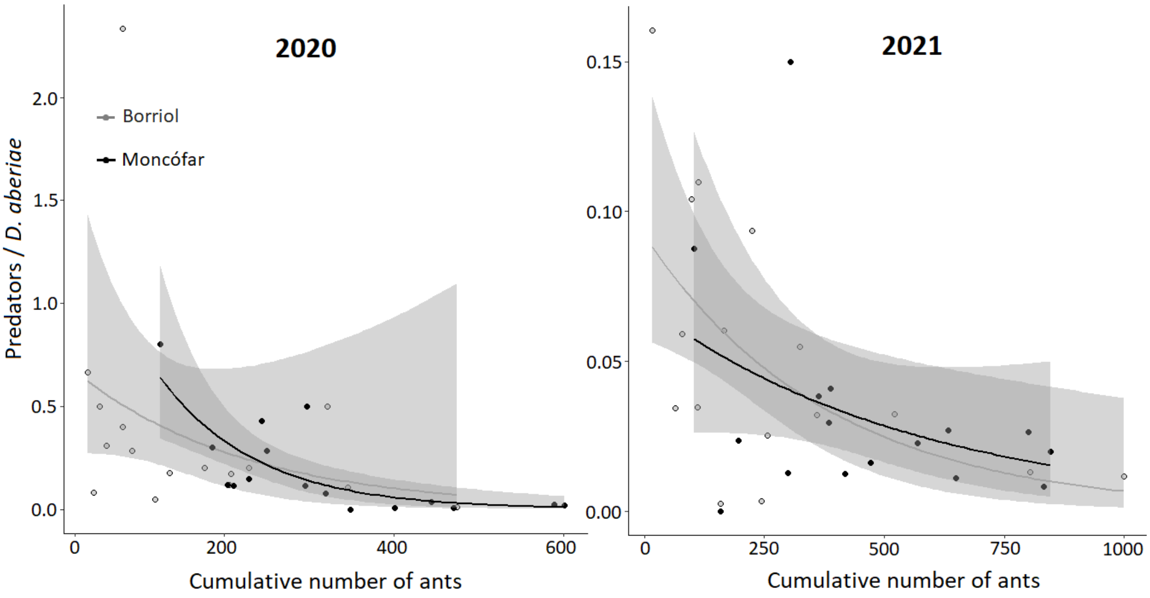


Figure 3. Cumulative number of ants to 'relative predator density' (cumulative number of predators divided by cumulative number of *D. aberiae*) in ant-allowed trees from two citrus orchards in the main citrus producing area of Europe (eastern Spain) in 2020 and 2021. Each point represents an ant-allowed tree. Generalized linear models based on quassipoisson distribution.

In ant-allowed trees, relative predator density decreased as the number of ants increased ($F_{1,\ 62} = 32.54$, $P < 0.001$). This effect was independent of orchard (ant-exclusion × orchard interaction: $F_{1,\ 59} = 0.21$, $P = 0.65$) or year (ant-exclusion × year interaction: $F_{1,\ 58} = 2.35$, $P = 0.13$) (**Figure 3**). Predator relative density was not affected by orchard ($F_{1,\ 61} = 0.27$, $P = 0.61$) but it was affected by year ($F_{1,\ 60} = 23.23$, $P < 0.001$), and this effect was independent of orchard (interaction orchard × year: $F_{1,\ 59} = 0.17$, $P = 0.67$).

Effect of ant-exclusion on *Delottococcus aberiae* density and fruit damage

The seasonal trend of *D. aberiae* was similar in both orchards and in both years. The mean number of *D. aberiae* per sample increased from May-June to September-October (**Figure 4a**). At Borriol, however, mealybug density remained very low throughout 2020.

In 2020, the average in-season density of mealybugs was lower in ant-excluded trees than in ant-allowed trees ($F_{1,\ 60} = 13.51$, $P < 0.001$). This effect was independent of orchard (ant-exclusion × orchard interaction: $F_{1,\ 60} = 1.94$, $P = 0.17$) (**Figure 4a**). In 2020, the average in-season density of the mealybugs was weakly affected by orchard ($F_{1,\ 60} = 3.88$, $P = 0.053$). Likewise, in 2021, the average in-season density of mealybugs was lower in ant-excluded trees than in ant-allowed trees ($F_{1,\ 60} = 24.96$, $P < 0.001$), and this effect was independent of orchard (ant-exclusion × orchard interaction: $F_{1,\ 60} = 0.25$, $P = 0.62$). In 2020, the average in-season density of the mealybug was strongly affected by orchard ($F_{1,\ 60} = 10.87$, $P = 0.0017$).

The ratio of damaged fruits at harvest was lower in ant-excluded trees than in ant-allowed trees ($F_{1,\ 126} = 15.97$, $P < 0.001$). This effect was independent of orchard (ant-exclusion × orchard interaction: $F_{1,\ 123} = 1.46$, $P = 0.23$) and year (ant-exclusion × year interaction: $F_{1,\ 122} = 0.12$, $P = 0.72$) (Fig. 4b). The ratio of damaged fruit was also strongly affected by orchard ($F_{1,\ 125} = 12.78$, $P < 0.001$), year ($F_{1,\ 124} = 11.97$, $P < 0.001$) and their interaction ($F_{1,\ 123} = 52.32$, $P < 0.001$).

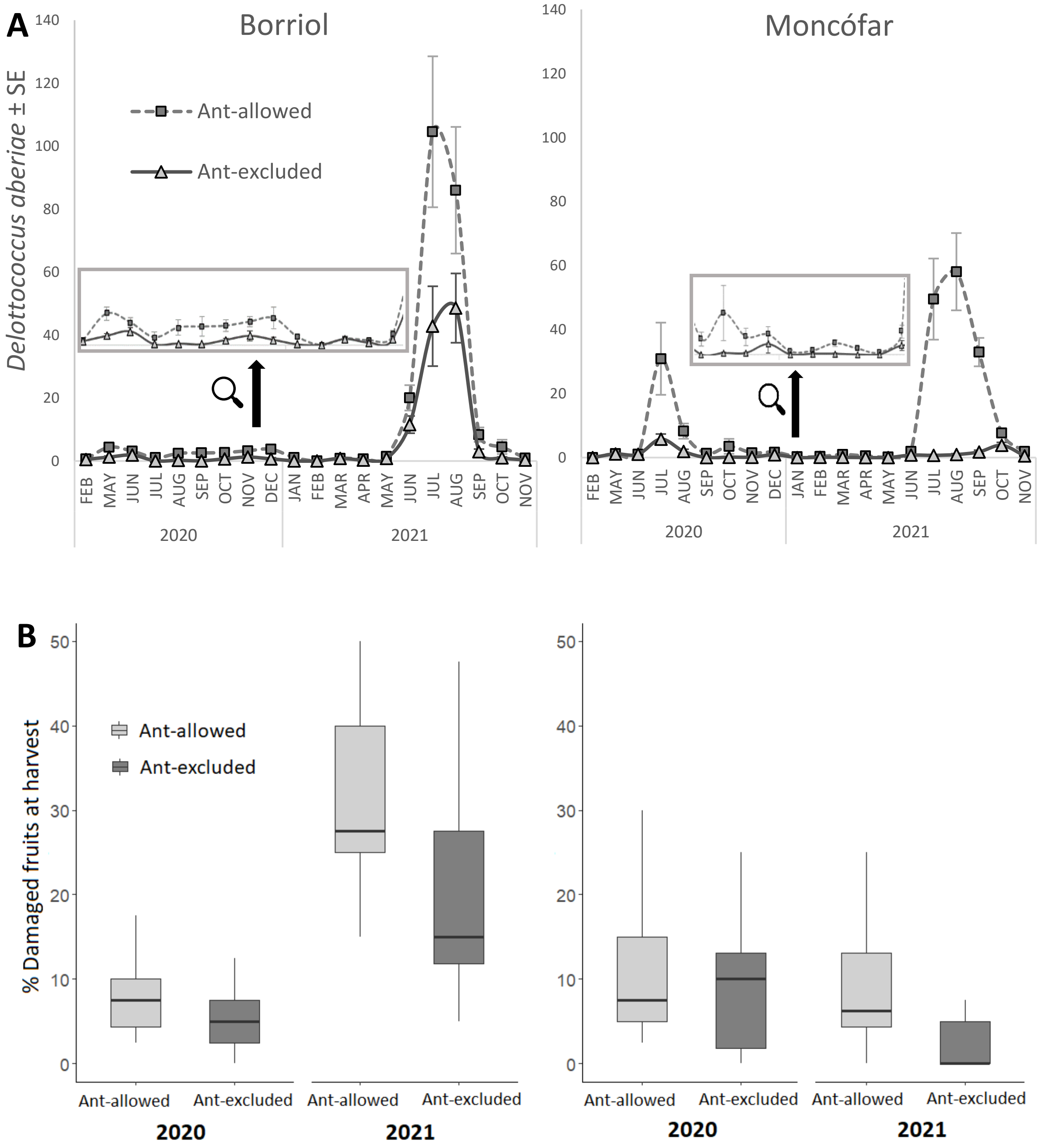


Figure 4. (A) Seasonal trend of *Delottococcus aberiae* in ant-excluded and ant-allowed trees. Represented by the number mealybugs (Mean ± SE) sampled in the canopy. (B) Ratio of damaged fruits at harvest in ant-allowed and ant-excluded trees. Each rectangle represents the second and third quartiles and the inner horizontal line indicates the median value. The lower and upper quartiles are shown as vertical lines either side of the rectangle.

4. Discussion

The Mediterranean ant *L. grandis* is the dominant ant species tending *D. aberiae* in citrus orchards (Plata et al., 2024a). This work demonstrated that *L. grandis* presence decreased the density of generalist predators, especially spiders and neuropterans, which may have benefited *D. aberiae*. Moreover, the physical exclusion of ants from citrus canopies reduced the density of the invasive mealybug *D. aberiae* in Mediterranean citrus crops. The decrease of *D. aberiae* density observed in ant-excluded trees can be due to the negative effects of ants on generalist predators but also to the effect of sticky barriers on mealybugs. Some mealybugs that climb down were trapped in the sticky barriers, increasing the mortality of adult mealybugs. Likewise, sticky barriers also prevented colonization by walking mealybugs infesting adjacent trees or by ants actively moving mealybugs to new feeding site.

In the two commercial citrus orchards sampled here, *L. grandis* represented more than the 95% of the ants attending *D. aberiae*. Ant-attendance and absolute ant-attendance (*i.e.*, number of ants per attended colony) indicated that approximately 30% of the mealybug colonies were attended by ants, with approximately 1.8 ants per tended mealybug colony (Plata et al., 2024a). The relative ant attendance (*i.e.*, number of ants per mealybug) was 0.4-0.7. These ant-attendance rates can be higher in orchards where mealybug density is lower (Plata et al., 2024a). Ant-attendance may decrease when mealybugs population outbreak because, beyond a certain number of honeydew producers, the number of foraging ants might not be enough to saturate honeydew sources (Cushman & Addicott, 1989). Although we observed that only one third of the mealybug colonies were attended by ants, we think that the presence of ants would have affected more than one third of the mealybug population for two reasons. Firstly, colonies of *D. aberiae* with high number of individuals are attended by ants more frequently than small colonies (Plata et al., 2024a). In the current study, 44% of the mealybugs were in ant-attended colonies. Secondly, mealybug colonies that were not attended during our observations between 10:00 and 14:00 can be attended at another time of the day, particularly at night, when ant-activity increases in Mediterranean citrus (Pekas et al., 2011). Some natural enemies can detect ants cuticular hydrocarbons within hemipteran colonies and avoid foraging in these colonies, thus mealybugs can benefit from ant-attendance even when ants are not present (Mouratidis et al., 2021).

Ant-exclusion reduced *D. aberiae* density and fruit damage at harvest. It is hypothesized that increased natural enemy activity was responsible for this observation. Additionally, tending ants might provide *D. aberiae* with other beneficial services such as transport to new feeding sites (Ho & Khoo, 1997), removal of excess honeydew (Queiroz & Oliveira, 2001), and sanitizing via removal of fungal contaminated-individuals (Nielsen et al., 2010). Furthermore, honeydew collecting ants can stimulate feeding and reproduction of some honeydew-producing hemipteran species and reduce developmental times (Morales, 2000; Xu et al., 2021). However, among the services that ants can provide to their trophobiont partners, the defense against natural enemies is the most important from a pest control perspective. Tending ants often attack mealybug natural enemies, decreasing their efficacy as biological control agents (*e.g.,* Daane et al., 2007; Mgocheki & Addison, 2009b; Nechols & Seibert 1985; Tanga et al., 2015). *Delottococcus aberiae* does not have effective native and naturalized parasitoids in Mediterranean citrus (Tena et al., 2017). For this reason, the parasitoid *Anagyrus aberiae* Guerrieri (Hymenoptera: Encyrtidae) has recently been imported and released in the invaded area of Valencia (Soto et al., 2021). The parasitoid was released in an adjacent orchard in Moncófar in 2020. However, no parasitized *D. aberiae* were detected in either of the two study orchards over the two years this project was conducted. Therefore, the potential disruption of *D. aberiae* biological control by ants is likely due to the negative effect of ants on predators.

Ant-exclusion increased the relative numbers of predators in both orchards and years. Previous studies in citrus have demonstrated that ant exclusion tends to increase the number of generalist predators, whereas specialists are generally not affected (Anjos et al., 2021; Calabuig et al., 2015). In our study, the most abundant orders of generalist predators were spiders and lacewings, and both benefited from ant-exclusion. Lacewings are important predators of mealybugs (Koutsoula et al., 2023; Tapajós et al., 2016), and we observed lacewings larvae preying frequently on *D. aberiae* in the field. Calabuig et al. (2015) observed that ants reduce lacewing abundance in citrus. This reduction could be due to the predation of lacewing eggs by ants and/or the protection of their potential prey (Morris et al., 1998). Spiders were frequently observed on mealybug colonies, however their role as biological control agents of mealybugs is less clear. While some species are known to prey on mealybugs (Carroll, 2013; Costello et al., 1995; Hajer & Hrubá, 2007;), other species may benefit mealybugs through intraguild predation (Dinter, 1998; Hambäck et al., 2021; Heimpel et al., 1997; Michalko et al., 2019). Spiders can be roughly divided into active hunters, ambush spiders, and web spiders. Among web spiders, some silk web spider species can prey on nymphs and adults of mealybugs, but their effect in the field has not been evaluated (Hajer & Hrubá, 2007). Silk web spiders are very abundant in Mediterranean citrus and we have observed some *D. aberiae* stuck in cobwebs (*e.g.,* therididae webs) or sheetwebs (*e.g.,* linyphidae webs). Among hunting and ambush spiders, some species have been observed eating mealybugs, (Carroll, 2013; Costello et al., 1995) but they can also feed on biological control agents (Dinter, 1998; Hambäck et al., 2021; Heimpel et al., 1997; Michalko et al., 2019). Therefore, further research should evaluate the effect of Mediterranean spiders on the biological control of mealybugs in citrus. Finally, the only specialist predator of mealybugs observed was *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae). This coccinellid species preys on *D. aberiae* and reduces its population density (Pérez-Rodríguez et al., 2019). *Cryptolaemus montrouzieri* abundance, however, was very low in both orchards (Supplementary material: **Table S1**), likely because this species is affected by insecticides that were used over the field experiment (Planes et al., 2013).

Ant-exclusion by sticky-barriers can have an additional effect on the control of *D. aberiae* because it prevents mealybug transit between soil (roots and adjacent trees) and citrus canopy. Nymphs and adults of *D. aberiae* usually moves across the citrus trunk (Martínez-Blay et al., 2018b). Adult females migrate from tree canopy to the soil in two periods, March-April and June-July, presumably for feeding or ovipositing on roots. We observed that sticky barriers trapped some of these adult females, increasing their mortality. Moreover, we also observed that some of these females were not trapped, but laid the ovisacs on the trunk, just above the sticky barrier. This behavior might be either positive or negative for mealybug management. While it might disrupt the hypothetical advantages that mealybugs obtain when they descend to the soil, it might also increase reinfestation of the canopy, reduce predation in soil (Pérez-Rodríguez et al., 2018). Moreover, these sticky-barriers may exclude non-flying predators such as earwigs (Piñol et al. 2009). Earwigs are important predators of aphids (Carroll & Hoyt, 1984; Romeu-Dalmau et al., 2012) and they can also prey on mealybugs (Navasero, 2006). We observed very low number of earwigs in our study (Supplementary material: **Table S1**), likely because these insects forage at night and hide during the day (Lamb, 1975). The other main group of wingless predators are spiders and, as explained above, we recorded a higher number in trees with sticky barriers. Overall, the impact of sticky barriers in this citrus ecosystem needs further study to better understand their effects on ground-canopy-ground movement of mealybugs and natural enemies over long periods (Mestre et al., 2013; Piñol et al. 2009). Night observations or digital video recording over multiple consecutive 24h periods would help to identify natural enemy species, their abundance and association and impacts on mealybug colonies at night (Grieshop et al., 2012; Kistner et al., 2017). These natural enemies can be specially affected by ants because they increase their activity at night in Mediterranean citrus (Pekas et al., 2011).

This study demonstrates that ant exclusion with sticky barriers attached to citrus trunks and installed at the end of winter, before ant activity began, reduced *D. aberiae* density and damage likely because ant-exclusion enhances the efficacy of generalist predators attacking this pest mealybug. However, this ant-exclusion method is laborious and expensive, and might have negative long-term effects on wingless predators that ascend trunks from the soil to search the citrus canopy for prey. Alternative control methods for suppressing sugar feeding ants need to be developed and made it commercially available for the citrus industry. These alternative methods to control ants should target only pest tending-ants because other ant species (mainly soil-dwelling ants) may have a positive role in controlling key citrus pests (Pérez-Rodríguez et al., 2021; Urbaneja et al., 2006). Other options that have been studied to control tending ants are micro-encapsulated insecticide paints (Juan-Blasco et al., 2011), liquid baits (Daane et al. 2008; McCalla et al. 2023; Rust et al., 2004; Samways, 1985), hydrogel baits (McCalla et al. 2020; Sunamura et al., 2022), or the provision of alternative liquid sugar sources to distract tending ants from honeydew-producing hemipterans (Offenberg, 2001; Parrilli et al., 2021; Wäckers et al., 2017). Further research should assess these more economically viable methodologies to reduce tending ants in order to enhance biological control of *D. aberiae* and decrease the level of crop damage caused by this mealybug.

Data accessibility

Data from this study are available at the IVIA Digital Repository:
https://doi.org/10.58582/redivia.8477

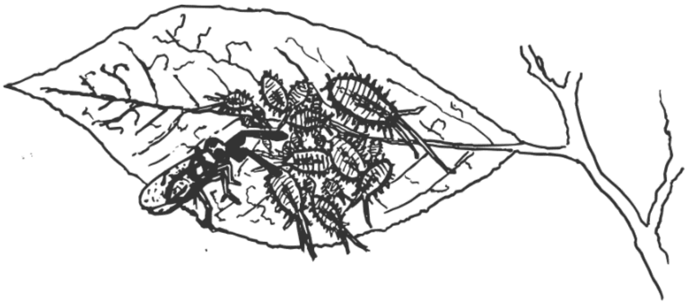
Supplementary material

Table S1. Number of predators sampled in the canopy in two citrus orchards located in the main citrus producing area of Europe (eastern Spain) between May 2020 and November 2021. The number of predators observed on *Delottococcus aberiae* colonies are represented in brackets. Percentages represent the proportion of each predator with respect to the total number of predators sampled in each orchard and year.

		Borriol		**Moncófar**	
Predator	**Preferred prey**	**2020**	**2021**	**2020**	**2021**
Araneae	Generalist	42 (4) 34.1%	103 (13) 33.2%	47 (3) 45.2%	56 (5) 38.9%
Neuroptera					
Chrysopidae	Generalist	46 (5) 37.4%	124 (8) 40%	50 (7) 48.1%	70 (7) 48.6%
Coniopterygidae	Generalist	6 (0) 4.9%	8 (1) 2.6%	2 (0) 1.9%	3 (0) 2.1%
Coleoptera					
Coccinellidae					
Propylea quatuordecimpunctata	Generalist	6 (0) 4.9%	3 (3) 1%	0	5 (0) 3.5%
Rodolia cardinalis	*Icerya purchasi*	12 (0) 9.8%	12 (0) 3.9%	1 (0) 1%	0
Cryptolaemus montrouzieri	Mealybugs	0	6 (4) 1.9%	3 (2) 2.9%	5 (4) 3.5%
Hemiptera					
Reduviidae					
Zelus renardii	Generalist	6 (0) 4.9%	37 (0) 11.9%	1 (0) 1%	1 (0) 0.7%
Miridae					
Pilophorus sp.	Generalist	2 (0) 1.6%	1 (0) 0.3%	0	0
Campyloneura virgula	Generalist	0	10 (3) 3.2%	0	0
Diptera					
Syrphidae					
Episyrphus balteatus	Aphids	1 (0) 0.8%	1 (0) 0.3%	0	2 (0) 1.4%
Cecidomyiidae					
Aphidoletes aphidimyza	Aphids	0	3 (0) 1%	0	2 (1) 1.4%
Dermaptera					
Forficulidae					
Forficula auricularia	Generalist	1 (0) 0.8%	0	0	0
Total predators		**123 (9)**	**310 (32)**	**104 (12)**	**144 (17)**

Section II

Improving the management of mealybugs in persimmon



Chapter 3

Mealybugs in Mediterranean persimmon: damage, seasonal trend and effect of climate change

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Abstract

Mealybugs (Hemiptera: Pseudococcidae) are the main pest of persimmon in Spain, the second producer in the world. In order to develop an Integrated Pest Management (IPM) program, it is necessary to identify the main mealybug species, determine their phenology, and develop tools to predict damage. To do this, we sampled 17 orchards from the main persimmon producing area in Spain over two years. *Pseudococcus longispinus* (Targioni-Tozzeti) was the most abundant and widely distributed species. This mealybug species completed three generations per year and reached peak density just before harvest. Fruit infestation at harvest was highly correlated with mealybug density in spring and summer. The estimated thermal constants to complete development and one generation were 512.5 and 956.3 degree days, respectively. Based on climate change predictions, crop damage caused by the third generation of *P. longispinus* will increase in 2040 and the mealybug will complete a fourth generation by 2080. *Pseudococcus longispinus* has become the main pest for Mediterranean persimmon and damage produced by this mealybug may be exacerbated by climate change. This work provides essential data to design a sampling protocol and determine intervention times and thresholds against this mealybug.

1. Introduction

Pest pressure tends to increase after the first years of planting a new crop in a new area (Panizzi & Correa-Ferreira, 1997). This trend has also occurred with the expansion of persimmon in Spain. The area dedicated to growing persimmons has increased sixfold in the last 25 years in this country, that has become the largest producer of persimmon in the Mediterranean basin and the second producer in the world (FAO, 2022). In the last decade, the density of some pests has increased, and mealybugs stand out among these pests (Fernandez-Zamudio et al., 2020; García-Martínez, 2019).

Mealybugs represent the main threat for Spanish persimmons. In 2021, farmers reported a 20-25% decrease in production that represented 40€ million losses due to the damage produced by mealybugs (ASAJA, 2021). The damage is caused by the honeydew excreted by mealybugs which supports the growth of sooty mold, depreciating the fruits (García-Martínez, 2019). Additionally, persimmon fruits can lose commercial value due to the mere presence of mealybug at harvest. The three most abundant mealybug species in Spanish persimmon orchards are *Pseudococcus longispinus* (Targioni-Tozzeti), *Pseudococcus viburni* (Signoret) and *Planococcus citri* (Risso) (García-Martínez et al., 2017; Prieto, 2016). The cryptic behavior of mealybugs and their difficult identification make their management problematic. In order to develop Integrated Pest Management (IPM) programs against mealybugs, it is important to identify the mealybug species that are currently causing the damage in the main persimmon producing area and to study their seasonal trend and phenology.

As with other insects, temperature is the driving abiotic factor for mealybug development, and can strongly alter mealybug phenology (Martínez-Ferrer et al., 2003; Walton et al., 2013). Although other abiotic factors such as humidity may also affect mealybug development, the magnitude of their effect seems to be lower than temperature (Raja et al., 2009). From the mid-20th century to the present, there has been a global increase in the average annual temperature and most models predict that greenhouse gas emissions will accelerate this warming during the 21st century (IPCC, 2014). The Mediterranean basin is especially susceptible to this phenomenon (IPCC, 2014; Zittis et al., 2019). Consequently, insects can be pervasively affected by climate change and the incidence of several insect pests can increase (Jactel et al., 2019; Skendžić et al., 2021). In the case of mealybugs, temperature increase can expand their distribution, decrease their generation time, and increase their fecundity and survival in winter (Fand et al., 2014; Jara et al., 2013; Ji et al., 2020). Therefore, it is necessary to study mealybug responses under the projected climate scenarios to predict future damages.

The aims of this work were to: i) determine the spatio-temporal dynamics of mealybug species in the main persimmon producing area of the Mediterranean basin; ii) determine seasonal trend, phenology, and fruit infestation of the main mealybug species; and, iii) predict the effect of climate change on mealybug phenology. To achieve the third aim, the thermal constant necessary to complete a generation for the main mealybug species identified was estimated under field conditions.

2. Material and methods

Spatio-temporal dynamics of mealybugs

Seventeen persimmon orchards located in Valencia, Spain, were selected (Figure 1). All persimmon orchards were managed under IPM guidelines, details of which are provided in Supplementary material: **Table S1**. Within each orchard, a plot consisting of 40 trees (8 × 5) was established. This standardized size was used because it was the maximum area that could be applied to all the orchards of our study (Table 1). From each 40-trees plot, nine alternate trees were sampled (Supplementary material: **Figure S1**) in spring (between May 10 and 22), summer (between July 14 and July 27) and early-autumn (between September 27 and October 10) across two consecutive years (2020 and 2021). For each tree and date, 120 leaves (30 per cardinal direction), 40 fruits (10 per cardinal direction) and the surface of the trunk (from the base to a 50 cm height) were surveyed. Fruits and leaves were randomly chosen from those closest to the ground up to a height of 2 meters and from the external and internal part of the tree, and the trunk was inspected for 30 seconds. All mealybugs were counted and identified to species using taxonomic keys (Miller & Giliomee, 2011; Williams & Granara, 1992). When necessary, insects were collected and transported to the laboratory for identification under stereo- or compound- (young nymphs of mealybugs) microscopy. All field observations were made between 9 a.m. and 4 p.m.

To study the seasonal trend of mealybugs, mealybug density was calculated for each mealybug species as the mean number of mealybugs (sum of mealybugs observed in 120 leaves, 40 fruits, and trunk) per orchard (mean of nine trees) and sampling date. To evaluate whether mealybug density was affected by season and year, a factorial generalized linear model (GLM) with Poisson distribution was used, with mealybug density (all mealybug species together) as the response variable and season (spring, summer and autumn) and year (2020 and 2021) as explanatory variables. Multiple comparisons to assess differences among seasons were based on Tukey's post hoc tests.

To study the geographical distribution of mealybugs, the relative abundance of each mealybug species for each orchard and year was calculated.

Fruit infestation of *P. longispinus*

To estimate the damage caused by the most abundant mealybug species, *P. longispinus*, the proportion of fruit infested by this mealybug at harvest was calculated. The proportion of fruit infested by mealybugs at harvest can provide a good estimation of damage because these fruits lose commercial value due to mealybug presence. This proportion was calculated for each orchard (mean of 9 trees; 40 fruits per tree) and year as the number of fruits infested by *P. longispinus* divided by the number of sampled fruits. To determine whether the proportion of fruits infested by *P. longispinus* at harvest (autumn) can be predicted with early season sampling, in spring or summer, two GLMs with binomial distribution were used. The proportion of fruits infested in autumn was the response variable, while the proportion of fruits infested in spring or summer were the explanatory variables. Year (2020 and 2021) was also included as fixed factor in both models. The assumed error structures were then assessed using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If an over- or an underdispersion was detected, we re-evaluated the significance of the explanatory variables using an *F*-test after re-scaling the statistical model by a Pearson's $\chi 2$ divided by the residual degrees of freedom. Significance was assessed by the change in deviance when a variable was removed from the model using a $\chi 2$ test with binomial error. Significant values are given for the minimal model, while the non-significant values are those obtained before we deleted the variable from the initial model (Crawley, 2007).

Seasonal trend and phenology of *P. longispinus*

An orchard that had high density of *P. longispinus* (39°48'12.1"N 0°09'58.7"W 15 m. a.s.l) was selected to study the seasonal trend and phenology of this mealybug species over 2021 and 2022. In this orchard, 16 trees were selected and sampled twice per month. The same trees were sampled over the two years. From each tree, 40 leaves (ten per cardinal direction), 20 fruits (five per cardinal direction) and the surface of the trunk (from the base to a 50 cm height) were sampled, and the number of *P. longispinus* was recorded.

The mean number of mealybugs per tree and sampling date was recorded, as well as the developmental instar of each mealybug individual. Adult males were rarely seen and were not considered. The phenology was described based on the proportion of each mealybug instar per sample, and it was recorded only when more than 10 mealybugs were observed per sampling date. The number of generations per year was estimated by counting the number of peaks of first instar nymphs (herein after "peak of nymphs").

Degree-days necessary to complete a generation and reach adulthood

Based on the calculated temperature thresholds for development under laboratory conditions (Costa et al., 2011; Gillani et al., 2011), the degree-day curve of *P. longispinus* for the two study years was determined. For this, the daily mean temperature recorded in the closest weather station was used (SIAR Carlet, 6.5 km from the sampled orchard). Each day, the number of degrees above the lower threshold temperature for development of *P. longispinus* (8ºC) was accumulated, up to 27ºC, which is the optimal development temperature (Costa et al., 2011; Gillani et al., 2011; **Figure S2**). The upper temperature threshold is unknown for *P. longispinus*, but it is known that is above 32ºC (Costa et al., 2011; Gillani et al., 2011). As daily mean temperature in the study area does not exceed this temperature in any of our models, it was assumed that the development rate decreased linearly above the optimum temperature. Therefore, when the daily mean temperature exceeded 27ºC, each degree above was subtracted from the optimal 19 degree-days (see supplementary material: **Figure S2** for details).

The number of accumulated degree-days from January 1st until the first peak of nymphs was determined for the two years (see **Figure 4**). January 1st was taken because the lowest temperatures in the area are reached in January (see supplementary material: **Figure S3**).

To calculate the degree-days necessary to complete a generation, the degree-days between consecutive peaks of nymphs within a year were calculated. Therefore, the degree-days between the first and second peaks of nymphs and between the second and the third peaks of nymphs for both years were calculated. Then, the mean for the four generations, two from each year, was calculated.

To calculate the degree-days necessary to reach adulthood, the number of accumulated degree-days between the peaks of nymphs and the subsequent peak of adult females was estimated. Then, the mean accumulated degree-days for four generations, two from each year, was calculated.

Effect of climate change on *P. longispinus* phenology

Different degree-day curves were generated with the temperature thresholds of *P. longispinus*, described in the previous section, but assuming changes in the daily mean temperature. Firstly, a degree-day curve that represents the current annual temperature trend was generated. To do this, the climate data from the last 10 years in the closest weather station was used (Carlet, 6.5 km from the sampled orchard; MAPAMA, 2022b). For each day, the average daily mean temperature in the last 10 years was calculated. Secondly, three additional curves were generated to simulate different scenarios. One degree-day curve was generated to simulate a recent past scenario (1972-1996), when this mealybug species was not problematic to Spanish persimmon. This curve was calculated with a daily mean temperature 1.1ºC below the current temperature (*i.e.*, each day was considered 1.1 ºC cooler than the average daily mean temperature in the last 10 years). This 1.1ºC was the difference between the annual average temperature of the last 25 years (1997-2021) and the previous 25-year series (1972-1996) from the closest station recording temperatures in 1972. This was the Meteorological station #8416 of Valencia, 35 km from the sampled orchard (MAPAMA, 2022c).

Then, two degree-day curves with the most probable scenarios of temperature in the short-medium (2040-2060) and medium-long term (2080-2100) were generated. The curves were based on the predictions of the IPCC 2014 for the Mediterranean basin: an increase of ~1.5ºC for the period 2040-2060 and ~3ºC for 2080-2100 (IPCC, 2014; Zittis et al., 2019). As the models agree that the temperature will increase more in summer than in winter in the Mediterranean area (Cos et al., 2022), the 2040-2060 curve was calculated with a daily mean temperature 1ºC above the current temperature from December to February; +1.5ºC from March to May; +2ºC from June to August; and +1.5ºC from September to November. The curve for the 2080-2100 scenario was based on the predictions of the IPCC 2014 for the Mediterranean basin (an increase of ~3ºC) (IPCC, 2014; Zittis et al., 2019). The 2080-2100 curve was calculated with a daily mean temperature 2ºC above the current temperature from December to February; +3ºC from March to May; +4ºC from June to August; and +3ºC from September to November.

Based on the number of degree-days necessary to reach the first peak of nymphs, to complete a generation and to reach adulthood under field conditions, which had been previously estimated, the number of generations per year and when they would occur under the three climate scenarios were estimated.

3. Results

Spatio-temporal dynamics of mealybugs

To study the geographical distribution and seasonal trend of the main mealybug species, 17 persimmon orchards were sampled in spring, summer and autumn of 2020 and 2021 . A total of 66,065 mealybugs were counted and identified during the two years, 20,465 in 2020 and 45,600 in 2021. Of these mealybugs, 131 were observed on trunk (0.2%), 15,855 (24%) on leaves and 50,079 (75.8%) on fruit. Mealybug density, measured as mean number of mealybugs per tree per orchard, was higher in 2021 than in 2020 ($F_{1, 98} = 5.17$, $P = 0.025$) and was strongly affected by season ($F_{2, 99} = 6.6$, $P < 0.001$), independently of the year (Interaction season × year: $F_{2, 96} = 0.26$, $P = 0.77$) (**Figure 1**). Mealybug density was significantly higher in summer than in spring, and in autumn than in summer.

Three species of mealybugs were identified: *P. longispinus*, *P. viburni* and *P. citri*. *Pseudococcus longispinus* was the most abundant species over the two-year study: 88.8% of the sampled individuals in 2020 and 98.2% in 2021. Moreover, *P. longispinus* was also the species with the widest distribution. It was present in 14 and 16 out of the 17 sampled orchards in 2020 and 2021, respectively (**Figure 2**). *Pseudococcus viburni* was the second species in relative abundance (8.5% in 2020 and 1.4% in 2021). In both years, it was present across all seasons. *Pseudococcus viburni* was observed in nine and eight orchards in 2020 and 2021, respectively, but two of these orchards had very low abundance. *Planococcus citri* was present only in summer and autumn in both years and observed in seven orchards, but with low relative abundance (2.6% in 2020 and 0.4% in 2021).

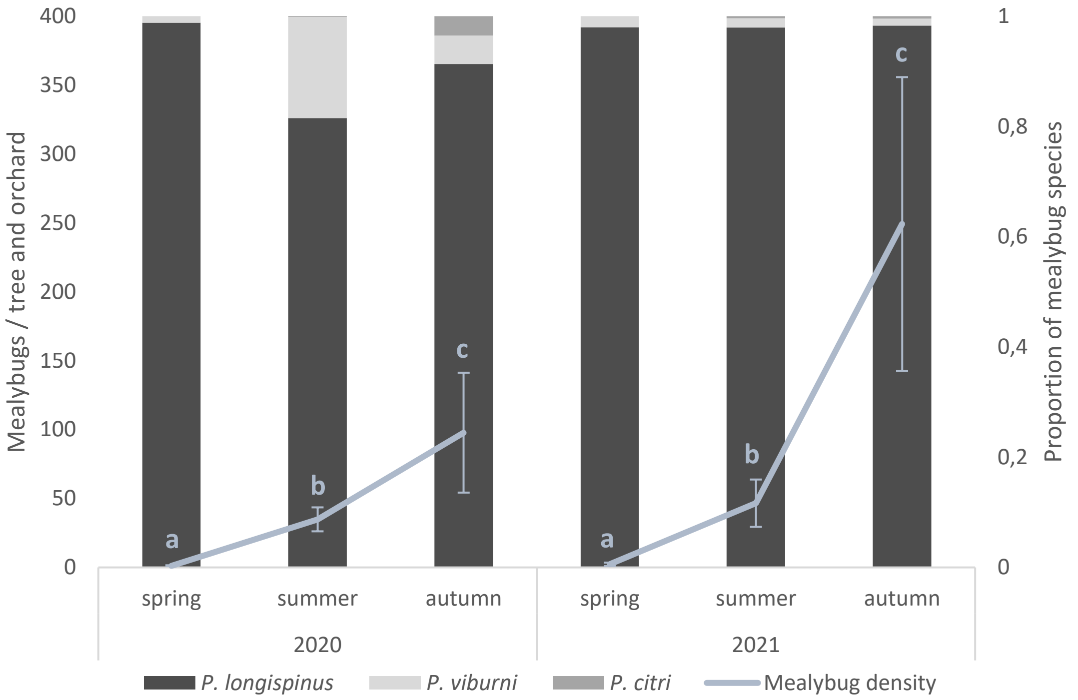

Figure 1. Seasonal mealybug density in the 17 persimmon orchards in Valencia during 2020 and 2021. Presented as the mean number of mealybugs sampled per tree per orchard (± SE). Different letters represent significant differences between the mean densities of mealybugs in the three seasons of each year (Tukey test, $P < 0.05$). Bars represent the seasonal relative abundance of each mealybug species.

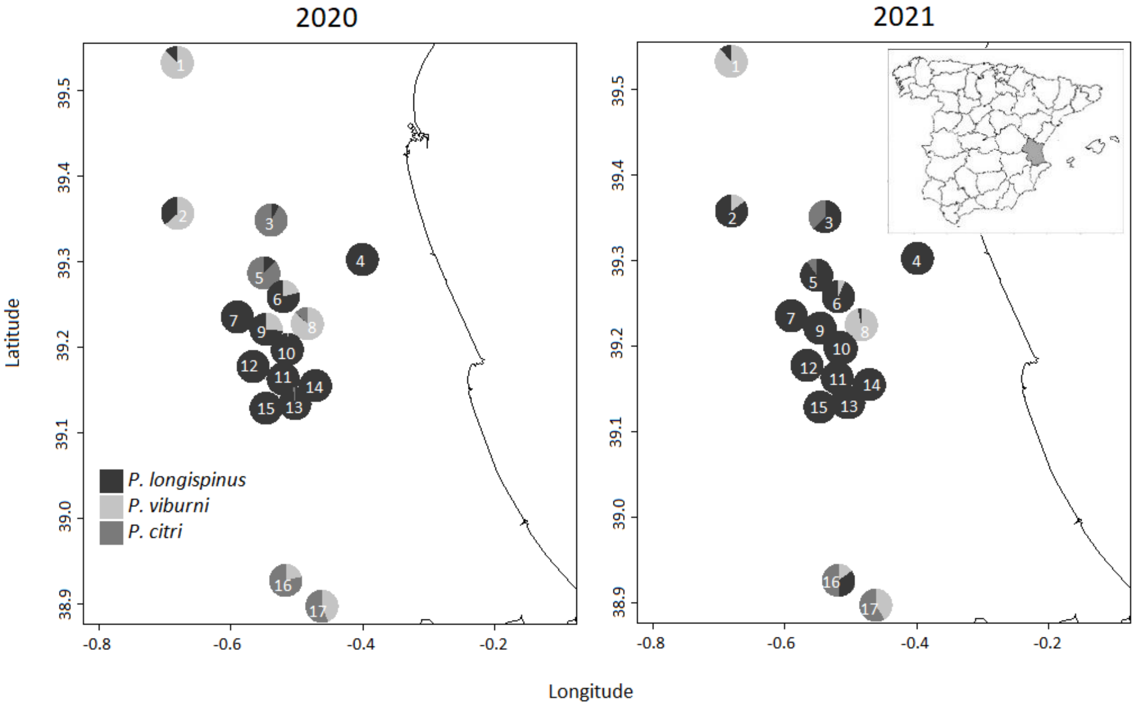

Figure 2. Complex of mealybug species in the 17 persimmon orchards sampled in the main persimmon producing area of the Mediterranean basin (eastern Spain). The different shades represent the relative abundance of each mealybug species in each orchard and year (including the three sampling seasons: spring, summer and autumn). Geographical reference system: WGS84.

Fruit infestation of *P. longispinus*

The proportion of fruit infested by *P. longispinus* was calculated in spring, summer and autumn of 2020 and 2021. These data were used to determine whether the infestation at harvest (autumn) can be predicted with the infestation levels in spring or summer. The proportion of fruit infested by *P. longispinus* per tree and orchard in spring averaged 0.017 ± 0.006 in 2020 and 0.03 ± 0.015 in 2021. In summer, this proportion increased to 0.067 ± 0.048 in 2020 and 0.217 ± 0.064 in 2021. During the harvest period in autumn, the proportion of fruit infested by *P. longispinus* averaged 0.206 ± 0.053 in 2020 and 0.341 ± 0.093 in 2021.

The proportion of fruit infested by *P. longispinus* in autumn was positively correlated with the proportion in spring ($F_{1, 32} = 9.98$, $P < 0.001$), without a significant effect of the year ($F_{1, 31} = 1.44$, $P = 0.24$) nor a significant interaction ($F_{1, 30} = 0.39$, $P = 0.54$). The model explained 43.8% of the total deviance (**Figure 3A** and **Table S2**). Likewise, the proportion of fruit infested by *P. longispinus* in autumn was positively correlated with this proportion in summer ($F_{1, 32} = 60.18$, $P < 0.001$), without a significant effect of the year ($F_{1, 31} = 2.4$, $P = 0.13$) nor a significant interaction ($F_{1, 30} = 0.57$, $P = 0.46$). The model explained 65% of the total deviance (**Figure 3B** and **Table S2**).

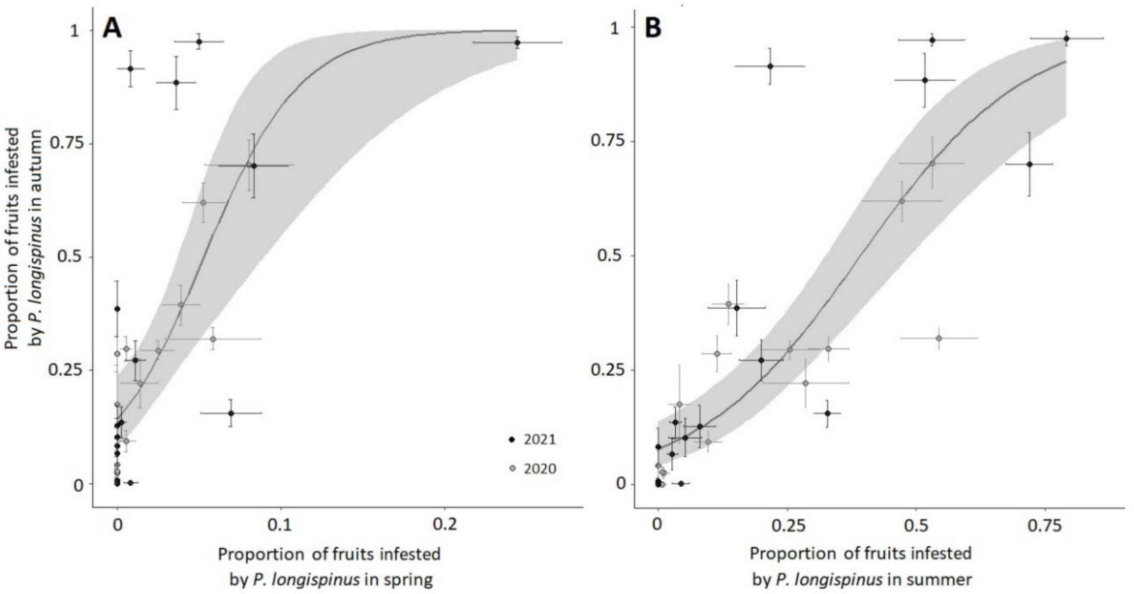

Figure 3. The proportion of fruit infested by *P. longispinus* in spring (A) and in summer (B) to the proportion of fruit infested by *P. longispinus* in autumn. Each point represents the mean (± SE) proportion per tree and orchard in 2020 and 2021. Tree was the sampling unit for each orchard and there were nine trees per orchard. Lines represent the generalized linear models (GLMs) with binomial distribution. The year was not included as a factor in the model as neither the year nor its interaction with season were significant.

Density and phenology of *P. longispinus*

To describe the density and phenology of *P. longispinus* in detail, a persimmon orchard was sampled twice per month in 2021 and 2022. *Pseudococcus longispinus* density was extremely low during the winter (**Figure 4**). The few individuals observed between December and May were mostly adult females hidden under the bark of the trunk. At the beginning of May, when persimmons are setting, female adults of *P. longispinus* were observed moving towards the tree canopy and settling under the sepals of the newly set fruits. Then, *P. longispinus* density increased exponentially until harvest, reaching a maximum in October. In 2022, there was a slight decrease in *P. longispinus* density during mid-August, but then it grew exponentially again until October.

Three discrete generations of *P. longispinus* were observed per year, as shown by peaks of nymphs (**Figure 4**). The first peak of nymphs was observed in mid-June of both years, with a proportion of first nymph instars of 0.64 in 2021 and 0.76 in 2022. The second peak of nymphs was observed in mid-August, when the proportion of first instar nymphs was 0.62 in 2021, and 0.49 in 2022. During 2022, the second peak of nymphs was taken as the intermediate date between two consecutive sampling dates, as the proportion of first instar nymphs and adults was similar on both dates. The third peak of nymphs was observed in early October both years, with a proportion of first instar nymphs of 0.73 in 2021, and 0.5 in 2022. The highest density of *P. longispinus* was reached at this third peak of nymphs both years (**Figure 4**).

Degree-days necessary to complete a generation and reach adulthood

The degree-days necessary to complete a generation and reach adulthood were calculated using the previous data. The first peak of nymphs was reached at 1176.7 and 1166.7 degree-days in 2021 and 2022, respectively (**Figure 4**). Therefore, the mean degree-days (herein after "DD") for the first peak of nymphs was 1171.7 ± 5 DD. In 2021, the second and third peaks of nymphs were reached at 2112.4 and 3081.8 DD (**Figure 4A**). In 2022, these peaks were reached at 2116.2 and 3086.9 DD (**Figure 4B**). Therefore, in 2021 there were 935.7 DD and 969.4 DD between peaks of nymphs, while in 2022 there were 949.5 DD and 970.7 DD between these peaks. These values averaged a thermal constant of 956.3 ± 8.4 DD to complete each generation.

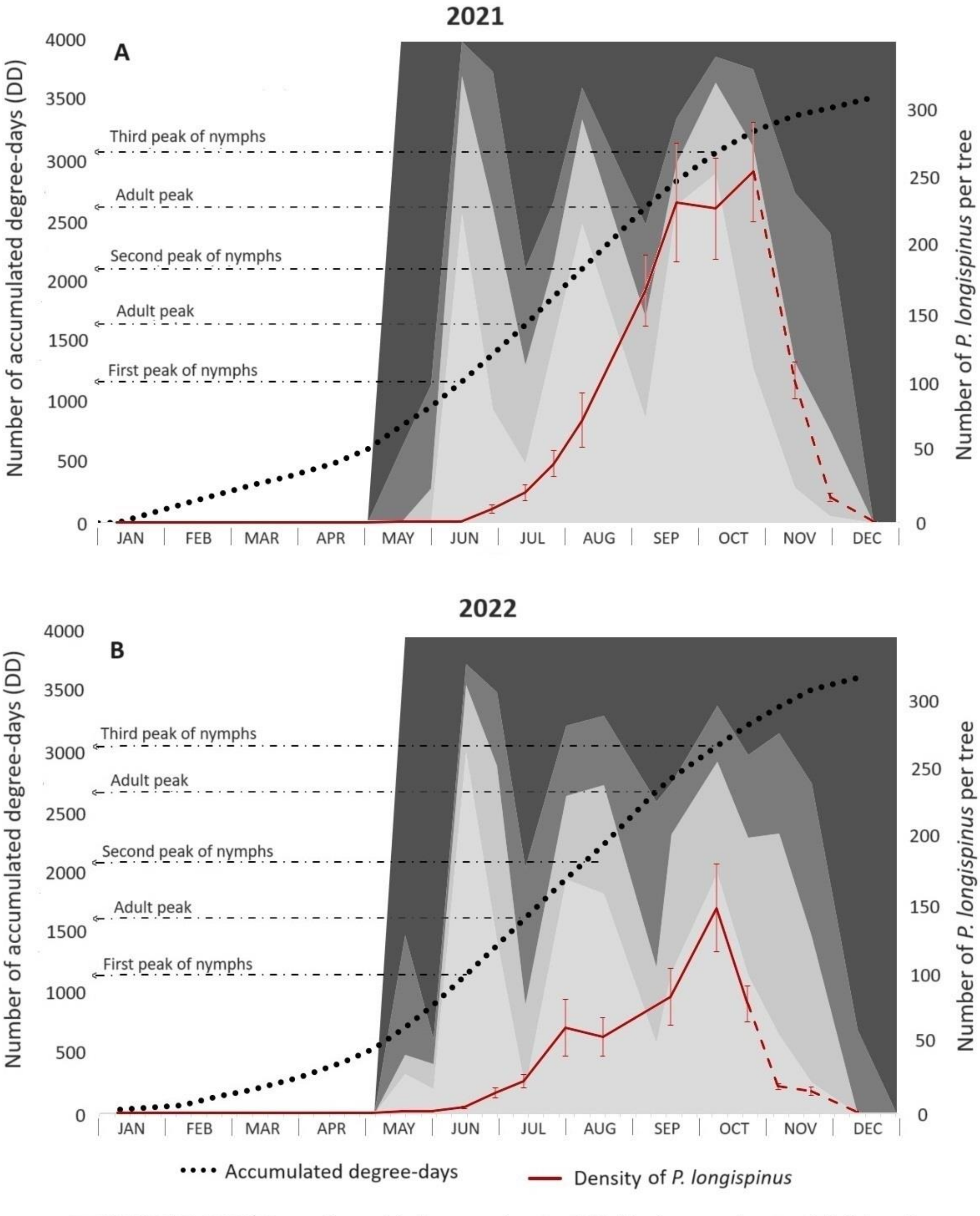

Figure 4. Red lines represent the seasonal density of *Pseudococcus longispinus* in the persimmon orchard throughout 2021 and 2022, represented by the number of mealybugs per tree (Mean of 16 trees ± SE). The trunk surface, 40 leaves and 20 fruits were sampled in each tree. The dashed line represents the trend after fruit harvest, when only the trunk and leaves were sampled because the trees had no fruit. Grey scale represent the seasonal phenology of *Pseudococcus longispinus* in the persimmon orchard throughout 2021 (A) and 2022 (B). The black dotted line represents the number of accumulated degree-days as of January 1st, considering the temperature thresholds for development of *P. longispinus*. Dashed horizontal lines represent the number of accumulated degree-days when the different peaks of first instar nymphs and adult females were reached.

In 2021, the first peak of adult females was reached at 1651.1 DD and the second at 2626.1 D (**Figure 4A**). In 2022, these peaks were reached at 1644 DD and 2702.2 DD, respectively (**Figure 4B**). Therefore, there were 474.4 and 512.3 DD between the peaks of nymphs and the subsequent peaks of adults in 2021, and 477.3 and 586 DD in 2022. These values averaged a thermal constant of 512.5 ± 26 DD to reach adulthood.

Effect of climate change on *P. longispinus* phenology

To evaluate the effect of climate change on *P. longpispinus* phenology, we generated three models. These models represent the temperature between 1972-1996, when *P. longispinus* was not considered a pest in persimmon, and the most probable scenarios of temperature in the short-medium (2040-2060) and medium-long term (2080-2100) based on the predictions of the IPCC 2014 for the Mediterranean basin.

1972-1996 scenario

The generated model shows that *P. longispinus* would reach the two first nymphal peaks approximately two weeks later under the 1973-1996 scenario (**Figure 5**; blue line vs. black line). The second generation would reach adulthood 20 days later (in late September instead of early September). The third peak of nymphs would be 40 days later, in November instead of October. This third generation would not reach the adulthood.

2040-2060 scenario

The generated model shows that *P. longispinus* will reach the three nymphal peaks approximately two weeks earlier under the 2040-2060 scenario (**Figure 5**; orange line vs. black line). The second generation will reach the adulthood 12 days earlier. The third generation will reach the adulthood 56 days earlier, in late October instead of late December.

2080-2100 scenario

The generated model shows that *P. longispinus* will reach the three nymphal peaks approximately three weeks earlier under 2080-2100 scenario (**Figure 5**; red line vs. black line). The second generation will reach adulthood 18 days earlier. The third generation will reach adulthood 70 days earlier (early October instead of late December). A fourth peak of nymphs will occur in November, so there will be one more generation constant of 512.5 ± 26 DD to reach adulthood.

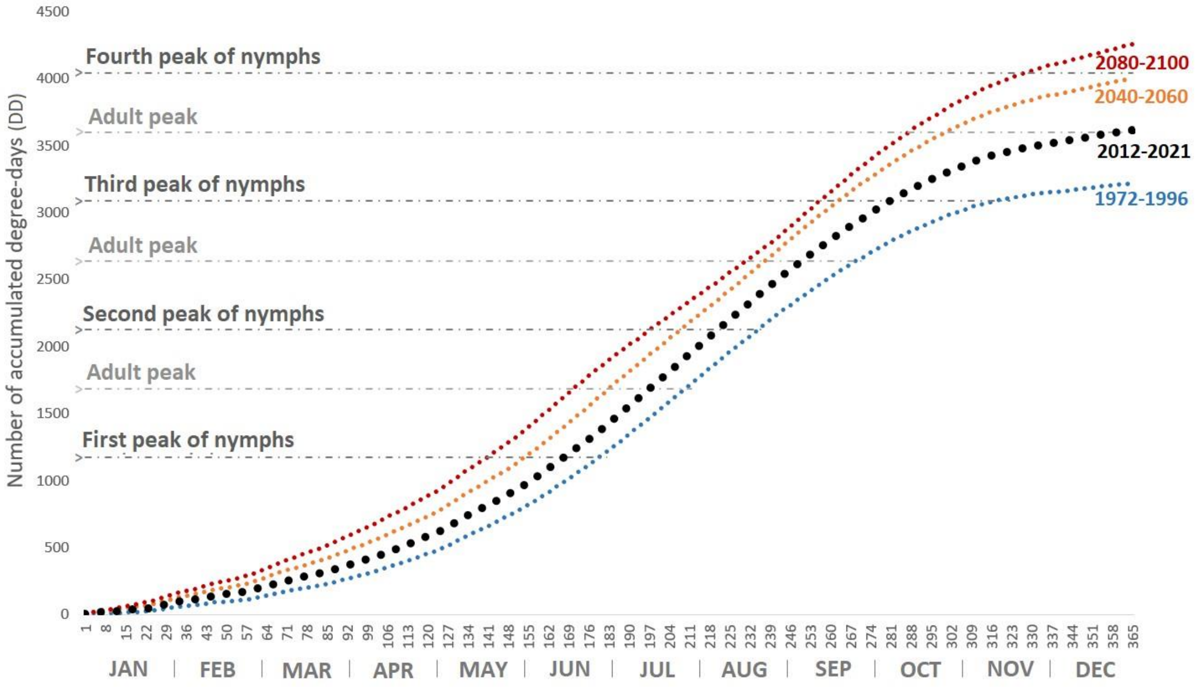

Figure 5. Potential effects of climate change on *Pseudococcus longispinus* phenology. The black dotted line represents the number of accumulated degree-days under the current temperature trend (2012-2021 daily mean temperatures). The blue dotted line represents the number of accumulated degree-days under the recent past scenario (1972-1996). The orange and red dotted lines represent the number of accumulated degree-days under the predicted scenarios in 2040-2060 and 2080-2100. The dashed horizontal lines represent the thermal constants (number of accumulated degree-days) calculated for *P. longispinus* to reach the nymphal and adult peaks in persimmon. The intersection between the dashed lines and the different dotted lines represents the day of the year in which *P. longispinus* would reach nymphal and adult peaks under the different scenarios.

4. Discussion

The number and density of pests has increased in persimmon after its expansion within the Mediterranean basin. This work demonstrates that *P. longispinus* was the main mealybug species in the main Mediterranean persimmon-producing area, and can cause high crop damage. In this manuscript, we have described the fruit infestation levels, the seasonal trend and the phenology of *P. longispinus*. We have also related these observations with current and expected temperature in the coming years under global warming. Finally, we used these data to suggest potential sampling protocols and management strategies against *P. longispinus* in persimmon.

The long-tailed mealybug *P. longispinus* was the most abundant and widely distributed mealybug species across the 17 persimmon orchards sampled in our two-year study. Previous studies had shown that *P. longispinus*, *P. viburni* and *P. citri* were equally abundant in this area until 2015 (García-Martínez et al., 2017; Prieto, 2016). Our results showed that *P. longispinus* has become the predominant mealybug species. In our study, more than 20% of the fruit was infested by *P. longispinus* at harvest and this percentage increased from 2020 to 2021. The second year of the study, four of the 17 sampled orchards exceeded 80% of fruit infested by this mealybug at harvest. These infestation levels of *P. longispinus* are strongly higher than those reported five years earlier (García-Martínez, 2019). Several factors could explain why *P. longispinus* has become the most abundant and damaging mealybug species in persimmon. Below, we discuss the effect of temperature and climate warming on the phenology and density of this mealybug.

Pseudococcus longispinus completed three annual generations per year in persimmon and reached the maximum density in the third generation that occurred in autumn, when persimmons are harvested. Field studies from other crops and countries with Mediterranean climate also observed three annual generations for *P. longispinus* (Charles, 1981; Furness, 1976;). The thermal constants estimated in the present study could be useful to predict the peaks of young nymphs of *P. longispinus*, which are susceptible to insecticides. Many active ingredients have low efficacy against adult females because they are protected by wax (Ulusoy et al., 2022). If it is necessary to spray with insecticides, we recommend spraying against the first nymphal peak (1200 DD from January 1st) because persimmon fruit is not fully developed, and mealybug developmental stages are more exposed and susceptible to insecticides. Another strategy to improve the control of *P. longispinus* could be the release of natural enemies. The main natural enemy of *P. longispinus* in Mediterranean persimmon is the encyrtid parasitoid *Anagyrus fusciventris* (Plata et al., 2023a; 2023b). This species parasitizes third instar nymphs and adult females of *P. longispinus*. Therefore, the optimal times for inoculative or inundative releases of this parasitoid would be when the proportion of adults is high (1650 and 2650 DD from January 1st).

To calculate the intervention thresholds against *P. longispinus*, it is first necessary to develop a sampling protocol. Our results demonstrate that fruit infestation at harvest can be predicted at the end of spring or mid-summer. Therefore, we suggest a binomial sampling based on direct observations of persimmon fruit in late spring (June) and repeat it in August, when the deviance explained by our model was higher. Monitoring the presence/absence of mealybugs by direct observation of fruit has been widely used in IPM programs of other mealybug species and in other crops (Pérez-Rodríguez et al., 2017). For Mediterranean persimmon, economic and environmental cost analysis must next be carried out to determine intervention thresholds.

Our models suggest that crop damage caused by the third generation of *P. longispinus* will increase in 2040 and the mealybug will complete a fourth generation by 2080 under the predicted climate models. Under the assumption of 1.5ºC increase in the annual average temperature, predicted in the short-medium term (2040-2060), our models showed that the third generation will reach adulthood two months earlier, in late October instead of late December, so it would develop and cause damage (honeydew excretion) before harvest. Under the assumption of 3ºC increase in the annual average temperature, predicted by the end of the century (2080-2100), *P. longispinus* will complete one more generation and a fourth peak of nymphs will occur in November. On the other hand, although the predicted average daily mean temperature does not exceed 32ºC (Figure 2), a high level of unpredictability is expected regarding heatwave events. *Pseudococcus longispinus* tolerates high temperatures (up to 39ºC) for a limited time (Hollingsworth & Armstrong, 2005), but prolonged periods above 36ºC can increase mortality of *P. longispinus* nymphs (Costa et al., 2011). Our models also show that the temperature increase over the two hottest months, July and August, will slightly slow down *P. longispinus* development by exceeding its optimum temperature for development (Fig. 6). The effect of climate warming on mealybugs have been studied for several species (Fand et al., 2014; Jara et al., 2013; Ji et al., 2020). However, this is the first time that this question is addressed for *P. longispinus*. This mealybug species is present in most regions of the world and can affect a wide variety of crops. Our findings showed that climate warming may be a key factor causing outbreaks of *P. longispinus* and facilitating its pest status in some crops from temperate ecosystems such as the Mediterranean basin. Further research should evaluate how the effect of climate warming on *P. longispinus* can be modulated by the interactions between the mealybug, its natural enemies, and mutualistic ants, as temperature related biological and behavioral changes of these organisms can be contrasting (Gutierrez et al., 2008; Zhou et al., 2017).

In conclusion, *P. longispinus* has become the most damaging pest in the main persimmon-producing area of the Mediterranean basin. The abundance and damage produced by *P. longispinus* may increase in the coming years because it might have a fourth generation due to rising temperatures. There is, therefore, an urgent need to develop an IPM program against this mealybug. Essential data to design a sampling protocol and determine intervention thresholds are provided in this manuscript. Our data show that fruit infestation at harvest can be predicted by sampling fruit infestation in late spring or mid-summer. The critical times to: i) spray insecticides against insecticide-susceptible instars; and ii) release the parasitoid *A. fusciventris* have also been determined. Further research should identify and evaluate the efficacy of this parasitoid in Mediterranean persimmon orchards, as well as the potential mutualistic relationship between *P. longispinus* and ants.

Data accessibility

Data from this study are available at the IVIA Digital Repository:
https://doi.org/10.58582/redivia.8674

Supplementary material

Figure S1. Plot of 40 trees selected in each orchard, the trees in black were sampled in spring, summer and autumn 2020 and 2021.

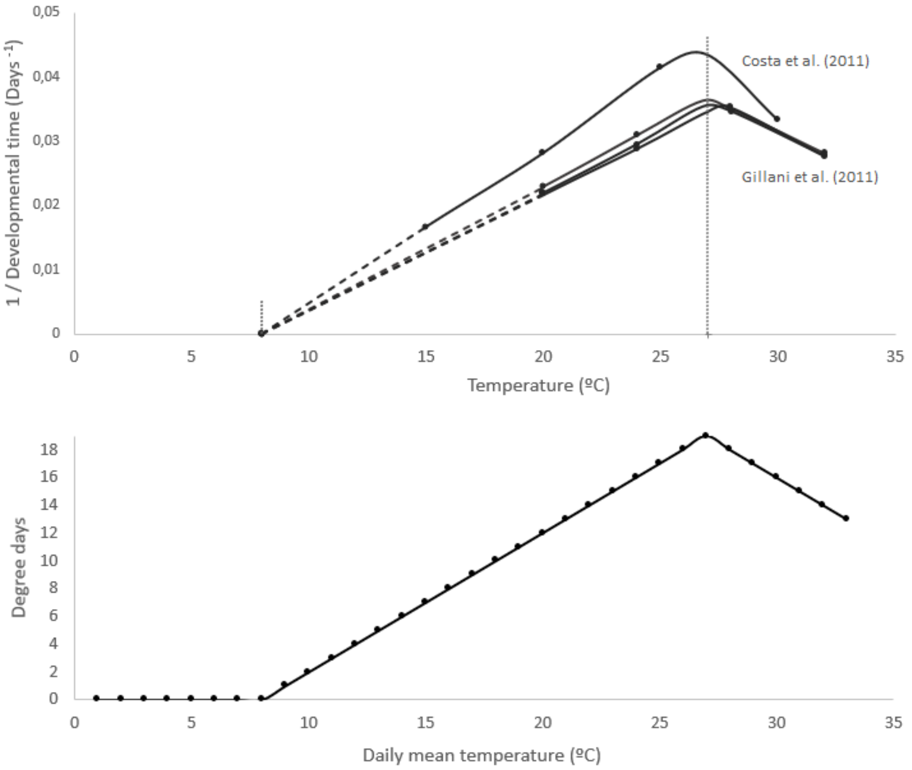

Figure S2. Top graph: Speed of development (1/ Time to reach adulthood) of *P. longispinus* at different temperatures on different plants. Based on laboratory tests carried out by Costa et al. (2011) and Gillani (2011). 8ºC is the minimum temperature for development and 27ºC is the optimum temperature for development. Bottom graph: Number of accumulated degree-days each day according to the daily mean temperature in our experimental orchard.

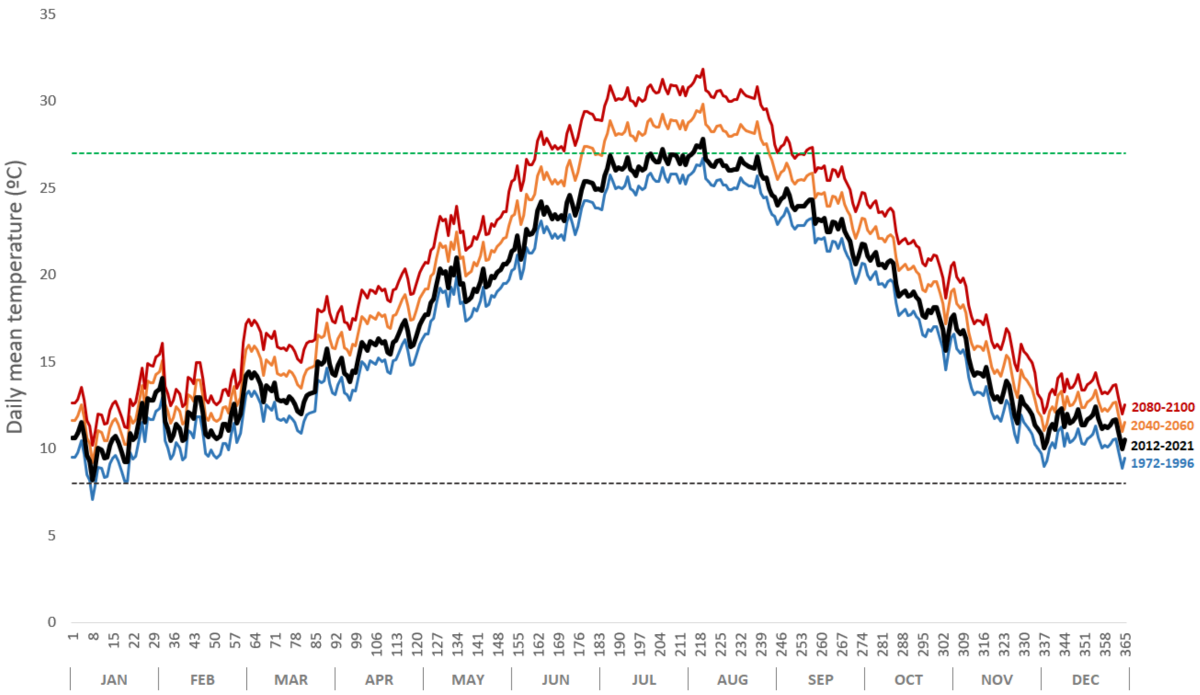

Figure S3. Black line represents the average daily mean temperature during the last 10 years (2012-2021) in the closest weather station to the sampled orchard (SIAR Carlet, 6.5 kms from the sampled orchard). Blue line represents the daily mean temperature under a recent past scenario (1972-1996). Orange and red lines represent the daily mean temperature under the predicted scenarios in 2040-2060 and 2080-2100. Grey dashed line represent the threshold temperature for development (8ºC) and green dashed line represents the optimum temperature for development (27ºC) of *P. longispinus*.

Table S1. Agronomic characteristics of the 17 persimmon orchards from the main persimmon producing area of the Mediterranean basin (eastern Spain) sampled in 2020 and 2021. Orchards were drip irrigated, the persimmon variety was 'Rojo brillante' and farmers followed the IPM guidelines in all the orchards (http://gipcaqui.ivia.es/).

Orchard	Altitude (m a.s.l)	Orchard size (ha)	Crop age (years)	Green cover	Surrounding landscape
1	178	3.64	10	Medium	Citrus/Forest/Persimmon /Other crops
2	180	1.46	10	Medium	Citrus/Persimmon/Forest
3	151	0.29	7	Very low	Citrus/Persimmon/Other crops
4	13	0.23	17	None	Citrus/Persimmon /Vegetables/Rice
5	105	0.42	6	Low	Citrus/Persimmon/Gardens
6	63	0.46	17	High	Citrus/Persimmon
7	113	0.56	6	Medium	Persimmon/Citrus
8	42	0.31	11	Medium-Low	Persimmon/Citrus
9	61	0.3	14	Medium	Persimmon/Citrus/Gardens
10	40	0.31	23	Low	Persimmon/Citrus
11	35	0.19	42	Very low	Persimmon/Citrus/Vegetables
12	49	0.91	25	None	Persimmon/Citrus
13	31	0.28	14	None	Persimmon/Citrus
14	26	0.42	10	None	Persimmon/Citrus
15	36	0.48	22	None	Persimmon/Citrus
16	204	0.52	15	High	Other crops/Persimmon
17	195	1.94	15	Very low	Other crops/Persimmon

Table S2. Values of the generalized linear models (GLMs) based on binomial distribution that correlate the proportion of fruit infested by *P. longispinus* in spring (A) and in summer (B) to the proportion of fruit infested by *P. longispinus* in autumn. Significant *P* values are given for the minimal model, while the non-significant *P* values are those obtained before we deleted the variable from the initial model.

A	**Proportion of fruit infested by *P. longispinus* in autumn**		
Variable	Parameter stimate	*t*	*P*
Intercept	**-1.8**	**-5.58**	**<0.001**
Proportion of fruit infested in spring	**33.9**	**3.85**	**<0.001**
Year			
2020	0.0	-	-
2021	-0.58	1.17	0.25
Proportion of fruit infested in spring × Year			
2020	0.0	-	-
2021	-10.8	-0.62	0.54
Null deviance: 17.76 Residual deviance: 9.98			

B	**Proportion of fruit infested by *P. longispinus* in autumn**		
Variable	Parameter stimate	*t*	*P*
Intercept	**-2.47**	**-7.6**	**<0.001**
Proportion of fruit infested in summer	**6.29**	**6.48**	**<0.001**
Year			
2020	0.0	-	-
2021	0.64	1.53	0.135
Proportion of fruit infested in summer × Year			
2020	0.0	-	-
2021	1.55	0.75	0.46
Null deviance: 17.76 Residual deviance: 6.21			

Chapter 4

Do hyperparasitoids disrupt the biological control of *Pseudococcus longispinus* in persimmon?

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Abstract

The long-tailed mealybug, *Pseudococcus longispinus*, represents a threat for several crops worldwide and has become the main pest of Mediterranean persimmon. Parasitoids are the primary natural enemies of *P. longispinus*, but their efficacy is highly variable among crops and countries. Here, we tested whether hyperparasitoids hinder the biological control of *P. longispinus* in Mediterranean persimmon. For this aim, we sampled 16 orchards across two consecutive years and measured the impact of primary parasitoids and hyperparasitoids on *P. longispinus*. *Anagyrus fusciventris* was the most abundant and widely distributed primary parasitoid and it reduced the population growth rate of *P. longispinus*. The hyperparasitoids *Chartocerus* sp. and *Prochiloneurus* sp. emerged mostly from large mealybug mummies that were used by *A. fusciventris* females. However, and contrary to our hypothesis, hyperparasitism did not affect the population growth rate of *P. longispinus* within the same year, suggesting that the high abundance of hyperparasitoids did not disrupt the biological control of this invasive mealybug. Based on these results, we propose several strategies based on conservation and augmentative biological control to improve the management of *P. longispinus* in persimmon.

1. Introduction

Human trade has facilitated the spread of some polyphagous mealybug (Hemiptera: Pseudococcidae) species that feed on a wide variety of crops. Some of these mealybugs can produce high crop damage and have become a global concern (Franco et al., 2004; Mani & Shivaraju, 2016; Miller et al., 2002). The long-tailed mealybug, *Pseudococcus longispinus* (Targioni-Tozzeti), feeds on a wide range of important crops such as vegetables, coffee, vines, citrus and other fruit trees (Barrass, 1993; Charles, 1993; Daane et al., 2012; Franco et al., 2004; Furness, 1976; Wysoki et al., 1977). This mealybug species is native to Australia (Flanders, 1940), but it is currently present in Europe, New Zealand, America and Southern Africa (Daane et al., 2012). In the Mediterranean basin, *P. longispinus* has been present since 19th century (Pellizzari & Germain, 2010), and it was considered a dangerous pest due to its potential to transmit diseases in vineyard and the high damage it caused to avocados from Israel in the 1970s (Bertin et al., 2010; Krüger et al., 2015; La Notte et al., 1997; Swirski et al., 1980). However, *P. longispinus* did not cause severe damage in other Mediterranean crops until its recent detection in Spanish persimmons (García-Martínez et al., 2017; Plata et al., 2023c).

Pseudococcus longispinus has become the most damaging pest in Spanish persimmon, a booming crop in eastern Spain, where this mealybug has spread rapidly (Plata et al., 2022; 2023c). This mealybug settles under the fruit sepal and excretes honeydew that promotes the growth of sooty mold on the fruit. The mealybug has three generations per year in Spanish persimmon. The maximum density is reached in the last generation, that occurs in September, just before harvest, when fruit infestation by *P. longispinus* can reach up to 80%. Moreover, it is expected that the global warming predicted for the coming years will increase the density and damage produced by *P. longispinus* (Plata et al., 2023c).

Critically, the high densities of *P. longispinus* in some crops have been associated with impaired biological control (Furness, 1976; Wysoki et al., 1977; Swirskii et al., 1980). Parasitoids are considered the primary natural enemies of *P. longispinus* and can be highly effective in controlling this mealybug pest (Flanders, 1940; Swirskii et al., 1980). In Australia, its area of origin, *P. longispinus* is mainly parasitized and controlled by *Tetracnemoidea* spp., *Anagyrus fusciventris* (Girault) (Hymenoptera: Encyrtidae) and *Coccophagus gurneyi* Compere (Hymenoptera: Aphelinidae) (Flanders, 1940). These and other parasitoid species are now present in other countries, sometimes through intentional importations. However, the establishment and efficacy of these species differ among the invaded regions. (Charles, 1993; Charles et al., 2015; Daane et al., 2008; Flanders, 1940; Furness, 1977). One factor determining this efficacy might be the presence of hyperparasitoids attacking primary parasitoids of *P. longispinus* in some of these regions (DeBach, 1949; Shaw et al., 2017; Wakgari & Giliomee, 2003). Hyperparasitoids can cause significant mortality to their primary parasitoid hosts and, therefore, potentially disrupt biological control (Gómez-Marco et al., 2015; Poelman et al., 2012). However, although hyperparasitoids are present in most studies evaluating biocontrol strategies of invasive pests such as *P. longispinus*, the impact of these hyperparasitoids on biological control has been rarely assessed, and the debate about the extent to which hyperparasitoids can disrupt biological control remains open (Schooler et al., 2011; Tougeron & Tena, 2019).

Herein, we tested whether hyperparasitoids disrupt the biological control of *P. longispinus* in Mediterranean persimmon. For this purpose, we sampled 16 orchards over two consecutive years. In these orchards, we first identified the primary parasitoids and hyperparasitoids that attack *P. longispinus* in persimmon and measured the rates of parasitism and hyperparasitism. Then, we evaluated whether parasitism and/or hyperparasitism rates affect the population growth rate of *P. longispinus*. Finally, we determined whether hyperparasitoids emerged from mealybug hosts of similar size than females of the primary parasitoids. These and other biological traits of the primary parasitoids and hyperparasitoids were analyzed to better understand their coexistence and their effects on *P. longispinus*. These insights aim to serve in implementing a biological control program against *P. longispinus* in persimmon.

2. Material and methods

Sampling sites and protocol

Sixteen persimmon orchards located in the main persimmon producing area of the Mediterranean basin (Valencia, Spain) were selected (**Figure S1** from supplementary material). All persimmon orchards were managed under IPM guidelines, and authorized insecticides were used when necessary. Other details are provided in Supplementary material: **Table S1**. In each orchard, nine trees were selected and sampled in spring (between May 10 and 22), summer (between July 14 and 27) and early-autumn (between September 27 and October 10) across two consecutive years (2020 and 2021). For each tree and date, 120 leaves (30 per cardinal direction), 40 fruits (10 per cardinal direction) and the surface of the trunk (from the base to a height of 50 cm) were surveyed. All mealybugs were counted and identified to species level. Mummified mealybugs and mealybugs with parasitism symptoms (*i.e.*, loss of wax and/or black dot) found in *P. longispinus* colonies were counted and collected. Mummies with an operculum from which parasitoids had already emerged were not considered. In the laboratory, the length of the mummies was measured using a millimeter ruler with a precision of 0.1 mm. Finally, the mummies were individualized and kept in a glass vial sealed with cotton at room temperature until the emergence of parasitoids.

Primary parasitoids and hyperparasitoids of *Pseudococcus longispinus*

Emerged parasitoids from each mummy were counted and identified. Primary parasitoids were identified to species level, while secondary parasitoids were identified to genus level. Identifications were carried out based on taxonomic keys (Andreason et al., 2019; Bouček & Rasplus 1991; Goulet & Huber, 1993; Noyes & Hayat, 1994; Noyes, 2003; Woolley 1988). The relative abundance of each parasitoid species was calculated for each season and year. To calculate the relative abundance of parasitoid species, mummies were used as the experimental unit because one of the identified parasitoid species was gregarious. In addition, the number of orchards where each species was recorded over the two years study is presented as a measure of its geographical distribution.

Effect of parasitism on *Pseudococcus longispinus*

Mean parasitism of *P. longispinus* was calculated for each season and year using orchard as the experimental unit. Orchard was used as the experimental unit instead of tree because mealybug density was too low in many trees during spring and summer. For each orchard, the sum of parasitized *P. longispinus* divided by the total number of *P. longispinus* suitable for parasitism (parasitized and non-parasitized) was calculated. First instar nymphs were excluded from parasitism calculations because this instar was not parasitized. Orchards with fewer than ten *P. longispinus* per year were excluded from this analysis. Parasitism variability among orchards was analyzed in autumn, when mealybug density was high, using tree as the experimental unit.

Finally, we tested whether the population growth rate of *P. longispinus* is affected by parasitism. To do this, we evaluated the effect of parasitism in autumn on the population growth rate of *P. longispinus* between summer and autumn in each orchard. Population growth rate was expressed as the intrinsic rate of growth, r, calculated as ln(Nt+1/Nt) (Pérez-Rodríguez et al., 2019), in which N represents the total number of all developmental stages of *P. longispinus* per orchard at time t (summer) and t+1 (autumn). The r-value was expressed using the units d^{-1} by dividing ln(Nt+1/Nt) by the period of time in days between the survey date t and the subsequent survey date t+1 days (≈75 days between summer and autumn surveys).

Effect of hyperparasitism on *Pseudococcus longispinus*

Hyperparasitism of *P. longispinus* was also calculated using orchards as the experimental unit. For each orchard, the sum of mummies from which hyperparasitoids emerged was divided by the total number of mummies from which either primary parasitoids or hyperparasitoids emerged. Variability in hyperparasitism among orchards was studied in autumn, when mealybug density and parasitism were high, using tree as the experimental unit. To test whether *P. longispinus* density is affected by hyperparasitism, we evaluated the effect of hyperparasitism in autumn on the population growth rate of *P. longispinus* between summer and autumn.

Biology of primary parasitoids and hyperparasitoids

To test whether hyperparasitoids emerged from hosts of similar size to the females of the primary parasitoids, we evaluated the relationship between the host size (measured as mummy length) and the species of emerged parasitoids, its sex and brood size. For this purpose, we counted, identified and determined the sex of the emerged parasitoids from each previously measured mummy. Sex ratio was calculated as proportion of males. Mealybug mummy was used as the experimental unit.

Statistical analysis

Linear Models (LMs) were conducted to evaluate the effect of: i) season and year on parasitism and ii) hyperparasitism; iii) orchard on parasitism and iv) hyperparastisim in autumn; and v) parasitism or vi) hyperparasitism in autumn on population growth rate of *P. longispinus*. Generalized Linear Models (GLMs) with binomial distribution were not used due to underdispersion of the data, and linear models explained a higher proportion of deviance. The data met the assumption of normality. Multiple comparisons to assess differences among seasons or orchards were performed using Tukey’s post hoc tests. To evaluate the effect of season and year on parasitism and hyperparasitism, season, year and their interaction were considered as categorical fixed factors and orchard was used as the experimental unit in both analyses. To evaluate the effect of orchard on parasitism and hyperparastisim in autumn, orchard, year and their interaction were considered as categorical fixed factors and tree was used as the experimental unit in both analyses. To evaluate whether parasitism or hyperparasitism in autumn affect *P. longispinus* population growth rate, year was included as categorical fixed factor, and orchard was used as the experimental unit in both analyses.

To determine whether each "parasitoid type" (species and sex) emerged from mummies of different sizes, we used a one-way ANOVA. The response variable was mummy length, and the predictor variable was parasitoid type. Mealybug mummy was used as the experimental unit. Multiple comparisons to assess differences among parasitoid type were conducted using Tukey's post hoc tests. We used "parasitoid type" because we were specifically interested in the sex of each species.

Generalized Linear Models (GLMs) with binomial error variance were used to evaluate the effect of mummy length on i) the sex ratio of *A. fusciventris,* and ii) the probability of hyperparasitoid emergence. Mealybug mummy was used as the experimental unit. For the sex ratio, male emergence was the response variable,while for hyperparasitism probability, hyperparasitoid emergence was the response variable. In both cases, mummy length was the predictor variable.

A GLM with Poisson error variances was used to evaluate the effect of mummy length on the brood size (number of emerged parasitoids from each mummy) of the gregarious parasitoid *Chartocerus* sp. Mealybug mummy was used as the experimental unit. The number of emerged individuals was the response variable and mummy length was the predictor variable.

For all the GLMs, the assumed error structures were assessed using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. In case of over- or an underdispersion, the significance of the explanatory variables was re-evaluated using an F-test after re-scaling the statistical model by a Pearson's $\chi2$ divided by the residual degrees of freedom. Significance was assessed by the change in deviance when a variable was removed from the model using a $\chi2$ test with binomial error. Significant values are reported for the minimal model, while the non-significant values are those obtained before we deleted the variable from the initial model (Crawley, 2007; Mayhew & van Alphen, 1999).

3. Results

Primary parasitoids and hyperparasitoids of *Pseudococcus longispinus*

Across the two-years study, 1,392 mealybug mummies were collected from *P. longispinus* colonies, and parasitoids emerged from 1,060 of these mummies. Eight parasitoid species were identified, including five primary parasitoids: *A. fusciventris*, *Anagyrus vladimiri* Triapitsyn, *Anagyrus aligarhensis* Agarwal & Alam, *Cryptanusia comperei* (Timberlake) and *T. peregrina* (Hymenoptera: Encyrtidae). Additionally, three hyperparasitoids were found: *Chartocerus* sp (Hymenoptera: Signiphoridae), *Prochiloneurus* sp. (Hymenoptera: Encyrtidae), and *Pachyneuron* sp. (Hymenoptera: Pteromalidae). *Anagyrus fusciventris* was the most abundant species, accounting for 47.6% and being widely distributed in 14 of the 16 sampled orchards. The other primary parasitoids, *C. comperei* (4.1%), *A. vladimiri* (2.5%) and *A. aligarhensis* (1.5%) were less abundant and were present in a fewer number of orchards (**Table 1**). The primary parasitoid *T. peregrina* (0.3%) was only found in one orchard in autumn 2021. On the other hand, two hyperparasitoid species, *Chartocerus* sp. and *Prochiloneurus* sp., were highly abundant, emerging from 23.6% and 17.5% of the mummies, respectively. Both species were present in 12 out of the 16 sampled orchards. Finally, the other hyperparasitoid species, *Pachyneuron* sp. (2.9%), was found in five orchards. All the parasitoids are solitary, with one single parasitoid emerged from each mummy, except *Chartocerus* sp., which is gregarious (**Table 1**).

The relative abundance of parasitoids differed among seasons but followed a similar trend in both years (**Figure 1**). *Anagyrus fusciventris* was the most abundant species in all seasons, but its relative abundance decreased from 100% in spring to 44.9% and 44% in autumn of 2020 and 2021, respectively. The primary parasitoid *C. comperei* was present in summer and autumn, but its relative abundance was lower than 10% in both seasons and years. The primary parasitoid *A. vladimiri* was only present in autumn and represented less than 4% of the identified parasitoids. The three hyperparasitoid species were found in summer and autumn. In summer, they represented the 35.2% of the mummies in 2020 and 18.7% in 2021. *Prochiloneurus* sp. was the most abundant hyperparasitoid in summer (19 % of mummies in summer 2020 and 9.3% in summer

2021). The highest number of parasitoid species was recovered in autumn. The relative abundance of hyperparasitoids was highest in autumn (48.8% in autumn 2020 and 46.8% in autumn 2021), whith the gregarious hyperparasitoid *Chartocerus* sp. being the most abundant (32.7% of mummies in autumn 2020 and 23.8% in autumn 2021).

Table 1. Primary parasitoids and hyperparasitoids of *Pseudococcus longispinus* found in 16 persimmon orchards located in the main persimmon producing area of Mediterranean basin across two consecutive years (2020 and 2021). The biology of each species is presented based on previous studies.

			Recovered in this study		**Recovered in previous studies**	
Family	**Species**	**Biology**	**Number of mummies**	**Orchard presence**	**Upon *Pseudococcus longispinus***	**Upon other mealybug species**
Encyrtidae	*Anagyrus fusciventris*	Primary solitary parasitoid	505	14/16	3*,5,6,7,9,14,15,17,18	1*,17, 28
	Anagyrus vladimiri	Primary solitary parasitoid	27	8/16	3*,4,13	1*,4,19,24*
	Anagyrus aligarhensis	Primary solitary parasitoid	16	5/16	-	1*,20,21,23
	Cryptanusia comperei	Primary solitary parasitoid	43	8/16	2*	18,26
	Tetracnemoidea peregrina	Primary solitary parasitoid	3	1/16	2*,3*,5,6,8,11,12,13,15,16,17	-
	Prochiloneurus sp.	Solitary hyperparasitoid	185	12/16	4,11	4, 19*,22,23,25,27
Pteromalidae	*Pachyneuron* sp.	Solitary hyperparasitoid	31	5/16	-	17,19*,24
Signiphoridae	*Chartocerus* sp.	Gregarious hyperparasitoid	250	12/16	3,4	17,19*,22,23,25*,27

References: **(1)*** Soler et al. 2021; **(2)*** Soler et al. 2015; **(3)*** Swirski et al. 1980; **(4)** Wakgari & Giliomee 2003; **(5)** Charles et al. 1993; **(6)** Charles et al. 2010; **(7)** Charles et al. 2015; **(8)** Shaw et al. 2017; **(9)** McAlpine 2019; **(11)** DeBach 1949; **(12)** Bartlett & Lloyd 1958; **(13)** Daane et al. 2008; **(14)** Furness 1976; **(15)** Bennet & Hughes 1959; **(16)** Flanders 1944; **(17)** Flanders 1940; **(18)** Timberlake 2019; **(19)*** Beltrá et al. 2013b; **(20)** van Alphen1990; **(21)** Pijls and van Alphen 1996; **(22)** Fischer et al. 1991; **(23)** Tamoli Torfi et al. 2020; **(24)** Berlinger 1977; **(25)*** Japoshvili et al. 2018; **(26)** Gordh 1974; **(27)** Triapitsyn et al. 2014; (28) Viggiani & Battaglia, 1983

*Study from the Mediterranean basin.

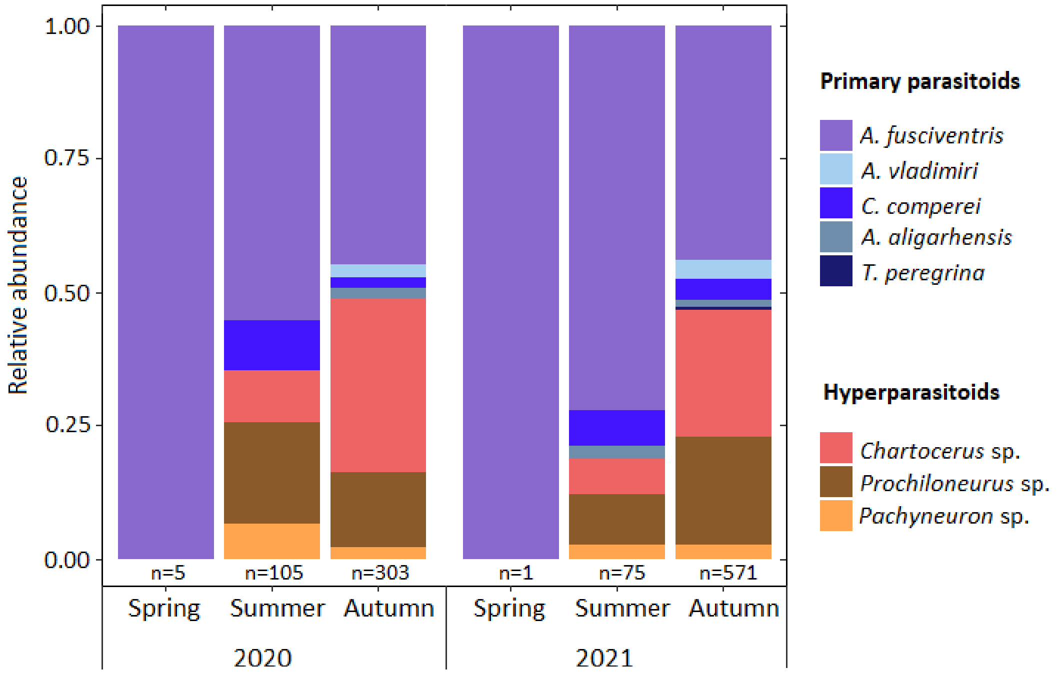


Figure 1. Seasonal abundance of *Pseudococcus longispinus* parasitoids collected in 16 persimmon orchards located in the main persimmon producing area of Mediterranean basin across two consecutive years (2020 and 2021). The total number of mealybugs parasitized by each parasitoid species in each season is displayed under the columns.

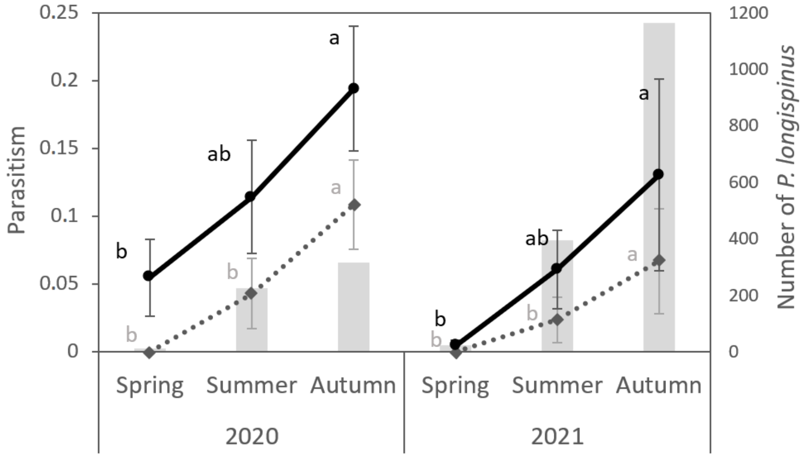


Figure 2. Mean parasitism and hyperparasitism rates of *Pseudococcus longispinus* (+SE) in 16 persimmon orchards located in the main persimmon producing area of Mediterranean basin across two consecutive years (2020 and 2021). Black and grey dashed lines represent parasitism and hyperparasitism, respectively. Different letters represent significant differences of parasitism (black) and hyperparasitism (grey) among seasons within each year (Tukey test, P < 0.05). Bars represent the mean abundance of P. longispinus (mean per orchard).

Effect of parasitism on *Pseudococcus longispinus*

Parasitism varied strongly among seasons ($F_{2,\,67} = 5.77$, *P* = 0.005), it increased from spring (mean parasitism = 0.03) to autumn (mean parasitism = 0.16) (**Figure 2**). Parasitism was not affected by year ($F_{1,\,66} = 3.31$, *P* = 0.073) or the interaction between year and season ($F_{2,\,64} = 0.017$, *P* = 0.98).

In autumn, when parasitism was higher, it was strongly affected by orchard ($F_{14, 192} = 15.68$, $P < 0.001$), year ($F_{1, 191} = 11.8$, $P < 0.001$) and the interaction between orchard and year ($F_{12, 179} = 7.41$, $P < 0.001$). Mean parasitism per orchard ranged from 0 to 0.49, in autumn 2020, and from 0 to 0.83 in autumn 2021 (**Figure S2** from supplementary material).

The population growth rate of *P. longispinus* between summer and autumn was strongly affected by parasitism in autumn ($F_{1, 25} = 25.8$, $P < 0.001$) (**Figure 3A**). The population growth rate was not affected by year ($F_{1, 24} = 0.26$, $P = 0.61$) or the interaction between parasitism and year ($F_{1, 23} = 0.23$, $P = 0.63$). Therefore, the population growth rate of *P. longispinus* decreased when parasitism in autumn was higher, independently of the year. The generated model explained 50.7% of the deviance (**Figure 3A** and **Table 2**).

Table 2. Values of the linear model based on binomial distribution that evaluates the effect of parasitism in autumn on the population growth rate of *Pseudococcus longispinus* between summer and autumn. Significant p-values are reported for the minimal model, while the non-significant p-values are those obtained before deleting the variable from the initial model.

Population growth rate of *P. longispinus* between summer and autumn (d^{-1})			
Variable	Parameter estimate	*t*	*P*
Intercept	**0.027**	**6.08**	**<0.001**
Parasitism in autumn	**-0.096**	**-5.08**	**<0.001**
Year			
2020	0.0	-	-
2021	0.003	0.51	0.61
Parasitism in autumn × Year			
2020	0.0	-	-
2021	0.002	0.48	0.63

Null deviance: 0.0146
Residual deviance: 0.0072

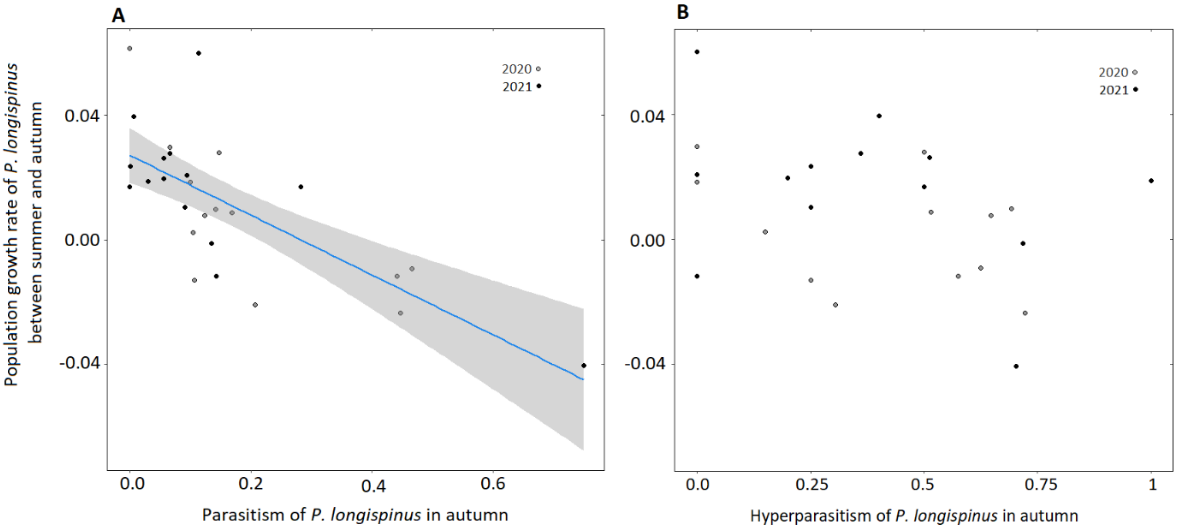
Effect of hyperparasitism on *Pseudococcus longispinus*

Hyperparasitism varied strongly among seasons ($F_{2, 46} = 6.46$, $P = 0.003$), it increased from spring (mean hyperparasitism = 0) to autumn (mean hyperparasitism = 0.54) (**Figure 2**). Hyperparasitism was not affected by year ($F_{1, 45} = 0.89$, $P = 0.35$) or the interaction between year and season ($F_{2, 43} = 0.14$, $P = 0.87$).

Hyperparasitism in autumn strongly differed among orchards ($F_{13, 122} = 3.3$, $P < 0.001$), but it was not affected by year ($F_{1, 121} = 0.027$, $P = 0.87$) or the interaction between orchard and year ($F_{10, 111} = 1.79$, $P = 0.07$). Hyperparasitism in autumn ranged from 0 to 0.77.

The population growth rate of *P. longispinus* between summer and autumn was not affected by hyperparasitism in autumn ($F_{1, 23} = 3.13$, $P = 0.09$) (**Figure 3B**). The population growth rate was not affected by year ($F_{1, 22} = 2.42$, $P = 0.13$) or the interaction between hyperparasitism and year ($F_{1, 21} = 0.0004$, $P = 0.98$).

Figure 3. (A) Effect of parasitism and (B) hyperparasitism in autumn on the population growth rate of *Pseudococcus longispinus* between summer and autumn. Each point represents an orchard. Grey points represent the orchards sampled in 2020 and black points in 2021. For panel (A): line represents the linear model, and grey area represents the 0.95 confidence interval. Year was not included as a factor in the final model because population growth rate was not affected by year or the interaction between year and parasitism.



Biology of the main primary parasitoids and hyperparasitoids of *Pseudococcus longispinus*

The host size used by primary parasitoids and hyperparasitoids, measured as mummy length, differed strongly between species and sexes ($F_{1,\,14} = 37.7$, $P < 0.001$) (**Figure 4**). Females of the primary parasitoid species emerged from larger mummies than males (*T. peregrina* was excluded from the analysis due to its very low abundance). The sex ratio (measured as proportion of males) was female biased in all primary parasitoid species, ranging from 0.25 ± 0.11 in *A. aligarhensis* to 0.42 ± 0.02 in *A. fusciventris* (Figure 4). For the most abundant primary parasitoid, *A. fusciventris*, the sex ratio decreased with host size [Sex ratio = 1/(1+Exp (-(11.9-6.3(mummy length [mm])))) ($F_{1,\,503} = 204.2$, $P < 0.001$)]; and its sex ratio was female biased when the mummy length was higher than 1.9 mm (**Figure 5A**).

All hyperparasitoids, except males of *Prochiloneurus* sp., emerged from mummies of the same size as female primary parasitoids (Figure 4). The probability of hyperparasitoid emergence increased with host size [Hyperparasitism = 1/(1+Exp(-(-1.88+0.79(mummy length [mm])))); ($F_{1,\,1053} = 22.9$, $P < 0.001$)] (**Figure 5B**).

In the case of the most abundant hyperparasitoid, *Chartocerus* sp., which is gregarious, 4.07 ± 0.14 individuals emerged from each mummy. Brood size ranged from 1 to 12 individuals, and it increased significantly with host size [Brood size = Exp (-0.77+1.02 (mummy length [mm])); ($F_{1,\,248}$ = 301.4, $P < 0.001$)] (**Figure 5C**).

The sex ratio of the solitary hyperparasitoids *Prochiloneurus* sp. and *Pachyneuron* sp. was female (0.4 ± 0.04) and male biased (0.52 ± 0.09), respectively (**Figure 4**). The sex ratio of the gregarious *Chartocerus* sp. was female biased (0.29 ± 0.01).

(Next page)

Figure 4. On the left, morphological details of the parasitoid species that emerged from Pseudococcus longispinus regarding the size of the mummy from which they emerged. Scale is shown at bottom. In the middle, bar plots represent the sex ratio (± SE) for each parasitoid species, presented as proportion of males. On the right, the host length (mm) of the mealybugs from which each parasitoid species and sex emerged. Each point represents a mummy. Rectangles represent the second and third quartiles and the inner vertical line represent the median value. The lower and upper quartiles are displayed as horizontal lines on either side of the rectangle. Half-violin plots represent the probability density of each data set. Different letters represent parasitoid "types" (species and sex) that emerged from mummies of different sizes (Tukey Test, P < 0.05).

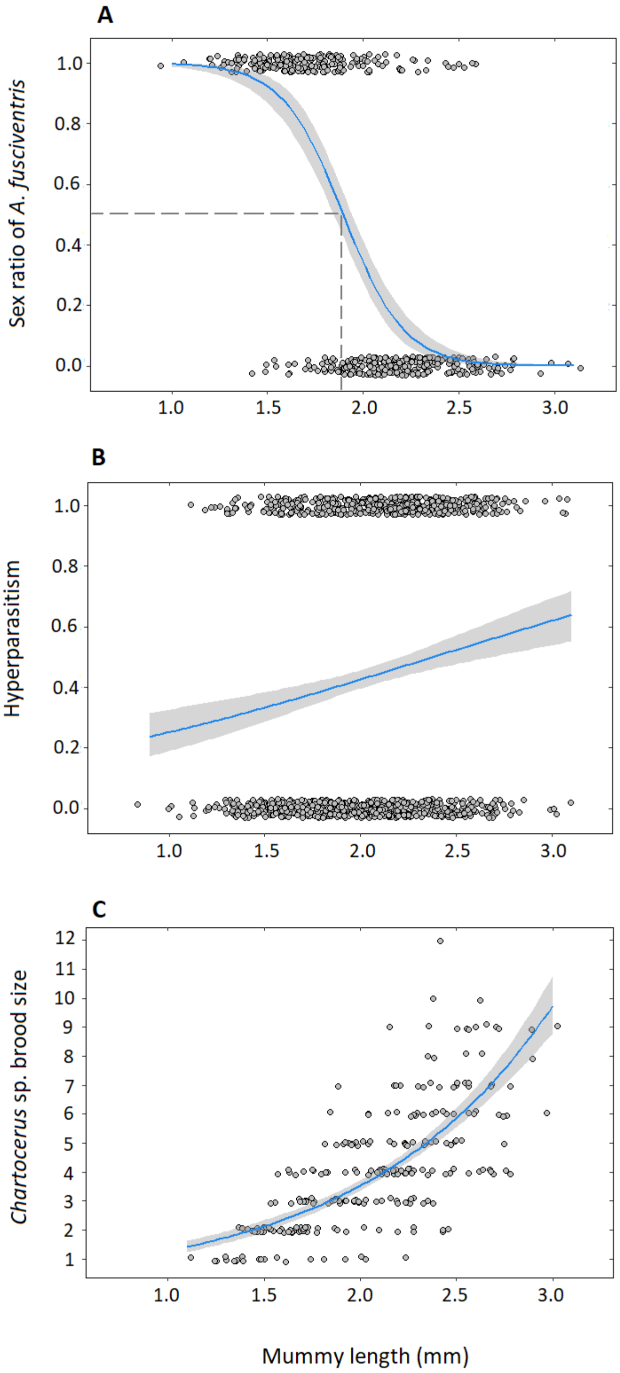
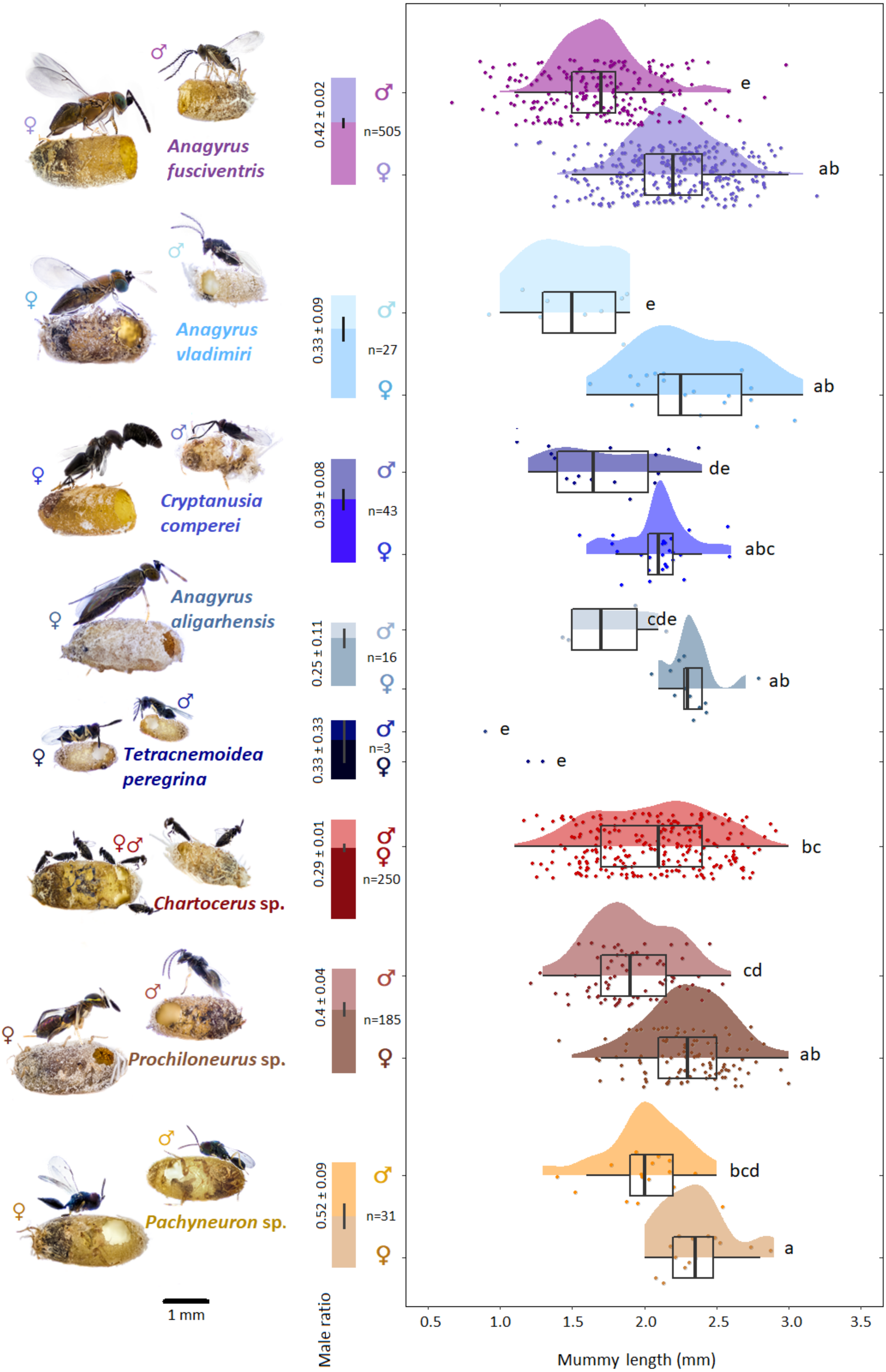


Figure 5. Effect of mummy length (mm) on: (A) sex ratio of the primary parasitoid *Anagyrus fuscivENTris*, presented as number of males divided by number of females and males; (B) probability that a hyperparasitoid species emerged from a parasitized *Pseudococcus longispinus*; (C) brood size (number of emerged individuals) of the hyperparasitoid *Chartocerus sp.* Lines represent the generalized linear model, andgrey areas represent the 0.95 confidence interval.


♀
♂
Anagyrus fusciventris
0.42 ± 0.02
n=505
♂
♀
e
ab
♀
♂
Anagyrus vladimiri
0.33 ± 0.09
n=27
♂
♀
e
ab
♀
♂
Cryptanusia comperei
0.39 ± 0.08
n=43
♂
♀
de
abc
♀
Anagyrus aligarhensis
0.25 ± 0.11
n=16
♂
♀
cde
ab
♂
♀
Tetracnemoidea peregrina
0.33 ± 0.33
n=3
♂
♀
e
e
♀♂
Chartocerus sp.
0.29 ± 0.01
n=250
♂
♀
bc
♂
♀
Prochiloneurus sp.
0.4 ± 0.04
n=185
♂
♀
cd
ab
♂
♀
Pachyneuron sp.
0.52 ± 0.09
n=31
♂
♀
bcd
a
1 mm
Male ratio
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Mummy length (mm)

4. Discussion

Our two-years study demonstrates that several native and naturalized hymenopteran species parasitize *P. longispinus* in persimmon. Among the primary parasitoid, *A. fusciventris* was the most abundant and widely distributed species. This parasitoid reduced the population growth rate of *P. longispinus* in summer, but parasitism rates and parasitoid abundance were highly variable among years and orchards. The high abundance of hyperparasitoids that emerged from mealybugs of the same size as *A. fusciventris* females might partially explain this variable parasitism. However, our analysis demonstrates that hyperparasitoids did not affect the population growth rates of *P. longispinus*. These results suggest that factors others than hyperparasitism reduce the efficacy of the biological control of *P. longispinus* in persimmon.

Our study confirms the establishment and widespread distribution of *A. fusciventris* in eastern Spain. *Anagyrus fusciventris* was found in all the persimmon orchards where *P. longispinus* was present. Moreover, it was the most abundant and widely distributed primary parasitoid throughout the two years. This species is native to Australia and has been imported to several countries for the control of *P. longispinus* in various crops, including Bermudas, Hawaii, California, South Africa and New Zealand (Bartlett & Lloyd, 1958; Bennett & Hughes, 1959; Charles, 1993; Charles et al., 2010; DeBach, 1949; Flanders, 1940; Noyes & Hayat, 1994; Wakgari & Giliomee, 2003). In the Mediterranean basin, *A. fusciventris* was introduced in Israel for the control of *P. longispinus* in avocados (Swirski et al., 1980). The parasitoid successfully established and, a few years later, it was observed on *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) in Italy (Viggiani & Battaglia, 1983). In Spain, *A. fusciventris* was first recorded in 2011 on ornamental plants in Andalusia, southern Spain (Marín, 2011). In 2016, *A. fusciventris* was found on *P. longispinus* and *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae) in sago palm (*Cycas revoluta*) and persimmon (Soler et al., 2021). The abundance of the other four primary parasitoids, *C. comperei*, *A. vladimiri*, *A. aligarhensis* and *T. peregrina*, was very low, and they were recovered in only a few orchards (see supplementary material for a detailed review of their origin and biology).

The high relative abundance and wide distribution of *A. fusciventris*, along with its effect on the population growth rate of *P. longispinus*, suggests that this parasitoid might regulate *P. longispinus* density in persimmon. This parasitoid attacked nymphs and adult females of *P. longispinus*, and its sex ratio was female biased in the field (see supplementary material for a detailed description of its biology). However, its efficacy was highly variable among orchards with very similar climatic conditions.

Hyperparasitoids can cause significant mortality on their primary parasitoid hosts (Gómez-Marco et al., 2015; Poelman et al., 2012), and they were very abundant in our study. The most abundant hyperparasitoid species were *Prochiloneurus* sp. and *Chartocerus* sp., which might reduce the biological control of *P. longispinus* in South Africa (Wakgari & Giliomee, 2003). In our study, hyperparasitoids emerged from mealybug mummies of the same size as *A. fusciventris* females, suggesting that they primarily hyperparasitize female rather than male primary parasitoids. This may further increase the negative impact of hyperparasitoids on the efficacy of *A. fusciventris*. Further laboratory trials or molecular analysis could corroborate this (Gómez-Marco et al., 2015).

Although hyperparasitoids increase the mortality of primary parasitoids, the presence of hyperparasitoids does not always reduce biological control (Schooler et al., 2011). For instance, *Chartocerus hyalipennis* Hayat (Hymenoptera: Signiphoridae) and *Prochiloneurus insolitus* Alam (Hymenoptera: Encyrtidae) were supposed to reduce the efficacy of *Anagyrus lopezi* (De Santis) (Hymenoptera: Encyrtidae) against *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae) (Goergen & Neuenschwander, 1990; 1994), but exclusion experiments showed that the impact of these hyperparasitoids on the mealybug densities was low (Goergen & Neuenschwander, 1992). Similarly, in this study, hyperparasitism did not affect the population growth rate of *P. longispinus*, which suggests that hyperparasitoids are not a key factor in reducing the biological control of *P. longispinus*. Nevertheless, the negative impact of hyperparasitoids on *P. longispinus* parasitism should be further evaluated using exclusion experiments and long-term studies that evaluate the effect of hyperparasitism on mealybug density over consecutive years. Unfortunately, few techniques have been developed to reduce hyperparasitism (Cusumano et al., 2020; Tougeron & Tena, 2019).

Other biotic and/or agricultural factors might explain the variability of parasitism by *A. fusciventris*. First, parasitism of *P. longispinus* may be negatively affected by insecticides. Outbreaks of *P. longispinus* have been linked to the application of broad-spectrum insecticides that can be toxic to parasitoids (Furness, 1977; Swirski et al., 1980; Wysoki et al., 1981). This toxicity, however, can vary among insecticides and parasitoid species (Calvo-Agudo et al., 2021; DeBach, 1949; Wakgari & Giliomee, 2003). The most effective insecticides against mealybugs, chlorpyrifos and methyl-chlorpyrifos, were banned in the European Union (European Commission, 2020). The alternative insecticides that have been used in Mediterranean persimmon in recent years, such as spirotetramat and sulfoxaflor, have shown lower efficacy against mealybugs, as reported by farmers and our own observations. Secondly, the presence of ants that exploit mealybug honeydew may affect the parasitism rates of *P. longispinus*. Ants typically attend mealybugs and other honeydew-producing species and protect them from their natural enemies (Anjos et al., 2022; Daane et al., 2007; Milosavljević et al., 2021; Rohrbach et al., 1988). The most abundant ant species in Mediterranean persimmon is *Lasius grandis* Forel (Hymenopera: Formicidae) (Plata et al., 2022). In citrus, the presence of *L. grandis* disrupts biological control of mealybugs by reducing the abundance and efficacy of predators and parasitoids (Campos et al., 2006; Mouratidis et al., 2021; Plata et al., 2023d). Finally, the lack of food or shelter resources for adult parasitoids might reduce their abundance, nutritional state and efficacy (Daane et al., 2018; Mockford et al., 2022; Roschewitz et al., 2005; Tena et al., 2015). Persimmon is a deciduous crop where these resources are scarce, especially during winter and the early spring. Therefore, the absence of non-crop habitats within or near the crop may hinder the establishment of the parasitoids at the beginning of the year. For all these reasons, we propose further research to evaluate the effect of three conservation biological control measures on the early establishment and efficacy of *A. fusciventris*: i) reduction of insecticide treatments; ii) exclusion of mutualistic ants; and iii) provision of non-crop habitats.

Overall, our results show that *A. fusciventris* is the main parasitoid of *P. longispinus* in Mediterranean persimmon, although its efficacy is variable. The development and implementation of conservation (as explained above) or augmentative biological control strategies could be options to improve the management of this mealybug. To our knowledge, only a preliminary assay tested the efficacy of augmentative releases of *A. fusciventris* against *P. longispinus*,and it was conducted under greenhouse conditions (Goolsby, 1994). In persimmon, inoculative or inundative releases of *A. fusciventris* in spring and early summer, when *P. longispinus* abundance is still very low, could facilitate the establishment of the parasitoid and prevent outbreaks of the mealybug in summer and autumn. These releases would be especially recommended after insecticide treatments that can negatively affect *A. fusciventris* or in orchards where the parasitoid is scarce.

Moreover, it is important to highlight that a companion study has described the seasonal trend of the mealybug, which can be used to schedule the releases of *A. fusciventris* (Plata et al., 2023c).

Data accessibility

Data from this study are available at the IVIA Digital Repository:
https://doi.org/10.58582/redivia.8672

Supplementary material

Parasitoid complex of *Pseudococcus longispinus*

The diversity of the parasitoid complex of *P. longispinus* in persimmon increased from spring to autumn. Five species of primary parasitoids were identified, all of which belonged to the family Encyrtidae (Hymenoptera). In order of abundance, these species were *A. fusciventris*, *C. comperei*, *A. vladimiri*, *A. aligarhensis* and *T. peregrina*. *Cryptanusia comperei* has been associated with *Pseudococcus calceolariae* (Maskell) (Hemiptera: Pseudococcidae) (Noyes & Hayat, 1984) and, more recently, with *P. longispinus* in New Zealand (Charles et al., 2010) and with *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) and *P. longispinus* in Spain (Soler & Guaita, 2014). The geographical distribution of *C. comperei* has increased this century. The other *Anagyrus* species, the native *A. vladimiri* and the introduced *A. aligarhensis*, were recovered in very low numbers in our study, likely because these parasitoids attack other mealybug species. *Anagyrus vladimiri* is the most abundant parasitoid species of *P. citri* in the Mediterranean area (Andreason et al., 2019; Jacas and Urbaneja, 2010; Martínez-Ferrer, 2003). *Anagyrus aligarhensis* was introduced in Africa to control *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae) (van Alphen et al., 1990). In Mediterranean persimmon, this species was recorded on *P. viburni* (Soler et al., 2021), but this is the first time that *A. aligarhensis* is associated to *P. longispinus*. Finally, only three individuals of *T. peregrina* were recovered from our samples. This parasitoid is considered specific of *P. longispinus* and has been imported to several regions, including the Mediterranean basin, where it was introduced in Israel in 1954 (Charles et al., 2010; Flanders, 1940; Shaw et al., 2017; Swirski et al., 1980). The low abundance of *T. peregrina* in our persimmon study contrasts with its high abundance in avocado (Swirski et al., 1980) and sago palm (Soler & Meliá, 2015). The same occurred in New Zealand, where *T. peregrina* was abundant in citrus, but it was absent in persimmon (Charles, 1993). These results suggest that some factors related to the crop might affect the establishment and/or performance of *T. peregrina* in persimmon. *Tetracnemoidea peregrina* was considered a superior competitor than *A. fusciventris* because it uses hosts of smaller size (Bartlett & Lloyd, 1958; Charles, 1993; Flanders, 1940). Another important parasitoid of *P. longispinus* is *Tetracnemoidea sydneyensis* (Timberlake) (Hymenoptera: Encyrtidae). This species is very abundant in New Zealand and California, and it is considered among the most effective parasitoids of *P. longispinus*, where it is present (Barrass, 1993;

Charles, 1993; Charles et al., 2015; DeBach, 1949; Flanders, 1940) (Supplementary material: **Table 2**). Nonetheless, *T. sydneyensis* has not been previously recorded in Mediterranean Basin and we did not recover it (Soler & Guaita, 2014; Swirski et al., 1980). Therefore, the absence of *T. sydneyensis* in the Mediterranean basin and the low abundance of *T. peregrina* in persimmon might explain the high relative abundance of *A. fusciventris* as parasitoid of *P. longispinus* in Mediterranean persimmon.

Hyperparasitoids were very abundant in summer and autumn. Three species of hyperaparasitoids were identified. The most abundant hyperparasitoid species were *Prochiloneurus* sp. and *Chartocerus* sp. in summer and autumn, respectively. These hyperparasitoid species are also very abundant in South Africa, where high hyperparasitism rates were also reported (Wakgari & Giliomee, 2003), suggesting that they can reduce the biological control of *P. longispinus*. The complex of hyperparasitoids is, however, totally different in New Zealand and California, where the facultative hyperparasitoid *Coccophagus gurneyi* Compere (Hymenoptera: Aphelinidae) was the most abundant species (Bartlett & Lloyd, 1958; Charles, 1993; Charles et al., 2010; DeBach, 1949; Flanders, 1940; Mcalpine, 2019; Shaw et al., 2017) (Supplementary material: Table 2).

Anagyrus fusciventris biology

Anagyrus fusciventris parasitized large-sized mealybugs, mainly third instar nymphs and adult females of *P. longispinus*. Previous studies found similar host size use (Charles, 1993; Goolsby, 1994; Sandanayaka et al., 2021). Females emerged from larger hosts than males, and sex ratio became female biased when mummy length was higher than 1.9 mm. The effect of host size on *A. fusciventris* sex ratio was also studied upon *Pseudococcus calceolariae* (Hemiptera: Pseudococcidae). *Anagyrus fusciventris* sex ratio became female biased when *P. calceolariae* mummies were higher than 2.1 mm (Sandanayaka et al., 2021). In other *Anagyrus* species, the relationship between sex ratio and host size is modulated by temperature (Daane et al., 2004). Further research should study this effect on *A. fusciventris*. Other biological traits of *A. fusciventris*, such as developmental time, longevity and fecundity under different temperatures, have been studied under laboratory conditions to design rearing protocols and mass-releases in the field (Goolsby, 1994; Mcalpine, 2019; Sandanayaka et al., 2022).

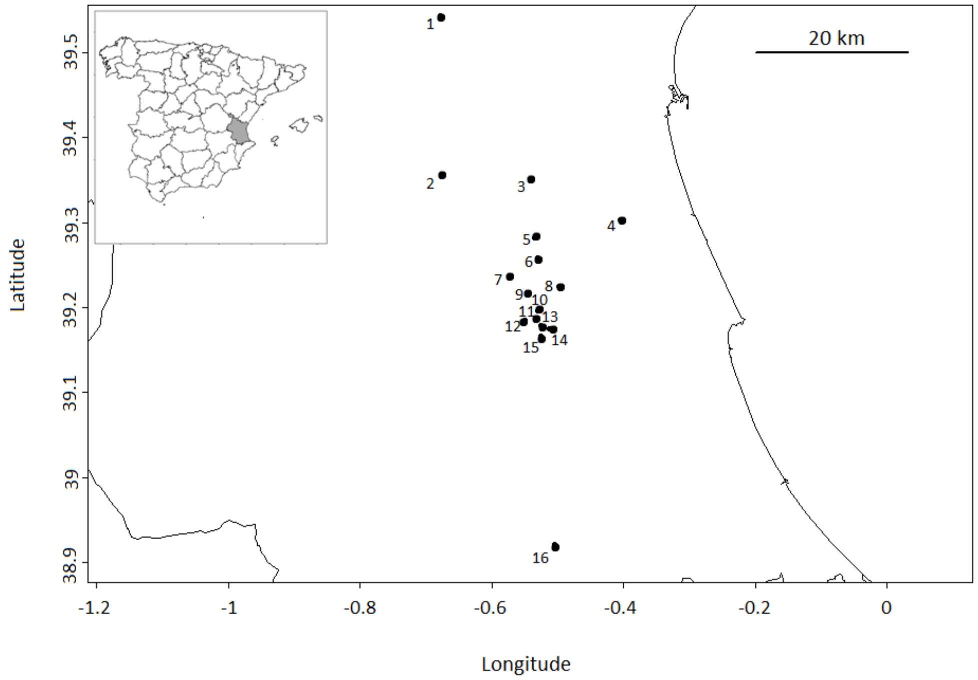


Figure S1. Geographical distribution of the 16 persimmon orchards in the main persimmon producing area from Mediterranean Basin (eastern Spain) sampled in 2020 and 2021. Geographical reference system: WGS84.

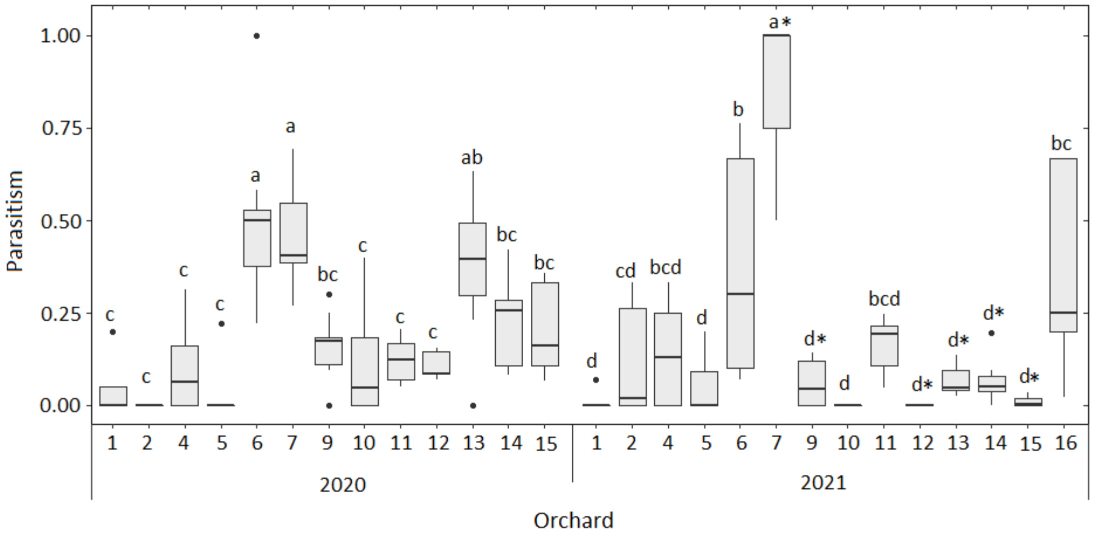


Figure S2. Mean parasitism rates of *Pseudococcus longispinus* in autumn in different persimmon orchards sampled in 2020 and 2021. Rectangles represent the second and third quartiles and the inner horizontal line indicates the median value per sampled tree in each orchard. The lower and upper quartiles are shown as vertical lines either side of the rectangle. Letters represent differences among orchards within each year. Asterisks represent differences between the first and the second year within each orchard (Tukey test, $P < 0.05$).

Table S1. Agronomic details of the 16 persimmon orchards sampled in eastern Spain in 2020 and 2021. The persimmon variety was 'Rojo brillante' in all the orchards and farmers followed the IPM guidelines (http://gipcaqui.ivia.es/). Seventeen orchards were sampled, but one orchard was not included in this study because *P. longispinus* was not present.

Orchard	**Altitude (m a.s.l)**	**Orchard size (ha)**	**Crop age (years)**
1	178	3.64	10
2	180	1.46	10
3	151	0.29	7
4	13	0.23	17
5	105	0.42	6
6	63	0.46	17
7	113	0.56	6
8	42	0.31	11
9	61	0.3	14
10	40	0.31	23
11	35	0.19	42
12	49	0.91	25
13	31	0.28	14
14	26	0.42	10
15	36	0.48	22
16	204	0.52	15

Table S2. Parasitoids of *Pseudococcus longispinus* recovered in other studies that have not been found in our study. References and details are provided in **table S3** from supplementary material.

Family	**Species**	**Biology**	**Upon *Pseudococcus longispinus***
Encyrtidae	*Gyranusoidea advena*	Primary solitary parasitoid	5,6,7,8
	Tetracnemoidea sydneyensis	Primary solitary parasitoid	5,6,7,8,10,11,12,16,17
	Tetracnemoidea brevicornis	Primary solitary parasitoid	5,6
	Tetracnemoidea pretiosa	Primary solitary parasitoid	14
	Parectromoides varipes	Primary solitary parasitoid	5,6,7
	Leptomastidea rubra	Primary solitary parasitoid	3*
	Coccidoxenoides perminutus	Primary solitary parasitoid	13
	Pseudaphycus angelicus	Primary gregarious parasitoid	11,12,17
	Chrysoplatycerus splendens	Primary solitary parasitoid	11,12
	Leptomastix sp.	Primary solitary parasitoid	3*
Aphelinidae	*Coccophagus gurneyi*	Facultative hyperparasitoid	4,5,6,8,9,11,12,16,17
Platygastridae	*Allotropa* sp.	Primary gregarious parasitoid	3*
Encyrtidae	*Achrysopophagus oviductus*	Solitary hyperparasitoid	14
Pteromalidae	*Ophelosia* sp.	Solitary hyperparasitoid	5,6,9,14,17
Ceraphronidae	*Lygocerus sp.*	Hyperparasitoid	11
Signiphoridae	*Thysanus sp.*	Hyperparasitoid	11,14,17

*Study carried out in the Mediterranean basin.

Table S3. Previous references that have identified potential parasitoids and hyperparasitoids of *Pseudococcus longispinus.* References 1-18 have identified potential parasitoid species of *Pseudococcus longispinus.* References 19-27 have identified parasitoid species found in our study, but on other mealybug species.

Id	Reference	Mealybug species	Region	Crop	Methodology
1	Soler et al. 2021	*P. longispinus*, *P. viburni*	Spain (Mediterranean)	Citrus, persimmon, sago palm	Chromatic + pheromone traps
2	Soler et al. 2015	*P. longispinus*	Spain (Mediterranean)	Sago palm	Chromatic + pheromone traps
3	Swirski et al. 1980	*P. longispinus*	Israel (Mediterranean)	Avocado	Collected mealybugs + evolutionary
4	Wakgari and Giliomee 2003	*P. longispinus*, *P. citri*, *P. calceolariae*	South Africa	Citrus	Collected mealybugs + evolutionary
5	Charles et al. 1993	*P. longispinus*, *P. calceolariae*	New Zealand	Apple, pear, citrus, vines, persimmon	Collected mealybugs + evolutionary
6	Charles et al. 2010	*P. longispinus*, *P. citri*	New Zealand	Vines, citrus	Collected mealybugs + evolutionary
7	Charles et al. 2015	*P. longispinus*	New Zealand	Forest	Sentinel mealybugs
8	Shaw et al. 2017	*P. longispinus*, *P. calceolariae*	New Zealand	Apple	Sentinel mealybugs
9	McAlpine 2019	*P. longispinus*, *P. calceolariae*	New Zealand	Vines	Sentinel mealybugs / Chromatic + pheromone traps
10	Barras 1993	*P. longispinus*	Australia	Pears	Collected mummies + evolutionary
11	DeBach 1949	*P. longispinus*	California	Citrus	Collected mealybugs + evolutionary
12	Bartlett and Lloyd 1958	*P. longispinus*, *P. citri*, *P. maritimus*	California	Citrus	Unknown
13	Daane et al. 2008	*P. longispinus*, *P. viburni*	California	Vines	Sentinel mealybugs
14	Furness 1976	*P. longispinus*	California	Citrus, vines, pear	Collected mummies + evolutionary
15	Bennet and Hughes 1959	*P. longispinus*	Bermudas	Sago palm	Unknown
16	Flanders 1944	*P. longispinus*	California	Avocado	Unknown
17	Flanders 1940	*P. longispinus*	Several	Several	Review
18	Timberlake 1919	*P. longispinus*	Hawaii	Several	Unknown
19	Beltrá et al. 2013	*P. peruvianus*	Spain (Mediterranean)	Urban gardens	Collected mummies + evolutionary
20	van Alphen 1990	*P. manihoti*	Africa	Cassava	Unknown
21	Pijls and van Alphen 1996	*P. manihoti*	South America	Cassava	Unknown
22	Fischer et al. 1991	*P. manihoti*	Togo	Cassava	Collected mealybugs + evolutionary
23	Tamoli Torfi et al. 2020	*P. solenopsis*	Irán	Cotton	Collected mealybugs + evolutionary
24	Berlinger 1977	*P. ficus*	Israel (Mediterranean)	Vines	Collected mealybugs + evolutionary
25	Japoshvili et al. 2018	*P. ficus*	Turkey (Mediterranean)	Vines	Collected mummies + evolutionary
26	Gordh 1974	*P. cryptus*	Philippines	Unknown	Unknown
27	Triapitsyn et al. 2014	*Hypogeococcus* spp.	Argentina	Unknown	Unknown

Section III

Improving the management of mealybugs through habitat management

Chapter 5

Habitat heterogeneity reduces abundance of invasive mealybugs in subtropical fruit crops

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Abstract

Simplification of agricultural landscapes have been associated with an increase in pest pressure. While monocultures increase resources available for pests and may facilitate their dispersion, the lack of non-crop habitats may reduce the resources for pest natural enemies. Herein, we tested which of these hypotheses, namely “resource concentration” and “natural enemies”, can better explain the abundance of invasive mealybug pests in two subtropical fruit crops. For this aim, seventeen persimmon orchards and sixteen citrus orchards were sampled during three different seasons across two consecutive years. Using a model selection approach, we assessed the effects of surrounding landscape (proportion of focal crops and semi-natural habitats at different distances) and inter-row ground cover vegetation on the abundance of mealybugs and their natural enemies. The proportion of focal crop in the landscape increased the abundance of mealybugs attacking both crops. This effect was found at closer distances (up to ~ 600 m) in citrus, and both closer and further distances (up to 1250 m) in persimmon. Non-crop habitats, both surrounding semi-natural habitats and ground cover vegetation, decreased the abundance of mealybugs by increasing the activity of their parasitoids in persimmon. Conversely, non-crop habitats did not decrease the abundance of the main mealybug species attacking citrus, likely because this mealybug species was not attacked by native or naturalized parasitoids. Our models show that the increase of habitat heterogeneity at local and landscape scales can reduce the abundance of invasive mealybugs in subtropical crops via “resource concentration” and “natural enemies” mechanisms. Therefore, habitat diversification strategies should be considered in conservation biological control of invasive mealybugs. Importantly, our findings also show that the presence of efficient natural enemies is critical to maximize their control through habitat diversification strategies.

1. Introduction

The intensification of agriculture has led to reduced habitat heterogeneity at both local and landscape levels, contributing to an increase in pest problems, as documented in numerous studies (*e.g.*, Bianchi et al., 2006; Chaplin-Kramer et al., 2011; Letourneau et al., 2011; Paredes et al., 2021; Tscharntke et al., 2005). Two concurrent hypotheses have been put forward to account for this phenomenon. The first, referred to as the "resource concentration hypothesis" (Root, 1973), proposes that monoculture systems, characterised by vast areas of a single crop, promote the spread of herbivore pests due to the absence of barriers hindering their movement (Martinson & Fagan, 2014; O'Rourke & Petersen, 2017). The second hypothesis, known as the "natural enemy hypothesis," asserts that natural habitat patches offer essential resources for pest natural enemies, thus enabling effective pest control (Chaplin-Kramer et al., 2011; Landis et al., 2000; Russell, 1989).

Based on these hypotheses, strategies that increase habitat heterogeneity at both, local and landscape, scales could improve pest control (Gurr et al., 2017; Landis et al., 2000). These strategies involve introducing inter-row ground cover vegetation within agricultural plots and preserving or restoring natural vegetation in the surrounding landscape. The idea is that pests would react unfavourably to the presence of natural habitats and ground cover vegetation because these areas can provide resources, such as nectar, for natural enemies. Conversely, pests are expected to respond positively to monoculture surroundings because of the available resources, which could promote pest population growth and dispersion. However, the outcomes are not always the expected and these responses highly vary across different crops, pests, and natural enemies (Heimpel 2019; Karp et al., 2018; Tamburini et al., 2020). Several factors contribute to this variability: dispersal behaviour of pests, lack of efficient natural enemies or effectiveness of non-crop habitats for providing resources to key natural enemies (González-Chang et al., 2019; Tscharntke et al., 2016). Critically, the high stochasticity inherent to agricultural systems is a major reason for the inconsistent responses, making it challenging to implement habitat heterogeneity strategies in pest management programs (Chaplin-Kramer et al., 2013; Paredes et al., 2021).

Habitat heterogeneity's impact on important pests attacking crops in temperate regions, such as aphids, has been extensively studied (*e.g.*, Bosem Baillod et al., 2017; Gómez-

Marco et al., 2016; Redlich et al., 2018; Rusch et al., 2016). However, there is little literature addressing this issue on mealybugs, which are major pests in subtropical crops and are expanding into subtropical and temperate ecosystems (Miller et al., 2002; Pellizzari & Germain, 2010). In Spain, invasive mealybugs have become key pests in two important subtropical crops, citrus and persimmon (Martínez-Blay et al., 2018a; Plata et al., 2023c). *Delottococcus aberiae*, native to South Africa, is the prevalent mealybug in citrus, while *Pseudococcus longispinus*, from Australia, is the prevalent species in persimmon. Although parasitoids regulate these mealybugs in their native habitats (Flanders, 1940; Guerrieri & Cascone, 2018), parasitism is highly variable in Spain. The effects of habitat heterogeneity on mealybug abundance may be mediated by the presence of these specific parasitoids, which could be one of the reasons explaining differences in mealybug responses to habitat context (Le et al., 2018; Moore, 1988; Muneret et al., 2018; Shapira et al., 2018). *Delottococcus aberiae* was first detected in Spain in 2009 (Beltrà et al., 2013a; 2015) and is not parasitized by native or naturalized parasitoids (Tena et al., 2017), thus the parasitoid *Anagyrus aberiae* was introduced (Soto et al., 2021). On the other hand, *P. longispinus* has been in Spain for over a century (Pellizzari & Germain, 2010), and naturalized parasitoids parasitize this mealybug species (Plata et al., 2023a; 2023b). Additionally, monoculture landscapes could facilitate mealybug dispersion and benefit both mealybug species regardless their natural enemies. Immatures and adult females of mealybugs do not fly (Grasswitz & James, 2008), which can make ecological barriers more effective at disrupting mealybug dispersion than for flying pests (Grez & González, 1995).

Given the differences in responses to habitat heterogeneity among pest species, the lack of studies on mealybugs, and their economic significance, it is crucial to explore the mechanisms involved in mealybug conservation biological control. Additionally, it remains uncertain whether resource concentration or natural enemies hypothesis is more influential for invasive mealybugs. In the case of recently introduced species, such as *D. aberiae* in citrus, resource concentration may play a more significant role (Tamburini et al., 2020). In contrast, both resource concentration and natural enemies could be significant factors in controlling *P. longispinus* in persimmon because its specific natural enemies are well-established in the invaded area.

Here, we first tested whether habitat heterogeneity at local and landscape scales reduces the abundance of invasive mealybug species in subtropical crops from Spain. Second, we evaluated which of the hypotheses, namely “resource concentration” or “natural enemies”, best explained mealybug abundance. Third, we assessed different pathways that may explain the observed effects of habitat heterogeneity on mealybug abundance. Finally, based on our findings, we defined habitat management strategies that can improve biological control of these invasive pests in subtropical crops.

2. Material and methods

Sampling design

The study was conducted in 17 persimmon and 16 citrus orchards in Eastern Spain (**Figure 1**). The selection of the orchards was based on a gradient of surrounding monoculture (persimmon or citrus) and semi-natural habitats and were separated at least 1 kilometre from each other of the same crop (see **Figure S1** in supplementary material). All orchards were at least 5 years old, drip irrigated, and followed Integrated Pest Management (IPM) guidelines; therefore, insecticide usage was similar across them (MAPAMA, 2022d; 2022e).

In each orchard, a plot of 40 (5 x 8) trees was established and sampled during two consecutive years (2020 and 2021) across three seasons: late spring, mid-summer, and mid-autumn. Nine alternate trees were selected per plot and sampled across the study (see **Figure S2**). For each tree and sampling date, we counted and identified the mealybugs present in 120 leaves (30 per cardinal direction), 40 fruits (10 per cardinal direction), and the trunk surface (from ground level to 50 cm height) (Plata et al., 2023c; 2024a). All observed mealybugs were identified up to the species level using taxonomic keys (*e.g.*, Miller & Giliomee, 2011). The number of mealybugs with parasitism symptoms was recorded from all the individuals surveyed (**Figure S3**). We also registered the abundance of potential mealybug predators such as *Cryptolaemus montrouzieri*, an important biological control agent against mealybugs present in the study area (Pérez-Rodríguez et al., 2019), however, its abundance was very low (see **Table S1**).

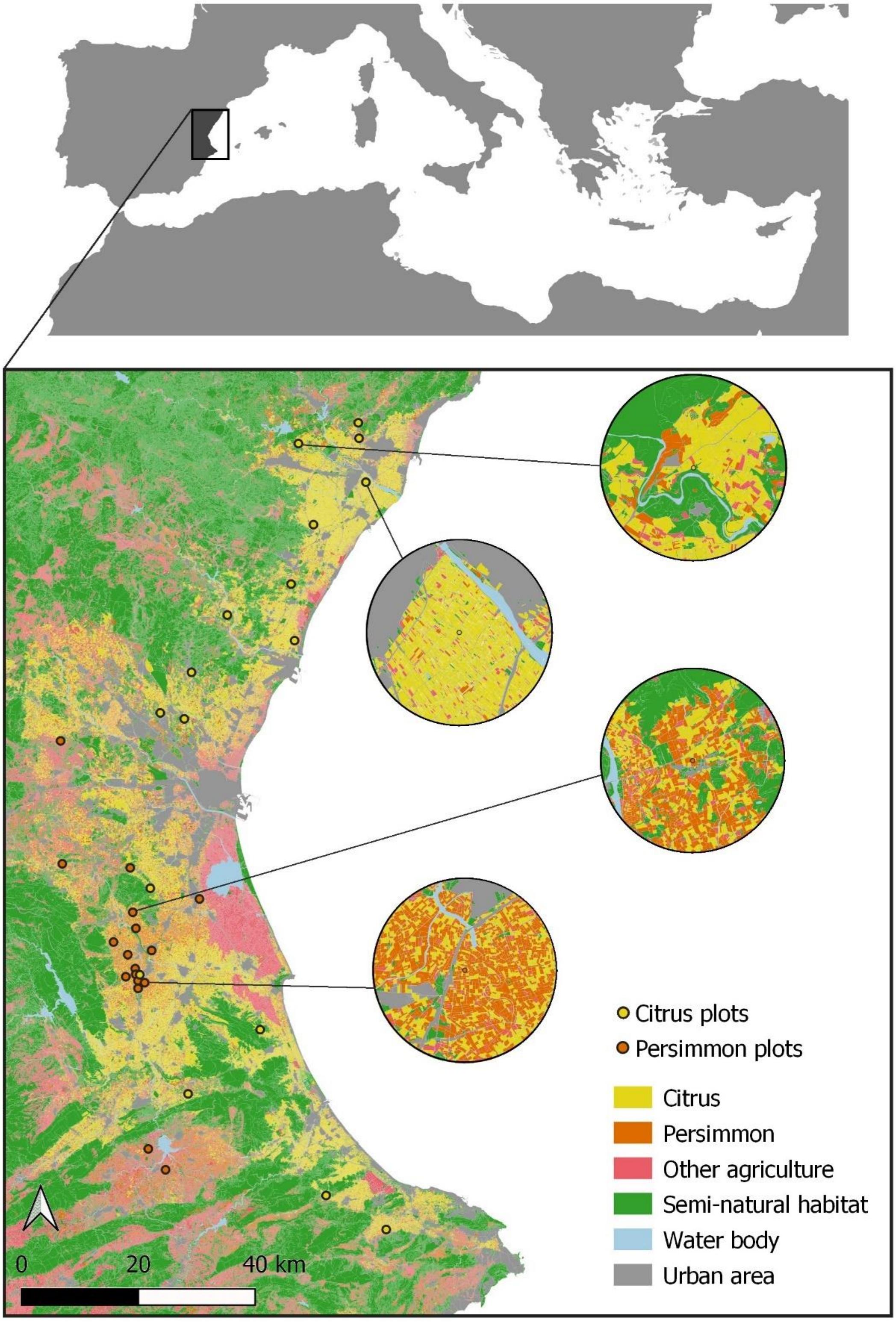


Figure 1. Location of citrus and persimmon orchards in eastern Spain. Landscape composition varied considerably, as indicated by the enlarged plots within the circles.

Habitat heterogeneity characterization

Habitat heterogeneity at local and landscape scale was characterized based on the inter-row ground cover vegetation within the plots and the proportion of land use types in the landscape surrounding the plots, respectively. For ground cover vegetation, we assigned values between 0 and 6 based on the percentage of ground covered by spontaneous vegetation per plot and sampling date: <3% = 0; 3-10% = 1; 10-20% = 2; 20-30% = 3; 30-50% = 4; 50-75% = 5; >75% = 6.

We characterized the landscape around sampled plots using Geographic Information System of Agricultural Plots (SIGPAC) for 2020 and 2021. The original SIGPAC categories were grouped into six new classes: Semi-natural habitat, Persimmon, Citrus, Other agriculture, Urban areas, and Water bodies. Semi-natural habitats included forests, shrublands, public parks, gardens, grasslands, and set-aside areas with spontaneous vegetation. When calculating the proportions of the focal crop (persimmon or citrus) and the semi-natural habitat, we initially, employed a 2000-meter buffer which caused significant overlap, particularly in persimmon plots. Hence, 10 out of 17 persimmon plots overlapped, with some exceeding 70%. To reduce this, we adjusted the buffer to 1250 meters, impacting only six plots, none exceeding 50% overlap. Additionally, we examined spatial autocorrelation using Moran's I Index and no significant autocorrelation was detected. To calculate the surrounding land use proportions for each plot, we assigned varying weights to landscape patches based on their proximity to the sampling location. This involved assessing land use within 25 concentric rings, each 50 meters wide, within a 1250-meter radius. Then, based on Karp et al. (2016), we calculated a weighted average of all rings around each location using Gaussian decay functions. Each ring was assigned a weight denoted as "*W*", as follows: $W = \exp(-I^2/(2*d^2))$. "*I*" represents the distance between the centre of the plot and the outer edge of the ring, while "*d*" is the decay rate that indicates the speed at which weightings decrease with a longer distance. We calculated the total weighted area for each land use type by summing the weighted proportions within the concentric rings. For comparison, we used two different decay rates to create small and large influence areas. In the first (d=250), closer landscape features held greater significance, rendering elements beyond 600 meters irrelevant (see **Figure S4A**). In the second (d=1250), the weight gradually decreased with increasing distance from the buffer's center (I), keeping all landscape elements relevant (see **Figure S4B**).

Modelling

Effect of habitat heterogeneity on mealybug abundance and parasitism

For statistical analysis, we employed Generalized Additive Models (GAMs) with mealybug abundance and parasitism as response variables. Predictors included proportions of the surrounding focal crop (persimmon or citrus), surrounding natural habitat, ground cover vegetation index, and the sampling year. Additive models were preferred due to the potential nonlinear responses of insects to different habitats. In exploratory analyses, we initially allowed an unrestricted number of knots for each fixed effect. However, to prevent overfitting, we ultimately capped the maximum number of knots at three for each predictor (Taylan et al., 2007). Due to potential predictor collinearity, we set a correlation limit of 0.5 to prevent it. As a result, landscape variables (focal crop and natural habitat proportion) were excluded from the same models due to exceeding this limit (see **Figure S5**). We treated the plot as the experimental unit, aggregating the number of insects sampled from the nine trees on each plot and sampling date. This approach was necessary because single-tree sample sizes were often too small for several variables on certain dates and plots, reducing the prevalence of zero values in our dataset. For the abundance variable, we first opted for a Poisson distribution. However, after checking for overdispersion with scaled simulated residuals and perform outlier, dispersion and KS test using the R Package “DHARMa” (Hartig, 2022), we decided to move to a negative binomial distribution with a log link function as this error distribution better fitted model assumptions (see **Figure S6**). For parasitism, we used a binomial distribution with a logit link function because the variable nature involved the number of individuals parasitized out of the total mealybugs per plot. We excluded first instar nymphs as they were not parasitized.

For each response variable, we created various models comprising all potential fixed factors along with a null model. Model selection employed the Akaike Information Criterion corrected for small sample size (AICc; Motulsky & Christopoulos, 2004). We used a Δ AICc < 2 threshold to decide if a model better explain variability than the following. This often resulted in multiple models for a single response variable, indicating substantial model selection uncertainty (Burnham & Anderson, 2002). Consequently, we conducted model averaging with the "Zero Method" to identify the variables with the most pronounced effect on the response variables (Grueber et al., 2011; Nakagawa & Freckleton, 2011). We assessed whether the variables included in the averaged model significantly affected the response variable and determined the size effect of each variable in the averaged model. When several models with a landscape factor (crop or natural habitat proportion) met AICc criteria at small and large influence area (250 and 1250 decay models), only the one with the lowest AICc was included in the averaged model. In such case, we assessed the effect of small and large influence area by comparing the size effect of both influence areas. GAM models were generated using the R Package "mgcv" (Wood 2011) and were averaged using the R Package "MuMIn" (Barton, 2016).

Mediating effects of parasitism on P. longispinus abundance

Based on the models (GAMs) selected in the analysis described above and our biological and ecological knowledge of the system, we hypothesized that the negative effects of ground cover vegetation and surrounding natural habitat on *P. longispinus* abundance were partially explained by the positive effects of these factors on *P. longispinus* parasitism, which is supposed to negatively affect mealybug abundance. Moreover, we hypothesized that the positive effect of surrounding persimmon on *P. longispinus* abundance in autumn was not mediated by parasitism, as parasitism was not negatively affected by this factor.

Our hypotheses were verified by structure equation modelling (SEM) for confirmatory factor analysis (CFA). CFA checks for unidimensionality, internal consistency and convergent and discriminant validity for each construct (Kline, 2023). We built three structure equation models, one for each season. In each season, *P. longispinus* abundance and *P. longispinus* parasitism were supposed to be affected by the year and the factors included in the selected GAM models. These factors were considered exogenous variables, *i.e.*, no other parameters causing or affecting these variables. Furthermore, mealybug abundance was also influenced by parasitism (endogenous variable, *i.e.*, affected by the other parameters and affecting the response variable). Mealybug abundance (number of *P. longispinus* per plot and season) was log transformed to build these models, while for mealybug parasitism we calculated the proportion of parasitized mealybugs per plot and sampling date (*Parasitized / Parasitized+Non-parasitized*). To enable the comparison of the relative strengths of the different pathways of the final model, path coefficients were standardized by mean and variance. These path coefficients were used to calculate and compare the strengths of direct and indirect links among variables.

We checked the model's goodness-of-fit using different indices (chi-square, GFI, RMSEA, CFI and SRMR; see Table S2) which provide sufficient basis for model evaluation (Byrne, 1994; Kline, 2023). R Packages 'lavaan' (Rosseel, 2012) and 'effectsize' (Ben-Shachar et al., 2020) were used to generate SEMs and calculate fit indices, respectively.

All statistical analyses were conducted in R (R Core Team, 2021). To visualize results, we used the R Package 'ggplot2' (Wickham, 2016).

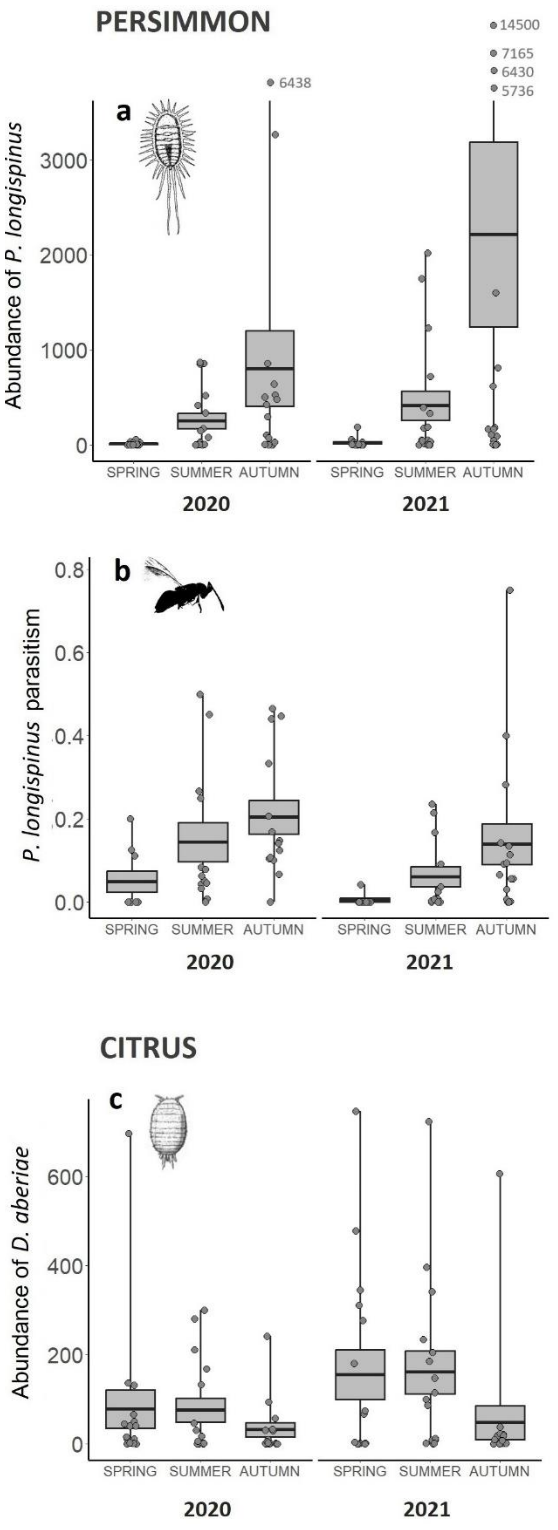
3. Results

Effects of habitat heterogeneity on mealybug abundance

Persimmon

Pseudococcus longispinus was the most abundant species, representing 88.8% of mealybugs in 2020 and 98.2% in 2021. Abundance per plot increased from spring (mean number of *P. longispinus* = 14.7) to summer (331.5) and autumn (1508.1; **Figure 2a**). *Pseudococcus longispinus* abundance in each season correlated significantly with the following season within a year, and autumn 2020 abundance also correlated significantly with spring 2021 (see **Table S3**).

Figure 2. Abundance (a) and parasitism (b) of *Pseudococcus longispinus* in persimmon, and abundance of *Delottococcus aberiae* in citrus (c). Each rectangle represents the mean ± standard error per sampled plot and sampling season. The points represent the abundance per plot, and the vertical lines on either side of rectangles represent their range.



Selected models indicated that all predictors explained *P. longispinus* abundance, but they varied by season (see **Tables S4** and **S5**). The year was not included in spring and summer models but in autumn, *P. longispinus* abundance was significantly higher in 2021 than in 2020 (p = 0.01). A higher proportion of surrounding semi-natural habitats in the larger influence area significantly reduced spring (p = 0.024) and summer (p < 0.001) mealybug abundance. This effect was most pronounced in summer, when mealybugs per plot decreased from about 750 when semi-natural habitats were absent to roughly 40

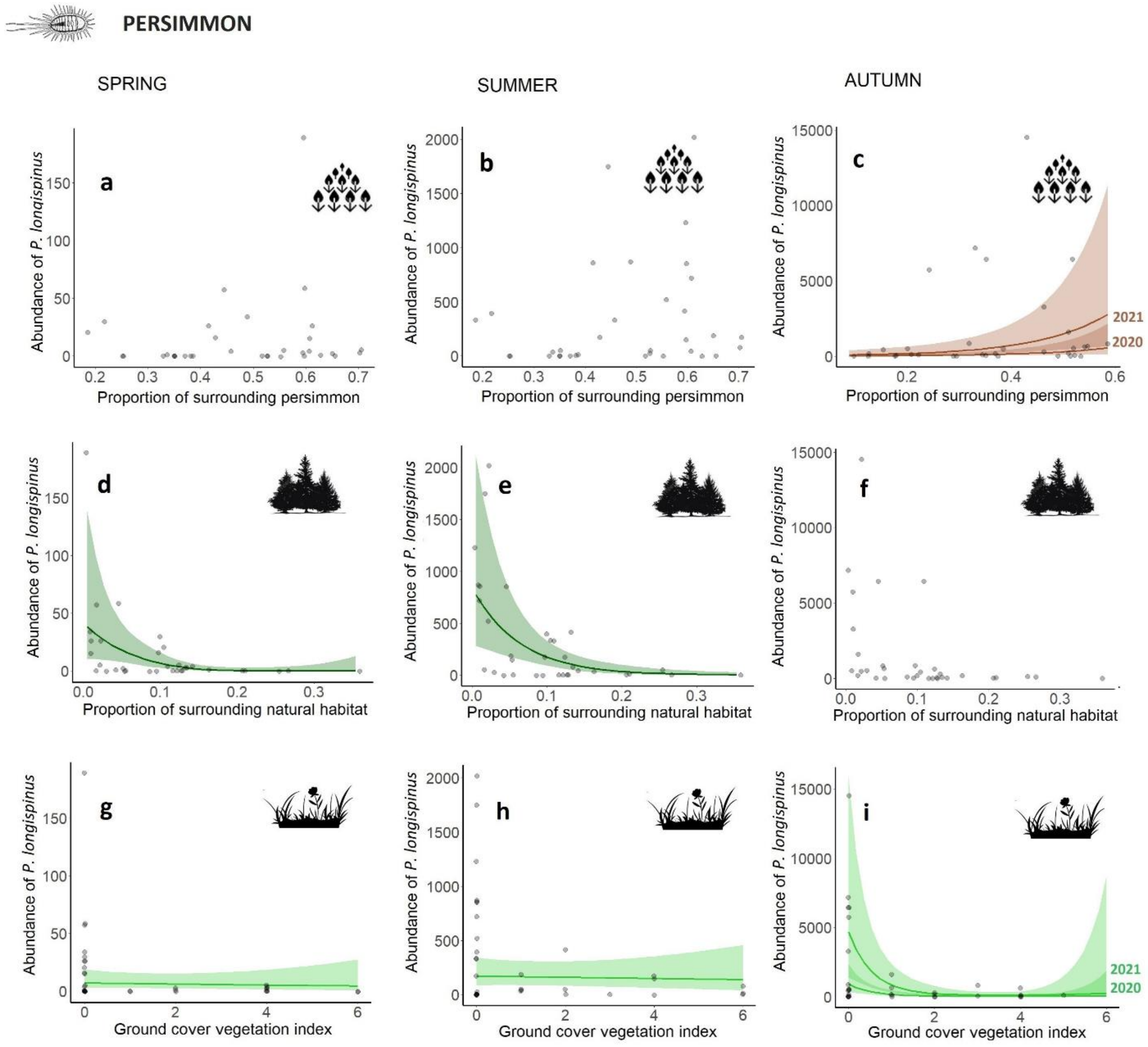


Figure 3. Effect of surrounding persimmon (a-c), semi-natural habitat (d-f), and ground cover vegetation (g-h) on *Pseudococcus longispinus* abundance in persimmon. Each season's mealybug abundance was determined by the variables in the selected averaged models (ground cover vegetation index in spring, summer, and autumn; proportion of semi-natural habitat in the large influence area in spring and summer; proportion of persimmon in the large influence area in autumn). Lines represent predictions from averaged GAMs with shaded regions denoting 95% confidence intervals. When the year effect was significant, multiple lines depict each year. For variables not included in the models, point dispersion is shown without predictions.

mealybugs per plot when the semi-natural habitat proportion reached 0.2 (**Figure 3e**; see **Table S5**). In contrast, the proportion of focal crop was the selected variable for autumn, when mealybug abundance increased nearly 20-fold when the surrounding persimmon proportion decreased from 0.7 to 0.3 (**Figure 3c**; see **Table S5**). The positive impact of persimmon proportion on *P. longispinus* abundance was consistent across both small and large influence areas (see **Table S4** and **Figure S7**). Moreover, ground cover vegetation had a significant impact on autumn mealybug abundance (p = 0.007). During this season, mealybug numbers dropped nearly tenfold as the ground cover vegetation index increased from 0 (bare soil) to around 10-20% coverage (**Figure 3i**; see **Table S5**).

Citrus

In citrus, the most abundant mealybug species was *D. aberiae*, representing 76.1% in 2020 and 91.6% in 2021. Abundance per plot was maximum in spring (mean number of *D. aberiae* = 116.2) and summer (117.3), and decreased in autumn (39.1; **Figure 2b**). *Delottococcus aberiae* abundance per plot each season did not significantly correlate with the next season's abundance, except for spring-summer in 2020 and summer-autumn in 2021 (see **Table S6**).

The proportion of surrounding focal crop and semi-natural habitat explained *D. aberiae* abundance, with no inclusion of ground cover vegetation in any selected models (Figure 4; see **Table S7**). The year was part of the selected models for spring, but the year's effect was not significant (p = 0.127; see **Table S8**). A higher proportion of surrounding citrus in the smaller influence area positively influenced *D. aberiae* abundance in spring (p = 0.008), summer (p = 0.002), and autumn (p = 0.002). Mealybug abundance exhibited a hump-shaped trend, initially increasing to a peak at about 0.6 proportion of surrounding citrus orchards, then decreasing (**Figure 4a-c**; see **Table S8**). In autumn, mealybug abundance was also influenced by citrus proportion in the larger influence area (see **Table S7**), decreasing as citrus proportion increased (see **Figure S8**). Semi-natural habitat proportion had a positive impact on *D. aberiae* abundance in spring (**Figure 3d**), although this effect was not significant (p = 0.053; see **Table S8**).

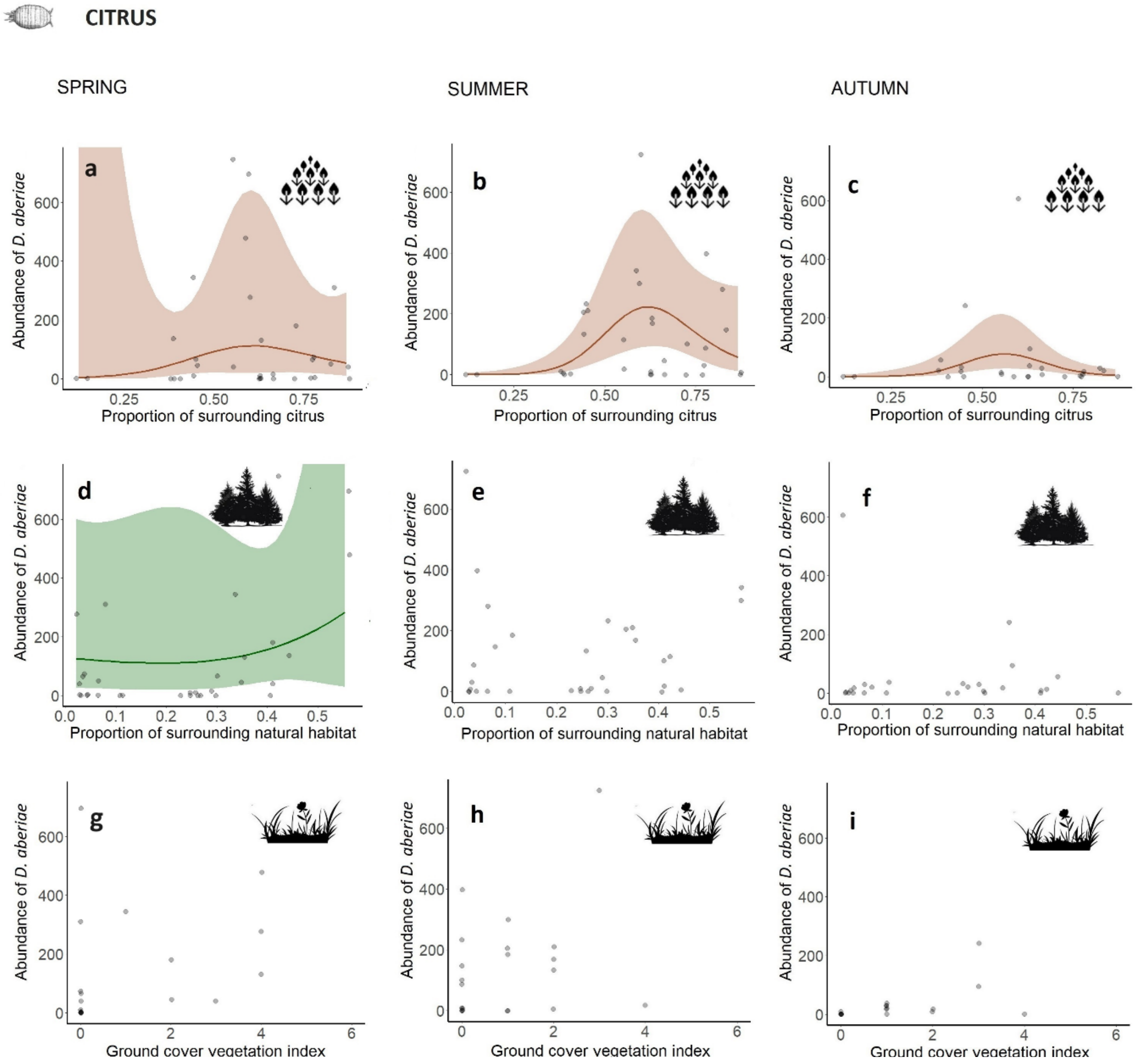


Figure 4. Effect of surrounding citrus (a-c), semi-natural habitat (d-f), and ground cover vegetation (g-h) on the abundance of *Delottococcus aberiae* in citrus. In each season, mealybug abundance was explained by the variables included in the averaged selected models (proportion of natural habitat in the large influence area in spring; proportion of persimmon in the small influence area in spring, summer and autumn). Lines represent predictions from averaged GAMs; shaded regions correspond to 95% confidence intervals. For variables not included in the models, only point dispersion is shown, with no predictions.

Effects of habitat heterogeneity on mealybug parasitism

In citrus, no parasitized *D. aberiae* was found during the study. In persimmon, the proportion of parasitized *P. longispinus* was very low in spring (mean parasitism = 0.025), increased in summer (0.102) and reached its maximum in autumn (0.169; **Figure 2c**). In spring, parasitism of *P. longispinus* was explained by the proportion of surrounding persimmon, but parasitism was only detected in a few plots and this effect was not significant ($p = 0.095$; **Figure 5a**; see **Table S4**). The year was included in the selected models explaining *P. longispinus* parasitism in summer and autumn, being significantly higher in 2020 than in 2021 ($p < 0.001$). In summer, parasitism significantly increased with the proportion of semi-natural habitat in the small influence area ($p <$

0.001). The proportion of parasitized *P. longispinus* increased almost eight times when the proportion of surrounding semi-natural habitats increased from 0% to 20% (**Figure 5e**; see **Table S9**). Furthermore, mealybug parasitism significant increased along with ground cover during the summer (p < 0.001) and autumn (p < 0.001), nearly quadrupling when ground cover ranged from 0% to 30-50% (**Figure 5h-i**; see **Table S9**). In autumn, *P. longispinus* parasitism was also affected by the proportion of persimmon (*p* = 0.001), displaying a U shape pattern with minimum parasitism at a proportion of 0.4 (**Figure 5c**; see **Table S9**).

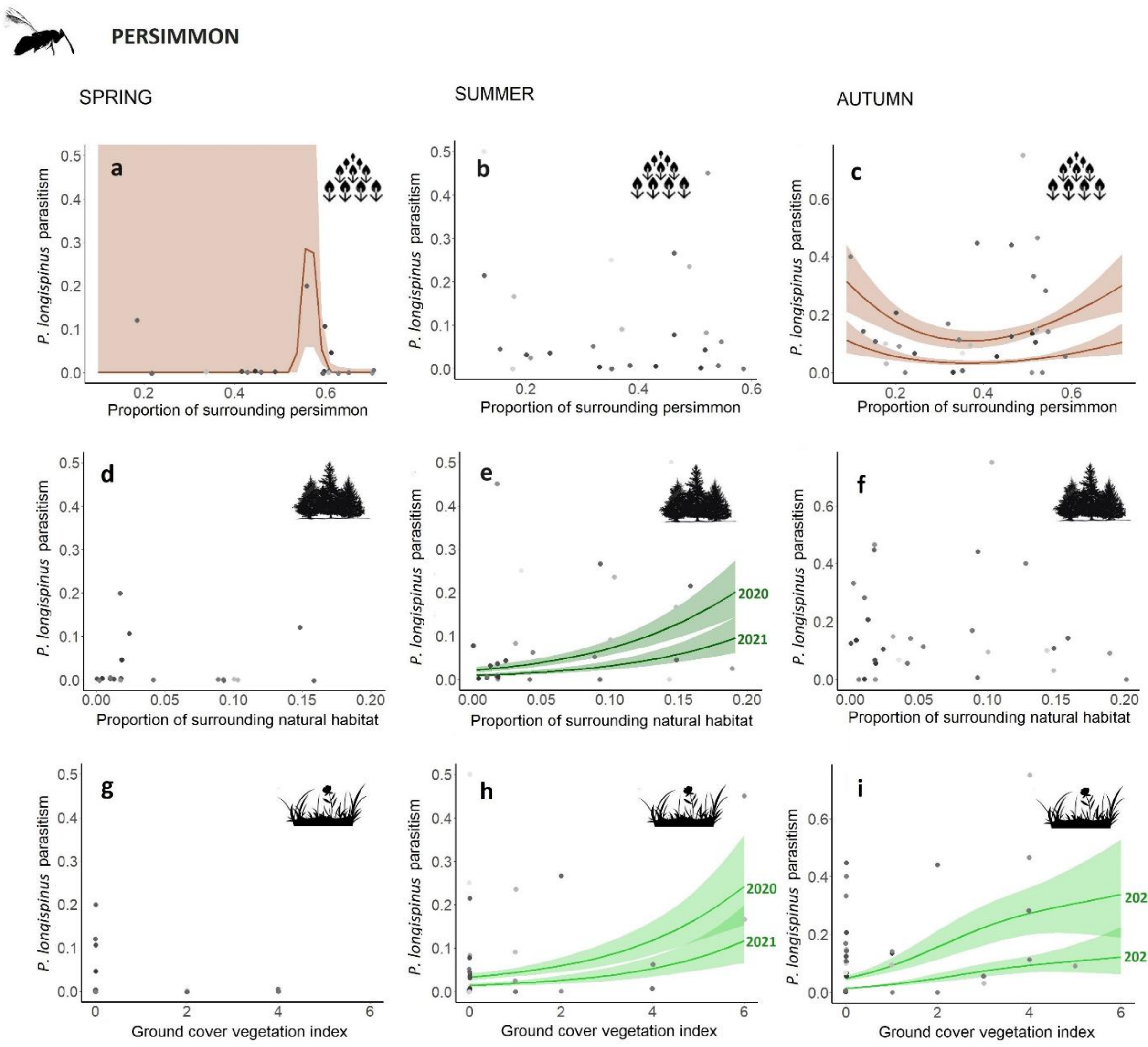


Figure 5. Effect of surrounding persimmon (a-c), semi-natural habitat (d-f), and ground cover vegetation (g-h) on *Pseudococcus longispinus* parasitism. In each season, mealybug parasitism was explained by the variables included in the averaged selected models (ground cover vegetation index in summer and autumn; proportion of natural habitat in the small influence area in summer; proportion of persimmon in the small and large influence area in summer and autumn, respectively). Lines represent predictions from averaged GAMs; shaded regions denote 95% confidence intervals. Darker points represent plots with higher sample size (number mealybugs). If the year effect was significant, different lines represent each year. For variables not included in the models, only point dispersion is shown, with no predictions.

Mediating effects of parasitism on *P. longispinus* abundance

Structure equation models confirmed that the proportion of semi-natural habitat negatively affected *P. longispinus* abundance in spring ($p = 0.001$; **Figure 6a**) and summer ($p < 0.001$; **Figure 6b**), while ground cover vegetation index negatively affected *P. longispinus* abundance in autumn ($p = 0.038$; **Figure 6c**). SEMs also confirmed that *P. longispinus* parasitism was positively affected by the proportion of semi-natural habitat in summer ($p < 0.001$; **Figure 6b**), and by the ground cover vegetation index in autumn ($p = 0.042$; **Figure 6c**). SEMs showed that *P. longispinus* abundance was not affected by parasitism in spring ($p = 0.962$; **Figure 6a**) and summer ($p = 0.835$; Figure 6b). In autumn, however, *P. longispinus* abundance was negatively affected by parasitism ($p = 0.030$; **Figure 6c**).

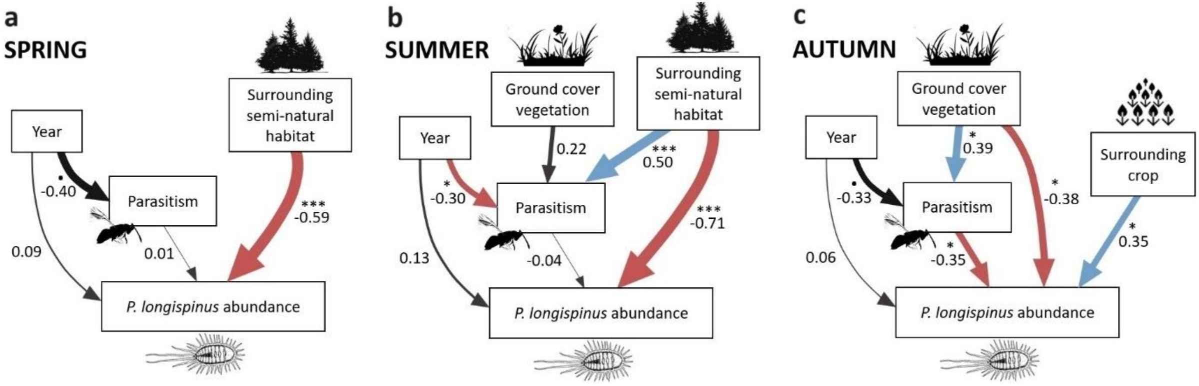


Figure 6. Structural equation models explaining *Pseudococcus longispinus* abundance in persimmon orchards in spring (a), summer (b) and autumn (c). Each season, the year of study and variables from previously selected models, explaining mealybug abundance, were considered exogenous variables influencing mealybug parasitism and abundance. Mealybug abundance was affected by parasitism (endogenous variable). Standardized path coefficients are provided along the arrows, with significance levels marked using stars (·$p < 0.08$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Arrow width indicates the strength of the relationship, with blue arrows representing positive relationships and red arrows representing negative relationships.

4. Discussion

Our study shows that habitat simplification can increase invasive mealybug abundance, a growing threat for subtropical crops worldwide. The proportion of focal crop (*i.e.*, monoculture) increased mealybug abundance in persimmon and citrus, while non-crop habitats strongly decreased mealybug abundance in persimmon by enhanced parasitism. Our models suggest that resource concentration and natural enemies hypotheses can work sequentially along the growing season and explain the incidence of mealybugs in persimmon. In contrast, only resource concentration hypothesis was supported in citrus, likely due to the absence of parasitoids.

The prevalence of crop monoculture in the landscape was found to increase mealybug abundance in persimmon and citrus. This effect was most pronounced when the densities of *P. longipinus* and *D. aberiae* were at their peak. Both mealybug species thrived in monoculture landscapes, likely because such environments facilitated their dispersion (Grez & González, 1995; Martinson & Fagan, 2014; O'Rourke & Petersen, 2017). Mealybugs, in contrast to winged insects, disperse primarily through walking and airborne movement, and their numbers decrease exponentially with greater distances between host plants (Grasswitz & James, 2008). Accordingly, the study found that the positive impact of monoculture on mealybug abundance was evident within a relatively small influence area, up to approximately 600 meters in citrus orchards. The resource concentration hypothesis varies depending on the organism, with oligophagous herbivores expected to respond more strongly than polyphagous ones to host concentration (Martinson & Fagan, 2014; O'Rourke & Petersen, 2017). While both *Pseudococcus longispinus* and *Delottococcus aberiae* can feed on various plants (Miller & Gilliomee, 2011; Wysoki et al., 1977), their positive response to persimmon and citrus monoculture, respectively, suggests that these host plants are particularly suitable for them.

Semi-natural habitats surrounding the crop and ground cover vegetation, were found to reduce the abundance of *P. longispinus* in persimmon orchards. This outcome supports the natural enemies hypothesis, as these non-crop habitats were associated with increased parasitism of *P. longispinus*. In Mediterranean persimmon, a complex of encyrtid parasitoids, particularly *Anagyrus fusciventris*, targets this mealybug (Plata et al., 2023b). Our study revealed that during spring and, especially, summer, semi-natural habitats and ground cover vegetation led to reduced mealybug abundance and increased parasitism. Additionally, the positive effects of these non-crop habitats on mealybug parasitism had a subsequent impact on mealybug abundance in autumn. This sequential pattern suggests potential cascading effects from higher trophic levels, supporting the theory of top-down control (Mills, 2001). Non-crop habitats can serve as shelters and provide resources like nectar, alternative hosts, and pollen to parasitoids (Bianchi et al., 2006; Landis et al., 2000; Rusch et al., 2010), improving their nutritional condition and, subsequently, their effectiveness as mealybug biocontrol agents (Mockford et al., 2022; Tena et al., 2015). It's important to note that persimmon is a deciduous tree with limited resources during winter and early spring. Nearby semi-natural habitats may host *P. longispinus* during these seasons (Wysoki et al., 1977), allowing parasitoid populations to develop before moving to areas with high pest density. The study further revealed that the impact of semi-natural habitats on *P. longispinus* parasitism was more significant in smaller influence areas. This aligns with previous research showing that specialist enemies like parasitoids may respond more strongly to landscape heterogeneity at smaller scales (Chaplin-Kramer et al., 2011; Thies et al., 2005). This study provides valuable insights into the role of non-crop habitats in enhancing mealybug parasitism and reducing mealybug density.

In contrast to persimmon orchards, non-crop habitats did not affect mealybug abundance in citrus. This is likely because of the lack of effective natural enemies (Tscharntke et al., 2016). *Delottococcus aberiae* is a recent introduction to Spain, and resident parasitoids do not parasitize it (Tena et al., 2017). Although the parasitoid *A. aberiae* was introduced in 2020 (Soto et al., 2021), our study showed that this parasitoid has not established or dispersed in the area. We did not find any parasitized *D. aberiae* during the study. Similarly, the abundance of predatory species like the coccinellid *C. montrouzieri*, which attacks mealybugs in the study area (Pérez-Rodríguez et al., 2019), was exceptionally low. Critically, the time since the arrival of an exotic pest plays a crucial role in how non-crop habitats affect these pests (Tamburini et al., 2020). Recent invaders like *D. aberiae* are less likely to have specific natural enemies in the invaded area. On the other hand, long-established invaders like *P. longispinus*, that are attacked by well-established parasitoids, are affected by non-crop habitats in terms of top-down control by these natural enemies.

The results of this study offer specific recommendations for managing invasive mealybugs in subtropical crops. First, it is advisable to reduce monoculture at the landscape scale to counter the advantages that mealybugs gain from resource concentration. Orchards surrounded by less than 30% of monoculture had significantly lower mealybug abundance in both studied crops, which suggests that orchard isolation can decrease mealybug incidence, even in the absence of strong top-down regulation by natural enemies. Furthermore, following the natural enemies' hypothesis, increasing the proportion of certain non-crop habitats at local and landscape scales can enhance mealybug biological control. Negative effects of semi-natural habitats and ground cover vegetation on mealybug abundance were observed in persimmon, but not in citrus, where mealybugs were not parasitized. The models showed that maintaining more than 10% ground cover by non-crop vegetation within the orchards and more than 15% of semi-natural habitats in the surrounding landscape can suppress *P. longispinus* by increasing its parasitism in persimmon. Although economic injury levels of *P. longispinus* in persimmon have not been yet determined (Plata et al. 2023c), these recommendations could significantly reduce mealybug abundance, and fruit damage. However, long-term landscape planning remains a challenge, emphasizing the need for policymakers and stakeholders to optimize the management of invasive pests such as mealybugs. In the short term, implementing ground covers at the field level can be a practical solution for reducing mealybug damage, particularly in the case of *P. longispinus*.

In summary, this research indicates that enhancing habitat diversity within both local and broader landscapes can significantly decrease the populations of invasive mealybugs in subtropical crops. This reduction is achieved by addressing both "resource concentration" and "natural enemies" aspects. While the presence of efficient parasitoids remains crucial for effective biocontrol, the study underscores that habitat diversification strategies represent an ideal approach to promote the sustainable cultivation of these crops.

Data accessibility

Data from this study are available at the IVIA Digital Repository:
https://doi.org/10.58582/redivia.8759

Supplementary material

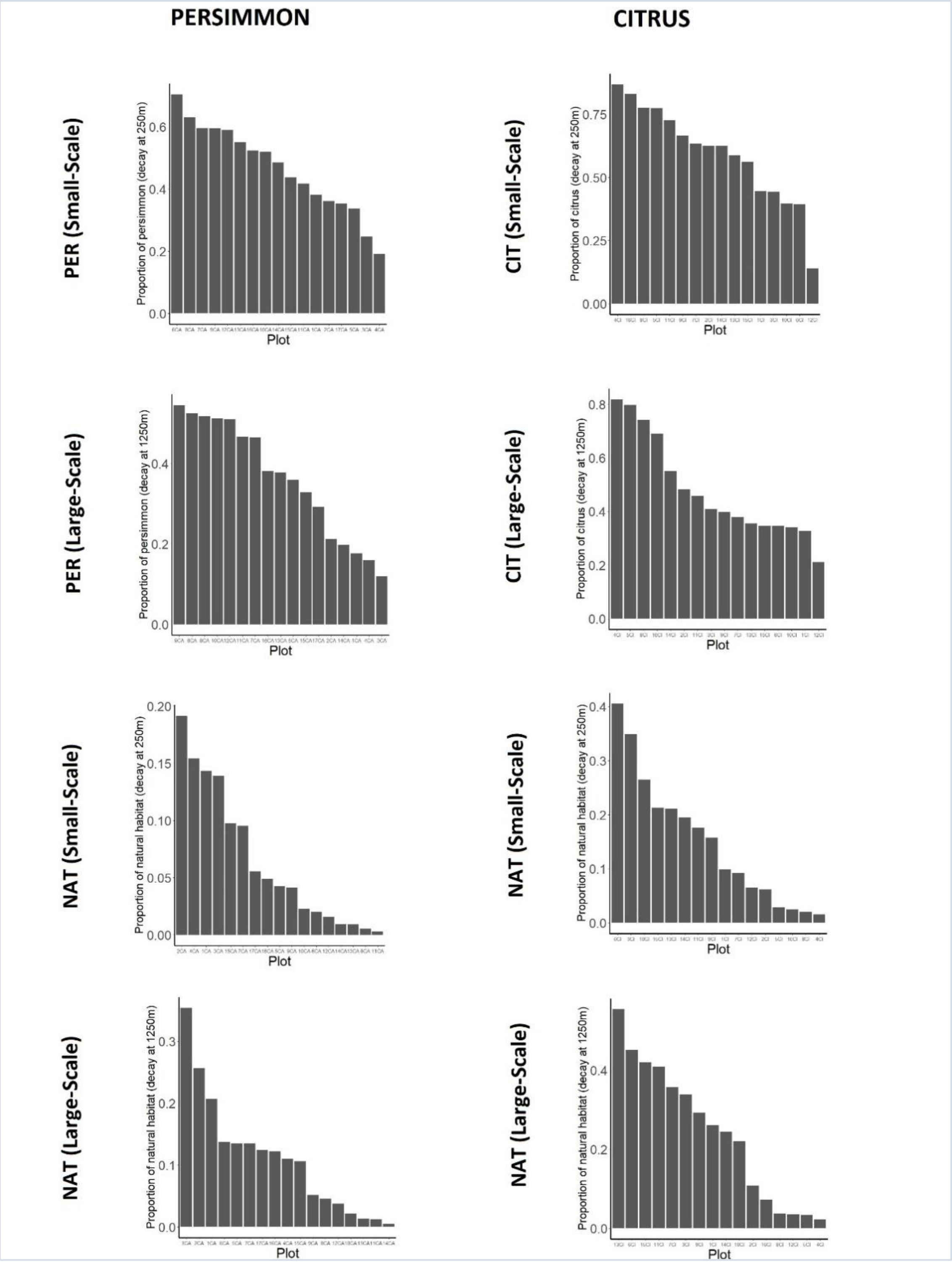


Figure S1. Gradient of surrounding monoculture (persimmon or citrus) and surrounding natural habitat proportion across all sampled orchards. PER (Small-scale) and PER (Large-scale): proportion of surrounding persimmon in the small influence area (250 decay rate) and in the large influence area (1250 decay rate); CIT (Small-scale) and CIT (Large-scale): proportion of surrounding citrus in the small influence area (250 decay rate) and in the large influence area (1250 decay rate); NAT (Small-scale) and NAT (Large-scale): proportion of surrounding natural habitat in the small influence area (250 decay rate) and in the large influence area (1250 decay rate).

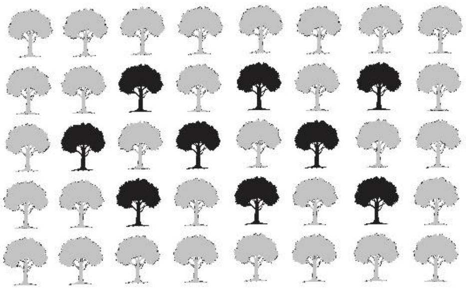


Figure S2. Plot of 40 trees selected in each orchard. Nine alternate trees (black colour) were randomly selected excluding the border and sampled across the three seasons of the study.

Figure S3. Adult female of healthy *Pseudococcus longispinus* (above), alongside a parasitized third-instar nymph (below). The signs of parasitism are evident; the mealybug mummifies, losing its white waxes and turning yellow-brown.

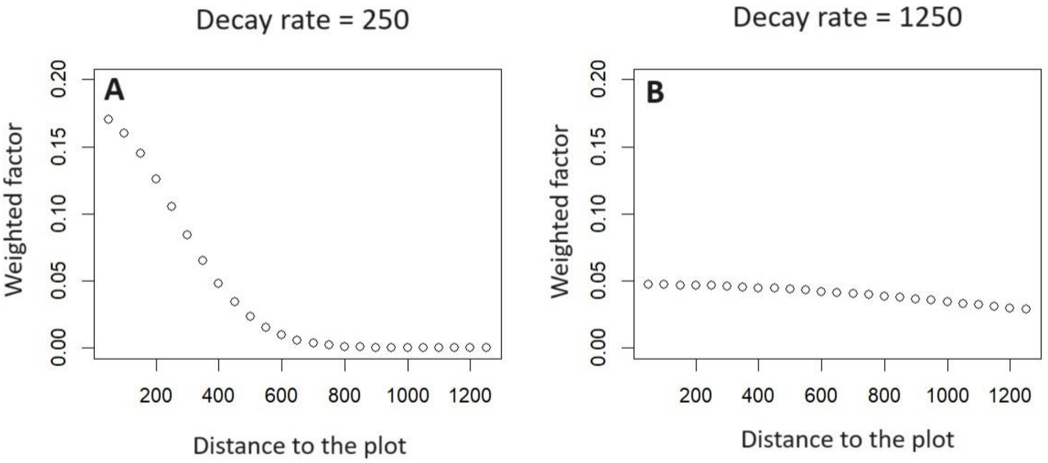


Figure S4. Weight of surrounding landscape according to the distance from each location using Gaussian decay functions with two different decay rates (250 and 1250) to vary the relative influence of closer versus further areas from the farm. (A) When the decay rate was 250, a higher weight was assigned to nearby landscape features, and this weight decreased exponentially as the distance to the plot increased, such that landscape elements beyond 600 meters were not relevant. (B) When the decay rate was 1250, a higher weight was assigned to nearby landscape features, but this weight decreased very gradually as the separation distance (i) increased, such that all landscape elements at a 1250-meter distance remained relevant.

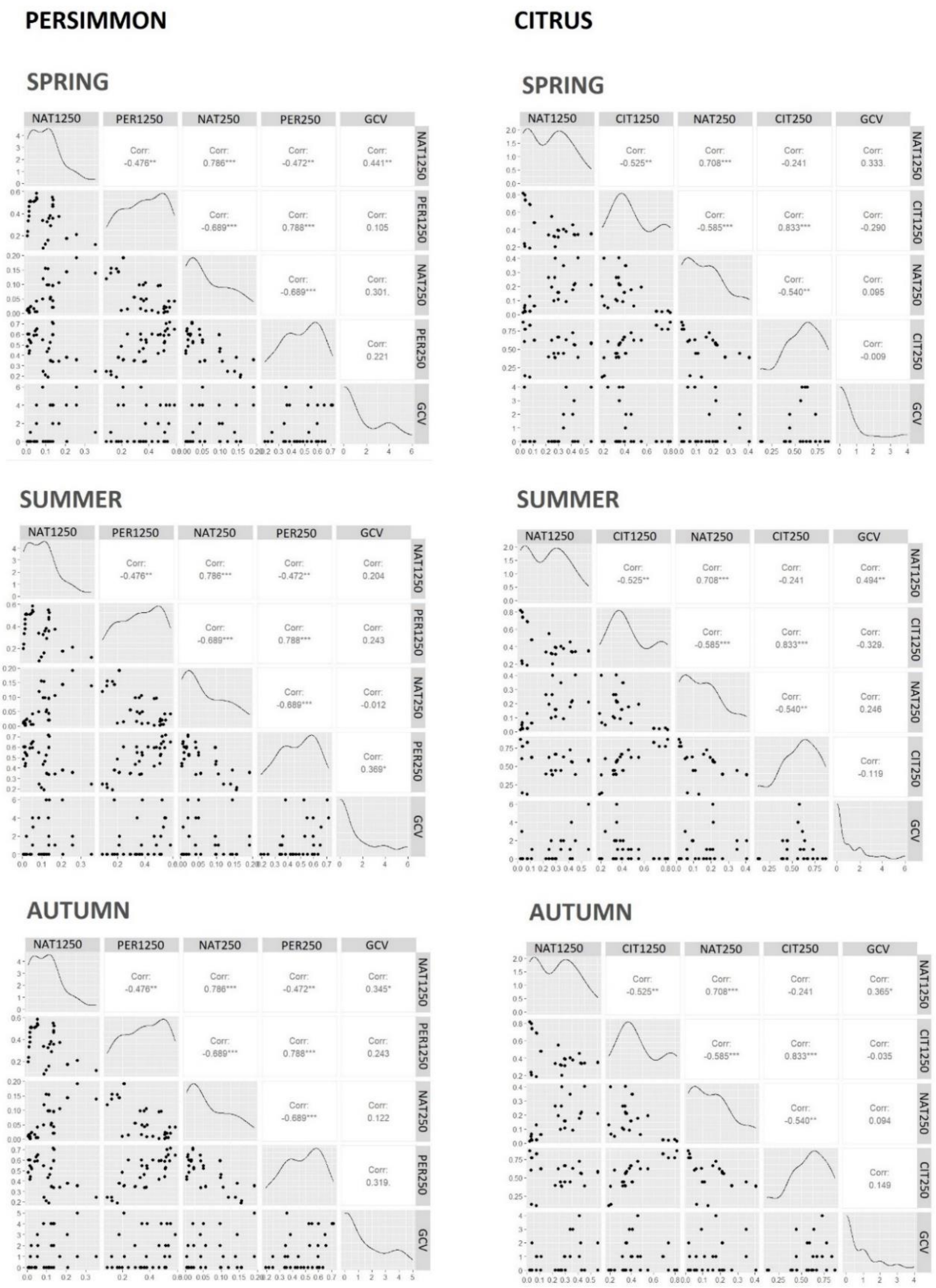


Figure S5. Correlation matrix among candidate variables to build GAM models predicting mealybug abundance and mealybug parasitism. PER250 and PER1250; proportion of surrounding persimmon at small-scale (decay of 250) and large-scale (decay of 1250); CIT250 and CIT1250; proportion of surrounding citrus in the small influence area (decay of 250) and in the large influence area (decay of 1250); NAT250 and NAT1250; proportion of surrounding semi-natural habitat in the small influence area (decay of 250) and in the large influence area (decay of 1250); GCV: Ground cover vegetation index.

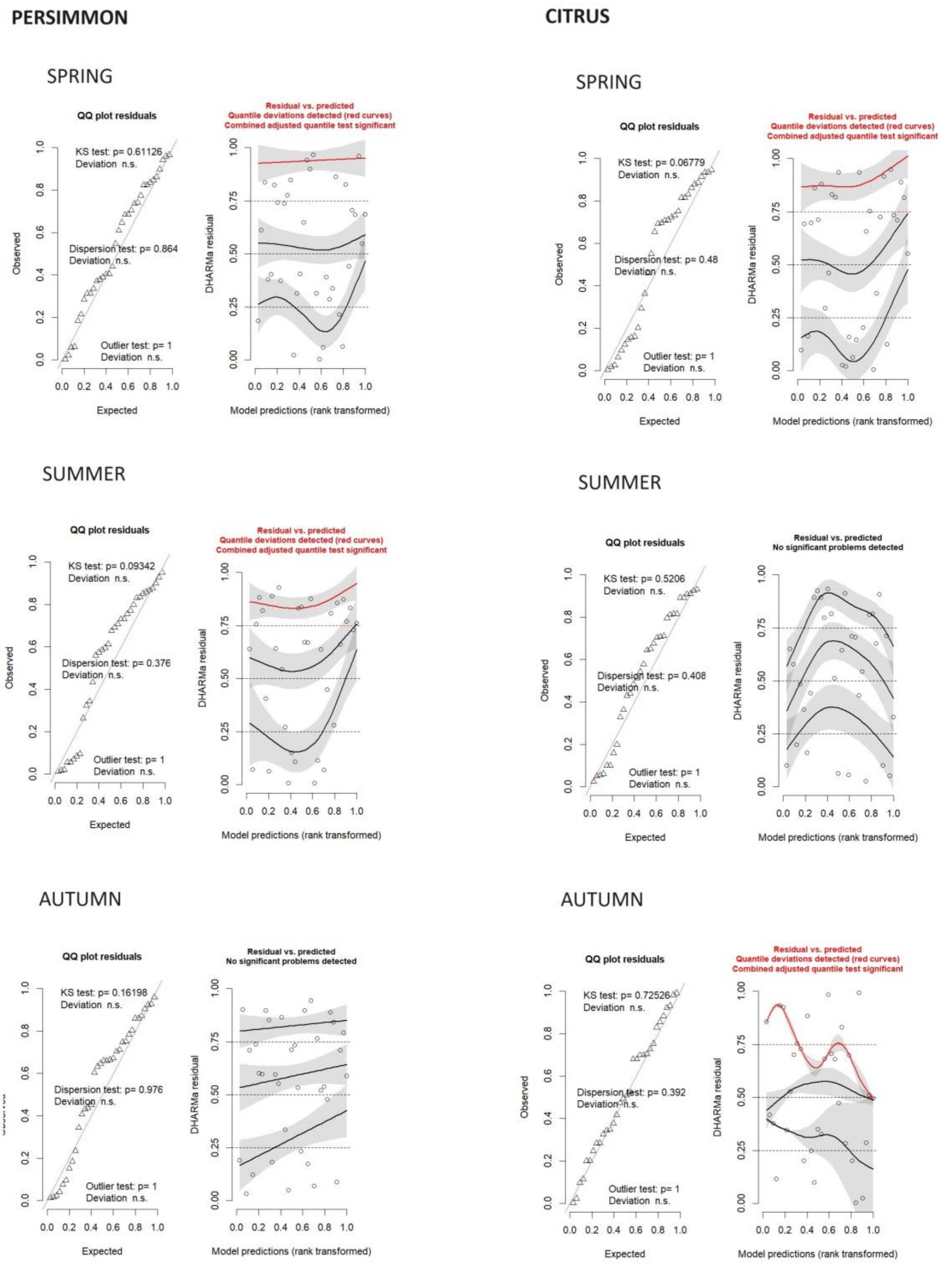


Figure S6. Dharma residuals of the GAM models predicting *Pseudococcus longispinus* abundance in persimmon and *Delottococcus aberiae* abundance in citrus.

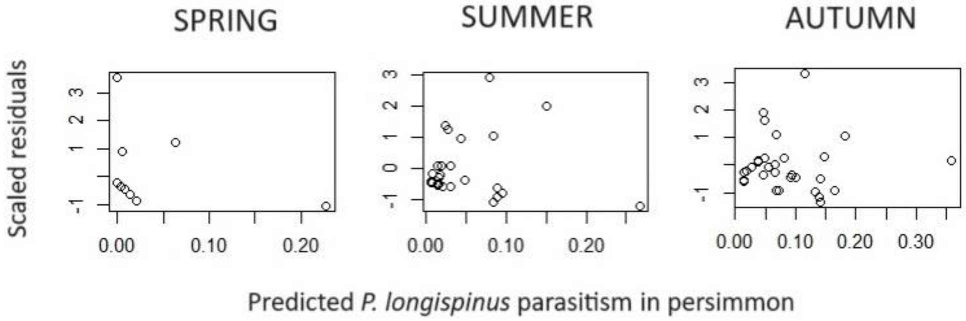


Figure S7. Scaled residuals versus predicted values of *P. longispinus* parasitism in persimmon by the GAM models generated for each season.

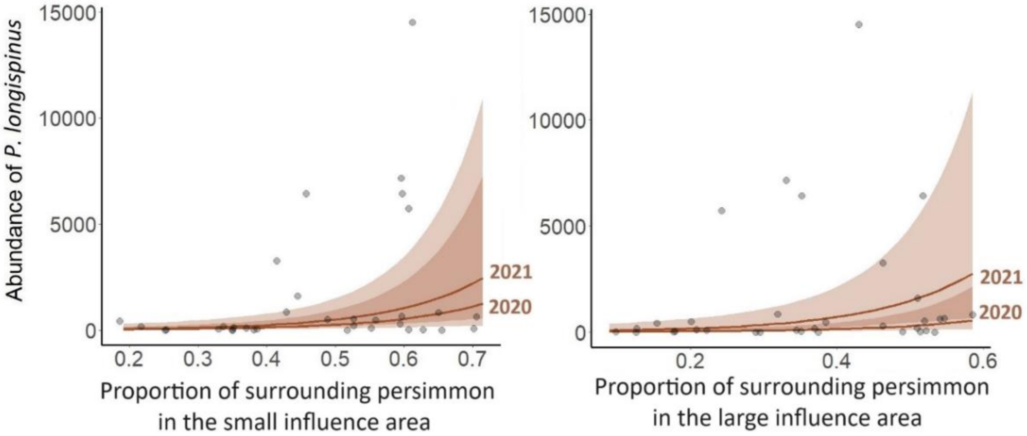


Figure S8. Effect of surrounding persimmon proportion in the small influence area (250 decay) and in the large influence area (1250 decay) on the abundance of the mealybug *Pseudococcus longispinus* in persimmon in autumn. Brown lines represent predictions from averaged GAMs; shaded regions correspond to 95% confidence intervals.

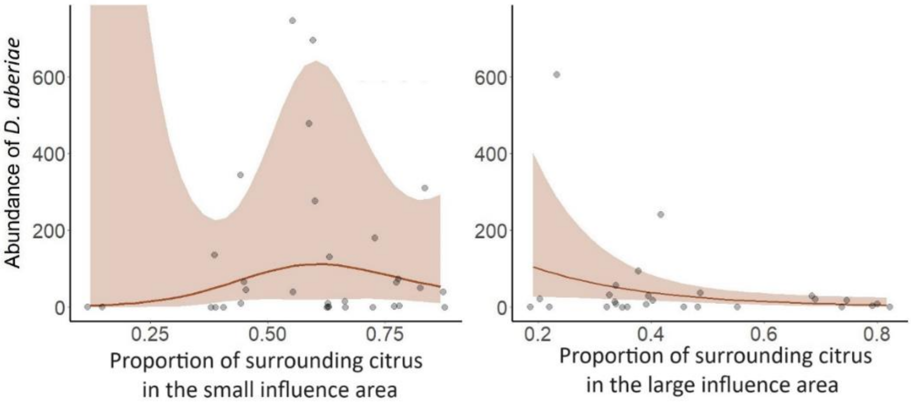


Figure S9. Effect of surrounding citrus proportion in the small influence area (250 decay) and in the large influence area (1250 decay) on the abundance of the mealybug *Delottococcus aberiae* in citrus in autumn. Brown lines represent predictions from averaged GAMs; shaded regions correspond to 95% confidence intervals.

Table S1. Total number of the mealybug predator, *Cryptolaemus montrouzieri*, observed in mealybug colonies across the two years study in all the sampled persimmon and citrus orchards.

Crop	Year	Season	Total number of *C. montrouzieri*
Persimmon	**2020**	Spring	0
		Summer	0
		Autumn	2
	2021	Spring	0
		Summer	0
		Autumn	1
Citrus	**2020**	Spring	0
		Summer	1
		Autumn	0
	2021	Spring	2
		Summer	1
		Autumn	0

Table S2. Fit indices of the SEM models predicting *Pseudococcus longispinus* abundance in persimmon for each season. All the models met the conditions described by Byrne 1994.

Fit index	Condition	Spring	Summer	Autumn
P-value (Chi-square)	>0.05	0.358*	0.512*	0.450*
GFI	>0.95	0.964*	0.994*	0.982*
CFI	>0.90	>0.999*	>0.999*	>0.999*
RMSEA	<0.05	<0.001*	<0.001*	<0.001*
SRMR	<0.08	0.069*	0.020*	0.035*

Table S3. Pearson correlation matrix of abundance of *Pseudococcus longispinus* in persimmon among the different sampling seasons.

	Spring 20	Summer 20	Autumn 20	Spring 21	Summer 21	Autumn 21
Spring 20	1					
Summer 20	0.84***	1				
Autumn 20	0.85***	0.70**	1			
Spring 21	0.87***	0.67**	0.96***	1		
Summer 21	0.57*	0.80***	0.59*	0.57*	1	
Autumn 21	0.44	0.56*	0.35	0.41	0.78***	1

Table S4. AICc of candidate GAMs models predicting *Pseudococcus longispinus* abundance and parasitism in persimmon. Models with less than two points above the model with the lowest AICc are marked in bold. GCV: Ground cover vegetation index; PER (Small-area) and PER (Large-area): proportion of surrounding persimmon in the small influence area (250 decay rate) and large influence area (1250 decay rate); NAT (Small-area) and NAT (Large-area): proportion of surrounding natural habitat in the small influence area (250 decay rate) and large influence area (1250 decay rate).

	***P. longispinus* abundance**			***P. longispinus* parasitism**		
GAM Model	**Spring**	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**
Null	205.9	403.3	493.6	31.6	408.1	1202.2
Year	207.7	405.3	494	27.6	353.7	915.6
GCV	199.7	401	488	33.6	341.4	890.2
GCV+Year	200.8	403.5	485.8	30	303.6	606.1
PER (Small-area)	207.8	404.8	490.3	**19.2**	342.5	1132.3
PER (Large-area)	208.4	405.3	490.9	27.9	344.8	831.6
PER (Small-area)+Year	209.9	407.1	492.4	22	291	880.6
PER (Large-area)+Year	210.3	407.5	493.1	26.2	303.5	733.8
PER (Small-area)+GCV	199.3	400	**481.2**	22.2	272	832.8
PER (Large-area)+GCV	201	401.9	483.7	29.3	284.9	788.9
PER (Small-area)+GCV+Year	201.2	402.8	**480.6**	25.2	248.5	593.7
PER (Large-area)+GCV+Year	202.2	404.4	**480.1**	28.6	256	**589.5**
NAT (Small-area)	206.6	402.1	489.7	32.6	291.5	1179.2
NAT (Large-area)	**194**	**393.9**	483.1	22.9	261.1	1024.7
NAT (Small-area)+Year	209.1	403.9	490.2	28.5	262.1	907.3
NAT (Large-area)+Year	196.7	396.3	483.5	24.3	238.1	801
NAT (Small-area)+GCV	199.9	399.5	486	35.2	247.1	887.6
NAT (Large-area)+GCV	**195.9**	**395.7**	483.5	25.3	258.9	854.8
NAT (Small-area)+GCV+Year	201.4	402.3	485.3	31.5	**222.4**	595.1
NAT (Large-area)+GCV+Year	198.6	398.4	483.7	27.8	236.7	607.6

Table S5. Proportion of deviance explained by the candidate GAMs models predicting *Pseudococcus longispinus* abundance and parasitism in persimmon. Selected models with less than two points above the model with the lowest AICc are marked in bold. GCV: Ground cover vegetation index; PER (Small-area) and PER (Large-area): proportion of surrounding persimmon in the small influence area (250 decay rate) and large influence area (1250 decay rate); NAT (Small-area) and NAT (Large-area): proportion of surrounding semi-natural habitat in the small influence area (250 decay rate) and large influence area (1250 decay rate).

	***P. longispinus* abundance**			***P. longispinus* parasitism**		
GAM Model	**Spring**	**Summer**	**Autumn**	**Spring**	**Summer**	**Autumn**
Null	0	0	0	0	0	0
Year	2.2	1.13	4.5	28.8	16.9	26.7
GCV	23.3	11	19.3	2.1	20.6	29.2
GCV+Year	26.9	11.1	26.5	30.9	32.6	55.7
PER (Small-area)	1.9	2.3	12.0	**75.7**	21.0	6.9
PER (Large-area)	0	1.1	16.0	25.6	20.3	34.6
PER (Small-area)+Year	3.3	3.0	13.2	76.8	37.1	30.4
PER (Large-area)+Year	2.2	2.1	17.1	47.6	33.4	43.9
PER (Small-area)+GCV	35.0	18.7	**37.2**	76.7	43.4	35
PER (Large-area)+GCV	26.6	16.3	33.5	34.0	39.5	38.8
PER (Small-area)+GCV+Year	37.2	18.6	**42.4**	67.1	51.3	57.3
PER (Large-area)+GCV+Year	30.2	16.2	**43.2**	51.8	49.2	**57.7**
NAT (Small-area)	11.5	10.3	13.2	6.6	36.1	2.5
NAT (Large-area)	**40.4**	**25.5**	25.5	57.6	45.2	16.8
NAT (Small-area)+Year	11.9	10.4	17.5	37.5	45.6	27.8
NAT (Large-area)+Year	40.7	25.8	29.4	64.1	32.8	37.6
NAT (Small-area)+GCV	29.0	19.6	29.4	7.8	50.7	29.9
NAT (Large-area)+GCV	**42.0**	**27.1**	33.5	54.9	47.4	33
NAT (Small-area)+GCV+Year	32.0	19.7	34.1	38.8	**58.0**	57.2
NAT (Large-area)+GCV+Year	42.9	27.3	36.7	64.1	54.9	55.8

Table S6. GAMs models predicting *Pseudococcus longispinus* abundance in persimmon. Estimates and p values of the parameters included in the averaged models for each season are shown. PER (Small-area) and PER (Large-area): proportion of surrounding persimmon in the small influence area (250 decay rate) and large influence area (1250 decay rate); NAT (Small-area) and NAT (Large-area): proportion of surrounding semi-natural habitat in the small influence area (250 decay rate) and large influence area (1250 decay rate). GCV: Ground cover vegetation index.

	Spring		**Summer**		**Autumn**	
Variable	**Estimate**	***P***	**Estimate**	***P***	**Estimate**	***P***
Intercept	1.41 ± 0.47	0.004	5.14 ± 0.31	<0.001	5.32 ± 0.48	<0.001
Year					1.64 ± 0.64	0.010
PER (Small-area)						
PER (Large-area)					1.07 ± 0.32	<0.001
NAT (Small-area)						
NAT (Large-area)	-2.73 ± 1.17	0.024	-1.29 ± 0.32	<0.001		
GCV	-0.51 ± 0.40	0.227	-0.34 ± 0.32	0.307	-1.09 ± 0.38	0.004

Table S7. Pearson correlation matrix of abundance of *Delottococcus aberiae* in citrus among the different sampling seasons.

	Spring 20	**Summer 20**	**Autumn 20**	**Spring 21**	**Summer 21**	**Autumn 21**
Spring 20	1					
Summer 20	0.61*	1				
Autumn 20	-0.03	0.44	1			
Spring 21	0.42	0.52*	0.24	1		
Summer 21	0.37	0.52*	0.29	0.32	1	
Autumn 21	0.07	0.24	0.29	0.15	0.78***	1

Table S8. AICc of candidate GAMs models predicting *Delottococcus aberiae* abundance in citrus. Models with less than two points above the model with the lowest AICc are marked in bold. GCV: Ground cover vegetation index; CIT (Small-area) and CIT (Large-area): proportion of surrounding citrus in the small influence area (250 decay rate) and large influence area (1250 decay rate); NAT (Small-area) and NAT (Large-area): proportion of surrounding natural habitat in the small influence area (250 decay rate) and large influence area (1250 decay rate).

	***D. aberiae* abundance**		
GAM Model	**Spring**	**Summer**	**Autumn**
Null	**304.3**	327.4	243.3
Year	306.1	328.8	245.5
GCV	306	328	246.5
GCV+Year	308.3	329.6	247.1
CIT (Small-area)	**302.9**	**324.9**	**240**
CIT (Large-area)	305.8	329.7	**240.7**
CIT (Small-area)+Year	305.3	327.2	242.9
CIT (Large-area)+Year	307.8	331.3	243.4
CIT (Small-area)+GCV	305.8	327.5	242.4
CIT (Large-area)+GCV	308.1	330.4	242.6
CIT (Small-area)+GCV+Year	310.6	332.2	245.5
CIT (Large-area)+GCV+Year	310.6	332.2	245.5
NAT (Small-area)	306	329.5	244.2
NAT (Large-area)	**303.7**	330.5	244.8
NAT (Small-area)+Year	307.8	331.3	247.2
NAT (Large-area)+Year	**304**	331.4	247.3
NAT (Small-area)+GCV	307.7	329.7	245.8
NAT (Large-area)+GCV	305.7	330.6	247.3
NAT (Small-area)+GCV+Year	310.1	331.8	248.4
NAT (Large-area)+GCV+Year	306.1	332.6	249.6

Table S9. Proportion of deviance explained by the candidate GAMs models predicting *Delottococcus aberiae* abundance in citrus. Selected models with less than two points above the model with the lowest AICc are marked in bold. GCV: Ground cover vegetation index; CIT (Small-area) and CIT (Large-area): proportion of surrounding citrus in the small influence area (250 decay rate) and large influence area (1250 decay rate); NAT (Small-area) and NAT (Large-area): proportion of surrounding semi-natural habitat in the small influence area (250 decay rate) and large influence area (1250 decay rate).

	***D. aberiae* abundance**		
GAM Model	**Spring**	**Summer**	**Autumn**
Null	**0**	0	0
Year	2.0	3.0	0.8
GCV	2.3	18.9	1.2
GCV+Year	3.4	7.7	4.7
CIT (Small-area)	**17.1**	**18.9**	**21.4**
CIT (Large-area)	2.7	0.5	**13.4**
CIT (Small-area)+Year	18.5	20.3	21.4
CIT (Large-area)+Year	4.7	3.5	13.4
CIT (Small-area)+GCV	17.1	19.5	22.6
CIT (Large-area)+GCV	3.7	5.8	21.4
CIT (Small-area)+GCV+Year	18.4	21	22.8
CIT (Large-area)+GCV+Year	5.2	8.5	21.2
NAT (Small-area)	2.2	1.1	11.3
NAT (Large-area)	**14.8**	2.2	2.7
NAT (Small-area)+Year	4.6	3.5	11.1
NAT (Large-area)+Year	**21.1**	3.6	3.15
NAT (Small-area)+GCV	4.8	7.62	14.6
NAT (Large-area)+GCV	16.8	5.4	8.0
NAT (Small-area)+GCV+Year	6.3	9.6	15.8
NAT (Large-area)+GCV+Year	23.6	7.7	7.4

Table S10. GAMs models predicting *Delottococcus aberiae* abundance in citrus. Estimates and *p* values of the parameters included in the averaged models for each season are shown. CIT (Small-area) and CIT (Large-area): proportion of surrounding citrus in the small influence area (250 decay rate) and large influence area (1250 decay rate); NAT (Small-area) and NAT (Large-area): proportion of surrounding semi-natural habitat in the small influence area (250 decay rate) and large influence area (1250 decay rate). GCV: Ground cover vegetation index.

	Spring		**Summer**		**Autumn**	
Variable	**Estimate**	***P***	**Estimate**	***P***	**Estimate**	***P***
Intercept	4.21 ± 0.58	<0.001	4.30 ± 0.35	<0.001	3.07 ± 0.38	<0.001
Year	1.21 ± 0.76	0.127				
CIT (Small-area)	5.15 ± 1.84	0.005	5.05 ± 1.75	0.002	5.78 ± 1.77	0.002
CIT (Large-area)						
NAT (Small-area)						
NAT (Large-area)	0.86 ± 0.44	0.053				
GCV						

Table S11. GAMs models predicting *Pseudococcus longispinus* parasitism in persimmon. Estimates and *p* values of the parameters included in the averaged models for each season are shown. PER (Small-area) and PER (Large-area): proportion of surrounding persimmon in the small influence area (250 decay rate) and large influence area (1250 decay rate); NAT (Small-area) and NAT (Large-area): proportion of surrounding semi-natural habitat in the small influence area (250 decay rate) and large influence area (1250 decay rate). GCV: Ground cover vegetation index.

	Spring		**Summer**		**Autumn**	
Variable	**Estimate**	***P***	**Estimate**	***P***	**Estimate**	***P***
Intercept	-100.77 ± 60.3	0.108	-2.95 ± 0.10	<0.001	-1.90 ± 0.08	<0.001
Year			-0.87 ± 0.17	<0.001	-1.29 ± 0.09	<0.001
PER (Small-area)	274.87 ± 158.3	0.095				
PER (Large-area)					-0.19 ± 0.07	<0.001
NAT (Small-area)			0.76 ± 0.08	<0.001		
NAT (Large-area)						
GCV			0.72 ± 0.09	<0.001	0.69 ± 0.07	<0.001

General discussion

This thesis provides basic and applied knowledge about the biology and ecology of mealybugs on two important subtropical fruit crops in Mediterranean basin, citrus and persimmon, where invasive mealybugs have become major pests that cause severe crop losses. Different strategies that can enhance mealybug biological control have been identified and evaluated. The findings of this thesis should be considered in the design of Integrated Pest Management programs against mealybugs in citrus and persimmon. In addition, this thesis has tested and reinforced several ecological hypotheses which outcomes might be relevant beyond the present study system, being useful to improve the management of mealybugs in other crops and geographical regions. Several questions arising from this thesis should be further addressed to improve the biological control of mealybugs in subtropical fruit crops.

1. Mutualism between ants and the invasive mealybug *Delottococcus aberiae* in Mediterranean citrus

The trophobiotic relationships between ants and honeydew producing hemipterans has drawn wide attention from evolutionary ecologists, as these relationships are considered drivers of the evolution of both ants and associated hemipterans (Nelson & Mooney, 2022). The increase in the number of invasive species has led to non-coevolved ants and hemipterans coming into contact and potentially establishing novel trophobiotic interactions that benefit both partners (Wang et al., 2021). Although the relationships between ants and invasive hemipteran pests of agricultural importance have been widely studied, the interactions between ants and *Delottococcus aberiae* had never been studied because this mealybug is an emerging pest that has not caused significant economic losses until its recent invasion in Mediterranean citrus. The first section of this thesis reveals that the trophobiotic relationships between *D. aberiae* and native Mediterranean ants may be one of the factors explaining the invasive success of this mealybug in Mediterranean citrus. Importantly, it also emphasizes that the management of these mutualistic ants can be an effective strategy to enhance the control of *D. aberiae*.

In the **first chapter**, I described the interactions between Mediterranean native ants and the invasive mealybug *D. aberiae*, a decade after the first detection of this invasive mealybug in the Mediterranean basin (Plata et al. 2024a). My findings highlight the importance of resident ant communities in the establishment and spread of honeydew-producing exotic insects (Abbot & Green, 2007; Helms & Vinson, 2003; Tena et al., 2013; Wang et al., 2021; Zhou et al., 2014a). Specifically, this study showed that the Mediterranean ant *L. grandis*, the most abundant in Mediterranean citrus orchards, and the invasive mealybug *D. aberiae* have established a trophobiotic relationship that can benefit both partners (**Figure 1**). I described this relationship over two years and three different seasons, representing one of the most detailed descriptions of an ant-mealybug interaction. The tendency of *L. grandis* to establish mutualistic relationships with honeydew producers could strengthen its hegemonic role as dominant ant in Mediterranean citrus orchards (Pekas et al., 2011; Zina et al., 2020). From a practical standpoint, I have demonstrated a strong density dependence between *L. grandis* and *D. aberiae* at both colony and orchard levels, with high densities of *D. aberiae* associated with increased activity of *L. grandis*. Interestingly, our study suggests that monitoring ants can help detect *D. aberiae* infestations, as other studies have proposed for other mealybug species (Castracani et al., 2023; Mgocheki & Addison, 2009c). Our finding also suggested that managing the Mediterranean ant *L. grandis* may enhance the control of *D. aberiae* in citrus orchards, which was tested in the following chapter.

Figure 1. The native ant *Lasius grandis* and the invasive mealybug *Delottococcus aberiae* have established a mutualistic relationship in Mediterranean citrus. In the image, two *Lasius grandis* ants are attending a colony of the mealybug.

In the **second chapter**, I assessed whether the physical exclusion of ants from citrus canopies may enhance the control of *D. aberiae* (Plata et al. 2023d). This study further confirmed that the application of sticky barriers can successfully exclude Mediterranean ground-nesting ants, such as *L. grandis*, from the citrus canopies (Juan-Blasco et al., 2011; Piñol et al., 2009; Pekas et al., 2010). As hypothesized in the first chapter, physical exclusion of ants resulted in a sharp reduction in *D. aberiae* density and damage in citrus. The experiment revealed that the presence of the native ant *L. grandis*, which attended the mealybug in the control trees, facilitates *D. aberiae* to reach high densities in citrus canopies. My results suggest that *D. aberiae* benefit from *L. grandis* because the ant disrupts the mealybug biological control. Although *D. aberiae* was not parasitized by the native parasitoids nor by the recently invaded *Anagyrus aberiae*, it was attacked by generalist predators that increased in abundance when ants were excluded. The lack of parasitism in this study increases its novelty, as parasitoids usually mediate the effects of ants on mealybugs (*e.g.*, Anjos et al., 2021; 2022; Fanani et al., 2020; Feng et al., 2015; Mgocheki & Addison, 2009b). Beyond a higher abundance of generalist predators, other factors likely contributed to the reduction in mealybug abundance. First, sticky barriers used in the study may serve to avoid the recolonization of citrus canopies by *D. aberiae* through the trunk. Additionally, the exclusion of ants might make the mealybug to lose other services not mediated by natural enemies that ants might provide, such as transportation or cleaning. These additional services that ants can provide to hemipterans have been much less studied, and it would be worthwhile for future studies to delve into their understanding. Overall, this work demonstrates that sticky barriers applied in citrus trunk can be efficient in reducing the damage caused by *D. aberiae*. However, sticky barriers may require a significant investment of time and money, and other ant-control techniques might be more cost-effective (Plata & Tena 2022; Schifani et al., 2024), including sugar dispensers and insecticide baits (Milosavljević et al., 2024; Pérez-Rodríguez et al., 2021).

2. *Pseudococcus longispinus* as the main mealybug pest in Mediterranean persimmon

The density of pests can increase following the expansion of a crop in new area. In a few years, mealybugs have become one of the most damaging pests in Mediterranean persimmon (ASAJA, 2021; García-Martínez, 2019). In the second section of the thesis, I identified the mealybug complex attacking persimmons and delved into understanding their biology and ecology. First, I found that *P. longispinus* has become the most abundant mealybug species in this crop and, remarkably, climate warming may exacerbate the damage caused by this mealybug. From a practical standpoint, the study of the *P. longispinus* seasonal trend and phenology can serve for designing sampling protocols and treatment thresholds for this mealybug. Finally, I identified the parasitoid complex of *P. longispinus* in Mediterranean persimmon and I found that the parasitoid *Anagyrus fusciventris* has a high potential to control the mealybug.

In the **third chapter**, I described the complex and seasonal trend of mealybugs attacking Mediterranean persimmon (Plata et al. 2022; 2023c). This study revealed that *P. longispinus* has become the most abundant species in the crop (**Figure 2**), displacing other mealybug species with which it shared prominence a few years ago (García-Martínez et al., 2017; Prieto, 2016). Interestingly, this polyphagous mealybug species is naturalized in the region and occurs in various crops, but it has rarely become a significant pest in the Mediterranean region (Bertin et al., 2010; Pellizzari & Germain, 2010; Swirski et al., 1980). The study showed that *P. longispinus* can cause severe crop losses in persimmon. To gain a better understanding of the biology and ecology of *P. longispinus* on this crop, I also investigated its seasonal phenology.

Figure 2. The long-tailed mealybug, *Pseudococcus longispinus*, has displaced other mealybug species and has become the most significant pest of Mediterranean persimmons. In the image, an adult female and a third instar nymph on a persimmon leaf.

I found that *P. longispinus* completed three generations and reached peak density in the last generation that appears in autumn, posing a significant threat to harvested fruits. Additional findings can serve to enhance the management of *P. longispinus* in persimmon. Firstly, I observed that the mealybug is usually located beneath the sepals of persimmon fruit, potentially protecting them from insecticides and natural enemies (Berlinger and Gol'berg, 1978; Daane et al., 2002). This suggest that systemic insecticides may be more effective than contact insecticides against *P. longispinus* and the mealybug would be more vulnerable to insecticides and natural enemies before fruit development. Furthermore, I found that the proportion of fruits infested by *P. longispinus* in spring and summer positively correlates with the infestation levels at harvest, thus samplings in spring-summer can help predict harvest damage and make decisions on whether to implement different control treatments. Binomial sampling of fruit has been used in IPM programs of other mealybug pests (Cavalloro & Prota, 1983; Pérez-Rodríguez et al., 2017). This study can serve to design a binomial sampling protocol to predict *P. longispinus* damage in persimmon. Further research should determine intervention thresholds against this mealybug.

In the third chapter, I also studied the phenology of *P. longispinus* in persimmon, which can highly contribute to improve the management of the mealybug. First, this work enabled the identification of optimal times for implementing insecticide treatments or releasing natural enemies targeting specific developmental stages of *P. longispinus*, as certain mealybug developmental stages are less vulnerable to insecticides or natural enemies (Bartlett & Lloyd, 1958; Ulusoy et al., 2022). In this sense, I would like to explain that the main cooperatives of persimmon are using these data to establish the sampling and treatment dates since 2022, and the damages produced by *P. longispinus* have been much lower in 2022 and 2023 than they were in previous years. I also assessed the potential effects of climate change on mealybug phenology, revealing that the rise in temperature may increase the incidence of *P. longispinus* in persimmon. The Mediterranean basin is particularly sensitive to climate warming, and it is anticipated that temperature increase will be more severe than in other parts of the world (IPCC, 2023; Zittis et al., 2019). This temperature warming could be a factor escalating the damage caused by *P. longispinus* by accelerating its development, potentially leading to a fourth generation that could have catastrophic consequences on crop losses. This discovery contributes to the growing body of evidence indicating that global warming may intensify the harm caused by mealybugs in certain crops (Fand et al., 2014; Jara et al., 2013; Ji et al., 2020), and it underscores the urgency of developing an Integrated Management Program targeting *P. longispinus* and other pests that could potentially thrive under future climate scenarios.

In the **fourth chapter**, biological control agents of *P. longispinus* in persimmon were identified and evaluated (Plata et al. 2023b). To achieve this, I focused on parasitoids, as they are considered the most effective natural enemies against this and other mealybug species (Bartlett & Lloyd, 1958; Charles et al., 2010; Flanders, 1940; Moore, 1998). I hypothesized that the high densities of *P. longispinus* could be attributed to the inefficacy of its parasitoids, as has been suggested in other crops and geographic areas where *P. longispinus* has become a pest. I found that *P. longispinus* was parasitized by a diverse complex of parasitoids in Mediterranean persimmon. This complex was dominated by one encyrtid species, *A. fusciventris* (**Figure 3**). Interestingly, this parasitoid has not been intentionally imported to Spain, thus its presence in the study area indicates that it has spread spontaneously, likely from the eastern Mediterranean Basin where it was introduced in the 1970s (Swirski et al., 1980). Our results demonstrated that the parasitoid is well-established in eastern Spain as it was present in all the orchards infested by *P. longispinus*, but parasitism rates highly varied among orchards. Critically, I observed that higher rates of parasitism were associated with lower pest densities at orchard level. Therefore, this parasitoid seems to have a high potential to be used in augmentative biological control programs (Plata et al. 2023a). In this regard, I identified the pest susceptible stages to *A. fusciventris*. Coupled with the phenology study conducted in the previous chapter, this would enable scheduling parasitoid releases at times when *P. longispinus* is most vulnerable.

In the **fourth chapter**, I also hypothesized that the high variability in *P. longispinus* parasitism among different persimmon orchards could be explained by the presence of hyperparasitoids attacking primary parasitoids (Gómez-Marco et al., 2015; Poelman et al., 2012). In this study system, hyperparasitoids were abundant and targeted mealybug hosts of the same size as *A. fusciventris* females. This suggest that hyperparasitoids may negatively affect biological control of *P. longispinus* and ultimately on the population growth rate of the pest. However, contrary to my hypothesis, higher rates of hyperparasitism were not associated with increased pest population growth during the crop cycle, suggesting that hyperparasitoids were not a determining factor for the biological control of *P. longispinus*. In Mediterranean persimmon hyperparasitoids are not abundant until late summer, when crop cycle was nearing its end. This may explain why I did not find a significant effect on the population growth rate of the pest. Our work suggests that, even when hyperparasitoids are abundant, hyperparasitism may not necessarily be a key factor diminishing the effectiveness of primary parasitoids. This adds to other studies questioning the significant negative impact of hyperparasitoids on biological control under certain circumstances (Goergen & Neuenschwander, 1992; Schooler et al., 2011).

Figure 3. A diverse complex of parasitoid wasps attacks *Pseudococcus longispinus* in Mediterranean persimmon. In the image, an adult female of the encyrtid *Anagyrus fusciventris*, the most abundant and widely distributed parasitoid species. This parasitoid can spontaneously control the mealybug, but its efficacy varies among orchards.

Overall, the debate on the potential disruption of biological control by hyperparasitoids remains open and further studies should address this question in different systems (Schooler et al., 2011; Tougeron & Tena, 2019). Here, factors other than hyperparasitism must explain the low parasitism levels of *P. longispinus* observed in some persimmon orchards. These factors need to be explored in the future. One such factor is the presence of mutualistic ants that can disrupt parasitism (Anjos et al., 2022; Nelson & Mooney, 2022). Future studies should identify the ant complex attending *P. longispinus* in Mediterranean persimmon and the effect of these ants on mealybug parasitism and abundance. Another factor that requires in-depth evaluation is the impact of insecticides on *P. longispinus* parasitoids. Several insecticides are known to negatively affect the parasitoids of other mealybug species (Mansour et al., 2018). Insecticides usage has been linked to outbreaks of *P. longispinus* and it has been postulated that this is because of parasitism disruption (Furness, 1977; Swirski et al., 1980; Wysoki et al., 1981). Finally, another factor potentially affecting *P. longispinus* parasitism is the habitat context, which was studied in the final chapter of this thesis.

3. Mealybugs are affected by habitat context

The low diversity of habitats in modern monocultural systems has been associated with increased density of some agricultural pests (Bianchi et al., 2006; Chaplin-Kramer et al., 2011; Letourneau et al., 2011). In the **fifth chapter**, I assessed whether habitat heterogeneity affects the density of mealybugs in citrus and persimmon (Plata et al., 2024b). Having two crops attacked by different mealybug species in the same geographic area enabled this study to yield interesting results, that differed among both crops. On one hand, I found that the proportion of surrounding monoculture increased the density of both *D. aberiae* in citrus and *P. longispinus* in persimmon. This can be explained by the resource concentration hypothesis, that poses that herbivorous pests can directly benefit from crop concentration because of higher dispersal and survival rates (Martinson & Fagan, 2014; O'Rourke & Petersen, 2017; Root, 1973). Remarkably, insects with lower dispersal capabilities, such as wingless mealybugs, may be especially favoured by the concentrations of their host plants (Grez & González, 1995). On the other hand, non-crop habitats, both semi-natural habitats surrounding the crop and inter-row ground cover vegetation, reduced the abundance of *P. longispinus* in persimmon by increasing parasitism, supporting the natural enemies' hypothesis (Chaplin-Kramer et al., 2011; Landis et al., 2000; Russell, 1989). My results suggest that the efficacy of the main parasitoid of *P. longispinus*, *A. fusciventris*, can be highly increased by providing different types of non-crop habitats.

This work highlights the importance of these non-crop habitats in providing key resources to biological control agents such as parasitoids (Mockford et al., 2022; Tena et al., 2015). In contrast, non-crop habitats did not affect the density of *D. aberiae* in citrus. This was likely because *D. aberiae* was not parasitized in the study area. As logically assumed, my work supported that the presence of effective natural enemies, such as mealybug parasitoids, is critical for non-crop habitats to improve the biological control of a pest.

The findings of this chapter allowed to make specific recommendations on habitat management to improve mealybug control in citrus and persimmon. In general, it would be advisable to reduce the proportion of monoculture to diminish the benefits that mealybugs obtain from resource concentration. Furthermore, when key natural enemies are present, as in the case of *A. fusciventris* attacking *P. longispinus*, providing different types of non-crop habitats is highly recommended to enhance mealybug biological control. While increasing crop diversity and maintaining patches of semi-natural habitats at landscape level would require regional cooperation among farmers, ground cover vegetation and hedgerows on field margins can be easily implementable at orchard level (**Figure 4**).

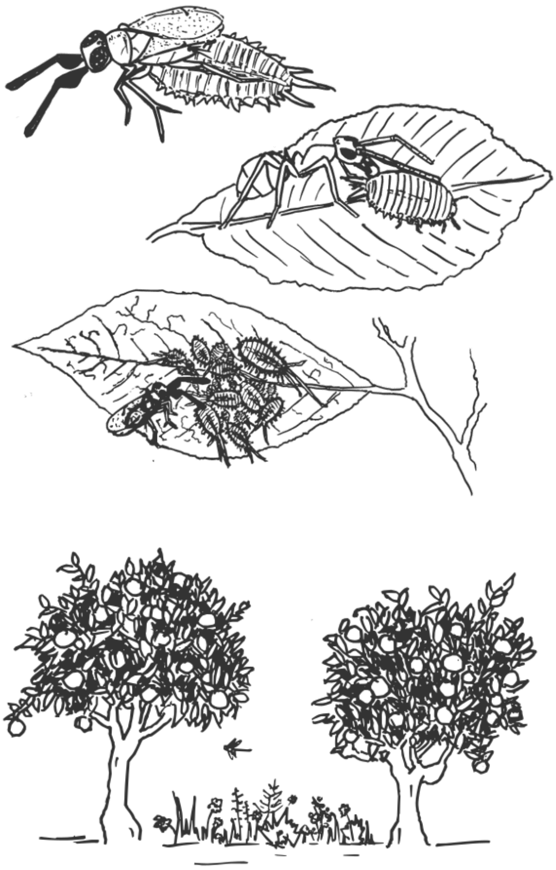
Figure 4. The presence of non-crop habitats can enhance the biological control of pests by providing resources to natural enemies. In the image, inter-row ground cover vegetation established in a persimmon orchard. Ground cover vegetation and semi-natural habitats reduced the abundance of *P. longispinus* by increasing its parasitism.

4. Mealybugs as emerging pests in subtropical crops

Overall, this thesis emphasizes that mealybugs have become primary pests that can cause high economic losses in several subtropical fruit crops in the Mediterranean basin, such as citrus and persimmon (Plata & Tena, 2023). Consistent with these findings, several studies in other geographic regions have also shown an increased incidence of mealybugs on a wide variety of tropical and subtropical crops throughout the world (Fand & Suroshe, 2015; Mani & Shivaraju, 2016; Miller et al., 2002; 2005; Neuenschwander, 2001). This trend makes mealybugs a growing threat to economic development and food security. One of the factors exacerbating mealybugs incidence is undoubtedly the increase in the number of invasive mealybug species in the last decades, that has been likely intensified because of global trade (Miller et al., 2002; Paini et al., 2016; Pellizari & Germain, 2010). Numerous studies suggest that outside their native range is where mealybugs become a problem. Indeed, all mealybug species reaching pest status in Mediterranean basin are exotic species, including *D. aberiae*, the recent invader in Mediterranean citrus (Franco et al., 2004; Pellizari & Gemain, 2010). This mealybug species comes from South Africa, a citrus-producing country that exports large quantities of fruit and plant material to Europe (Beltrà et al., 2015). This underscores the importance of surveillance and quarantine protocols to prevent the spread of mealybugs. Besides the lack of efficient natural enemies in its invasive range, the success of mealybugs in invaded areas may be achieved because of the mutualism that mealybugs can establish with resident ants (Anjos et al., 2022; Wang et al., 2021). This hypothesis have been reinforced by this thesis in the case of *D. aberiae* and the Mediterranean ant *L. grandis*. In addition to recently introduced invasive mealybug species, naturalized mealybug species can increase in abundance becoming in resurgent pests, as exemplified by *P. longispinus* in Mediterranean persimmon. This thesis shows that temperature warming may contribute to the increase in the incidence of this long-established mealybug species. Finally, changes in agricultural landscapes may also affect mealybug outbreaks (Bianchi et al., 2006; Muneret et al., 2018; Shapira et al., 2018). As shown in the final chapter, the trend to make agricultural landscapes more homogeneous, which is increasing the proportion of monoculture at the expense of non-crop habitats, can lead to an increase in mealybug density through different mechanisms.

In general, this thesis showed that the increase in mealybug incidence is caused by multiple factors, and all of them must be considered to design mealybug management strategies. Remarkably, this thesis has identified strategies that can be implemented to enhance the biological control of mealybugs in Mediterranean citrus and persimmon, including the management of mutualistic ants, the augmentative release of parasitoids, and the management of habitat at local and landscape scales. The findings of this thesis must be considered within the Integrated Pest Management programs against mealybugs in subtropical crops.

Conclusions



- Mealybugs have become key pests in various subtropical crops from Spain, such as citrus and persimmon. *Delottococcus aberiae*, a recently introduced species, is the dominant species in citrus, while *Pseudococcus longispinus*, a species long naturalized in the Mediterranean area, is the dominant species in persimmon.
- *Delottococcus aberiae* is attended by several native ant species in Mediterranean citrus. The Mediterranean ant *Lasius grandis* and *D. aberiae* have established a strong mutualistic relationship that can facilitate the invasion of the mealybug.
- Ant-exclusion from citrus canopies can reduce *D. aberiae* density and damage, likely because of an increase in the abundance of generalist predators. Therefore, ant management should be considered in Integrated Pest Management (IPM) programs against *D. aberiae*.
- *Pseudococcus longispinus* has displaced other species of mealybugs in the main persimmon-producing area from Spain, where *P. longispinus* can reach high fruit infestation levels and cause important economic losses.
- The phenology of *P. longispinus* in persimmon has been described. The moments to adopt different IPM strategies to control *P. longispinus* have been identified and have been already implemented by the main persimmon cooperatives in the Valencian Community.
- Climate change predicted in the coming years may increase the damage caused by *P. longispinus* in persimmon. Based on climate change predictions and *P. longispinus* biology, crop damage caused by the third generation of *P. longispinus* will increase in 2040 and the mealybug will complete a fourth generation by 2080.
- *Pseudococcus longispinus* is attacked by several parasitoid species in Mediterranean persimmon, among which the species *Anagyrus fusciventris* stands out. This parasitoid can effectively reduce the density of the pest despite the high abundance of hyperparasitoids. Therefore, *A. fusciventris* is a good candidate as a biological control agent to be reared and released in IPM programs against *P. longispinus*.
- Monoculture proportion in agricultural landscapes increases the abundance of mealybugs in Mediterranean citrus and persimmon. The presence of non-crop habitats, both inter-row ground cover vegetation and surrounding semi-natural habitats, can increase the parasitism of *P. longispinus* in persimmon, ultimately reducing mealybug abundance. Therefore, habitat diversification strategies can improve the control of mealybugs in citrus and persimmon.

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