UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Biology and management, by application of classical biological control, of the invasive mealybug *Delottococcus aberiae* (Hemiptera: Pseudococcidae) in citrus orchards in Spain.

DOCTORAL THESIS

Presented by: Victoria Martínez Blay
Directed by: Dr. Antonia Soto Sánchez

Valencia, May 2018

A mis padres

Aknowledgements

En primer lugar quiero expresar mi reconocimiento a Antonia Soto, por darme la oportunidad de realizar la presente tesis doctoral. Sus directrices, esfuerzo y paciencia, así como el haberme sabido transmitir su ilusión y vitalidad por la entomología han sido fundamentales durante estos años.

A todas las personas que han colaborado en la búsqueda de parcelas y a las que nos las han cedido desinteresadamente para realizar los estudios de esta tesis, mención especial a Maribel Deval, Plácido Calabuig y Vicent Soriano.

A Alejandro Tena, por sus consejos y todos los nuevos y originales puntos de vista aportados durante el transcurso de esta tesis.

A Jose Carlos Franco, y su equipo del ISA, por acogerme en Lisboa y enseñarme a comprender mejor el comportamiento de los pseudocóccidos. A Pia Addison, Jan Giliomee y a BioBee Letsitele (en especial a Rami Friedman, Desi Fourie y Lezel Beetge) por recibirme en Sudáfrica y ayudarme con todo lo necesario durante mi estancia allí.

Agradecer también a Ferran García, Francisco Ferragut, Paloma Pérez y Carmela Gisbert toda la ayuda prestada durante estos años. A todos los compañeros del IAM con los que he trabajado y compartido momentos de tertúlia alrededor de la mesa redonda: Alex, Altea, Amparo, Cristina, Emilio, Javi, Jorge, Juan Antonio, Juan Carlos, Leti, Marco, María, Natalia, Sergio, Stefano,…Mención especial a Alex por transmitirme sus conocimientos de pseudocóccidos al inicio de esta etapa, a Marco por su ayuda incondicional con la cría y las mangas, a Javi por su paciencia durante nuestros meses en Sudáfrica y a los "torris" por su amistad y por tantos momento de risas.

Por último, a mi familia. A mis padres, por habérmelo dado todo. Por sus esfuerzos, apoyo y comprensión y por transmitirme el placer de aprender. A mi padre, porque sin su ayuda incondicional en los muestreos y conteos habría sido imposible que acabara esta tesis. A mi madre, de quien he heredado la afición por la biología y estoy segura que le habría encantado poder leer y discutir sobre esta tesis. A mis abuelos y a Vicenta. Y como no, a Dani, por todo, por hacer este camino mucho más agradable.

A todos y todas, gracias.

Index

Figure index

Table index

Abstract

Delottococcus aberiae (Hemiptera: Pseudococcidae) is an invasive mealybug native to sub-Saharan Africa that was detected causing significant damage to citrus fruits in eastern Spain in 2009. Due to the lack of knowledge about this species, the management of *D. aberiae* has been carried out by the application of authorized insecticides against mealybugs. However, the latest European Directive (2009/128 / EC) on the sustainable use of pesticides stipulates that chemical treatments in agroecosystems must be reduced, promoting more sustainable management strategies such as the application of biological control methodologies. In addition, when an invasive species arrives for the first time in a territory it is necessary to study its biology, behavior, damage caused and control possibilities. This thesis presents for the first time these studies of biology and behavior of the pest as well as a characterization of the damage produced by *D. aberiae*. The possibilities of implementing a classical biological control program against this species in citrus in Spain have also been studied.

To analyze the biology and behavior of *D. aberiae*, several citrus orchards infested with the mealybug have been sampled for three years in the Valencian Community (eastern Spain). Samples have been collected periodically and the number of mealybugs, their developmental stage as well as the infested stratum and organ where they were present have being recorded. The period of damage to the fruit was studied in semi-field and field conditions by the artificial infestation with *D. aberiae* of fruits of different diameter. Finally, the behavior and possibilities of biological control of *D. aberiae* were studied by sampling several citrus orchards in the native area of the mealybug (South Africa).

Results showed that the density of *D. aberiae* populations in citrus orchards is high in spring and summer, decreasing to lower levels in autumn and winter. In addition, the insect completes several generations throughout the year and two of them are clearly defined and result in high population levels. Regarding its distribution, *D. aberiae* was mostly installed in the canopy of the tree and migrations were observed between different organs, showing a clear preference for the developing fruit. From February to September some mealybugs were found in the trunk and soil, moving upwards or downwards depending on the phenology of the plant and the climatic conditions.

The comparison between sampling techniques revealed that corrugated cardboard band traps provide a quantitative measurement of *D. aberiae* density in the orchards. On the other hand, sticky traps, baited with *D. aberiae* females were able to detect the main male flight periods. *D. aberiae* caused direct damage to the fruit (deformation and/or reduction in size) by feeding on the ovary of the flower or on small fruits in development. These damages are probably due to their interference with the process of cell division.

Finally, in South Africa, native area of the pest, the highest density levels of *D. aberiae* were found in summer and the highest parasitism rates occurred in autumn. Among the complex of *D. aberiae* natural enemies, the two most abundant species were *Anagyrus* sp. nov. 1 (Hymenoptera: Encyrtidae) and *Allotropa* sp. nov. (Hymenoptera: Platygastridae). Both parasitoids could play an important role in a biological control program against *D. aberiae* in Spain. For now, *Anagyrus* sp. nov. 1 seems the best candidate because of its higher rates of parasitism in South Africa.

Resumen

Delottococcus aberiae (Hemiptera: Pseudococcidae) es un pseudocóccido invasor originario del África subsahariana que fue detectado causando graves daños en cítricos del este de España en el año 2009. Debido al desconocimiento existente sobre esta especie, la gestión de *D. aberiae* se ha llevado a cabo mediante el uso de tratamientos químicos autorizados contra este tipo de insectos. Sin embargo, la última Directiva Europea (2009/128/EC) sobre el uso sostenible de productos fitosanitarios estipula que la aplicación de plaguicidas en el ámbito agrícola debe reducirse, promoviendo estrategias de manejo más sostenibles como es la aplicación del control biológico de plagas. Además, cuando una especie invasora llega por primera vez a un territorio es necesario estudiar su biología, comportamiento, daños causados y posibilidades de control. En esta tesis se presentan por primera vez estos estudios de biología y comportamiento de la plaga así como un análisis de los daños producidos por *D. aberiae.* También se han realizado estudios con el objetivo de implementar la aplicación de un programa de control biológico clásico sobre esta especie en cítricos en España.

Para analizar la biología y comportamiento de *D. aberiae* se han muestreado durante tres años varias parcelas de cítricos con poblaciones de *D. aberiae* en la Comunidad Valenciana. En estas parcelas se han recogido muestras y contabilizado todos los pseudocóccidos presentes, su estadío de desarrollo y el estrato y órgano del árbol donde se encontraban. El periodo de daños al fruto fue estudiado en semicampo y campo mediante la infestación artificial con *D. aberiae* de frutos de distintos tamaños de diámetro. Por último se estudió el comportamiento y las posibilidades de control biológico de *D. aberiae* muestreando diversas parcelas de cítricos en su zona de origen (Sudáfrica).

Los resultados mostraron que la abundancia de las poblaciones de *D. aberiae* en cítricos es elevada en primavera y verano, reduciéndose a niveles mucho más bajos en otoño e invierno. Además el insecto completa varias generaciones a lo largo del año, estando dos de ellas muy claramente definidas y siendo las que dan lugar a elevadas poblaciones de la plaga. En cuanto a su distribución, *D. aberiae* se instaló principalmente en la copa del árbol y se observaron migraciones entre los distintos órganos, mostrando una clara preferencia por el fruto en desarrollo. Entre febrero y septiembre parte de las poblaciones de *D. aberiae* se encontraron en tronco y suelo, existiendo movimientos de subida y de bajada a la copa en función de la fenología de la planta y las condiciones climáticas.

La comparativa entre técnicas de muestreo reveló que las trampas de cartón corrugado proporcionan una medida cuantitativa de la abundancia de *D. aberiae* en las parcelas. Por su parte, las trampas pegajosas, provistas de hembras de *D. aberiae*, fueron capaces de detectar los principales vuelos de machos. Por otro lado, *D. aberiae* causó daños directos al fruto (deformación y/o reducción de tamaño) al alimentarse del ovario de la flor o de los primeros estados de desarrollo de éste. Estos daños son posiblemente debidos a su interferencia con el proceso de división celular.

Por último, en Sudáfrica, lugar de origen de la plaga, los mayores niveles poblaciones de *D. aberiae* se encontraron en verano y la tasa de parasitismo fue máxima en otoño. Entre el complejo de enemigos naturales de *D. aberiae* encontrados destacaron dos especies, *Anagyrus* sp. nov. 1 (Hymenoptera: Encyrtidae) y *Allotropa* sp. nov. (Hymenoptera: Platygastridae). Ambos parasitoides podrían tener un papel importante en un programa de control biológico contra *D. aberiae* en España. Por ahora, *Anagyrus* sp. nov. 1 parece el mejor candidato por sus mayores tasas de parasitismo en Sudáfrica.

Resum

Delottococcus aberiae (Hemiptera: Pseudococcidae) és un pseudocòccid invasor originari de l'Àfrica subsahariana que va ser detectat causant greus danys en cítrics de l'est d'Espanya l'any 2009. A causa del desconeixement existent sobre aquesta espècie, la gestió de *D. aberiae* s'ha dut a terme mitjançant l'ús de tractaments químics autoritzats contra aquest tipus d'insectes. No obstant això, l'última Directiva Europea (2009/128/EC) sobre l'ús sostenible de productes fitosanitaris estipula que l'aplicació de plaguicides en l'àmbit agrícola ha de reduir-se, promovent estratègies de maneig més sostenibles com és l'aplicació del control biològic de plagues. A més, quan una espècie invasora arriba per primera vegada a un territori és necessari estudiar la seua biologia, comportament, danys causats i possibilitats de control. En aquesta tesi es presenten per primera vegada els estudis de biologia i comportament de la plaga així com una anàlisi dels danys produïts per *D. aberiae*. També s'han realitzat estudis amb l'objectiu d'implementar l'aplicació d'un programa de control biològic clàssic sobre aquesta espècie en cítrics a Espanya.

Per a analitzar la biologia i comportament de *D. aberiae* s'han mostrejat durant tres anys diverses parcel·les de cítrics amb poblacions de *D. aberiae* a la Comunitat Valenciana. En aquestes parcel·les s'han recollit mostres i comptabilitzat tots els pseudocòccids presents, el seu estadi de desenvolupament i l'estrat i òrgan de l'arbre on es trobaven. El període de danys al fruit va ser estudiat en semicamp i camp mitjançant la infestació artificial amb *D. aberiae* de fruits de diferents mides de diàmetre. Finalment es va estudiar el comportament i les possibilitats de control biològic de *D. aberiae* mostrejant diverses parcel·les de cítrics en la seua zona d'origen (Sud-àfrica).

Els resultats van mostrar que l'abundància de les poblacions de *D. aberiae* en cítrics és elevada a la primavera i estiu, reduint-se a nivells molt més baixos a la tardor i hivern. A més l'insecte completa diverses generacions al llarg de l'any, estant dos d'elles molt clarament definides i sent les que donen lloc a elevades poblacions de la plaga. Quant a la seua distribució, *D. aberiae* es va instal·lar principalment en la copa de l'arbre i es van observar migracions entre els diferents òrgans, mostrant una clara preferència pel fruit en desenvolupament. Entre febrer i setembre part de les poblacions de *D. aberiae* es van trobar en tronc i sòl, existint moviments de pujada i de baixada a la copa en funció de la fenología de la planta i les condicions climàtiques.

La comparativa entre tècniques de mostreig va revelar que les trampes de cartró corrugat proporcionen una mesura quantitativa de l'abundància de *D. aberiae* en les parcel·les. Per la seua banda, les trampes apegaloses proveïdes de femelles de *D. aberiae* van aconseguir detectar els principals vols de mascles. D'altra banda, *D. aberiae* va causar danys directes al fruit (deformació i/o reducció de mida) en l'alimentar-se de l'ovari de la flor o dels primers estats de desenvolupament d'aquest. Aquest danys són possiblement deguts a la seua interferència amb el procés de divisió cel·lular.

Finalment, a Sud-àfrica, lloc d'origen de la plaga, els majors nivells poblacionals de *D. aberiae* es van trobar a l'estiu i la taxa de parasitisme va ser màxima a la tardor. Entre el complex d'enemics naturals de *D. aberiae* trobats van destacar dues espècies, *Anagyrus* sp. nov. 1 (Hymenoptera: Encyrtidae) i *Allotropa* sp. nov. (Hymenoptera: Platygastridae). Tots dos parasitoides podrien tindre un paper important en un programa de control biològic contra *D. aberiae* a Espanya. Per ara, *Anagyrus* sp. nov. 1 sembla el millor candidat per les seues majors taxes de parasitisme a Sud-àfrica.

CHAPTER 1.
Introduction

Chapter 1. Introduction

1.1. Mealybugs

1.1.1 General characteristics

The family Pseudococcidae, commonly known as mealybugs, constitutes the second largest family, after Diaspididae, within the group of scale insects (Hemiptera: Coccoidea), with 1987 species described worldwide in 259 genera (Hardy *et al.* 2008, Williams *et al.* 2011, Kaydan *et al.* 2015, García-Morales *et al.* 2016a). Mealybugs are small, with oval to elongated soft bodies. Their common name refers to the mealy wax secretion, usually white, that covers their bodies in most of the species (Kosztarab and Kozár 1988, Gullan and Martin 2009). They are widely distributed, occurring in different habitats in all zoogeographic areas of the world, and the Palaearctic Region has the highest number of recorded species (McKenzie 1967, Ben-Dov 1994, García-Morales *et al.* 2016a).

Similarly to other scale insects, mealybugs exhibit sexual dimorphism (Gullan and Kosztarab 1997, Franco *et al.* 2009, Gullan and Martin 2009). After hatching from the eggs, females go through three immature instars before reaching maturity (Fig 1.1.). Due to neoteny, adult females resemble and keep the morphology of the immature individuals, being wingless and with well-developed mouthparts. They continue feeding and growing until mating and may live for several months before laying the eggs. In contrast, males have clear morphological differences between immature and adult stages.

Males go through four immature instars; two of them are like the female ones, but at the end of the second nymphal instar they develop a waxy cocoon. Inside this cocoon they develop two pupa-like stages, pre-pupa and pupa (Fig 1.1.), from which a winged adult male, with distinct head, thorax and abdomen, will emerge (McKenzie 1967, Cox

1987, Kosztarab and Kozár 1988, Gullan and Kosztarab 1997, Franco *et al.* 2000, Franco *et al.* 2009, Gullan and Martin 2009, Beltrà and Soto 2012, Mani and Shivaraju 2016). Adult males do not feed and live only a few days, having a limited time to seek out the females for mating and being easily overlooked in the field (Kosztarab and Kozár 1988, Gullan and Martin 2009).

Most mealybug species reproduce sexually (Gullan and Kosztarab 1997, Mani and Shivaraju 2016). However, some mealybugs, such as *Phenacoccus solani* Ferris (Lloyd 1952) or *Ferrisia malvastra* (McDaniel) (Ben-Dov 2005), reproduce parthenogenically, with the absence of males. Gravid females usually lay their eggs (oviparity) in a waxy covering, the egg sac. Nevertheless, some species such as *Pseusodoccus longispinus* (Targioni-Tozzetti) may retain them in their reproductive tract until hatching (ovoviviparity) (Franco *et al.* 2000).

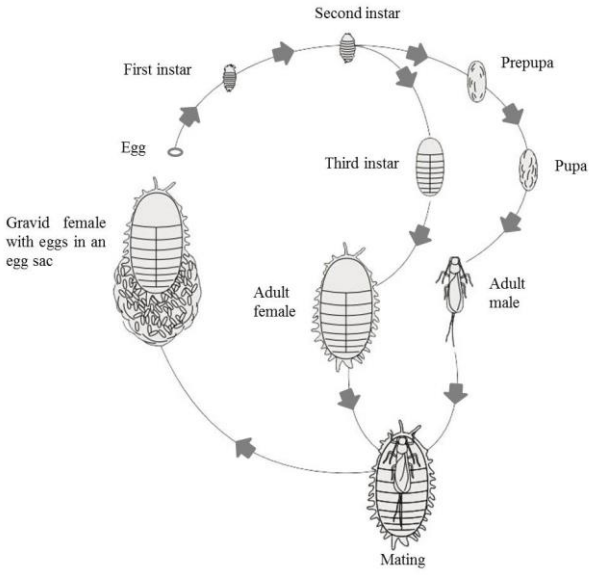

Fig.1.1. Life cycle of a mealybug, adapted from Beltrà and Soto (2012).

Taxonomy and identification of the Pseudococcidae family has traditionally been based on microscopic analysis of morphological structures present on the body surface of the adult female (Fig 1.2.), (Miller and Kosztarab 1979, Williams and Granara de Willink 1992). On the other hand, some attempts have also been made to classify adult males and immature stages (Beardsley 1960, Afifi 1968, Gullan 2000, Wakgari and Giliomee 2005). However, morphological identification involves several difficulties, such as being a time-consuming process. Besides, certain environmental conditions can induce morphological intra-specific variations in mealybugs, being sometimes impossible to differentiate between complexes of cryptic species (Cox 1983, Charles *et al.* 2000). Thus, morphological identification needs to be carried out by taxonomic specialists.

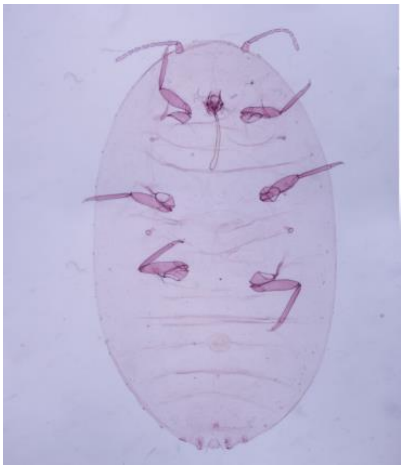

Fig. 1.2. General appearance, under microscope, of the body surface of an adult female mealybug.

The aforementioned difficulties have led to an increased interest in applying molecular techniques to complement mealybug taxonomy,

being, currently, DNA barcoding and multiplex PCR the most commonly used molecular techniques for mealybug identification (Hardy *et al.* 2008, Saccaggi *et al.* 2008, Rung *et al.* 2009, Park *et al.* 2010, Pieterse *et al.* 2010, Daane *et al.* 2011, Malausa *et al.* 2011, Park *et al.* 2011, Correa *et al.* 2012). Among their advantages are high accuracy and the feasibility of identifying nymphal and male stages in addition to females (Beltrà and Soto 2012). Thus, during recent years, several studies applying integrative taxonomy (combination of morphological and molecular characterization techniques) have been carried out to characterize mealybug species present in different regions worldwide, and also some of their natural enemies (Beltrà *et al.* 2012, Pacheco da Silva *et al.* 2014, Beltrà *et al.* 2015, Malausa *et al.* 2016, Pacheco da Silva *et al.* 2017).

1.1.2 Host plants

The family Pseudococcidae has adapted to a broad host range, from herbaceous plants to trees. Unlike other scale insect families, such as armored scales (Hemiptera: Diaspididae), mealybugs tend to attack predominantly herbaceous plants rather than woody plants (Kosztarab and Kozár 1988, Ben-Dov 1994, Miller 2005). The most common host family of Pseudococcidae is Poaceae, with 570 species of mealybugs associated, followed by Asteraceae, with 294 species and Fabaceae with 266. In a distant position, to complete the ten most common host families are Rubiaceae, Malvaceae, Myrtaceae, Rosaceae, Lamiaceae, Moraceae and Euphorbiaceae (García-Morales *et al.* 2016a) (Fig. 1.3.). In the Mediterranean Basin, mealybugs of major economic importance cause problems in woody crops of the familes Musaceae, Rosaceae, Rutaceae and Vitaceae; in horticultural crops of the families Solanaceae and Cucurbitaceae and in a wide range of families with ornamental plants (Beltrà and Soto 2011, Moreno-Salmerón 2011, Tena and García-Marí 2011, Beltrà and Soto 2012).

Some mealybug species are monophagous or oligophagous, this means quite specific with their hosts, such as *Chaetococcus phragmites* (Marchal), known only from reed (*Phragmites* and *Arundo* genera) or *Planococcus vovae* (Nasonov), which feeds almost exclusively on the family Cupressaceae (Kosztarab and Kozár 1988, García-Morales *et al.* 2016a). However, only a few mealybug species of narrow host range have commercial repercussions, being polyphagous the mealybugs considered as major pests worldwide. These polyphagous mealybugs present a serious threat because of their tendency to adopt new host plants easily (Franco *et al.* 2009). Some examples of major polyphagous mealybugs worldwide are Ferrisia virgata (Cockerell), *Maconellicoccus hirsutus* (Green), *Planococcus citri* (Risso), *P. longispinus*, *Pseudococcus viburni* (Signoret) or *Phenacoccus madeirensis* Green. Each one of the aforementioned species have been cited in about 80 different host botanical families (Ben-Dov 1994, García-Morales *et al.* 2016a).

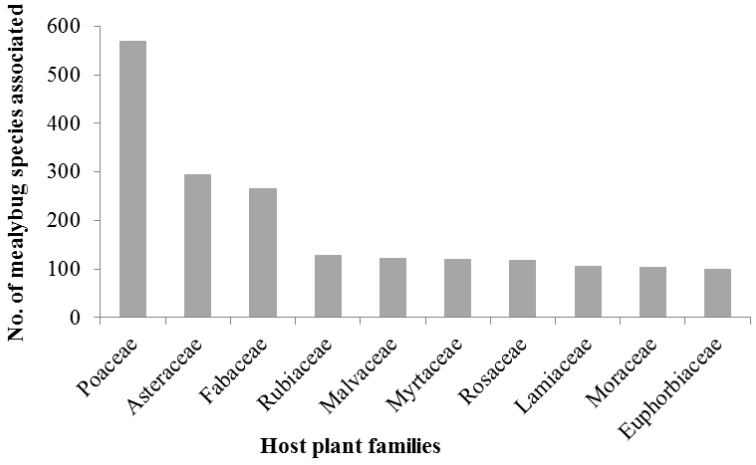

Fig. 1.3. Main host plant families of mealybugs. Made with information contained in García-Morales *et al.* (2016a).

1.1.3 Damages and economic importance

Most Pseudococcidae are phloem feeders, and the damage they cause is diverse according to the mealybug species and the host they attack. These insects may cause significant economic losses in the crops they infest and harm the aesthetic quality of ornamental plants (McKenzie 1967, Gullan and Martin 2009). Their feeding behavior, linked to sap-sucking, reduces plant vigor and the honeydew they secrete is associated with the growth of black sooty mold fungi that interferes with photosynthesis and affects fruit quality, especially in agricultural contexts (Fig. 1.4.) (McKenzie 1967, Douglas 2009, Franco *et al.* 2009). High densities or repeated infestations causes defoliation, fruit drop or even kill the plant (Franco *et al.* 2000, Franco *et al.* 2009). Indirect damage may also result from interations between mealybugs and other pests, such as Lepidoptera (Douglas 2009).

Fig. 1.4. Damage caused by mealybug's honeydew secretion in citrus fruits.

Several mealybug species can also act as vectors of virus in different commercial crops, such as banana or grapevine (Sforza *et al.* 2003, Watson and Kubiriba 2005, Cid *et al.* 2007, Tsai *et al.* 2010). Besides, some species are able to inject toxins that distort plant tissues, such as *Hypogeococcus pungens* Granara de Willink (McFadyen 1979, Carrera-Martínez *et al.* 2015), *M. hirsutus*

(Meyerdirk *et al.* 2001, Vitullo *et al.* 2009, Chong *et al.* 2015) or *Nipaeococcus viridis* (Newstead) (Thomas and Leppla 2008, Abdul-Rassoul 2014).

1.1.4 Population dynamics and distribution on the plant

Population dynamics differ according to mealybug species and environmental conditions. In Central Europe, native species generally complete one to three generations per year (Kosztarab and Kozár 1988). However, in the Mediterranean Basin, mealybug species with agricultural impact usually complete a high number of overlapping generations (Franco *et al.* 2000, Martínez-Ferrer *et al.* 2003, Beltrà and Soto 2012, Beltrà *et al.* 2013a).

Temperature, relative humidity and photoperiod are the environmental factors that most commonly influence the biology of mealybugs. Different laboratory assays show that mealybugs require approximately from 15 days to 3 months to complete a full life cycle at constant temperatures between 20 and 30 ºC (Amarasekare *et al.* 2008, Chong *et al.* 2008, Goldasteh *et al.* 2009, Varikou *et al.* 2010, Prasad *et al.* 2012, Kumar *et al.* 2013). Thus, in the Mediterranen Basin high mealybug population densities tend to occur in spring and early summer, whereas high summer temperatures together with dry winds may cause greater mortality of immature mealybug stages (Bartlett and Clancy 1972, Beltrà *et al.* 2013a). On the other hand, with colder temperatures mealybugs slow down their growth and may overwinter in the form of different stages (Miller 2005). Other factors, such as mechanical action of rainfall (Le Rü and Iziquel 1990), host nitrogen content (Hogendorp *et al.* 2006) or water-stressed plants (Calatayud *et al.* 2002) can also influence mealybug populations.

Within a host, mealybugs can feed on almost all plant strata, including leaves, flowers, fruits, stems, trunk and even roots (Mani and Shivaraju 2016). Due to their cryptic habits, mealybugs tend to feed in concealed areas (Miller 2005), congregating in small depressions or protected areas of plants. For example *Planococcus ficus* (Signoret) is frequently found under the bark of the vine (Geiger and Daane 2001) and *P. citri* under the calyx of citrus fruits (Martínez-Ferrer *et al.* 2003). Although all mealybug female stages are mobile, these insects have sedentary habits (Miller 2005). First nymphal instars or crawlers show the greatest mobility, being the main dispersal instar and seeking for suitable feeding sites. If conditions are favorable, crawlers usually settle in the natal host plant, close to their mothers, this resulting in a clumped spatial distribution (Nestel *et al.* 1995, Gullan and Kosztarab 1997) (Fig. 1.5.).

Fig. 1.5. Aggregated distribution of mealybugs in citrus.

On the other hand, some mealybug species move to different parts of the host for overwintering, feeding, ovipositing and molting (McKenzie 1967, Franco 1994, Miller 2005). This seasonal movements within the host have been reported for several mealybug

species, specially those associated with woody plants (Franco *et al.* 2009), such as *Ferrisia gilli* Gullan in pistachio (Haviland *et al.* 2012), *P. citri* in citrus (Franco 1994, Nestel *et al.* 1995, Martínez-Ferrer *et al.* 2003) or *Pseudococcus maritimus* (Ehrhorn) in grapevine (Geiger and Daane 2001). Franco (1994) suggested that immature feeding stages of mealybugs on citrus tend to settle at the major carbohydrate sinks of the plant, moving to different plant strata according to the phenology of the host. This hypothesis may also explain the migratory movements of other mealybug species. For example, Haviland *et al.* (2012) showed that feeding location of *F. gilli* corresponded with carbohydrate allocation in pistachio trees.

1.1.5 Mealybugs as invasive pests

Dispersion of mealybugs over longer distances occurs by human action, mainly with the movement of infested plant material, and wind action (Grasswitz and James 2008, Vitullo 2009). The introduction of alien species has increased during recent decades (Roques *et al.* 2009, Bellard *et al.* 2016). Globalization processes and the increase in the international trade of horticultural and ornamental plants worldwide have facilitated the introduction and spread of several insect pests (Meyerson and Mooney 2007, Hulme 2009, MacDonald *et al.* 2015). Within this context, the number of alien species is expected to increase in the near future (Pimentel *et al.* 2005, Roy *et al.* 2014). The impact of invasive alien species represents not only a major risk to biodiversity but also significant economic impacts, especially in agricultural ecosystems (Pimentel *et al.* 2000, Pimentel *et al.* 2001, Kenis *et al.* 2009, Sujay *et al.* 2010, Paini *et al.* 2016).

Mealybugs are frequent invasive species. Their small size and cryptic behavior allow them to pass unnoticed during quarantine inspections, being easily introduced into new territories. Besides, their high fecundity favors rapid spread (Miller *et al.* 2002, Hulme *et al.*

2008, Kenis *et al.* 2009, Pellizzari and Germain 2010, Mansour *et al.* 2017a). Population outbreaks are frequent when mealybugs are introduced into new areas without their specific natural enemies (Moore 1988, Miller *et al.* 2002, Franco *et al.* 2009). Several species have been involved in serious mealybug outbreaks in tropical and subtropical regions, such as *M. hirsutus* (Matile-Ferrero *et al.* 2000, Culik *et al.* 2013), *Phenacoccus manihoti* Matile-Ferrero (Herren and Neuenschwander 1991), *Phenacoccus solenopsis* Tinsley (Hodgson *et al.* 2008, Wang *et al.* 2010), *Paracoccus marginatus* Williams and Granara de Willink (Matile-Ferrero et al. 2000, Muniappan et al. 2008, Ahmed et al. 2015) or *Rastrococcus invadens* Williams (Han *et al.* 2007).

In Europe, mealybugs represent the third most numerous family of alien insects, after aphids and armoured scales, and the second within the scale insects (Hemiptera: Coccoidea) (Fig. 1.6.) (Roques *et al.* 2009, Pellizzari and Germain 2010).

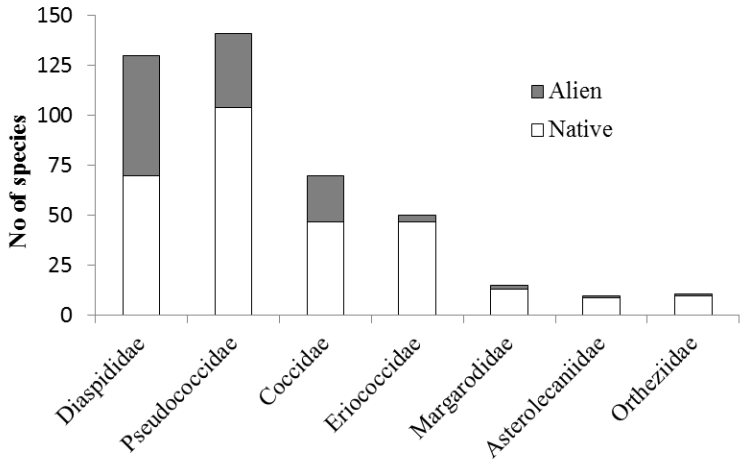

Fig. 1.6. Number of alien and native scale species in Europe. Adapted from Pellizzari and Germain (2010).

Within Europe, the Mediterranean Basin, due to its favorable climatic conditions, is especially susceptible to the establishment of tropical and subtropical non-native species (Roques *et al.* 2009, Walther *et al.* 2009). Therefore, since the 1990s many mealybug species have been recorded as new invaders in agricultural crops, urban environments and greenhouses in the Mediterranean Basin, being some examples *Dysmicoccus brevipes* (Cockerell) (Suma *et al.* 2015), *P. marginatus* (Mendel et al. 2016), *Phenacoccus defectus* Ferris (Mazzeo et al. 2014), *Phenacoccus peruvianus* Granara de Willink (Beltrà *et al.* 2010), *P. solani* (Mazzeo *et al.* 1999) or *Pseudococcus comstocki* (Kuwana) (Pellizzari 2005).

1.1.6 Sampling and monitoring

Sampling and monitoring mealybugs are processes based on different direct and indirect techniques. Direct sampling involves the visual examination of plant material, searching and counting live insects in different plant strata (Grimes and Cone 1985, Geiger and Daane 2001, Haviland *et al.* 2012, Wunderlich *et al.* 2013). There are different methodologies (Beltrà and Soto 2012): enumerative samplings count the number of mealybugs present per sampled organ, binomial samplings anotate the presence or absence of mealybugs per sampled organ and time-counts record for a certain time the number of mealybugs present in a particular part of the plant.

Alternative indirect monitoring techniques, mainly based on the use of different trap designs, have also been developed to determine the mealybug's seasonal occurrence, being the most common ones sticky traps, corrugated cardboard bands and sticky tapes (DeBach 1949, Furness 1976, Hill and Burts 1982, Goolsby *et al.* 2002, Millar *et al.* 2002, Walton *et al.* 2004, Roltsch *et al.* 2006, Cid *et al.* 2010, Beltrà and Soto 2012). Sticky traps are generally baited with sex pheromones to increase the captures, two types of lures being used to

attract the males: live virgin females or synthetic sex pheromones (Rotundo and Tremblay 1975, Moreno *et al.* 1984, Meyerdirk *et al.* 2001, Serrano *et al.* 2001, Millar *et al.* 2002, Walton *et al.* 2004, Mudavanhu *et al.* 2011). Corrugated cardboard band traps represent a nondestructive sampling method to monitor mealybug population densities (DeBach 1949, Furness 1976, Goolsby *et al.* 2002). The bands are wrapped around the trunk or main branches of the trees and serve as a refuge for gravid females to lay their eggs, or for second male instars to make their cocoon and develop into adults males (Beltrà and Soto 2012) (Fig. 1.7.). Sticky tapes are also wrapped around the trunk or branches of the plant and capture the mealybugs that pass over them (Vitullo 2009, Cid *et al.* 2010).

Sampling population dynamics is essential to understand the biology and ecology of arthropods and establish integrated pest management (IPM) programs (Stern 1973, Binns and Nyrop 1992). Monitoring protocols improve pest detection, provide information regarding their seasonal occurrence and determine the expected damaging periods. This information avoids unnecessary spraying and forms the basis of any IPM program (Gonzalez 1971, Binns and Nyrop 1992, De Villiers and Pringle 2007). Sampling and monitoring mealybugs have been widely developed to improve their control in many agricultural and ornamental ecosystems (Geiger and Daane 2001, Beltrà and Soto 2012, Haviland *et al.* 2012, Wunderlich *et al.* 2013, Kumar *et al.* 2014). Enumerative and binomial samplings have been used in IPM of many mealybug species affecting different crops and ornamental plants, such as *M. hirsutus, P. citri, P. ficus, P. peruvianus, P. longispinus* or *P. maritimus* (Furness 1976, Nestel *et al.* 1995, Geiger and Daane 2001, Goolsby *et al.* 2002, Walton and Pringle 2004, Martínez-Ferrer *et al.* 2006, Roltsch *et al.* 2006, Beltrà *et al.* 2013a). Time counts (1- 5 minutes) have also been carried out for some mealybugs, such as *P. citri* and *P. maritimus* (Geiger and

Daane 2001, Martínez-Ferrer *et al.* 2003). These methodologies can be quite laborious and time-consuming, but they usually allow us to obtain results with great precision (Grimes and Cone 1985, Geiger and Daane 2001, Haviland *et al.* 2012, Beltrà *et al.* 2013a).

Sticky traps have proved useful to monitor the seasonal flight periods of adult males of different mealybugs. In recent years, the development of synthetic pheromones for several mealybug species, of economic importance worldwide, such as *P. ficus*, *P. citri*, *P. viburni*, *M. hirsutus*, *P. longispinus* or *P. madeirensis*, has allowed its use in the form of lures for monitoring and sometimes detecting mealybug population outbreaks, simplifying sampling protocols (Millar *et al.* 2002, Vitullo *et al.* 2007, Martínez-Ferrer *et al.* 2008, Zada *et al.* 2008, Franco *et al.* 2009, Mudavanhu *et al.* 2011, Waterworth *et al.* 2011). Corrugated cardboard band traps have been tested with positive results to sample *P. viburni* (Mudavanhu 2009), *P. longispinus* (DeBach 1949, Furness 1976) or *M. hirsutus* (Goolsby *et al.* 2002, Roltsch *et al.* 2006). Sticky tapes are applicable to many crops but sometimes are difficult to adhere on the surface of the host. It has been used to sample mealybugs in vineyards (Cid *et al.* 2010), in pears (Hill and Burts 1982) and in ornamental plants (Vitullo 2009).

Fig. 1.7. Corrugated cardboard band traps for monitoring mealybugs in citrus.

Finally, during recent years, several attempts have been made to determine mealybugs economic injury levels and to establish intervention thresholds to improve the management of these pests (Walton *et al.* 2004, Martínez-Ferrer *et al.* 2006, Martínez-Ferrer *et al.* 2008, Mudavanhu *et al.* 2011, Beltrà *et al.* 2013a, Haviland *et al.* 2015).

1.1.7 Management

On the other hand, the Directive 2009/128/EC of the European Parliament and of the Council specifies a range of actions to achieve a sustainable use of pesticides in the European Union (EU) by reducing the risks of pesticide use on human health and the environment. This Directive promotes the minimization of heavy pesticides by using available alternative techniques (European Parliament and Council 2009). Thus, in recent years there has been an evolution towards more sustaible pest management systems in Europe and in Spain. In this way, there is an increasing interest in implementing IPM programs and, within this context, the evaluation and application of pesticides respectful and compatible with natural enemies, as well as the timing of those applications, are crucial (Mgocheki and Addison 2009b, Mansour *et al.* 2011, Mgocheki and Addison 2015). Besides, alternative strategies to chemical control such as cultural methods, sex pheromones and especially biological control, open new horizons for mealybug management.

Cultural methods may interfere with the phytosanitary status of agricultural and ornamental plants (Beltrà and Soto 2012). Factors, such as excessive nitrogen fertilization (Hogendorp *et al.* 2006) or water-stressed plants (Calatayud *et al.* 2002) can facilitate the proliferation of high mealybug populations. Thus, crop management is very important to avoid future problems. mealybug sex pheromones represent a promising and ecologically friendly way to reduce

mealybug population levels (Mansour *et al.* 2017b). However, in contrast to the increasing use of sex pheromones in controlling moth and beetle pests, sex pheromones are still in development for mealybugs (Franco *et al.* 2009, Beltrà and Soto 2012). In any case, pheromone-based control tactics, such as mass trapping or mating disruption, should be regarded as promising methods for mealybug management. In recent years, mating disruption has been tested with good results to control *P. ficus* in vineyards of California (USA), Israel, Sardinia (Italy) or Tunisia (Daane *et al.* 2006, Walton *et al.* 2006, Langone 2013, Cocco *et al.* 2014, Sharon *et al.* 2016, Mansour *et al.* 2017b) and should be considered as a control measure within the IPM programs in vineyards and as a future control measure to be tested against mealybugs affecting other crops. On the other hand, some mass trapping tactics have also been applied to mealybugs, but results were not very as this control tactic is still in development (Franco *et al.* 2004b, Suckling *et al.* 2015).

Biological control

Biological control of mealybugs has been widely studied due to the high number of invasive species introduced in crops of economic importance (McKenzie 1967). Mealybugs have many natural enemies, including parasitoids, predators and entomopathogenic fungi (Moore 1988, Franco *et al.* 2009).

Among parasitoids, encyrtids (Hymenoptera: Encyrtidae) are the largest and diverse group of natural enemies to control mealybugs (Noyes and Hayat 1994). Within this group of parasitoids, species belonging to the genera *Acerophagus* Smith, *Anagyrus* Howard, *Coccidoxenoides* Girault, *Gyranusoidea* Compere, *Leptomastidea* Mercet or *Leptomastix* Förster are used worldwide in biological control (Moore 1988, Franco *et al.* 2000) (Fig. 1.8.). They usually establish host-specific relationships with mealybugs and have a major

influence on their population dynamics (Charles 2011). Encyrtid parasitoids are primary endoparasitoids and their eggs develop inside the body of their host, giving place to a yellowish or brown cylindrical mummy from which will emerge one or more adult parasitoids (Franco *et al.* 2009, Beltrà and Soto 2012). Several important mealybug outbreaks have been solved by classical biological control, this is introducing encyrtid parasitoids from the native area of the mealybug. For example, *Anagyrus lopezi* (De Santis) has been introduced to control *P. manihoti* (Neuenschwander 2001, Parsa *et al.* 2012), *Anagyrus kamali* Moursi for *M. hirsutus* (Roltsch et al. 2006), *Acerophagus papayae* Noyes and Schauff and *Anagyrus loecki* Noyes for *P. marginatus* (Muniappan *et al.* 2006) or *Anagyrus mangicola* Noyes and *Gyranusoidea tebygi* Noyes to control *R. invadens* (Neuenschwander *et al.* 1994, Bokonon-Ganta *et al.* 2002). Encyrtid parasitoids are also used in augmentative biological control of mealybugs. In Spain this is a relatively common practice, and parasitoids are mass-released to control *P. citri* in citrus orchards and ornamental plants, *P. ficus* in vineyards or *P. solani* under greenhouse conditions (Lucas 2002, Villalba *et al.* 2006, Campos-Rivela 2008, Beltrà and Soto 2012).

Fig. 1.8. Adult female of *Anagyrus* sp. parasitizing a mealybug.

Regarding predators, most of them are generalist. This means that they show polyphagy, being able to subsist without pests and not showing a density-dependent response to their preys. In this way, when mealybug densities are still low, but start to increase, predators are already present in a crop and may play an essential immediate role, unlike specific natural enemies that take a longer time to arrive (Symondson *et al.* 2002). Therefore, they must be taken especially into account in conservation biological control practices (Beltrà and Soto 2012). Ladybird beetles (Coleoptera: Coccinellidae) stand out as the most important predators of mealybugs. Other primary groups are lacewings (Neuroptera) of the families Chrysopidae, Coniopterygidae and Hemerobiidae and flies (Diptera) of the families Cecidomyiidae and Chamaemyiidae (Franco *et al.* 2000, Franco *et al.* 2009).

Some coccinellids show specificity for mealybugs and are commonly used in classical and inundative biological control (Iperti 1999, Franco *et al.* 2004a, van Lenteren 2006). Among them, *Cryptolaemus montrouzieri* Mulsant (Fig. 1.9.), of Australian origin, has been introduced many times in a large number of countries, including Spain, with the aim of controlling different mealybug species (Moore 1988, Jacas *et al.* 2006). However, results are not always good, mainly due to overuse of non-selective insecticides and climate conditions (Franco *et al.* 2004a). This coccinellid is also mass reared by several biological control companies and is widely used in augmentative biological control (Franco *et al.* 2009). In the Mediterranean Basin, augmentative releases of the predator *C. montrouzieri* and the parasitoid *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae) are commonly used to control *P. citri* and have been reported to be effective in many countries (Franco *et al.* 2004a, Beltrà and Soto 2012).

The efficacy of the mealybug's natural enemies can be limited by different factors, such as chemical applications, climate conditions, the

lack of food sources, the absence of alternative hosts or the presence of ants. For these reasons, conservation biological control involves the manipulation of the environment to enhance the survival, fecundity, longevity and behavior of the existing natural enemies (Moore 1988, Landis *et al.* 2000, Davies *et al.* 2004, Franco *et al.* 2004a).

Fig. 1.9. *C. montrouzieri* adult (left) and larvae (right) feeding on *P. citri.*

Reducing pesticide applications is one of the actions that can play a vital role in increasing the efficacy of natural enemies. Thus, pesticides should only be used when strictly necessary and only selective compounds should be applied (Landis *et al.* 2000, Mansour *et al.* 2011). Besides, several experiments show that the longevity and fecundity of predators and adult parasitoids can be increased when they feed on sugars, such as nectar, pollen or insect honeydew (Landis *et al.* 2000, Sagarra *et al.* 2000, González-Hernández *et al.* 2005, Gurr *et al.* 2005, Heimpel and Jervis 2005, Chong and Oetting 2006, Sandanayaka *et al.* 2009, Beltrà *et al.* 2013b). Finally, the mutualism between ants and mealybugs also has an important and complex role in biological control. Ants feed on mealybug honeydew and provide them protection against predators and parasitoids (McKenzie 1967, Franco *et al.* 2004a, Beltrà and Soto 2012). Several studies have shown that the control of ants, their exclusion by physical barriers or

the provisioning of artificial sugars increase the action of natural enemies, improving biological control and helping to reduce mealybug densities (Nechols and Seibert 1985, Phillips and Sherk 1991, Campos *et al.* 2006, Mgocheki and Addison 2009a, Mgocheki and Addison 2010, Beltrà *et al.* 2017).

1.2. Mealybugs in citrus and their management

1.2.1 Citrus mealybugs

Seventy mealybug species are known to develop on Citrus worldwide, but only a few are regarded as significant pests (Ben-Dov 1994, García-Morales *et al.* 2016a). The Mediterranean Basin is one of the largest areas of citrus production and one of the leading exporting regions in the world (Lacirignola and D'Onghia 2009). Within this context, mealybug citrus pests affect fruit production and quality, influencing the economy of the citrus-growing countries in this region, especially when mealybug population outbreaks take place (Franco *et al.* 2004a).

In the Mediterranean Basin, six alien mealybug species have traditionally been reported as citrus pests, with different origins and histories of invasion (Bar-Zakay *et al.* 1987, Blumberg *et al.* 1999, Franco *et al.* 2000, Franco *et al.* 2004a): the citrus mealybug *P. citri*, the citriculus mealybug *Pseudococcus cryptus* Hempel, the longtailed mealybug *P. longispinus*, the citrophilus mealybug *Pseudococcus calceolariae* (Maskell), the obscure mealybug *P. viburni* and the spherical mealybug *N. viridis*. Recently, the mealybug *Delottococcus aberiae* (De Lotto) has also been added to the list of invasive mealybug species in the Mediterranean Basin citrus production area (Beltrà *et al.* 2013c). All the aforementioned species are highly polyphagous (Ben-Dov 1994, García-Morales *et al.* 2016a) and only the citrus mealybug, *P. citri* (Fig. 1.10.), is regarded as a major pest,

having a very wide distribution as a result of international trade (Franco *et al.* 2004a, García-Marí 2012).

P. citri is a cosmopolitan and polyphagous mealybug that has been found in 115 countries worldwide, attacking plants of 82 different botanical families (García-Morales *et al.* 2016a). It occurs in the tropical and subtropical zones worldwide, in large densities on perennial crops, among which are citrus, and ornamentals (Ben-Dov 1994, Franco *et al.* 2004a). Owing to its wide distribution, its origin remains unclear. It has been suggested that this species might be native to South America (Compere 1939a) or Eastern Asia (Bartlett 1978). However, the most recent hypothesis, involving its parasitoid *L. dactylopii*, suggests that has Afrotropical origin (Franco *et al.* 2004a, Franco *et al.* 2008, Bugila *et al.* 2014). According to Pellizzari and Germain (2010), *P. citri* arrived and established itselt in Europe during the nineteenth century and in Spain this species has been found, at least, since 1928, when Gómez-Clemente (1928) reported the introduction of the *C. montrouzieri* to control this mealybug.

Fig. 1.10. Adult female of *P. citri*.

The rest of species are considered minor pests in the Mediterranean Basin due to low population levels or because they are restricted to small geographic areas (Franco *et al.* 2004a, García-Marí

2012). *P. calceolariae, P. longispinus* and *P. viburni* usually appear isolated and at low population levels in citrus orchards (Franco *et al.* 2000, García-Marí 2012). *N. viridis* and *P. cryptus* are only relevant in Israel (Bar-Zakay *et al.* 1987, Blumberg *et al.* 1999) and *D. aberiae* is only present in Spain by now (García-Marí 2012, Beltrà *et al.* 2013c)

1.2.2 Management of citrus mealybugs

Mealybugs are regarded as occasional or minor pests of citrus, generally appearing at low density levels (Franco *et al.* 2004a, García-Marí 2012). However, some species can reach key pest status under certain conditions, especially when introduced into new areas without its main natural enemies. Therefore, numerous mealybug outbreaks in citrus orchards have been reported from several areas worldwide, particularly for the species *P. citri* (Clausen 1915, Bodenheimer 1951, Bar-Zakay *et al.* 1987, Hattingh *et al.* 1998, Blumberg *et al.* 1999, Franco *et al.* 2000, Beltrà *et al.* 2013c, Mansour *et al.* 2017a). When a citrus mealybug becomes a key pest, management strategies must be implemented to change its status to that of a minor or occasional pest. This may be achieved by reducing its populations below the economic injury level or by reducing the susceptibility of the plant host to mealybug injury. Different tactics, such as biological control, orchard management, direct chemical control or ant control, may be applied, depending on the mealybug pest situation and the occurrence of other key pests in the orchards (Franco *et al.* 2004a).

Classical biological control and augmentative releases have been widely developed against alien mealybug pests affecting citrus in the Mediterranean Basin, especially to control *P. citri* (Llorens 1994, Katsoyannos 1996, Blumberg *et al.* 1999, Franco *et al.* 2000, Villalba *et al.* 2006, Rahmouni and Chermiti 2013). However, the poor adaptation of several natural enemies to Mediterranean climatic conditions, means chemical control is still being widely used to control mealybug outbreaks (Sharaf and Meyerdirk 1987, Mendel *et*

al. 1999, Franco *et al.* 2000, Franco *et al.* 2004a). Other factors, such as characteristics of the citrus variety, mealybug's crytic behavior, interaction with ants, production system in the orchard or encapsulation, may also impact mealybug population outbreaks and the efficacy of mealybug's natural enemies (Berlinger and Gol'Berg 1978, Blumberg *et al.* 1995, Mendel *et al.* 1999, Campos and Martínez-Ferrer 2003, Campos *et al.* 2006, Hogendorp *et al.* 2006, Villalba *et al.* 2006, Suma *et al.* 2012).

The identification and synthesis of the sex pheromone of *P. citri* (Zada et al. 2004, Kukovinets et al. 2006), *P. calceolariae* (El-Sayed et al. 2010), *P. cryptus* (Nakahata et al. 2003), *P. longispinus* (Millar et al. 2009), or *P. viburni* (Millar and Midland 2007), has allowed for new opportunities to monitor and control mealybugs in citrus orchards. Thus, mass trapping, mating disruption or lure and kill should be considered for possible use in citrus IPM programs as alternative methods to chemical treatments (Franco *et al.* 2009). In citrus orchards of Israel and Portugal, a wo-year study for mass trapping *P. citri* males was carried out and results indicated that a significant reduction in male numbers can be achieved, but the reduction obtained with the experimental design was not enough to reduce fruit infestation significantly (Franco *et al.* 2004b). Besides, the complex structure of mealybug's pheromones limits large scale synthesis required for mating disruption (Franco *et al.* 2009). Therefore, the application of pheromones is still restricted to monitoring the evolution of citrus mealybugs in the orchards, in the case of those species whose sex pheromone is commercially available, being *P. citri* the most widely studied for now (Hwang and Chu 1987, Hefetz and Tauber 1990, Franco *et al.* 2001, Martínez-Ferrer *et al.* 2003, Zada *et al.* 2004, Levi-Zada *et al.* 2014).

Finally, enhancement of biological control through the management of ant populations is another promising tactic to control the density of mealybug pests in citrus orchards and has been tested

with good results during recent decades (Franco *et al.* 2004a, Villalba *et al.* 2006, Marras *et al.* 2008).

1.3. *Delottococcus aberiae* (De Lotto)

1.3.1 Genus *Delottococcus*

The genus *Delottococcus* was described by Cox and Ben-Dov (1986) for a range of African species that had been previously placed in several genera, including *Pseudococcus* (Brain 1915), *Planococcus* (Ezzat and McConnell 1956), *Allococccus* (De Lotto 1961) and *Paracoccus* (Williams 1958) (Miller and Giliomee 2011). This genus is mainly characterized as having an anal bar, presence of oral-rim tubular ducts, presence of abdominal cerarii with no more than two conical setae and no auxiliary setae, presence of translucent pores on hind tibia and absence on hind coxa and no circulus (Cox and Ben-Dov 1986, Miller and Giliomee 2011), it being included in the subfamily Pseudococcinae by Hardy *et al.* (2008). Unfortunately, none of these characters is consistently present in all specimens of each species. Therefore, due to morphological variation in species, it is a difficult group of mealybugs to identify and some specimens have in fact been misidentified. For example, *Delottococcus elisabethae* (Brain) was recorded from citrus and this appears to be a misidentification of *D. aberiae* (Miller and Giliomee 2011).

Miller and Giliomee (2011) reviewed the genus *Delottococcus* Cox & Ben-Dov and, currently, it includes nine mealybug species native to southern areas of the Afrotropical region: *D. aberiae*, *Delottococcus confusus* (De Lotto), *D. elisabethae*, *Delottococcus euphorbiae* (Ezzat & McConnell), *Delottococcus millari* Miller & Giliomee, *Delottococcus phylicus* (De Lotto), *Delottococcus proteae* (Hall), *Delottococcus quaesitus* (Brain) and *Delottococcus trichiliae* (Brain) (Miller and Giliomee 2011, García-Morales *et al.* 2016a).

Some *Delottococcus* species have been cited as invasive mealybugs and their economic impact can be substantial. These are the cases of *D. aberiae*, reported in Spain (Beltrà *et al.* 2013c), *D. confusus* detected in California and Hawaii (Watson 2007, Stocks 2014) or *D. euphorbiae* present in France and Italy (Matile-Ferrero 1983, Longo *et al.* 1995b, Foldi 2000, Pellizzari and Germain 2010).

1.3.2 The mealybug *D. aberiae*

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) (Fig. 1.11.) is a mealybug native to sub-Saharan Africa (Miller and Giliomee 2011). It has been found in plants of 25 different botanical families (García-Morales *et al.* 2016a) and it feeds on different tropical and subtropical crops, such as citrus, coffee, guava, pear, persimmon or olive (De Lotto 1961, Miller and Giliomee 2011, Beltrà *et al.* 2013c, Pérez-Hedo *et al.* 2018). *D. aberiae* is a common species in the country of South Africa (Miller and Giliomee 2011), where it is mainly found on wild olive trees and on the roots of the flowering shrub *Chrysanthemoides monilifera* (L.) T. Norl. However, it can also be found, irregularly distributed, in citrus orchards of the north of the country. There, it is considered a secondary pest that can go unnoticed for years (Hattingh *et al.* 1998, Miller and Giliomee 2011), but several mealybug outbreaks have been reported in recent years by the Citrus Research International (Moore and Hattingh 2012, Beltrà *et al.* 2015).

In 2009, *D. aberiae* was detected as an invasive species in eastern Spain (Les Valls, Valencia) (Fig. 1.12.), causing serious damage in citrus orchards and being this the first report of the mealybug as a significant citrus pest worldwide (Beltrà *et al.* 2012, García-Marí 2012, Beltrà *et al.* 2013c). Identification was confirmed by molecular and taxonomic techniques, and recent studies have shown that Spanish invasive populations are native from Limpopo, in northern South Africa (Beltrà *et al.* 2015). Since its arrival, the mealybug has continued spreading, slowly but steadily, towards adjoining areas and

has become a significant pest in eastern Spain (Soto *et al.* 2016b). Like other mealybug species, *D. aberiae* reduces plant vigor and excretes honeydew, promoting the growth of sooty mold fungi, interfering with plant photosynthesis and giving shelter to secondary pests, such as pyralid moths (Franco *et al.* 2000). However, feeding behavior of *D. aberiae* causes severe direct damage to young citrus fruits, distorting their shape and size (Fig. 1.13.), depreciating their commercial value and leading to significant crop losses (Beltrà *et al.* 2013c, Tena *et al.* 2014).

The complex of natural enemies of *D. aberiae* is practically unknown, and since its introduction in Spain the absence of effective biological control to manage population outbreaks has been reported (Tena *et al.* 2014, Soto *et al.* 2016a, Tena *et al.* 2017a). The existing parasitoids in Spain fail to control this pest (Tena *et al.* 2017a, Tena *et al.* 2017b) and the predators, mainly *C. montrouzieri*, appear late when the damage to the fruit has already been done (Soto *et al.* 2016a).Therefore, due to the necessity of the growers to control this pest, the management of *D. aberiae* relies on the use of insecticides (Tena *et al.* 2014, Pérez-Rodríguez *et al.* 2017).

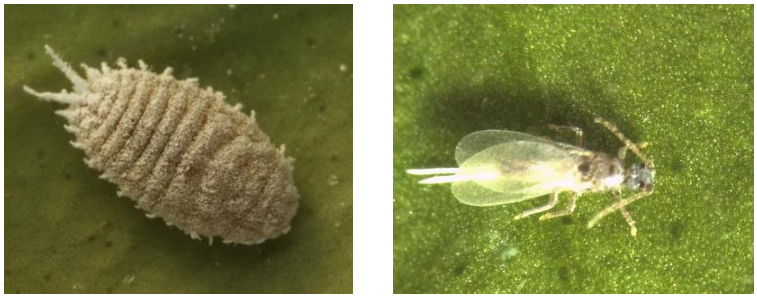

Fig. 1.11. *D. aberiae* adult female (left) and *D. aberiae* adult male (right).

Fig. 1.12. Colonies of *D. aberiae* in citrus orchards of eastern Spain.

Fig. 1.13. Distortions in fruit shape and size originated by *D. aberiae* in different orange and mandarine cultivars.

CHAPTER 2.
Justification and objectives

Chapter 2. Justification and objectives

Mealybugs are important crop pests because they are easily introduced into new areas due to their small size and cryptic behavior, especially through international plant trade. As such, they represent one of the insect groups with more alien species in Europe. Among the recently introduced invasive species, *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) is the latest mealybug found in citrus in Spain.

In 2009, *D. aberiae* was detected after causing significant direct damage to citrus fruits in the region of "Les Valls" (Valencia, eastern Spain), within the Mediterranean Basin, and thus poses a threat to citrus production in the area. Currently, *D. aberiae* is considered a citrus pest only in Spain and its native (Limpopo, northern South Africa) and given its recent designation as an invasive species, little is known about the biology, behavior and natural enemies of the insect in this crop.

Since its arrival to Spain, *D. aberiae* has been managed using insecticides. However, the latest European Directive on sustainable use of pesticides (2009/128/EC) stipulates the reduction in chemical applications which interfere with natural enemies and pollinators. Thus, a better understanding of the biology and behavior of *D. aberiae*, as well of its natural enemies, is needed to develop alternative management strategies. Therefore, in order to design accurate sampling protocols, facilitate an early detection of the pest and promote the biological control of *D. aberiae*, the following objectives were established for this doctoral thesis:

i. To study the behaviour of *D. aberiae* in citrus orchards throughout the year:

   a. To identify the seasonal trend (density and estructure) of *D. aberiae* by different sampling methods, comparing them.
   b. To analyze the seasonal distribution of *D. aberiae* in citrus trees.

ii. To determine the period of susceptibility of the citrus fruits to direct damage caused by *D. aberiae* and to characterize the damage.

iii. To study the feasibility of developing a classical biological control program to manage *D. aberiae* in Spain:
   a. To study the behaviour of *D. aberiae* in citrus orchards in its native area (South Africa).
   b. To characterize the complex of natural enemies of *D. aberiae* in its native area.

CHAPTER 3.

Density and phenology of the invasive mealybug *Delottococcus aberiae* on citrus: implications for integrated pest management

Chapter 3. Density and phenology of the invasive mealybug *Delottococcus aberiae* on citrus: implications for integrated pest management

Martínez-Blay, V., Pérez-Rodríguez, J., Tena, A. & Soto, A. 2017. J Pest Sci 91: 625-637. Adapted author's Post-print version.

Abstract

Delottococcus aberiae De Lotto (Hemiptera: Pseudococcidae) is a new invasive citrus pest in Spain. It causes severe fruit distortions and, as a new invasive mealybug, there is a lack of information about its biology. This research aims to examine the seasonal trend of D. *aberiae* in citrus, using several sampling methods, as a first step to develop an integrated pest management program. Ten citrus orchards from eastern Spain were periodically sampled during three years using absolute (plant material) and relative (corrugated cardboard band traps and sticky traps) sampling methods. The three sampling methods showed that *D .aberiae* completes multiple generations per year, two of them being clearly defined and resulting in high populations. *D. aberiae* peaked between May and June, damaging the developing fruit. Corrugated cardboard band traps were able to detect pre-pupa and pupa male instars and gravid females, providing a quantitative measurement of *D. aberiae* density at its first population peak. The use of corrugated cardboard band traps is recommended to monitor population levels and sticky traps to determine male flight periods, representing simple sampling techniques to monitor *D. aberiae*. These results will improve the sampling protocols and allow for the development of an integrated pest management program.

Keywords: corrugated and sticky traps, life cycle, sampling protocols, *D. aberiae*, citrus.

3.1. Introduction

The globalisation process and the increase in the international trade of ornamental and crop plants has led to an exponential rise in the introduction and establishment of alien and invasive insects in Europe (Roques *et al.* 2009, Pellizzari and Germain 2010, Pellizzari and Porcelli 2014). Mealybugs (Hemiptera: Pseudococcidae) are the second most diverse family of scale insects (Coccoidea), comprising around 2,000 species distributed worldwide and including many agricultural and ornamental pests which can cause substantial damage (Ben-Dov 1994, Hardy *et al.* 2008, García-Morales *et al.* 2016a). Due to their small size and cryptic behavior, many mealybug species live in hidden habitats and are often unnoticed during quarantine inspections. Therefore, they are easily introduced into new areas through international plant trade. Once in a new territory their high fecundity favours rapid invasion, constituting an ecological and economic threat to many agricultural and ornamental ecosystems (Pimentel *et al.* 2001, Miller *et al.* 2002, Hulme *et al.* 2008, Kenis *et al.* 2009, Pellizzari and Germain 2010, Mansour *et al.* 2017a).

In Europe, mealybugs represent the third most numerous family of alien insects; since the 1990s, several species have been recorded as new invaders in the Mediterranean Basin, some examples are *D. brevipes* (Suma *et al.* 2015), *P. marginatus* (Mendel *et al.* 2016), *P. defectus* (Mazzeo *et al.* 2014), *P. solani* (Mazzeo *et al.* 1999), *P. comstocki* (Pellizzari 2005) or *P. peruvianus* (Beltrà *et al.* 2010). Most of these mealybugs have established in anthropogenic habitats, such as cultivated agricultural lands, urban environments, nurseries or greenhouses (Roques *et al.* 2009, Pellizzari and Germain 2010). The Mediterranean Basin is one of the largest areas of citrus production and one of the leading exporting regions in the world

(Lacirignola and D'Onghia 2009). In this area, six alien mealybug species have been reported as citrus pests, with different origins and histories of invasion (Blumberg *et al.* 1999, Franco *et al.* 2000): *Planococcus citri* (Risso), *Pseudococcus cryptus* (Hempel), *Pseudococcus longispinus* (Targioni-Tozzetti), *Pseudococcus calceolariae* (Fernald), *Pseudococcus viburni* (Signoret) and *Nipaecoccus viridis* (Newstead). Among them, *P. citri*, is the most damaging species with a wide distribution due to international plant trade (Franco *et al.* 2004a).

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) is a mealybug of Southern African origin. It has been reported as a species that feeds on different tropical and subtropical crops, such as citrus, coffee, guava, pear or olive (De Lotto 1961, Miller and Giliomee 2011). In South African citrus orchards it is considered a secondary pest that can go unnoticed for years (Hattingh *et al.* 1998, Miller and Giliomee 2011). In 2009, nevertheless, *D. aberiae* was detected as an invasive species in eastern Spain, with serious damages in citrus (García-Marí 2012, Beltrà *et al.* 2013c), being identification confirmed by molecular and taxonomic techniques (Beltrà *et al.* 2012, Beltrà *et al.* 2015). Like other mealybug species, reduces plant vigour and excretes honeydew that promotes the growth of sooty mold fungi and interferes with plant photosynthesis (Franco *et al.* 2000). However, when *D. aberiae* develops on young citrus fruits causes severe distortions and fruit size reduction, leading to significant crop losses and representing a threat to Mediterranean citrus production (Beltrà *et al.* 2013c, Soto *et al.* 2016b). Since its establishment in Spain, different assays have revealed the absence of effective natural enemies to control *D. aberiae* outbreaks (Beltrà *et al.* 2013c, Soto *et al.* 2016a, Tena *et al.* 2017a). Therefore, the management of the

pest relies on the use of broad-spectrum insecticides, such as chlorpyrifos (Tena *et al.* 2014), which interferes with the biological control of other citrus pests (Franco *et al.* 2009, Tena and García-Marí 2011).

Monitoring protocols improve pest detection, provide information regarding their seasonal occurrence and determine the expected susceptible periods. This information avoids unnecessary spraying and forms the basis of any integrated pest management (IPM) program (Gonzalez 1971, De Villiers and Pringle 2007). Sampling and monitoring mealybugs are processes based on different techniques which have improved their control in agricultural and ornamental ecosystems (Geiger and Daane 2001, Walton *et al.* 2004, Martínez-Ferrer *et al.* 2006, Mudavanhu *et al.* 2011, Waterworth *et al.* 2011). However, for most mealybug species, sampling consist of laborious and time consuming visual examination of plant material, searching for live insects and counting all life stages (Grimes and Cone 1985, Geiger and Daane 2001, Walton *et al.* 2004, Waterworth *et al.* 2011). Alternative monitoring techniques, mainly based on the use of different trap designs, have been developed to determine the mealybug's seasonal occurrence, being the most common ones corrugated cardboard bands and sticky traps (Goolsby *et al.* 2002, Millar *et al.* 2002, Walton *et al.* 2004, Roltsch *et al.* 2006, Beltrà and Soto 2012).

Corrugated cardboard band traps represent a non-destructive sampling method to monitor mealybug population densities (DeBach 1949, Furness 1976, Goolsby *et al.* 2002). The bands are wrapped around the trunk or main branches of the trees and serve as a refuge for gravid females to lay their eggs, or for second male instars to make their cocoon and develop into adults males (Beltrà and Soto 2012). This first method has been tested with positive results to sample *P. viburni* (Mudavanhu

2009), *P. longispinus* (DeBach 1949, Furness 1976) or *Maconellicoccus hirsutus* (Green) (Goolsby *et al.* 2002, Roltsch *et al.* 2006). Sticky traps are used to monitor some flying pests, including the winged adult males of different mealybugs (Samways 1988, Grout and Richards 1991, Sun *et al.* 2002). These traps are generally baited with sex pheromones to increase male captures and monitor their seasonal flight periods (Moreno *et al.* 1984, Millar *et al.* 2002, Walton *et al.* 2004, Mudavanhu *et al.* 2011). This second method has proven useful when monitoring species such as *P. calceolariae*, *P. citri*, *P. comstocki* or *M. hirsutus* (Moreno *et al.* 1972, Rotundo and Tremblay 1975, Moreno *et al.* 1984, Serrano *et al.* 2001), and two types of lures may be used to attract the males: live virgin females or synthetic sex pheromones (Meyerdirk *et al.* 2001).

D. aberiae is up to now a significant citrus pest only in Spain. Due to its recent designation as an invasive species, little is known about the biology and behavior of the insect in this crop. The main objectives of this work are: (i) to determine the seasonal trend of *D. aberiae* throughout the year, by absolute sampling methods (visual examination of plant material) and (ii) to compare the obtained results with relative sampling procedures (corrugated cardboard band traps and sticky traps) in order to identify simpler monitoring methods to establish *D. aberiae* density. These results will be used to improve its control within the existing IPM programs for citrus in Spain.

3.2. Material and methods

3.2.1 Survey sites

Ten commercial citrus orchards, which presented visual evidence of more than 50% of damaged fruits during previous seasons (400 fruits were sampled randomly in each orchard),

were sampled in different areas of eastern Spain from March 2014 to November 2016. Orchards sampled were carefully selected to avoid mixture with other mealybug species and to ensure that they contained almost exclusively *D. aberiae* populations. They ranged from 0.16 to 2 ha, five of them included sweet orange trees (*Citrus sinensis* (L.) Osbeck; 'Lane late', 'Navelina' and 'Sanguinelli' varieties) and the other five clementine mandarin trees (*Citrus reticulata* Blanco; 'Oroval' and Clemenules varieties).

3.2.2 Absolute sampling protocol. Plant material

In each of the ten orchards, eight to ten trees were marked and sampled regularly between 2014 and 2016. In 2014 and 2015, samplings were done weekly, during the periods of most rapid mealybug development (March-August), and twice a month or monthly during the rest of the year, depending on population levels; in 2016 samplings were carried out at monthly intervals. No insecticide sprays were applied to the trees during the sampling period. For each sampling date, and at each sampling site, four 20-cm long twigs per marked tree, each one from a different cardinal orientation, were collected randomly from the middle and outer part of the canopy. A minimum of five orchards, fifty trees and two hundred twigs were always sampled simultaneously at each sampling date. Each twig included its leaves, flowers and fruits when these organs were available. Samples were bagged and transported to the laboratory inside a portable cooler. All the material was processed within the next 24 h. Each mealybug present on each twig, on four leaves per twig and on one to eight flowers or fruits (depending on their availability during the year) was counted under a stereomicroscope (Nikon SMZ645). Leaves, flowers and fruits to be examined from each twig were randomly selected. The sex and instar of each mealybug were

also recorded. To separate between developmental stages, a laboratory colony of D. aberiae was established at Universitat Politècnica de València (UPV) in 2013, using specimens collected from a clementine orchard located in Quart de les Valls (Valencia, Spain). Previous to starting field samplings, a laboratory assay was done. In this assay, direct observations were carried out, every 24h, in search of successful development from one instar to the following one, being the passage recognized by the presence of exuviae. Afterwards, 20 mealybugs of each instar, obtained from the laboratory colony and successfully molted, were measured (Martínez-Blay *et al*., in prep.). The following body length ranges were obtained and used to separate instars: first nymphal instar (0.40-0.69 mm), second nymphal instar (0.70-0.98 mm), third nymphal instar (0.99-1.40 mm) and females (>1.41 mm), in the latter case separating young from gravid females. Thus, for routine samplings, mealybugs were separated by measuring them with a stereomicroscope fitted with an ocular micrometer. Males and females of the first and second nymphal instars were pooled together as sex cannot be distinguished until the end of the second instar (Gullan and Martin 2009, Beltrà *et al.* 2013a).

3.2.3 Relative sampling methods. Traps

In the present study two types of traps were used to capture mealybugs: corrugated cardboard band traps and adapted sticky traps. Both types of traps were placed in five of the ten sites surveyed. Traps were sampled with the same periodicity as plant material. In 2014 and 2015, samplings were done weekly, fortnightly or monthly, depending on population levels. In 2016 samplings were carried out at monthly intervals. No insecticides were applied to the trees during the sampling period.

Corrugated cardboard band traps were placed in five marked trees (in each of the sampled orchards). Four corrugated cardboard bands, of approximately 20 cm wide each, were placed per tree: one around the trunk and three around the main branches. Traps were opened in the field at each sampling date, and the mealybugs were counted and separated into the following categories: nymphs (first, second and third instars), young females, gravid females and immature males (pre-pupa and pupa). After counting, each cardboard band was cleaned, with the help of a small brush, to remove all the present mealybugs and wrapped around the trunk and branches again.

In addition, two sticky traps were placed on two trees in each orchard (different from the ones used for corrugated cardboard band traps) at approximately 1.5 m above the ground in the southern external part of the canopy. Live virgin females were used as a bait to conform a special sticky trap, adapted from the ones previously used in similar studies (Moreno *et al.* 1972, Meyerdirk and Newell 1979, Meyerdirk *et al.* 1981, Grimes and Cone 1985, Meyerdirk *et al.* 2001, Serrano *et al.* 2001). Therefore, from this point on, these will be referred to as sticky sex pheromone traps. Each trap consisted of a 0.5L plastic bottle containing one or two lemons and ten new virgin females. Each bottle had a modified lid, consisting of a fine mesh cloth, to allow ventilation and dispersion of the female sex pheromone to attract adult males. Females were obtained from the laboratory established colony. A yellow sticky card, 20 × 12.5 cm (ECONEX S.L.) was attached to each bottle with two plastic clothes pins. At each sampling date, virgin females were replaced with new ones and all yellow sticky cards were changed and transported to the laboratory, where the male mealybugs were counted under a dissecting microscope (Nikon SMZ645). To confirm that males counted were *D. aberiae*, at

least ten males (fewer if 10 were not present) were removed from each trap and mounted following the procedure describe by Beardsley (1960). A drop of lemon extract was used to remove the males from the sticky surface of the traps. Afterwards, they were compared, based on the morphology of their genitalia (Beardsley 1960, Afifi 1968, Tremblay *et al.* 1977), with other mealybug species present in citrus in eastern Spain (*P. citri*, *P. longispinus* and *P. viburni*) and with *D. aberiae* males obtained from our established laboratory colony. It was possible to separate *D. aberiae* from the rest of species taken into account the anal pair of filaments and the form of the genital capsule and the genital style (Martínez-Blay *et al*., in prep.).

3.2.4 Data analysis

Data from the seasonal monitoring of *D. aberiae*, by both absolute and relative sampling methods, are presented graphically to show the seasonal abundance trends of the pest. The number of mealybug generations per year was determined by plotting the percentage of each developmental stage per sample unit over time. To compare differences in population abundance between the years 2014 and 2015, the mean number of mealybugs capture from March to December, per sample unit was calculated. Data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. As data were normally distributed but with unequal variances, an unequal variance t-test (Welch's t-test) was performed to compare means between the two years. An analysis of covariance test (ANCOVA) was made to check the potential effect of the year and the average number of mealybugs captured on traps at the first peak (corrugated cardboard band traps or sticky sex pheromone traps) on the average number of mealybugs per orchard and sample unit at the

first *D. aberiae* population peak. Depending on the influence of the factor year, the relationship between the average number of mealybugs per sample unit and the average number of mealybugs per trap at the first peak was plotted and compared, using regression analysis, pooling all data together or separating data by year (Fig. 3.4.). Data collected during 2016 were excluded from all the analysis because samplings were performed much less frequently than in 2014 and 2015. All statistical analyses were conducted using Statgraphics Centurion XVI.II (Statpoint Technologies Inc, Warrenton, USA).

3.3. Results

3.3.1 Seasonal trend by absolute sampling methods

The development of *D. aberiae* showed a similar trend over the three-year periods of study. Mealybugs completed multiple generations during the year, as illustrated by first nymph instars or crawler peaks (Fig. 3.1.). Two of these generations were clearly defined every year. The first one was recorded in spring, coinciding with a peak of crawlers between mid-May and early June, with a percentage of crawlers, of the total population, of 89.40 ± 4.04 %, 87.09 ± 4.88 % and 59.46 ± 4.16 % in 2014, 2015 and 2016 respectively. The second one was recorded in summer, between mid-July and mid-August, with percentages of 74.75 ± 3.69 %, 73.54 ± 9.91 % and 66.49 ± 9.86 % each consecutive studied year. These two main generations were those which resulted in a high population density of the pest. The rest of the crawler peaks were not so well defined, probably due to overlapping generations and the low population density after August (Fig. 3.2.).

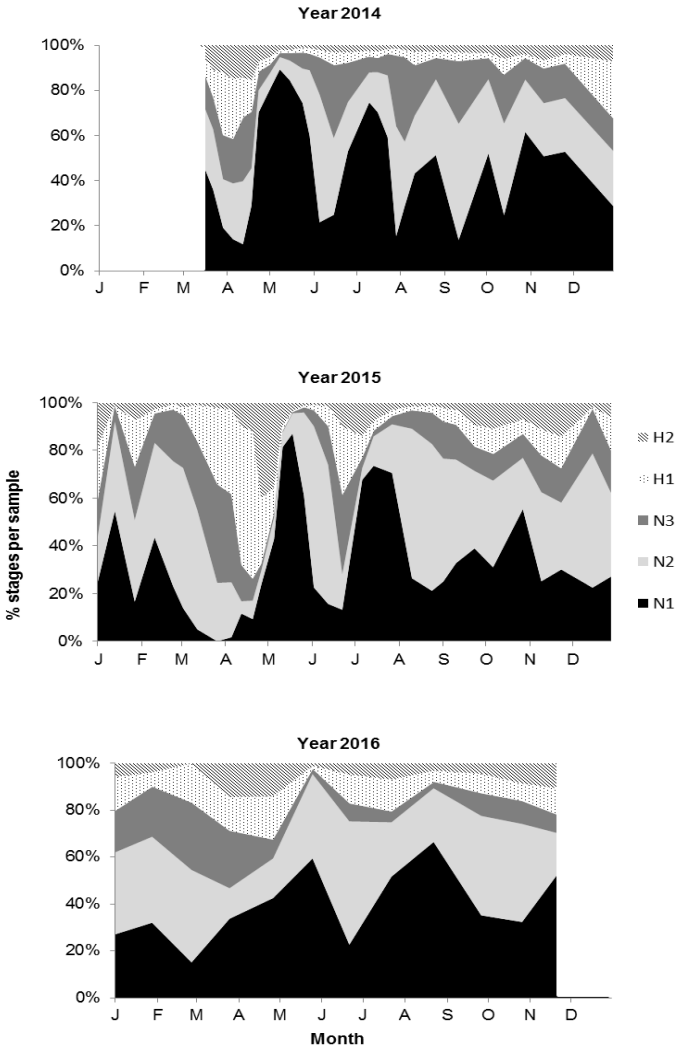

Fig. 3.1. Seasonal relative abundance of *D. aberiae* developmental stages in ten citrus orchards in eastern Spain. Percentage of each developmental stage per sample unit and date is represented for the years 2014, 2015 and 2016 (N1 = first nymphal instars, N2 = second nymphal instars, N3 = third nymphal instars, H1 = young females, H2 = gravid females).

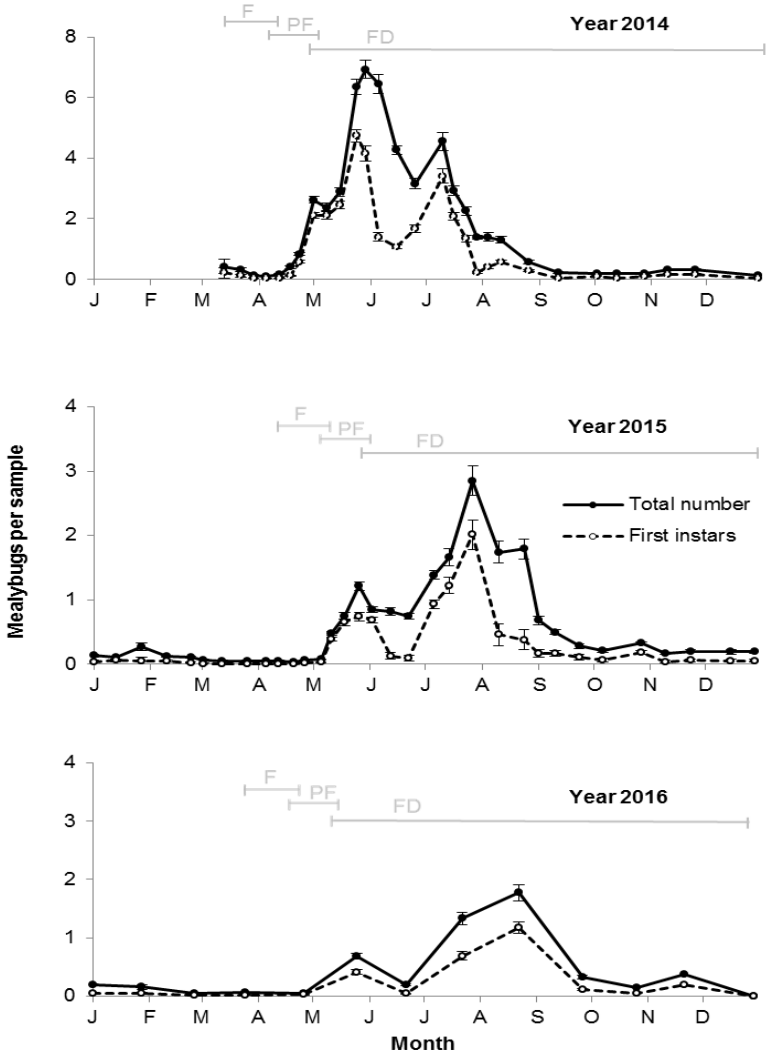

Fig. 3.2. Seasonal trend of *D. aberiae* populations in ten citrus orchards in eastern Spain. Mean number of mealybugs ± SE collected per sample unit (total number and first instars). Above each graph the length of the flowering period (F), petal fall period (PF) and fruit developing period (FD) is presented.

The density of *D. aberiae* populations started to increase rapidly in April and May, leading to a first population peak in spring, at the end of May-beginning of June, and a second one in summer, between July and August. Both peaks occurred at the crawler emergence periods (Fig. 3.2.). The spring peak population density reached an average (mean ± standard error, SE) of 6.92 ± 0.30, 1.21 ± 0.07 and 0.69± 0.05 mealybugs per sample unit in 2014, 2015 and 2016. In summer, *D. aberiae* population density recorded a mean value, respectively for each year, of 4.55 ± 0.29, 2.85 ± 0.23 and 1.77± 0.13 mealybugs. Afterwards, the population decreased and was almost undetectable in autumn and winter (Fig. 3.2.). Populations were more abundant in the year 2014 (mean ± SE: 1.86 ± 0.08 mealybugs per sample unit) than in 2015 (0.56 ± 0.03) ($t = 16.04$, $df = 444$, $P < 0.001$).

3.3.2 Seasonal trend by relative sampling methods

Corrugated cardboard band traps caught mainly gravid females and immature, pre-pupa and pupa, male instars (nymphs and young females were trapped at very low levels and are not represented on Fig. 3.3.), whereas sticky sex pheromone traps attracted adult males. Corrugated cardboard band traps captured immature male stages over the three-year study, captures being much more abundant in the year 2014. Two peaks for these male instars could be observed each year. The first one was recorded at the end of March-beginning of April, with 40.55 ± 2.51, 12.01 ± 1.42 and 8.79 ± 0.73 males per trap (mean ± SE) in 2014, 2015 and 2016 respectively. The second maximum was reached at the end of May-beginning of June, with an average of 69.58 ± 5.65, 6.15 ± 1.21 and 9.10 ± 0.91 males per year. Gravid females were very abundant in corrugated cardboard band traps

during certain periods of the year, especially in 2014. Two peaks of females with egg sacs were detected each year. The first one was reached at the end of April-beginning of May, with 67.84 ± 3.84, 22.40 ± 2.11 and 10.31 ± 0.97 females per trap (mean ± SE) in 2014, 2015 and 2016. The second one was recorded at the end of June, with an average of 107.64 ± 8.53, 9.42 ± 0.95 and 25.02 ± 1.79 females each consecutive studied year. During the rest of the year, female populations in corrugated cardboard band traps remained at undetectable levels.

D. aberiae was the only mealybug species collected and identified in the sticky sex pheromone traps. Two main peaks of captures were recorded both years of the study, corresponding with two distinct flights. The first one occurred at the end of March-beginning of April, with an average (mean ± SE) of 66.40 ± 7.9, 48.14 ± 14.41 and 9.33 ± 2.40 males in 2014, 2015 and 2016 respectively. The second one was between the end May and the beginning of June, with 41.20 ± 5.75, 17.57 ± 5.49 and 42.33 ± 9.10 males per trap in 2014, 2015 and 2016. The number of males out of those periods decreased considerably; sticky sex pheromone traps were only able to detect small increases in mealybugs between July and December, and at the beginning of the year, but captures were always below the average of 5 males per trap (Fig. 3.3.). Males and females were captured successively over time in the traps (Fig. 3.3.): firstly, males in the stages of pre-pupa and pupa were detected in corrugated cardboard band traps; secondly, adult males were found in sticky sex pheromone traps and finally gravid females were captured in corrugated cardboard band traps.

ANCOVA tests showed a significant relationship between the average number of mealybugs per sample unit, at the first *D. aberiae* population peak (end of May), and the average number

of *D. aberiae* males caught in sticky sex pheromone traps for each orchard ($F = 9.94$; $df = 1, 9$; $P = 0.02$) and the average number of gravid females ($F = 39.99$; $df = 1, 9$; $P < 0.001$) and immature male instars ($F = 12.81$; $df = 1, 9$; $P = 0.01$) captured in corrugated cardboard band traps. This relationship differed significantly between years for male captures in sticky sex pheromone traps ($F = 52.35$; $df = 1, 9$; $P < 0.001$) but not for gravid females ($F = 1.13$; $df = 1, 9$; $P = 0.32$) or immature male instars in corrugated cardboard band traps ($F = 52.35$; $df = 1, 9$; $P = 0.08$). Thus, the total average number of *D. aberiae* per plant sample unit and orchard at the first *D. aberiae* population peak was regressed, considering data from both years together, in comparison with the average number of gravid females ($y = 0.18x - 2.49$; $df = 1,9$; $F = 273.72$; $P < 0.001$; $r^2 = 0.97$) and immature male instars ($y = 0.25x - 1.45$; $F = 62.69$; $df = 1,9$; $P < 0.001$; $r^2 = 0.89$) per corrugated cardboard band trap and orchard, showing a significant and positive correlation (Fig. 3.4.).

Besides, the total average number of *D. aberiae* per plant sample unit and orchard at the first *D. aberiae* population peak was regressed in comparison with the average number of adult males per sticky sex pheromone trap and orchard, but for each year independently (Fig. 3.4.) (2014: $y = 0.12x + 1.99$; $F = 43.53$; $df = 1,4$; $P = 0.08$; $r^2 = 0.94$ / 2015: $y = 0.02x + 0.63$; $F = 56.88$; $df = 1,4$; $P = 0.01$; $r^2 = 0.95$).

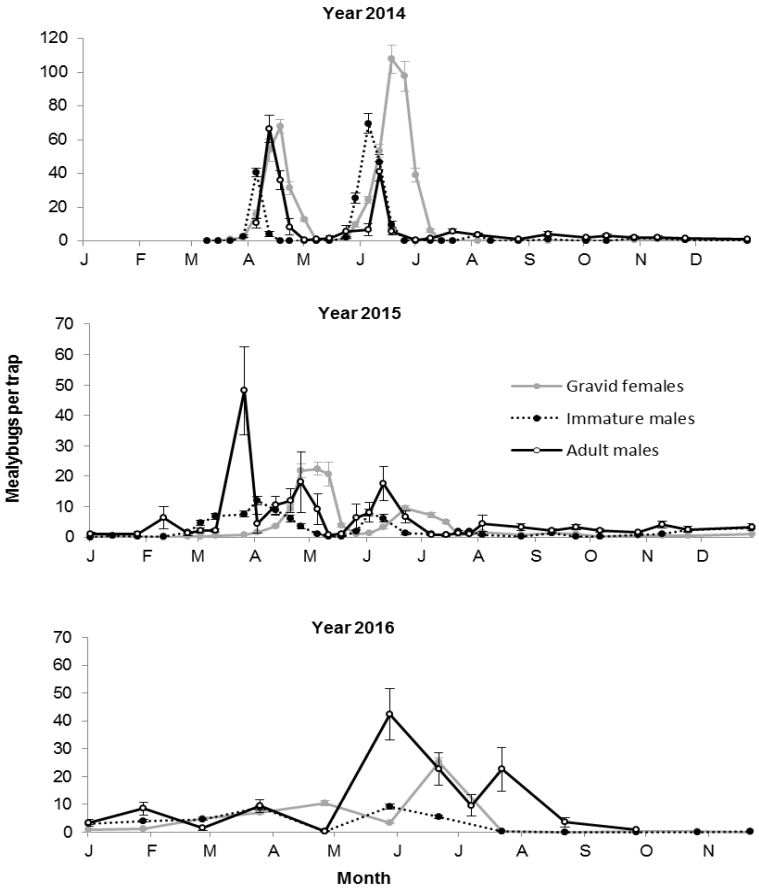

Fig. 3.3. Seasonal trend of *D. aberiae*, captured with two types of traps, during the years 2014, 2015 and 2016 in five citrus orchards in eastern Spain. Presented as mean number of mealybugs ± SE captured in corrugated cardboard band traps (gravid females and immature male instars) and in sticky sex pheromone traps (adult males). Note that y-axis scales are different for 2014 and 2015-2016.

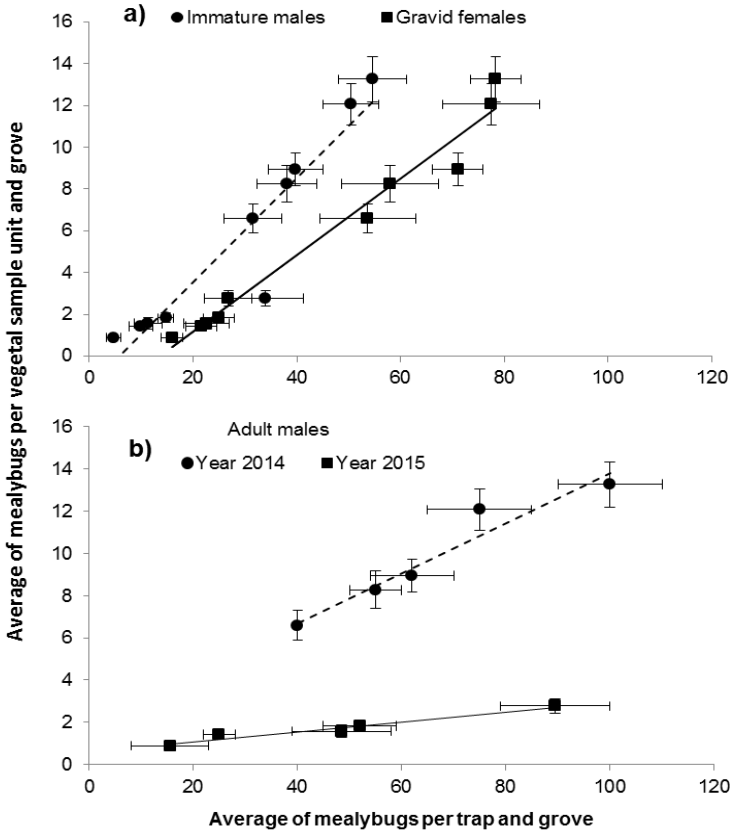

Fig. 3.4. Relationship between the mean number of *D. aberiae* per plant sample unit and the mean number of individuals collected in different traps at the first population peak. a) Average number of *D. aberiae* per plant sample unit correlated with average number of gravid females ($y = 0.18x - 2.49$; df = 1,9; $F = 273.72$; $P < 0.001$; $r^2 = 0.97$) and immature males ($y = 0.25x - 1.45$; $F = 62.69$; df = 1,9; $P < 0.001$; $r^2 = 0.89$) per corrugated cardboard band trap. b) Average number of *D. aberiae* per plant sample unit correlated with the average number of adult males per sticky sex pheromone trap and year (2014: $y = 0.12x + 1.99$; $F = 43.53$; df = 1,4; $P = 0.01$; $r^2 = 0.94$ / 2015: $y = 0.02x + 0.63$; $F = 56.88$; df = 1,4; $P = 0.01$; $r^2 = 0.95$).

3.4. Discussion

The main purpose of the current study was to determine the seasonal trend of the new invasive pest *D. aberiae*, on citrus, as a basis to design sampling protocols and improve its control. Our results reveal that *D. aberiae* density increased in spring, reaching its first significant maximum during May and June, coinciding with fruit development. High population levels developed on fruits until the end of August, when populations decreased and remained at very low levels for the rest of the year. These results are the first quantitative description of *D. aberiae* biology on any crop. The rapid decrease at the end of the summer, and significant differences in mealybug abundance between years, might be a consequence of different biotic and abiotic factors, such as climate, the action of natural enemies or the quality of the feeding substrate. The high temperatures and low humidity that frequently occur during summer, in countries with Mediterranean climate, may cause high mortality in mealybugs, especially of first instars (Browning 1959, Bartlett and Clancy 1972, Furness 1976). The population levels of *D. aberiae* were lower in 2015 than in 2014. In 2015, unusually low temperatures and heavy rains occurred at the end of March, followed by a period of very high temperatures with low humidity levels in April (Benavites data, IVIA SIAR's Weather Net, http://riegos.ivia.es/datos-meteorologicos).The combination of these two consecutive climatic factors might have negatively affected *D. aberiae* in May, as populations did not increase as much as in May of 2014. Moreover, this decrease occurred in all the sampled orchards. The effect of the natural enemies cannot explain this reduction as native and naturalized parasitoid species do not develop on *D. aberiae* (Tena *et al.* 2017a). The predator *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) attacks *D. aberiae*, but always after May (Pérez-

Rodríguez *et al*. in prep.). This predator is abundant in June and peaks at the beginning of August, contributing to the decline of mealybug populations at the end of summer and fall. Besides, in the year 2016, sampling was carried out only in five orchards, which already had low levels; this factor might also have contributed to the fact that population levels were even lower than in 2015.

However, the most limiting factor of mealybug populations feeding in citrus trees, during the second half of summer, seems to be the quality of the feeding substrate. As eurymeric species, mealybugs are able to feed on different organs of the host plant (Kozár 1989), but not all the organs constitute a food source of equal quality. Therefore, it is expected that the development and fecundity of the mealybugs vary according to the organ in which they are located (Franco *et al.* 2000). One of the factors affecting mealybug's distribution, in the different plant parts, is the phenology of the host. Franco (1994) suggested that immature feeding stages of mealybugs on citrus tend to settle at the major carbohydrate sinks of the host plant in each phenological period and Haviland *et al.* (2012) showed that feeding location of *Ferrisia gilli* Gullan corresponded with carbohydrate allocation in pistachio trees. Most mealybug species are phloem feeders (McKenzie 1967) and their populations follow the movement of plant nutrients. The developing fruit in citrus is a strong sink of carbohydrates, giving better conditions, in terms of food quality, for the development of mealybugs (e.g. higher fecundity) (Franco 1994). Thus, during the period of fruit set and development in citrus, mealybugs tend to aggregate and concentrate on fruits and *D. aberiae* is not an exception. However, in August the physiology of the citrus tree changes and fruits lose their intensive flow of nutrients (Franco 1994, Agustí 2003). This

supposes a decrease in the food quality of fruits and, therefore, in female's fecundity, these factors influencing the reduction of population levels.

In this study, absolute sampling methods showed that *D. aberiae* completed several generations per year, remaining active even during winter. Regarding the number of generations, two were clearly defined each year due to a concentrated and homogeneous crawler emergence (Fig. 3.1.): the first one took place in spring and the second in summer, those two generations being the only ones capable of causing fruit distortion and size reduction during fruit development (Martínez-Blay *et al.* 2018a). The other peaks of crawlers were heterogeneous and varied between years. These generations did not increase *D. aberiae* density and tended to overlap between them (Fig. 3.2.). These overlapping generations resulted in the mix of developmental stages present at the end of the year. Apparently, at least three more generations may occur depending on the year and the environmental conditions: one between January and February, another one between August and October and one more between October and December. Of these, the generation between August and October is the most remarkable, being frequently observed and better defined than the others (Fig. 3.1.). Afterwards, populations remain at very low levels. Similar studies carried out in the Mediterranean Basin with other mealybug species of agronomic and ornamental importance, such as *P. madeirensis* (Longo *et al.* 1995a), *P. peruvianus* (Beltrà *et al.* 2013a), *P. citri* (Santorini 1977, Martínez-Ferrer *et al.* 2003) or *P. viburni* (Panis 1986), showed a similar pattern with several, usually overlapping, generations throughout the year. The overlap of development stages has relevant implications for mealybug management. Host stage can influence the efficiency of natural enemies, especially

parasitoids, and must be taken into account when designing future biological control strategies (Islam and Copland 1997, Jervis *et al.* 2005, Beltrà *et al.* 2013a). If chemical control is required, to manage population outbreaks, we suggest monitoring just after petal fall, before fruits are damaged (Martínez-Blay *et al.* 2018a), when most of the individuals are in the first instar.

Monitoring *D. aberiae* populations by absolute sampling methods is a laborious and time-consuming process because it is necessary to count live insects present on plant material. In the present work, results based on plant material were compared with those obtained by simpler monitoring methods such as corrugated cardboard band traps and sticky traps. The two most harmful generations of *D. aberiae* were also detected by these relative sampling methods (Fig. 3.3.). Corrugated cardboard band traps were able to detect immature male instars and gravid females because these instars tend to use the bands as a shelter to develop into male adults or to lay their eggs, respectively. Moreover, these relative levels of *D. aberiae* were highly correlated with mealybug levels in the canopy at the first population peak (Fig. 4). Interestingly, this peak is also correlated with fruit damage at harvest (Pérez-Rodríguez *et al.* 2017). Therefore, corrugated cardboard band traps represent a suitable and simple sampling method to detect and quantify *D. aberiae* during this damaging period. This technique has been used in several biological control programs to monitor population densities of mealybugs and also to evaluate the impact of their natural enemies, mainly predators (DeBach 1949, Browning 1959, Furness 1976, Goolsby *et al.* 2002).

Our results indicate that *D. aberiae* virgin females use a sex pheromone to attract males, as a large number of them were captured. Sticky traps, baited with virgin females, provided

evidence of two important flights, confirming the two main generations of *D. aberiae*, one between March and May and another between June and July (Fig. 3.3.), matching subsequently periods of adult females producing egg sacs. The double peak of male captures in 2015 (March-April) has been considered to be part of the same flight and may be a consequence of the unusually low temperatures and heavy rains that occurred at the end of March and beginning of April. Mechanical action of rain drops and lower than expected temperatures, may have killed part of the population (especially young instars) and delayed the development of new males. Like corrugated cardboard band traps, sticky sex pheromone traps provided a quantitative measurement of *D. aberiae* density at its first population peak. However, and contrary to the former, there were significant differences between the sampled years, likely due to the effect of adverse conditions on male flights. Therefore, we would recommend the use of corrugated cardboard band traps to monitor population levels and sticky sex pheromone traps to determine flight periods. In fact, sticky sex pheromone traps are commonly used to monitor flight population peak periods (Suckling 2000, Way and van Emden 2000). Field trapping of males using sticky sex pheromone traps, with virgin females, has been carried out previously with good results for other mealybug species, including *M. hirsutus* (Serrano *et al.* 2001), *P. citri* (Rotundo and Tremblay 1975, Moreno *et al.* 1984), *P. calceolariae* (Rotundo and Tremblay 1975), *P. comstocki* (Moreno *et al.* 1972, Meyerdirk and Newell 1979, Meyerdirk *et al.* 1981) and *Pseudococcus maritimus* (Ehrhorn) (Grimes and Cone 1985). More recently, synthetic pheromones have been developed and tested for many mealybugs species such as *M. hirsutus* (Hall *et al.* 2008), *P. citri* (Martínez-Ferrer *et al.* 2003, Waterworth *et al.* 2011), *P. ficus*

(Millar *et al.* 2002, Walton *et al.* 2004), *P. longispinus* (Waterworth *et al.* 2011), *P. viburni* (Mudavanhu *et al.* 2011) or *P. maritimus* (Bahder *et al.* 2013). Identification of the female sex pheromone would allow for the use of pheromone traps to monitor *D. aberiae* in IPM Schemes.

We have shown that *D. aberiae* completes multiple generations per year, two of them being clearly defined and resulting in high populations. Moreover, *D. aberiae* peaks between May and June and causes damage to developing fruit. Corrugated cardboard band traps and sticky pheromone traps are able to identify peak periods of *D. aberiae* populations; corrugated traps provide a quantitative measurement of *D. aberiae* density and are recommended to monitor population levels while sticky traps can be used to determine male flight periods. Both systems represent simple monitoring techniques to detect mealybug population outbreaks. These results are the first description of *D. aberiae* seasonal trend in citrus and may serve to improve the sampling protocols and develop an IPM program.

3.5. Acknowledgements

We would like to thank the owners of the orchards for allowing us to use their plantations and P. Bru (IVIA) and J. Catalán (IVIA) for their help in sampling. This research was supported by two predoctoral grants (FPU to V. Martínez-Blay and Val I + d to J. Pérez-Rodríguez from the Spanish Ministry of Education, Culture and Sport and Generalitat Valenciana, respectively), the European Grants FP7-IAPP #324475 'Colbics' and FP7-IRSES #612566 'Biomodic,' and a national project provided by INIA (Project No. RTA2014-00067). The authors thank Debra Westall (UPV) for revising the manuscript.

CHAPTER 4.

Seasonal movement and distribution of the invasive pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae) in citrus: implications for its integrated management

Chapter 4. Seasonal movement and distribution of the invasive pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae) in citrus: implications for its integrated management

Martínez-Blay, V., Pérez-Rodríguez, J., Tena, A. & Soto, A. 2018. Submitted to J Econ Entomol. Adapted author's Pre-print version.

Abstract

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) is the latest invasive mealybug introduced in citrus in Spain. Its feeding behavior causes severe direct damage to citrus fruits, distorting their shape and/or causing reductions in size. There is no information available regarding its distribution within the citrus trees. The main objective of this study was to describe the seasonal distribution of *D. aberiae* within citrus trees and migration patterns. Ten citrus orchards from eastern Spain were periodically sampled during three years. In each orchard, the mealybug was sampled in different infested strata (canopy, trunk and soil) and canopy organs (flower, fruit, leaf and twig). Results showed that, within the sampled strata, *D. aberiae* was mostly in the canopy. Within the canopy, the feeding organ of *D. aberiae* changed throughout the year. *D. aberiae* overwintered in the twigs and moved to the flowers and fruits in spring. Once there, its populations started to increase exponentially until August. From February to September between 30 and 5% of the mealybugs migrated to the trunk and soil. These mealybugs were found moving upwards and downwards depending on the phenology of the plant and the climatic conditions. These results will facilitate an early detection of the pest in the areas where it is spreading and improve sampling protocols and pesticide applications.

Keywords: applied entomology, IPM, mealybug, migration

4.1. Introduction

Mealybugs (Hemiptera: Pseudococcidae) are considered one of the major agricultural pests worldwide, causing serious problems when introduced into new areas without their natural enemies (Miller *et al.* 2002, García-Morales *et al.* 2016b). These insects are small and live in hidden habitats, representing one of the families with many exotic species in Europe because they are frequently unnoticed during international plant trade (Roques *et al.* 2009, Pellizzari and Germain 2010). Within this context, *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) is the latest invasive mealybug introduced in citrus in Spain. In 2009, this species was detected causing significant damage in citrus orchards in the region of "Les Valls" (Valencia, eastern Spain), within the Mediterranean Basin citrus production area (Beltrà *et al.* 2012, García-Marí 2012, Beltrà *et al.* 2013c, Beltrà *et al.* 2015). Identification was confirmed by molecular and taxonomic techniques (Beltrà *et al.* 2012, Beltrà *et al.* 2015) and, after an unsuccessful eradication program, *D. aberiae* became established in the region. Since then, the mealybug has continued spreading, slowly but steadily, towards adjoining areas, becoming a significant pest in eastern Spain (Pérez-Rodríguez *et al.* 2017, Tena *et al.* 2017a, Martínez-Blay *et al.* 2018b).

D. aberiae is native to sub-Saharan Africa, being a common species in South Africa (Miller and Giliomee 2011). Recently, it has been confirmed that the invasive populations, present in Spain, are native to Limpopo province, in Northern South Africa (Beltrà *et al.* 2015). Like other mealybug species in Mediterranean conditions, *D. aberiae* completes several generations throughout the year, being two of them very clearly defined and resulting in high population levels between May and July (Pérez-Rodríguez *et al.* 2017, Martínez-Blay *et al.* 2018b). During this period, nymphs and adults settle and feed on

fruitlets. However, unlike other mealybugs, this feeding behavior causes severe direct damage to citrus fruits, distorting its shape (mainly protuberances around fruit calyx) and/or causing size reduction, which depreciates its commercial value (Pérez-Rodríguez *et al.* 2017, Martínez-Blay *et al.* 2018a, Martínez-Blay *et al.* 2018b). Direct damage has been observed in all citrus cultivated in eastern Spain (sweet oranges, mandarins and hybrids) (Pérez-Rodríguez *et al.* 2017, Martínez-Blay *et al.* 2018b). Recently, the duration of the damaging period has been established, including from flowering stage (March-April in eastern Spain conditions) to fruits with a diameter of 25-30 mm (around July in eastern Spain conditions) (Pérez-Rodríguez *et al.* 2017, Martínez-Blay *et al.* 2018b). It has also been shown that distortions appear during this period because *D. aberiae* interferes with the fruit cell division process (Martínez-Blay *et al.* 2018a). Afterwards, at the end of summer, populations decrease and remain at low levels, but active, for the rest of the year (Martínez-Blay *et al.* 2018b).

The complex of natural enemies of *D. aberiae* on its native area was practically unknown, and since its introduction in Spain no effective biological control has been found: the existing parasitoids in Spain fail to control this pest (Tena *et al.* 2017a) and the predators, mainly *Cryptolaemus montrouzieri* Mulsant, appear late when the damage to the fruit has already been done (Pérez-Rodríguez *et al.* 2017). Thus, the management of *D. aberiae* currently depends on the use of broad-spectrum insecticides (Pérez-Rodríguez *et al.* 2017). However, these applications interfere with the existing biological control of other citrus pests in the Mediterranean Basin (Franco *et al.* 2009, Tena and Garcia-Marí 2011), being essential the need to monitor the seasonal trend of the pest and avoid unnecessary spraying. Within this context, recent studies have shown that *D. aberiae* presents a clumped distribution in the organs it attacks and that fruit damage at harvest is strongly correlated with fruit

occupation in spring (Pérez-Rodríguez *et al.* 2017). Based on these results the Economic Injury Level (EIL) and the Economic Environmental Injury Level (EEIL) for *D. aberiae* have been calculated as 7.1 and 12.1% of occupied fruits in spring, respectively, being recommended to sample 275 fruits (binomial sampling) or 140 fruits (enumerative sampling) between petal fall and July (Pérez-Rodríguez *et al.* 2017).

Pest monitoring is a fundamental component of any integrated pest management program (IPM). The ability to predict future pest damage, based on early field counts, is valuable and necessary for good control decisions (Kogan 1998), especially in the case of cryptic species that can easily pass unnoticed. Previous research has shown that mealybugs migrate within the plant throughout the season (Geiger and Daane 2001, Haviland *et al.* 2012, Beltrà *et al.* 2013a, Wunderlich *et al.* 2013, Kumar *et al.* 2014). Thus, it is necessary to change sampling strategies throughout the year to detect and quantify the density levels of the pest in the infested stratum (canopy, trunk and soil) and organ (flower, fruit, leaf and twig) of the plant. This information has improved the control of many mealybug species affecting agricultural and ornamental ecosystems worldwide (Geiger and Daane 2001, Martínez-Ferrer *et al.* 2006, Mudavanhu *et al.* 2011, Haviland *et al.* 2012, Kumar *et al.* 2014). To date, there is no information available regarding *D. aberiae* distribution patterns within the citrus trees. This makes it difficult to detect early infestations of this mealybug, especially in the absence of typical damage symptoms. Thus, the main objective of this work was to describe the seasonal distribution of *D. aberiae* within citrus trees and migration patterns. This information will help to design better sampling protocols, facilitating an early detection of the pest and improving pesticide applications within the existing IPM programs for citrus in Spain.

4.2. Material and Methods

4.2.1 Sampling sites and general sampling protocol

Ten citrus orchards infested with *D. aberiae* and located in eastern Spain (region of Les Valls, Valencia) were sampled from March 2014 to December 2016. Five orchards included sweet orange trees (*Citrus sinensis* (L.) Osbeck: Lane late, Navelina and Sanguinelli varieties) and the other five clementine mandarin trees (*Citrus reticulata* Blanco: Oroval and Clemenules varieties). Within each orchard, eight to ten trees were marked and sampled regularly. These trees were not sprayed with pesticides during the whole sampling period. In 2014 and 2015, samplings were done weekly, during the periods of most rapid mealybug development (March-August), and bimonthly or monthly during the rest of the year. In 2016, to confirm previous results, samplings were done in five orchards at monthly intervals.

The following sections detail the different methodologies used to sample the seasonal distribution of *D. aberiae*, throughout the year, in the infested stratum (canopy, trunk and soil) and organ of the canopy (flower, fruit, leaf and twig). Canopy and trunk samplings were done in the ten studied orchards, whereas soil samplings were carried out in four of them.

4.2.2 Canopy sampling protocol

At each sampling date, four 20-cm long twigs (each one from a cardinal orientation), with its leaves and flowers or fruits, were collected randomly from the canopy of each marked tree per orchard. Samples were bagged individually and transported to the laboratory, being examined under a stereomicroscope (Nikon SMZ645) within the next 24h. Mealybugs present on each twig, on four leaves per twig and on one to eight flowers or fruits (depending on their availability during the year) were

counted. Leaves, flowers and fruits were randomly selected within the twigs. All developmental stages counted were pooled together, as data regarding the phenology of *D. aberiae* in the canopy of the tree has recently been published in a companion manuscript (Martínez-Blay *et al.* 2018b).

4.2.3 Trunk sampling protocol

Trunk samplings consisted of visual counts, during 2 minutes, of all the mealybugs present on the trunk and main branches of the trees (until 60 cm in height). Each mealybug counted was classified in one of the following categories: nymphs (first, second and third instars together), adult females, gravid females and immature males (pre-pupa and pupa).

To determine the direction of migration, the directionality of the movement was also recorded in 2015. That is if the mobile instars (nymphs and adult females) were ascending or descending the trunk. Immobile mealybugs were not considered for this analysis.

4.2.4 Soil sampling protocol

At each sampling date, four orchards and four trees per orchard were sampled from March 2014 to December 2015. From each tree, soil samples were collected at three distances horizontally from the base of the trunk (0-15 cm, 15-30 cm and 30-45 cm) and at each distance one per cardinal direction (North, South, East and West). This is 12 samples per tree and 48 samples per orchard. Each sample was collected from the soil surface and consisted of a circular area with a diameter of 10 cm and 2 cm depth that was bagged and transported to the laboratory. Once there, each sample was placed in a Berlese funnel for 48 hours. Mobile mealybug instars present in the soil moved away from the heat source, down the funnels, and fell into containers with 70% ethanol where they were preserved. Afterwards each container was checked, under a dissecting

microscope (Nikon SMZ645), for the presence of *D. aberiae*. Each mobile mealybug found was counted and classified into one of the following categories: first nymphal instar, second nymphal instar, third nymphal instar, adult females and adult males. Data from the three distances was used to determine the location of *D. aberiae* in the soil. Data from soil samples collected within a distance of 0 to 15 cm, horizontally from the base of the trunk, was used to analyze the seasonal trend of *D. aberiae* in the soil.

4.2.5. Data analysis

Sampling data of the different strata and organ were averaged per tree and afterwards per orchard, being the latter the sampling unit used for the graphics and statistical analysis. Results from the samplings carried out in 2014 and 2015 are presented in all the figures. Data from the year 2016 are presented for the figures 4.1. and 4.2. (general strata distribution and distribution on tree canopy).

The percentage of mealybugs is represented per unit area (cm^2) to be able to compare the abundance of *D. aberiae* in each sampled organ (flower, fruit, leaf and twig) or stratum (canopy, trunk and soil) (Fig. 4.1 and Fig. 4.2). The surface of the trunk and three main branches was calculated as the side area of cylinders, $2\pi RH$, being R an average radius of the trunk and branches and H the sampled height of the trunk and branches (60 cm in total). As mealybugs were found only a few centimeters in depth, the surface of the soil was estimated as the area of a circle, πR^2, considering R as the radius of each soil sample (5 cm), multiplied by two to consider both sides of the sample. For the twig, the surface was calculated as the side area of a cylinder, $2\pi RH$, being R an average radius of four twigs per sample and H the length of the sampled twigs (20 cm). Leaf surface was estimated as the area of an ellipse, πAB, multiplied by two to consider both sides of the leaves, being A an average

of half of the leaf length and *B* an average of half of the leaf width. Fruit surface was estimated as the area of a sphere, $4\pi R^2$, being *R* the average radius of the fruit. In flowers, as mealybugs were only found in the ovary, the surface taken was the area of a sphere, $4\pi R^2$, being *R* an average radius of the ovary. Afterwards, the surface of each organ or stratum was multiplied by the total number of sampled organs or strata. Finally, the number of mealybugs per unit area (cm^2) (Fig. 4.1 and Fig. 4.2) was calculated as the total number of mealybugs divided by the total surface in which those insects were counted.

The mean percentage of mealybugs per unit area (cm^2) and orchard in the soil between March and July [period in which *D. aberiae* causes damage to fruits and chemical treatments must be applied (Pérez-Rodríguez *et al.* 2017, Martínez-Blay *et al.* 2018a)] was compared using analysis of variance (ANOVA). Month was the explanatory variable and two ANOVAs were carried out, separately for 2014 and 2015, being means compared using Tukey tests (Fig. 4.1.). Data were tested for homogeneity of variances using Levene's test. If required, percentage data were subjected to an angular transformation before analysis to satisfy model assumptions regarding homogeneity of variances and to approximate a normal distribution (Kasuya 2004).

The directionality of the movement of the mobile instars present on the trunk was analyzed separately for nymphs and adult females (Fig. 4.4). The number of nymphs and adult females were first averaged per tree and afterwards per orchard, using the mean per orchard for the statistical analysis. Within each month of the year 2015, t-tests were used to determine whether the mean number of nymphs and adult females ascending or descending the trunk differed significantly from each other. Data were tested for homogeneity of variances using Levene's test. If required, data were log transformed, before the

analysis, to satisfy homogeneity of variances and to approximate a normal distribution.

Data collected during 2016 were excluded from all the analysis because samplings were performed much less frequently than in 2014 and 2015. All statistical analyses were conducted with the software Statgraphics Centurion XVI.II (Statpoint Technologies Inc, Warrenton, USA).

4.3. Results

4.3.1 Strata distribution

Population density of *D. aberiae*, per unit area (cm^2), started to increase in March and the maximum was reached in May for the three years of study (Fig. 4.1.). Afterwards, population density began to drop, and from September to February mealybugs remained at low levels. Within the sampled strata, *D. aberiae* was present mostly in the tree canopy (Fig. 4.1.). For each month, more than 70% of the total number of mealybugs found per unit area (cm^2) was located on the canopy during the three years of study. However, from February to September, some of the mealybugs were also detected in the soil and trunk (Fig. 4.1.). During this period, the percentage of *D. aberiae* in soil was always lower than 30% and remained below 5% in the case of the trunk.

From March to July, period in which *D. aberiae* causes damage to fruits, the percentage of *D. aberiae* in the soil differed significantly between months for 2014 and 2015 (ANOVA 2014: $F = 7.05$, df = 4, 15, $P = 0.002$; ANOVA 2015: $F = 2.08$, df = 4, 15, $P < 0.001$). Means compared using Tukey tests showed that the percentages of *D. aberiae* in the soil were significantly higher in March and April followed by June and July in 2014 and 2015. The lowest percentage was registered in May also in both years (Fig. 4.1.).

4.3.2 Distribution on tree canopy

Within the canopy, the feeding organ of *D. aberiae* changed seasonally, as it is shown by the differences in the percentage of mealybugs found per organ throughout the year (Fig. 4.2.). From November to March (winter), the highest percentage of mealybugs was always on twigs, with more than 60% of the mealybugs distributed on this organ, mainly protected in the insertion of the twigs with the leaves. In March and April, during the flowering period, a small percentage of mealybugs were present in flowers. Fruit set (April-May) and fruit development marked a change in *D. aberiae* feeding location preference (Fig. 4.2.). Thus, from May to August, more than 70% of the mealybugs settled and fed on the fruit, mainly underneath the calyces, coinciding with the period of highest mealybug density in the orchards.

4.3.3 Migration through the trunk

Mobile and immobile instars on trunk

Both, mobile (nymphs and adult females) and immobile instars (immature males and ovipositing females) were present on the trunk from March to August, coinciding with the period of high mealybug density in the canopy of the tree. Mobile and immobile instars peaked together and twice during this period (Fig. 4.3.). The first peak occurred between March and April, and the second from mid-May to July.

Directionality of mobile instars

Adult females migrated mostly from the tree canopy to the soil in two periods, March-April and June-July, as the number of adult females descending was significantly higher than ascending (March: t = -3.95, df = 18, P = 0.001; April: t = -2.54, df = 18, P = 0.02; June: t = -3.26, df = 18, P = 0.004; July: t = -2.86, df = 18, P = 0.01) (Fig 4.4a.). The rest of the year the number of adult females ascending and descending was similar

(January: t = -0.90, df = 18, P = 0.38; February: t = -0.41, df = 18, P = 0.07; May: t = -0.60, df = 18, P = 0.56; August: t = -0.90, df = 18, P = 0.38; September: t = -0.45, df = 18, P = 0.66; in October, November and December the number of adult females was null).

Nymphs migrated mostly from the tree canopy to the soil in two periods, March and May-June, as the number of nymphs descending was significantly higher than ascending (March: t = -2.40, df = 18, P = 0.02; May: t = -2.47, df = 18, P = 0.02; June: t = -2.52, df = 18, P = 0.02) (Fig 4.4b.). The rest of the year the number of nymphs ascending and descending was similar (February: t = -0.74, df = 18, P = 0.47; July: t = -0.34, df = 18, P = 0.74; August: t = -0.26, df = 18, P = 0.80; September: t = -1.00, df = 18, P = 0.33; November: t = 0.00, df = 18, P = 1.00; in January, October and December the number of nymphs was null), except in April when the number of nymphs ascending was significantly higher than descending (t = 2.81, df = 18, P = 0.01).

4.3.4 Distribution and seasonal trend on soil

97% of the total number (4567 mealybugs) of *D. aberiae* collected from soil samples were captured within a distance of 0 to 15 cm horizontally from the base of the trunk, 3% from 16 to 30 cm and 0% in samples separated more than 30 cm from the base of the trunk.

Within a distance of 0 to 15 cm horizontally from the base of the trunk, *D. aberiae* was present from March until August in the samples obtained with Berlese funnels (Fig 4.5.). Second instar nymphs and adult females peaked in March and June; first instar nymphs in April-May (with a maximum in April) and July and adult males in April and June.

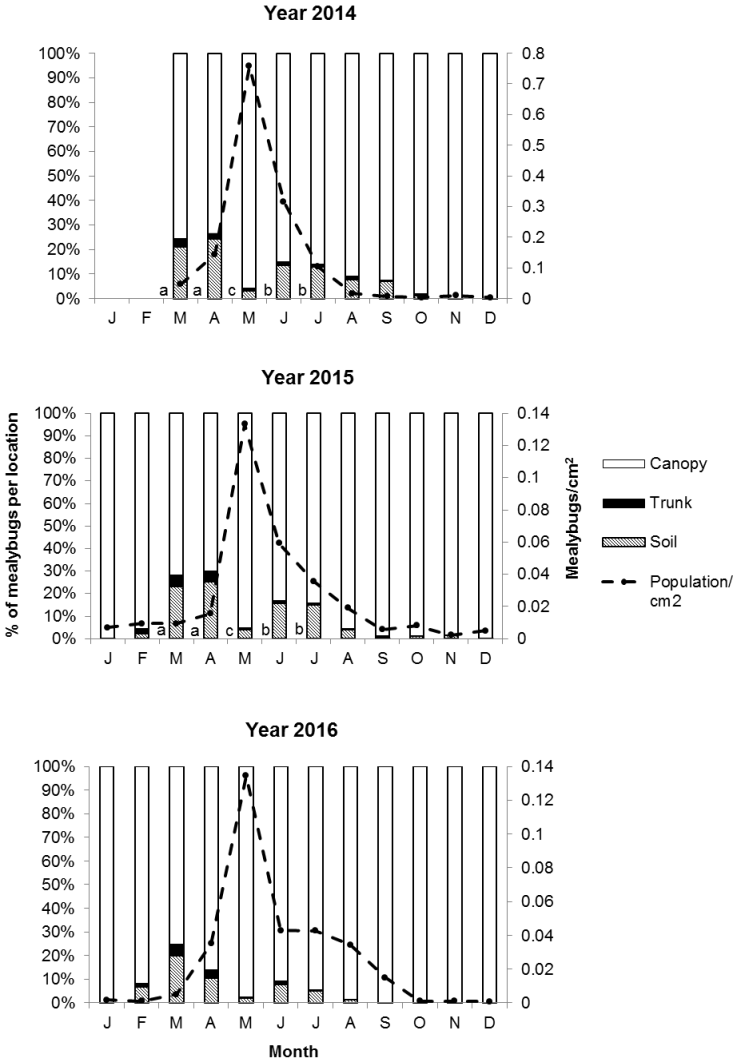

Fig. 4.1. Strata distribution of *D. aberiae* in ten citrus orchards in eastern Spain. Percentage of mealybugs per strata (canopy, trunk and soil) (primary Y axis) compared to the number of mealybugs per unit area (cm^2) (secondary Y axis) is represented per month for the years 2014, 2015 and 2016. Different letters, on the left of soil percentages, indicate that those proportions differed significantly between them (ANOVA 2014: $F = 7.05$, $df = 4, 15$, $P = 0.002$; ANOVA 2015: $F = 2.08$, $df = 4, 15$, $P = 0.01$), means compared by Tukey tests ($P<0.05$).

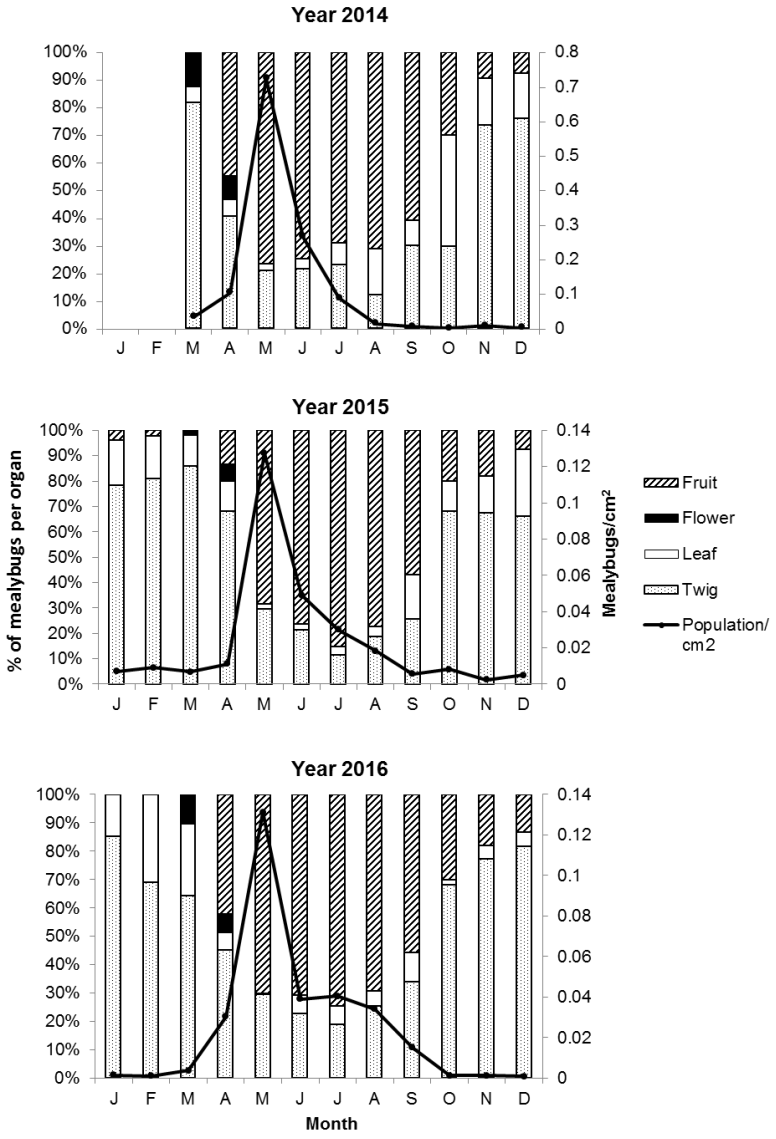

Fig. 4.2. Distribution of *D. aberiae* within the tree canopy in ten citrus orchards in eastern Spain. Percentage of mealybugs per organ (flower, fruit, leaf or twig) (primary Y axis) compared to the number of mealybugs per unit area (cm^2) in the canopy (secondary Y axis) is represented per month for the years 2014, 2015 and 2016.

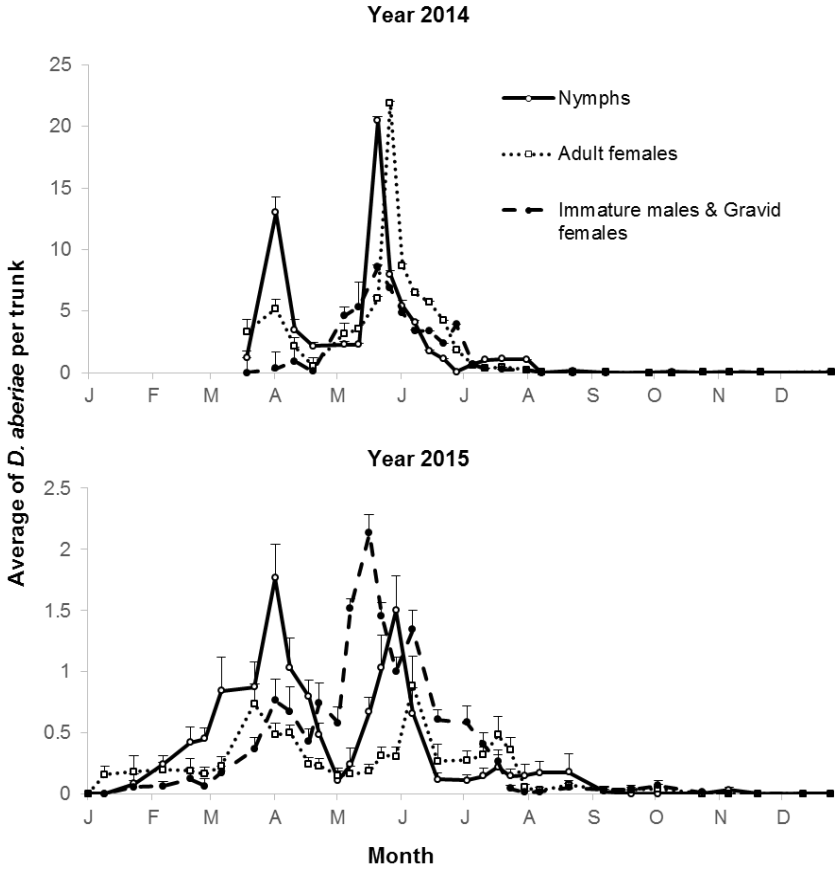

Fig. 4.3. Seasonal trend of mobile (nymphs and adult females) and immobile instars (immature males and ovipositing females) of *D. aberiae* on trunk in ten citrus orchards in eastern Spain in 2014 and 2015. Mean number of mealybugs counted visually in the orchards, during 2 minutes, is represented. Vertical bars represent the positive standard error (+SE). Note that y-axis scales are different for each year.

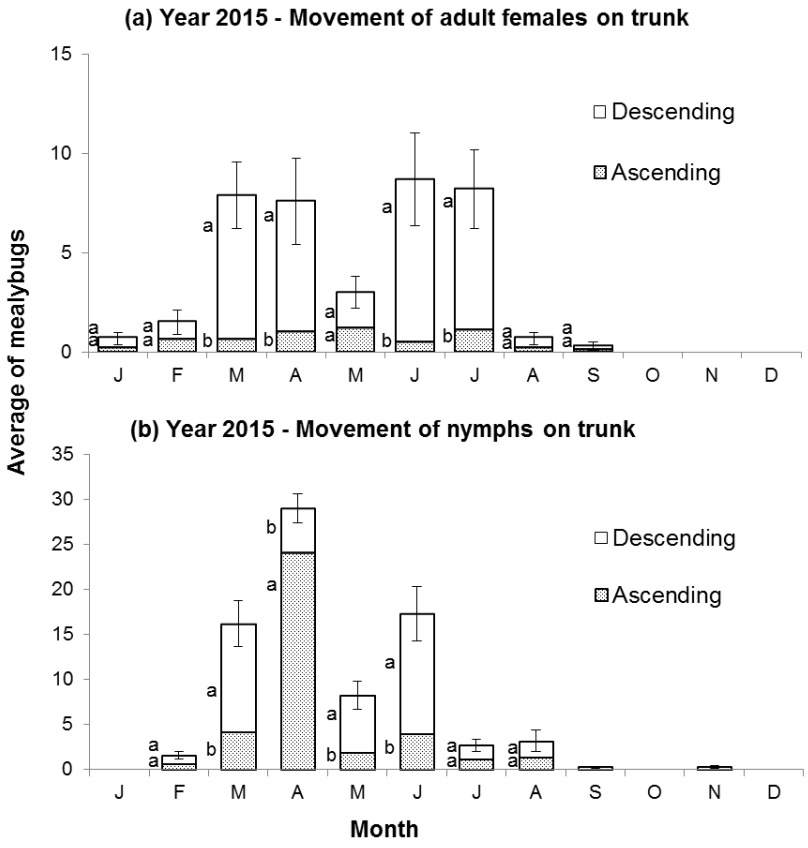

Fig. 4.4. Directionality of the mobile instars in the trunks during the 2-minutes visual samplings. The monthly mean number of mealybugs (± SE), ascending or descending, is represented for the year 2015, separating between (a) adult females and (b) nymphs. Within each month, different letters, on the left of each bar, indicate that the mean number of mealybugs ascending or descending differed significantly between them (t-tests).

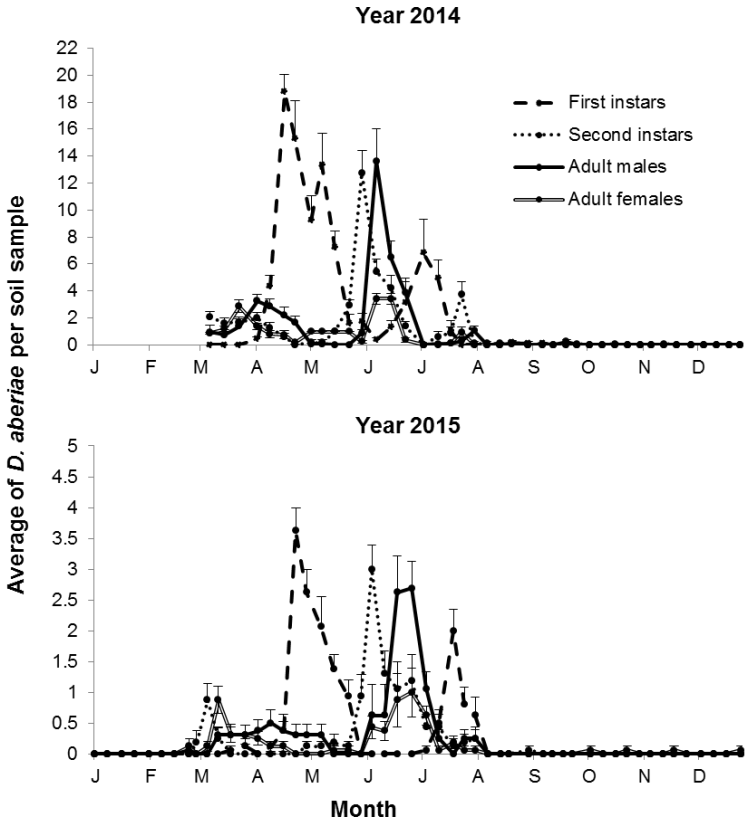

Fig. 4.5. Seasonal trend of *D. aberiae* in soil in ten citrus orchards in eastern Spain in 2014 and 2015. Mean number of mobile instars (nymphs, adult females and adult males) captured by Berlese funnels is represented. Vertical bars represent the positive standard error (+SE). Note that y-axis scales are different for 2014 and 2015.

4.4. Discussion

4.4.1 Strata distribution

Within the sampled strata (canopy, trunk, soil), *D. aberiae* was mostly found in the tree canopy. However, from February to September some mealybugs are also present and active in the trunk and soil. This result should be taken into account for the

management of the pest. Insecticide applications are currently recommended only if 12% or more of the fruit is infested by *D. aberiae* after petal fall (Pérez-Rodríguez *et al.* 2017). Since some mealybugs were present on the trunk and soil during that period, insecticides recommended against mealybugs in citrus in Spain should soak the trunk and the soil up to 20 cm from its base.

4.4.2 Distribution on tree canopy

Distribution patterns in scale insects are the result of its intrinsic and physiological behavior, morphological characteristics of the host-plant tissue and the activity of predators and parasitoids (Nestel *et al.* 1995). As most mealybug species are phloem feeders, they vary their feeding and settling locations throughout the year, searching the movement of nutrients in their hosts to find the best nutritional conditions for them (McKenzie 1967, Boavida *et al.* 1992, Geiger and Daane 2001, Cid *et al.* 2010, Haviland *et al.* 2012, Wunderlich *et al.* 2013, Kumar *et al.* 2014). This behavior results in the migration of mealybugs to different strata of their hosts, adapting to plant phenology (Browning 1959, Furness 1976, Franco 1994, Geiger and Daane 2001, Grasswitz and James 2008, Cid *et al.* 2010, Haviland *et al.* 2012, Wunderlich *et al.* 2013). Besides looking for food, mealybugs might also migrate to find protection against extreme weather conditions and natural enemies (Gutierrez *et al.* 2008, Daane *et al.* 2012, Mani and Shivaraju 2016).

The present study showed that the preferred feeding organ of *D. aberiae* changed seasonally. From November to February (winter), *D. aberiae* remained mainly in the twigs, where mealybugs were usually found in the insertion of the leaves. According to a companion manuscript, these individuals are mostly nymphs of second and third instar during winter (Martínez-Blay *et al.* 2018b). Afterwards, during the flush and

blossom period (from March to April in eastern Spain), *D. aberiae* moved from the twigs to the new shoots and flowers in blooming , where they reached the adult stage (Martínez-Blay *et al.* 2018b). In March and April, during flowering period, a small percentage of *D. aberiae* was present in flowers. This percentage was much lower than the percentage of mealybugs that infested fruit later on. Fruit set (April-May in eastern Spain conditions) and fruit development marked a significant change in *D. aberiae* feeding location preference. During this period, crawlers from the first generation of the pest emerged (Martínez-Blay *et al.* 2018b) and tended to migrate and settle in the new citrus fruitlets in development, mainly in the calyx area. Thus, from May to August most of the mealybugs developed on fruits, this coinciding with the period of highest *D. aberiae* density in the orchards. The developing citrus fruit is the preferred feeding location of mealybugs affecting this crop because it provides very good nutritional conditions for their development (Franco 1994). These results show that *D. aberie* tended to search for and settle at the major carbohydrate sinks of the citrus tree. During the three years of this study, the movement of mealybugs from overwintering sites to the shoots and flowers coincided with spring flush and blossom period, when carbohydrates are shift acropetally from the roots to the buds; afterwards, *D. aberiae* aggregated on the developing fruit, a strong sink of carbohydrates (Agustí 2003, Iglesias *et al.* 2007). The behavior of nymph's migration following the plant nutrients has also been reported for other mealybug species, such as *Ferrisia gilli* Gullan in pistachio trees (Haviland *et al.* 2012), *P. citri* in citrus (Franco 1994, Martínez-Ferrer *et al.* 2003) or *Pseudococcus maritimus* (Ehrhorn) in vineyards (Geiger and Daane 2001). *D. aberiae* was not an exception and herein we have described these movements within citrus trees.

4.4.3 Migration to the trunk and soil

Mealybugs are a group of insects that usually migrate to complete their life cycle in protected locations against bad weather conditions and natural enemies (Gutierrez *et al.* 2008, Daane *et al.* 2012, Mani and Shivaraju 2016). Herein, mobile and immobile instars of *D. aberiae* were present and active on the trunk and soil from February to September and during this period the mealybug peaked twice on both strata, simultaneously with the two main peaks in the canopy. Some studies mention that mealybugs might migrate and overwinter in the soil (Bodenheimer 1951, Rotundo *et al.* 1979, Franco *et al.* 2000). Our results, however, show that *D. aberiae* did not stay protected in this stratum in the coldest months.

Besides, adult females migrated mostly from the tree canopy to the trunk and soil in two periods, March-April and June-July. The peaks of first instars observed in this stratum after the presence of adult females indicate that crawlers emerged from eggs laid by ovipositing females. Results are in agreement with the migration of females to the trunk and soil for ovipositing in protected places previously reported for other citrus mealybug species, such as *P. citri* (Franco 1994, Franco *et al.* 2000, Martínez-Ferrer *et al.* 2003, García-Marí 2012).

On the other hand, nymphs migrated mostly from the tree canopy to the trunk and soil in two periods, March and May-June. The migration of nymphs downwards the trunk is not commonly reported in mealybugs but some previous studies have mentioned this descending movement in other mealybug species, reporting that it could be an adaptive strategy to facilitate the mating process between males and females (Browning 1959, Franco 1994, Franco *et al.* 2000). It seems to be also the strategy followed by *D. aberiae* as adult males were captured after the migration to the soil of second instars at the end of February-beginning of March. On the other hand, nymph

migration from the soil to the canopy was only observed in April. This month coincides with fruit set period. Thus, this ascending movement is probably a migration to the new fruitlets of the crawlers emerged in soil in the same way that has been aforementioned within the tree canopy. It is also remarkable that nymphs from the second generation did not ascend back to the canopy in summer. The high temperatures and low humidity of Mediterranean countries may cause high mortality in young mealybugs (Browning 1959, Bartlett and Clancy 1972, Furness 1976, Beltrà *et al.* 2013a). Therefore, soil could be a drain of *D. aberiae* in summer.

Finally, the fact that most mealybugs were found horizontally close to the base of the trunk, suggests that *D. aberiae* moves to the soil intentionally depending on the phenology of the plant and the climatic conditions (Browning 1959, Bartlett and Clancy 1972, Furness 1976, Franco *et al.* 2000, Martínez-Ferrer *et al.* 2003, Beltrà *et al.* 2013a); and not because insects fall by chance from the tree canopy.

4.4.4 General conclusion

Our results show that *D. aberiae* change its distribution patterns due to physiological and behavioral requirements. Chemical control programs against *D. aberiae* are likely to continue until more sustainable approaches, particularly biological control, can be implemented against this mealybug. Until then, these results will improve insecticide applications, which should take into consideration the migration and presence of *D. aberiae* in the soil in spring but not in summer, when crawlers likely die because of warmer and drier conditions. Moreover, our results will facilitate an early detection of the pest in those areas where *D. aberiae* is spreading in Spain, as technicians will be able to search in the correct plant strata and organ depending on the season.

4.5. Aknowledgements

We would like to thank the owners of the orchards for allowing us to use their plantations, especially Plácido Calabuig. The authors thank Debra Westall (UPV) for English corrections. This research was supported by two predoctoral grants (FPU to V. Martínez-Blay and Val I+d to J. Pérez-Rodríguez from the Spanish Ministry of Education, Culture and Sport and Generalitat Valenciana, respectively), a national project provided by INIA (Project no. RTA2014-00067) and the European grants FP7-IAPP #324475 'Colbics' and FP7-IRSES #612566 'Biomodic'.

CHAPTER 5.

Characterization and damage period to fruits caused by the invasive pest *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae)

Chapter 5. Characterization and damage period to fruits caused by the invasive pest *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae)

Martínez-Blay, V., Benito, M. & Soto, A. 2017. IOBC/WPRS Bulletin. Accepted, currently in press. Adapted author's Post-print version.

Abstract

Delottococcus aberiae De Lotto (Hemiptera: Pseudococcidae) is a mealybug of Southern African origin. In 2009, this species was detected in eastern Spain causing severe fruit distortions in citrus orchards. As a recent invasive mealybug, there is not enough information about its behavior on this crop. This research aims to study the period in which citrus fruits may be damaged by *D. aberiae*, as well as the characterization of these damages. To achieve these goals a trial was carried out on two different citrus trees varieties: 'Clemenules' (Citrus reticulata Blanco) and the hybrid 'Ortanique' (Citrus reticulata x Citrus sinensis). In this experiment, handmade mesh cloth sleeves were used to individually isolate flowers or fruits in different developmental stages. Afterwards, each flower or fruit was infested with four *D. aberiae* females and they were removed after seven days. In order to avoid any contact with *D. aberiae*, outside the chosen infestation period, the sleeves were maintained until fruit harvest. Afterwards, before harvesting, any kind of fruit damage was categorized taking into account its shape and size. Twenty replicates were done per each category tested. Results showed that the highest percentage of damages is recorded when *D. aberiae* attacks the initial stages of fruit development; however mature fruits obtained from infested flowers also appeared with distortions. No damage was observed when *D. aberiae* was in contact with fruits exceeding 3 cm in diameter. Percentages of damaged fruits, categorized by its shape and size, are also presented. These results will be very useful to set appropriate spraying treatments within the existing integrated pest management programs (IPM) for citrus in Spain.

Keywords: citrus, fruit distortions, mealybug, pest management.

5.1. Introduction

Mealybugs (Hemiptera: Pseudococcidae) represent a group of insects that cause significant losses in the plants they infest, especially when invading new regions as a result of accidental introductions (Miller *et al.* 2002, Hardy *et al.* 2008, Pellizzari and Germain 2010). In the Mediterranean Basin, they cause serious direct and indirect damages to many agricultural crops and to a wide range of ornamental plant families (Franco *et al.* 2009, Beltrà and Soto 2011, Mazzeo *et al.* 2014, Mansour *et al.* 2017a). Their feeding reduces plant vigor and the honeydew they secrete promotes the growth of black sooty mold fungi, which interferes with plant photosynthesis, gives shelter to other secondary pests, such as pyralid moths, and affects fruit quality. High population densities may also cause leaf fall, fruit loss or even the death of the plant (Franco *et al.* 2009, Gullan and Martin 2009). Some mealybugs can also transmit virus to commercial crops, causing serious damage (Watson and Kubiriba 2005, Cid *et al.* 2007, Tsai *et al.* 2010); others are able to inject toxins that distort plant tissues, such as *H. pungens* (Carrera-Martínez *et al.* 2015), *M. hirsutus* (Meyerdirk *et al.* 2001, Vitullo *et al.* 2009, Chong *et al.* 2015) or *N. viridis* (Thomas and Leppla 2008, Abdul-Rassoul 2014).

Delottococcus aberiae De Lotto (Hemiptera: Pseudococcidae) was first detected in citrus orchards of eastern Spain in 2009 (Beltrà *et al.* 2013c). Recently, it has been confirmed that Spanish invasive populations are native from Northern South Africa (Limpopo province) (Beltrà *et al.* 2015). There, however, this mealybug is not considered a significant pest and may remain unnoticeable for years (Hattingh *et al.* 1998, Miller and Giliomee 2011), being *D. aberiae*, up to now, a significant problem only in Spain. Unlike other citrus mealybug species present in Spain, when *D. aberiae* develops on citrus fruits causes severe direct damages to them, distorting its shape and size. These damages depreciate most of the affected fruits and

render them unmarketable, leading to significant crop losses (Beltrà *et al.* 2013c, Soto *et al.* 2016b). Damage have been observed in all citrus varieties cultivated in eastern Spain (sweet oranges, mandarins, hybrids and lemons), without observing a clear predilection for any particular group (Tena *et al.* 2014).

Currently, due to the absence of effective natural enemies in Spain and the necessity of growers to control this new pest, the management of the mealybug depends on the use of chemical treatments, mainly the broad-spectrum insecticide chlorpyrifos (Tena *et al.* 2014, Tena *et al.* 2017a). However, these applications are problematic and disrupt the existing biological control of other citrus pests (Franco *et al.* 2009). Besides, distortions in citrus fruits may be observed from flowering period until fruit harvest; however the exact moment in which these damages were caused and the duration of the damaging period are still unknown, being these factors very important to avoid unnecessary chemical spraying. Thus, this research aims to study the period in which citrus fruits are sensitive to *D. aberiae* attacks, as well as the characterization of these damages. Results will be very useful to set the appropriate moment for spraying treatments, trying to make it compatible with the existing integrated pest management programs (IPM) for other citrus pests in Spain.

5.2. Material and methods

5.2.1 Experiment sites and mealybug rearing

An experiment was carried out on two different citrus trees varieties: 'Clemenules' (*C. reticulata*) and the hybrid 'Ortanique' (*Citrus sinensis L.Osb. x C. reticulata Bl.*). Forty 'Clemenules' trees and twenty 'Ortanique' trees were used for the experiment. 'Clemenules' trees had between five and ten years old and were under greenhouse conditions, whereas the 'Ortanique' ones were part of a

commercial orchard and ranged between fifteen and twenty years old. All the trees were drop irrigated and free from *D. aberiae* and other mealybug species. No insecticide sprays were applied to any of them during the whole experiment.

D. aberiae specimens required for the experiment were obtained from a laboratory colony established, on organic pumpkins (*Cucurbita maxima* 'Castellana'), since the year 2013. This colony was mantained in darkness in a climatic chamber (25 ± 1ºC, 65 ± 5% RH) at Universitat Politècnica de València (UPV).

5.2.2 Experiment design and sampling protocol

The experiment consisted of the artificial infestation of citrus flowers and fruits, of different sizes, with *D. aberiae* females obtained from the laboratory colony established at UPV. Ten organ categories were established for the experiment, flower and the following nine fruit classes (equatorial diameter measured with a caliper graduated in millimeters): 0-5 mm, 6-10 mm, 11-15 mm, 16-20 mm, 21-25 mm, 26-30 mm, 31-35 mm, 35-40 mm and 40-45 mm. The final number of repetitions done, per each one of the aforementioned categories was twenty, being each repetition one flower or one fruit with the shoot were it was included. To reduce the effect of the abscission period, single flowers or fruits in leafy inflorescences (in terminal position or distributed along the shoot) were intentionally selected because they are commonly associated with higher fruit set (Agustí 2003, Iglesias *et al.* 2007). Afterwards, each selected flower or fruit was infested with four *D. aberiae* females, with the aid of a small brush, being the insects removed after seven days to ensure that infested organs continued in the same diametral class initially established. To avoid any contact with *D. aberiae*, or other pests, outside the chosen infestation period, each experimental unit was isolated with a specially designed structure for this purpose. Each structure consisted of a fine handmade mesh cloth bag, similar to a sleeve, of 60 cm long and 20

cm wide, with a 30 cm long zip closure and one opened end. Each bag was rolled around the branch, containing the selected shoot, and sealed by the opened end using wire and adhesive tape. Bags were maintained until fruit harvest to avoid any undesirable colonization. The zip allowed to make periodical observations, being each bag opened weekly to check the evolution of the growing fruit and the absence of external contamination. If the four females were not recovered after the seven days, inside the isolated area, these repetitions were removed from the experiment.

At the end of the growing season and before harvesting (September for 'Clemenules' and January for 'Ortanique'), any kind of fruit distortion was categorized taking into account its shape and size. The following damage categories were established: 0 = fruit without any deformation, 1 = one slight protuberance around fruit calyx and normal size, 2 = several protuberances around fruit calyx or fruit completely distorted with normal size, 3 = dwarf fruit (25 mm or less of diameter with any kind of distortion). Vegetative status and phenological evolution of the studied trees, was also recorded during the assay, using the aid of a plastic hoop (0.56 m in diameter and 0.25 m^2 of surface) (Soler and García-Marí 2016). Weekly, this plastic hoop was placed in the canopy of each tree (40 'Clemenules' and 20 'Ortanique') to count the number of flowers and/or fruits (also indicating its mean diameter) present inside the area, being later these data multiplied by a factor equivalent to the total surface of the tree canopy (variable depending on the size of the tree).

5.2.3 Data analysis

Percentages of distorted fruits are presented graphically. To check the effect of the organ category initially infested, on the total percentage of final matured fruits with distortions, these proportions were compared by pairs, and separately for each one of the two varieties tested, using a chi-square test (χ^2). Statistical analyses were

performed using IBM SPSS version 22.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

5.3. Results and discussion

5.3.1 Damage period and characterization of damages

Figure 5.1. shows that the highest percentage of damages in the variety 'Ortanique' is recorded when *D. aberiae* attacks the initial stages of fruit development. However, a high percentage of mature fruits developed from infested flowers also appears with distortions. 75%, 90%, 80% and 85% of the final matured 'Ortanique' fruits, grown from *D. aberiae* infested flowers or fruits of 1-5 mm, 6-10 mm and 11-15 mm of diameter respectively, showed distortions. None of the aforementioned percentages differed significantly between them: flower and fruit of 1-5 mm ($\chi^2 = 1.558$, df = 1, P = 0.212), flower and fruit of 6-10 mm ($\chi^2 = 0.143$, df = 1, P = 0.705), flower and fruit of 11-15 mm ($\chi^2 = 0.476$, df = 1, P = 0.490), fruit of 1-5 mm and fruit of 6-10 mm ($\chi^2 = 0.784$, df = 1, P = 0.376), fruit of 1-5 mm and fruit of 11-15 mm ($\chi^2 = 3.584$, df = 1, P = 0.062), fruit of 6-10 mm and fruit of 11-15 mm ($\chi^2 = 1.129$, df = 1, P = 0.288). However, when the mealybug changed from feeding on fruits of 11-15 mm to 16-20 mm in diameter the percentage of fruits with distortions decreased significantly ($\chi^2 = 12.907$, df = 1, P < 0.001), not being observed any kind of damage when *D. aberiae* attacked fruits exceeding 25 mm in diameter in the variety 'Ortanique'. This work shows that distorted fruits are only obtained when *D. aberiae* feeds on the ovary of the flower or on very small tender fruits. Growth and development of citrus fruits follows a sigmoid growth curve, divided into three stages. Phase I is characterized by cell division and slow growth (from anthesis until the end of the phisiological June fruit drop). In the phase II, the fruit experiences a huge increase in size due to cell enlargement and phase III corresponds with the maturation period (Agustí 2003,

Iglesias *et al.* 2007). Results confirm that when *D. aberiae* pierces the fruit with its stylet, during phase I, is able to interfere with cell division process, distorting the affected area while the rest of the fruit continues growing normally. Afterwards, when *D. aberiae* feeds on fruits exceeding 15-20 mm, the percentage of distortions decreases considerably, coinciding this fruit size with the end of phase I and a high decline in cell division. Thus, when cell division stage finishes, the fruit stops being susceptible to *D. aberiae* direct damages, this being very important for the management of the pest.

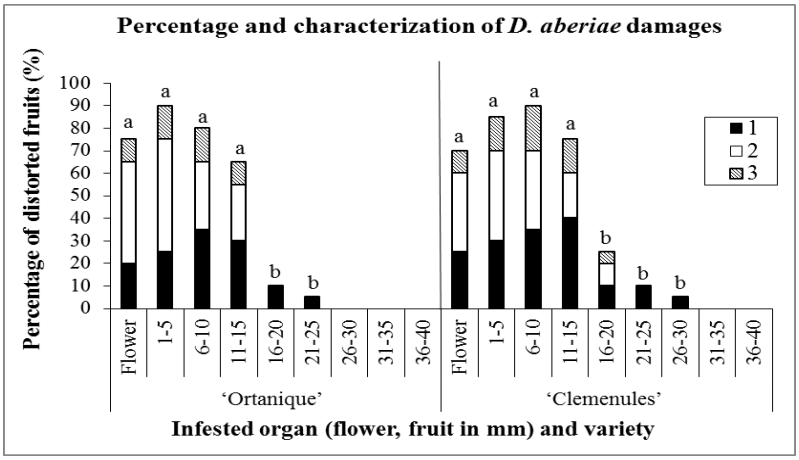

Figure 5.1. Final percentage of distorted mature fruits, by *D. aberiae*, obtained for each of the organ categories and varieties tested. Each percentage is divided in the established categories of fruit damage: 1 = one slight protuberance around fruit calyx and normal size, 2 = several protuberances around fruit calyx or fruit completely distorted with normal size, 3 = dwarf fruit (25 mm or less of diameter with any kind of distortion). Different letters above the columns denote statistically significant differences between the total percentage of distorted fruits for each of the organ categories initially infested at $P<0.05$ ($\chi 2$ test) and separately for each one of the two varieties tested.

The same conclusions have been obtained in the variety 'Clemenules'. 70%, 85%, 90% and 75% of the final matured fruits, grown from *D. aberiae* infested flowers or fruits of 1-5 mm, 6-10 mm and 11-15 mm of diameter respectively, show damages and none of these percentages differ significantly: flower and fruit of 1-5 mm (χ^2 = 1.290, df = 1, P = 0.256), flower and fruit of 6-10 mm (χ^2 = 2.500, df = 1, P = 0.114), flower and fruit of 11-15 mm (χ^2 = 0.125, df = 1, P = 0.723), fruit of 1-5 mm and fruit of 6-10 mm (χ^2 = 0.229, df = 1, P = 0.633), fruit of 1-5 mm and fruit of 11-15 mm (χ^2 = 0.625, df = 1, P = 0.429), fruit of 6-10 mm and fruit of 11-15 mm (χ^2 = 1.558, df = 1, P = 0.212). When the mealybug changes from feeding on fruits of 11-15 mm to 16-20 mm in diameter the percentage of fruits with distortions decreases significantly (χ^2 = 10.000, df = 1, P = 0.002), not being observed any kind of damage when *D. aberiae* attacks fruits 30 mm in the variety 'Clemenules'.

Figure 5.1. also shows that the percentage of distorted fruits, for each of the organs initially infested, does not differ between citrus varieties. Previous works said that fruit damage may be observed in all citrus cultivated in eastern Spain, without observing a predilection for any particular group (Tena *et al.* 2014). These results coincide with that observations; however, further research, considering more citrus varieties, is needed to generalize this fact.

Regarding the characterization of damages, the three categories of fruit distortions appear in similar percentages for both varieties tested and do not seem to follow a clear tendency in relation with fruit size. Fruits in the category 1 frequently comply with international export standards to be supplied fresh to the consumer and ranged from 5% to 40% in the variety 'Clemenules' and from 5% to 35% in 'Ortanique'. Fruits in the category 2 lose a significant part of their commercial value, being normally destined to industrial processing (OECD 2010) and varied from 10% to 40% in 'Clemenules' and from 25% to 50%

in ‘Ortanique’. The percentage of distorted fruits in the category 3 ranged from 5% to 20% in the variety ‘Clemenules’ and from 10% to 15% in the variety ‘Ortanique’. These fruits lose completely their commercial value, being excluded from fresh consumption and, due to their size, also from industrial processing (OECD 2010), thus they frequently are not even harvest from the trees. These different types of damage caused by *D. aberiae* that appear without a clear tendency, within the susceptible fruit sizes, could be due to the feeding mechanism used and the number of cells attacked by the mealybug. Further reseach, regarding the phisiology of the fruit, is needed to clarify this aspect.

Other citrus pests may also attack and distort developing tissues causing direct damages, for example the citrus bud mite *Aceria sheldoni* (Ewing) (Acari: Eriophidae) (Boyce and Korsmeier 1941, Phillips and Walker 1997, Vacante and Bonsignore 2016), the mealybug *N. viridis* (Nechols 2003, Thomas and Leppla 2008), the bayberry whitefly *Parabemisia myricae* (Kuwana) (Walker 1985) or the kelly’s citrus thrips *Pezothrips kellyanus* (Bagnall) (Webster *et al.* 2006). Frequently, these distortions are related with toxic compounds present in the insect’s saliva, being this the case of the mealybug *N. viridis* that has been cited feeding on immature citrus fruits and causing extensive protuberances around the stem end (Hattingh *et al.* 1998, Thomas and Leppla 2008). Different works mention that during its piercing and sucking feeding procces, nymphs and adult females of *N. viridis* inject toxic saliva into its host tissues, being able to injure buds, flowers, fruits, leaves, twigs, shoots and stems (Nechols 2003, Thomas and Leppla 2008, Abdul-Rassoul 2014). Contrarily to *N. viridis*, *D. aberiae* causes distortions in fruits but not to the rest of plant organs it feeds on. On the other hand, *D. aberiae* has been also cited feeding on other agricultural crops without causing any kind of fruit distortions, being this the case of the persimmon (Urbaneja *et al.*

2017). Two reasons may explain this fact: *D. aberiae* populations appear in a later period, not coinciding with developing tissues, or *D. aberiae* may have any kind of toxic substance on its saliva which affects citrus but not persimmon. The second reason can not be confirmed and further research is needed to determine if *D. aberiae* injects or not toxic substances on its hosts. But, by now, this work has shown that citrus fruit distortions appear if *D. aberiae* populations coincide with fruit cell division stage, interfering with the growth of the attacked cells.

5.3.2 Relation between damage period and *D. aberiae* population density

According to the results, *D. aberiae* can cause fruit distortions and size reduction in citrus orchards from flowering period until fruits sized between 25 and 30 mm for the varieties tested. Figure 5.2. shows that this period ranges from March to the beginning-mid July. It can also be observed that *D. aberiae* density increases considerably in May and June, being fruit damage observed mainly during this period. These results are very important for the mealybug management, because chemical spraying is forbidden during the flowering period. Thus, monitoring proceses should start after petal fall and, if populations reach the recently established Economic Environmental Injury Level (EEIL), recommended insecticides against mealybugs in Spain will be used (Pérez-Rodríguez *et al.* 2017).

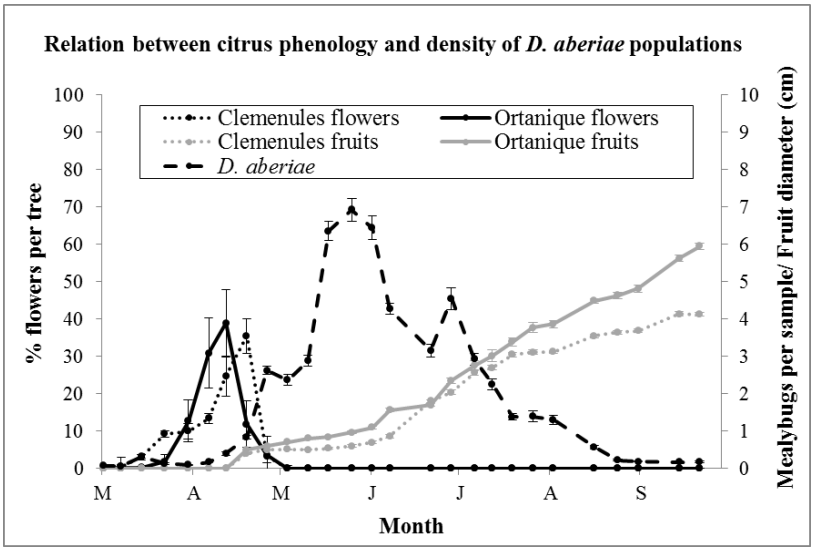

Figure 5.2. Relation between citrus phenology of the two studied varieties ('Clemenules' and 'Ortanique') and *D. aberiae* density between March and September [own data adapted from the publication of Martínez-Blay *et al.* (2018b)]. Primary Y-axis shows the percentage of flowers (± standard error, SE) per tree during the flowering period. Secondary Y-axis represents two parameters: the evolution of citrus fruit diameter ± SE (in cm) for each variety and the mean number of mealybugs (± SE) in citrus orchards of eastern Spain.

5.4. Acknowledgements

The authors thank the owners of the orchards for allowing us to use their plantations. This research was supported by a predoctoral grant (FPU to V. Martínez-Blay) from the Spanish Ministry of Education, Culture and Sport, by The Generalitat Valenciana (Program 714.80 of Direcció General D'Agricultura, Ramaderia i Pesca), and a European grant (FP7-IRSES #612566 'Biomodic').

CHAPTER 6. Application of classical biological control to manage the new invasive citrus pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae)

Chapter 6. Application of classical biological control to manage the new invasive citrus pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae)

Martínez-Blay, V., Addison, P., Beetge, L., Benito, M., Friedman, R., Guerrieri, E., Puig, J., Soto, A. Submitted to Biol Control. Adapted author's Pre-print version.

Abstract

Delottococcus aberiae De Lotto (Hemiptera: Pseudococcidae) is a mealybug species native to Southern Africa. It is an invasive citrus pest in Spain since 2009. Classical biological control is one of the most effective methodologies to control new pests in the invaded area, where no effective biocontrol agents are usually present. The introduction of natural enemies of *D. aberiae* from its native area represents a sustainable alternative to manage this pest. To develop a classical biological control program, to manage *D. aberiae* in Spain, this research aimed to characterize the behavior and complex of natural enemies of this mealybug in citrus orchards in its native area (South Africa). A total of 32 sites were surveyed from January 2017 to December 2017. Mealybugs present per sample were counted and examined for parasitism signs. Parasitized mealybugs were isolated and checked for parasitoid emergence. Identification of recovered specimens was done by morphological characterization. When necessary, molecular characterization was also carried out. Results showed that *D. aberiae* population density peaked in summer with all developmental stages overlapping. The highest parasitism rates occurred in autumn. A high biodiversity of species was found. The most abundant natural enemies of *D. aberiae* in its native area were the primary parasitoids *Anagyrus* sp. nov. 1 (Hymenoptera: Encyrtidae) and *Allotropa* sp. nov. (Hymenoptera: Platygastridae).

Keywords: *Allotropa* sp. nov., *Anagyrus* sp. nov. 1, behavior, parasitism, mealybug, South Africa

6.1. Introduction

The increase in international trade throughout recent decades has risen the number of exotic species entering Europe (Roques *et al.* 2009, Bellard *et al.* 2016). Mealybugs (Hemiptera: Pseudococcidae) are typical invasive pests, due to their small size and cryptic behavior, and have entered Europe at a high rate (Miller *et al.* 2002, Pellizzari and Germain 2010). Some examples are *Dysmicoccus brevipes* (Cockerell) (Suma *et al.* 2015), *Paracoccus marginatus* Williams & Granara de Willink (Mendel *et al.* 2016), *Phenacoccus defectus* Ferris (Mazzeo *et al.* 2014), *Phenacoccus solani* Ferris (Mazzeo *et al.* 1999), *Pseudococcus comstocki* (Kuwana) (Pellizzari 2005) or *Phenacoccus peruvianus* Granara de Willink (Beltrà *et al.* 2010, Beltrà *et al.* 2013a). Within this context, *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) was first detected in eastern Spain in 2009 and currently it is known that Spanish populations are native to Limpopo province, in Northern South Africa (Beltrà *et al.* 2012, García-Marí 2012, Beltrà *et al.* 2013c, Beltrà *et al.* 2015). *D. aberiae* completes several generations throughout the year, being two of them clearly defined and resulting in high population levels between May and July (Martínez-Blay *et al.* 2018b). Nymphs and adults settle and feed on fruitlets (Martínez-Blay *et al.* 2018b) and their feeding behavior causes severe direct damage to citrus fruits, distorting its shape and/or causing size reduction, depending on the cultivar, which depreciates its commercial value (Pérez-Rodríguez *et al.* 2017). Recently, the duration of the damaging period has been established, including from flowering stage (March-April in eastern Spain conditions) to fruits with a diameter of 25-30 mm (around July in eastern Spain conditions) (Martínez-Blay *et al.* 2018a).

At present, chemical control, based on the use of available insecticides against mealybugs, is the main strategy used to control *D. aberiae* in Spain. However, the economic and environmental impacts

of chemical control and its potential interference with the biological control of other citrus pests (Franco *et al.* 2009) make it necessary to look for alternative management strategies. Classical biological control is one of the most effective methodologies to control new pests in the invaded area, where no effective biocontrol agents are usually present. This management strategy has been previously implemented against other invasive insects in Spanish citrus orchards and, in combination with other management techniques, usually leads to a balance in the populations of the pest in the invaded area (Soto *et al.* 1999, Garcia-Marí *et al.* 2004, Vercher *et al.* 2005, García-Marí 2012, Sorribas *et al.* 2012).

The invasive condition of the family Pseudococcidae and the problems to control them by chemical methods have made this group of insects a target of biological control strategies (Moore 1988, Franco *et al.* 2009, Venkatesan *et al.* 2016). Population outbreaks are frequent when mealybugs are introduced into new areas without their specific natural enemies. Therefore, classical biological control programs, based on the introduction and release of exotic natural enemies from the native area of the pest, have been widely used for their management (Moore 1988, Miller *et al.* 2002, Franco *et al.* 2009). Classical biological control has been implemented with positive results for several mealybug species, such as *Maconellicoccus hirsutus* (Green) (Kairo *et al.* 2000, Roltsch *et al.* 2006), *Paracoccus marginatus* Williams and Granara de Willink (Muniappan *et al.* 2006, Amarasekare *et al.* 2009), *Phenacoccus manihoti* Matile-Ferrero (Neuenschwander 2001) or *Rastrococcus invadens* Williams (Neuenschwander *et al.* 1994, Agricola *et al.* 2009). Most of the successful classical biological control programs against mealybugs involve the use of insect parasitoids (Moore 1988, Charles 2011). Among them, encyrtid parasitoids (Hymenoptera: Encyrtidae) are the most important and diverse group of natural enemies to control

mealybugs (Noyes and Hayat 1994, Charles 2011), but species from the families Aphelinidae and Platygastridae also prove successful on several occasions (Moore 1988). Encyrtids usually establish host-specific relationships with mealybugs and have a major influence on their population dynamics (Charles and Allan 2002, Charles 2011, Beltrà *et al.* 2013b, Bugila *et al.* 2015).

Biological control of *D. aberiae* had never been investigated until this species was introduced into Spain. Recent studies (Tena *et al.* 2017a, Tena *et al.* 2017b) showed that native and naturalized parasitoids present in the Mediterranean Basin fail to control the mealybug. Thus, the best option is to search for effective parasitoids in the native area of the pest, South Africa. In this country, since the early 1990s, there has been an increasing emphasis on citrus integrated pest management (IPM), based on the conservation of natural enemies and bio-rational control strategies. However, it should be considered that in recent years the control of some pests and diseases is increasingly dependent again on the use of harmful chemical products to maintain quarantine pests at low enough levels to satisfy the increasing export market restrictions, which disrupts the IPM of many other important key pests (Grout 2015).

Within the aforementioned context, the implementation of a classical biological control, by the introduction of a natural enemy known to be effective against *D. aberiae* in its native area, seems to be the most promising strategy to control this pest. The survey made by Beltrà *et al.* (2015) showed that Spanish *D. aberiae* populations came from citrus orchards in Limpopo province (northern South Africa) and, there, they found an undescribed species of the genus *Anagyrus* (Hymenoptera: Encyrtidae). Thus, this geographic area should be considered as the first choice for collecting parasitoids to be introduced in Spain against *D. aberiae*. Herein, with the aim of developing a classical biological control program for the management

of *D. aberiae* in Spain, the following objectives were established: (1) to describe the behavior of *D. aberiae* in citrus orchards in its native area (Limpopo, northern South Africa) and (2) to characterize the complex of natural enemies of *D. aberiae* in citrus orchards in its original area, in particular parasitoids, as well as to determine their seasonal abundance in the field and their potential as candidate species for classical biological control.

6.2. Materials and Methods

6.2.1 Survey sites and sampling protocol

A total of 32 sites were surveyed in the province of Limpopo (northern South Africa) from January 2017 to December 2017. All surveyed sites comprised private citrus orchards, 22 of them included grapefruit trees (*Citrus* x *paradisi* Macfad) and the other 10 sweet orange trees (*Citrus sinensis* (L). Osbeck) (Table 6.1.). Sampled sites belonged to the municipality of Greater Tzaneen and were located in the surroundings of the town of Letsitele. All of them were selected for presenting *D. aberiae* during previous seasons. The orchards ranged from 2.2 to 15.30 ha and were drip-irrigated.

For each sampling date and at each sampling site, twenty trees per orchard were monitored and four 20-cm long twigs, each one from a different cardinal orientation, were collected randomly from each tree. Each twig included its leaves and flowers and/or fruits (depending on their availability during the year). Samples were bagged and transported to the laboratory inside a portable cooler. All the material was processed within the next 24 h according to the procedures described below.

Table 6.1. Surveyed sites: location, citrus varieties and sampling date.

Site	Citrus species	Citrus variety	UTM coordinates (grid: 36K)		Sampling date
1	*Citrus* x *paradisi*	Star Ruby	238949 m E	7358974 m S	January to December
2	*Citrus* x *paradisi*	Star Ruby	234518 m E	7357213 m S	January to December
3	*Citrus* x *paradisi*	Star Ruby	238178 m E	7360059 m S	February
4	*Citrus sinensis*	Valencia Late	238630 m E	7361595 m S	August
5	*Citrus* x *paradisi*	Star Ruby	240312 m E	7361398 m S	January to December
6	*Citrus* x *paradisi*	Star Ruby	241311 m E	7362395 m S	January, March, April
7	*Citrus* x *paradisi*	Star Ruby	240534 m E	7361103 m S	January to December
8	*Citrus* x *paradisi*	Star Ruby	238139 m E	7362368 m S	January to December
9	*Citrus* x *paradisi*	Star Ruby	242214 m E	7361897 m S	January to May
10	*Citrus* x *paradisi*	Star Ruby	242367 m E	7359775 m S	January to May
11	*Citrus* x *paradisi*	Star Ruby	241711 m E	7364021 m S	January to August
12	*Citrus sinensis*	Valencia Late	233568 m E	7355845 m S	January to May
13	*Citrus sinensis*	Valencia Late	237872 m E	7344548 m S	September
14	*Citrus sinensis*	Valencia Late	238572 m E	7359160 m S	June
15	*Citrus sinensis*	Valencia Late	239086 m E	7358943 m S	June
16	*Citrus sinensis*	Valencia Late	239175 m E	7358977 m S	June and December
17	*Citrus sinensis*	Valencia Late	238817 m E	7359040 m S	December
18	*Citrus sinensis*	Valencia Late	238151 m E	7361496 m S	June
19	*Citrus sinensis*	Valencia Late	238160 m E	7362361 m S	December
20	*Citrus sinensis*	Valencia Late	238643 m E	7361610 m S	October, November
21	*Citrus* x *paradisi*	Star Ruby	234492 m E	7357199 m S	July
22	*Citrus* x *paradisi*	Star Ruby	234869 m E	7356925 m S	August
23	*Citrus* x *paradisi*	Star Ruby	229849 m E	7358728 m S	July
24	*Citrus* x *paradisi*	Star Ruby	230194 m E	7358465 m S	July
25	*Citrus* x *paradisi*	Star Ruby	229703 m E	7359017 m S	July to November
26	*Citrus* x *paradisi*	Star Ruby	236206 m E	7359351 m S	October, November
27	*Citrus* x *paradisi*	Star Ruby	240347 m E	7361433 m S	September, November
28	*Citrus* x *paradisi*	Star Ruby	234708 m E	7357377 m S	July to November
29	*Citrus* x *paradisi*	Star Ruby	234653 m E	7357695 m S	December
30	*Citrus* x *paradisi*	Star Ruby	234996 m E	7356968 m S	October
31	*Citrus* x *paradisi*	Star Ruby	230040 m E	7359124 m S	September
32	*Citrus* x *paradisi*	Star Ruby	240536 m E	7361102 m S	September

6.2.2 Mealybug seasonal phenology

Among the 32 sites, 6 citrus orchards, under permission of their owners, were sampled monthly to study mealybug phenology. Mealybugs present on each twig, on four leaves per twig and on one to eight flowers or fruits (depending on their availability during the year) were counted under a stereomicroscope (Nikon SMZ645) and classified into one of the following developmental stages: first nymphal instar (N1), second nymphal instar (N2), third nymphal instar (N3), immature males (pre-pupa and pupa) (M1), adult males (M2), adult females (H1) or gravid females (H2). Leaves, flowers and fruits to be examined from each twig were randomly selected.

6.2.3 Complex of natural enemies

Collecting parasitoids

Samplings were done with a methodology similar to the previously explained in the former section. The complex of natural enemies and their abundance was determined by collecting parasitized mealybugs from twigs, leafs, flowers and/or fruits from 32 citrus orchards. Each month of the study, 10 citrus orchards, randomly selected among the total surveyed, were sampled. All the collected mealybugs were morphologically checked for parasitism and the developmental stage of each *D. aberiae* parasitized was recorded. A mealybug was considered to be parasitized when it was mummified or when it showed the first signs of mummification (body deformation and cuticle sclerotization) (Beltrà *et al.* 2013d). When a mummy was found, it was separated with a fine camel hair brush and placed into a 3.0 x 0.8-cm glass vial (1 mummy/per vial). The vials were covered with a cotton plug and stored in the laboratory at room temperature (25 ± 5ºC) and the natural outdoor photoperiod. Vials were checked daily for parasitoid emergence. Upon emergence, absolut ethanol was added into each tube to kill adult parasitoids and vials were stored

until identification of parasitoids. The number and sex of each parasitoid species emerged per mummy were recorded. Parasitism rates were estimated as the proportion of mummified mealybugs to the total number of mealybugs susceptible to parasitism (alive and mummified mealybugs of second instar, third instar and adult females). Parasitism rates per month were obtained.

Morphological and molecular characterization of parasitoids

For morphological characterization of parasitoids, card mounted and slide mounted specimens were prepared. For card mounting, parasitoids were killed in absolute ethanol and then placed in a 1:1 ethanol: xylene solution for 24 h, transferred to amyl acetate for 24 h and mounted on cards with water-soluble glue. For slide mounting (Noyes 1982), starting from a card prepared specimen, wings were dissected and directly mounted in Canada balm on the slide. The remaining of the specimen was processed in 10% KOH for 5 min at 100°C, transferred to acetic acid for 5 minutes, afterwards to increasing ethanol series (from 70% to absolute) and finally to clove oil. Dissected part (head, antennae, thorax, gaster, hypopygium and ovipositor) were mounted on the slide in Canada balm. The slide was put on hot plate at 100° for 2 hours and then Canada balm and cover slips added onto dissected parts. The card and slide-mounted specimens were compared with literature descriptions and authoritatively identified specimens deposited in the Natural History Museum of London (UK). Parasitoids species, belonging to the families Pteromalidae and Platygastridae, were sent to the British National History Museum for identification.

Additionally, when the number of recovered specimens allowed for it, molecular characterization was carried out to clarify the identity of the complex of *Anagyrus* species. DNA was extracted with non-destructive technique from females and males using ZR Tissue &

Insect DNA MicroPrep Kit (Zimoresearch) according to the manufacturer instructions. Amplifications of 5' region of Cytochrome Oxidase Complex I (COI) mitochondrial gene was obtained using the primers: TL2-N-3014 TCCAATGCACTAATCTGCCATATTA and C1-J-2183 CAACATTTATTTTGATTTTTTGG. Amplifications were carried out in 50,0 µl volume containing 50 ng template DNA, 1X DreamTaq Buffer, 0.2 µM dNTPs, 1.5 U DreamTaq DNA polymerase (Thermo Scientific) and 6.0 µM each primer; PCR was performed for 45 cycles using 45.0°C for 45 seconds as primers annealing conditions and 2.0 minutes as DNA polymerase elongation time. All amplifications were achieved using 2720 ThermoCycler (Applied Byosystem). PCR products were checked on 1.5% agarose gel stained with SYBR Safe (Invitrogen) and sequenced at CIBIACI (University of Florence - Italy.) and at Macrogen® (Seoul - Korea). Resulting sequences were aligned and compared with Gene Bank ones relative to the genus *Anagyrus* and particularly with those of close related species.

6.2.4 Statistical analysis

Statistical comparison to determine whether the mean percentage of total parasitism (all instars considered together) differed among different months of the year was performed using analysis of variance (ANOVA). Means were compared using Fisher's Least Significant Difference (LSD) tests. Data are presented as mean ± standard error (SE). Data were tested for homogeneity of variances using Levene's test. If required, data were subjected to an angular transformation, arcsine of the square root of the proportion, before analysis to satisfy model assumptions regarding homogeneity of variances and approximate a normal distribution (Kasuya 2004). To determine the preferred parasitized instar, a statistical comparison using analysis of variance (ANOVA) was performed separately for each one of the two most abundant parasitoids recovered in this study, *Anagyrus* sp. nov. 1

and *Allotropa* sp. nov. Means were compared using Fisher's Least Significant Difference (LSD) tests. Data were tested for homogeneity of variances using Levene's test. If required, data were log (x+1) transformed before analysis to satisfy model assumptions regarding homogeneity of variances (McDonald 2014).

Data were averaged per orchard, being this the sampling unit used for all the statistical analysis. The significance level was set at $\alpha = 0.05$. All the statistical analyses were conducted with the software Statgraphics Centurion XVI.II (Statpoint Technologies Inc, Warrenton, USA).

6.3. Results

6.3.1 *D. aberiae* seasonal phenology

D. aberiae population density started to increase in November (spring in South Africa) and reached a maximum in January and February (summer in South Africa). Afterwards in March (end of summer in South Africa) populations started to decreased and remained at practically undetectable levels in autumn and winter. All developmental stages tended to overlap between them (Fig. 6.1.). Besides, the seasonal trend and mealybug density throughout the year was very similar among instars (Fig. 6.2.).

6.3.2. Complex of natural enemies and seasonal trend

Parasitoids

Total parasitism rates (all susceptible instars considered together) in the field differed throughout the year (Fig. 6.1.) ($F = 4.80$, df = 11, 108, $P < 0.001$). The maximum percentage of total parasitism was reached in June, with a mean percentage ± standard error (SE) of 26.90 ± 0.69%, and was significantly higher than in any other month of the year. This percentage was followed by May (15.85 ± 0.49%),

April (14.05 ± 0.47%), March (10.44 ± 0.41%), February (9.39 ± 0.39%), July (6.25 ± 0.34%) and January (5.56 ± 0.31%) and none of these values differed among them. Parasitism rates from August to December were similar and practically null (Fig. 6.1.).

Parasitism rates per instar (Fig. 6.2.) showed that the maximum percentage of parasitism was reached in June for second instars (70.09 ± 0.71%) and gravid females (24.49 ± 0.66%), in April for third instars (45.45 ± 0.67%) and in February for adult females (12.97 ± 0.45%). Parasitism rates in adult and gravid females were more homogeneous throughout the year and always below 30%.

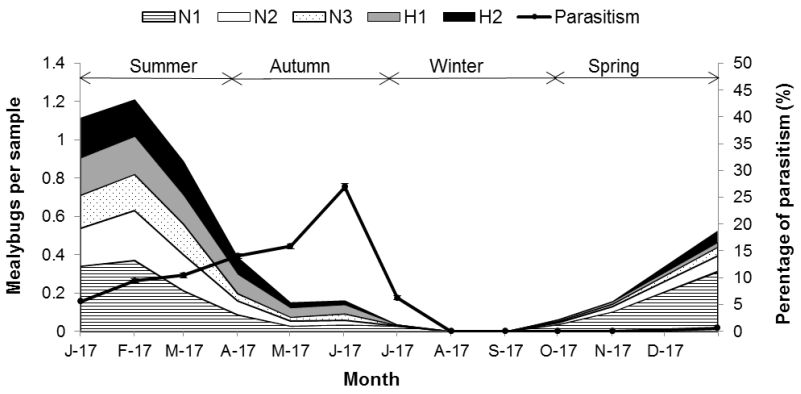

Fig. 6.1. Seasonal phenology of *D. aberiae* (N1 = first nymphal instar, N2 = second nymphal instars, N3 = third nymphal instars, H1 = young females, H2 = gravid females) and total monthly percentage of parasitism (all susceptible instars considered). Results are based on samples taken in citrus orchards in northern South Africa throughout 2017. Vertical bars represent the standard error (SE).

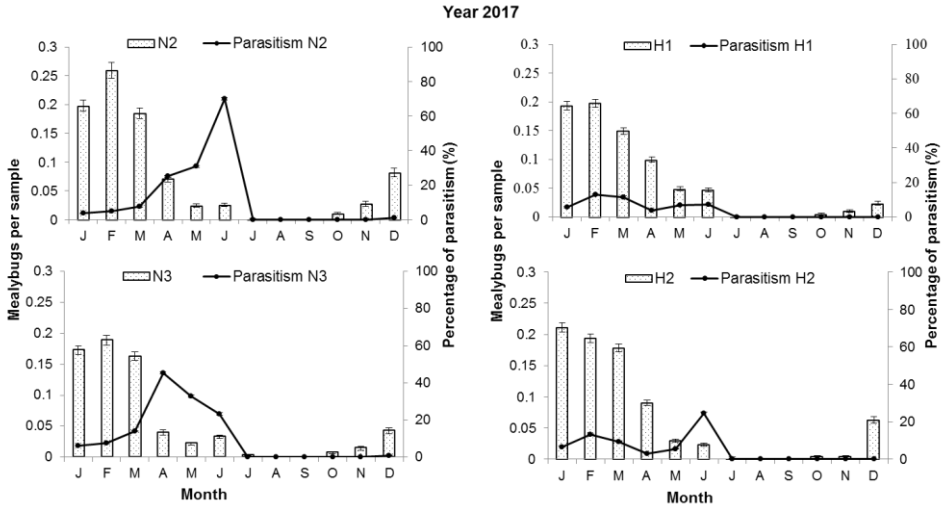

Fig. 6.2. Seasonal trend and parasitism rates for each one of the *D. aberiae* instars susceptible to parasitism (N2 = second nymphal instars, N3 = third nymphal instars, H1 = young females, H2 = gravid females). Results are based on samples taken in citrus orchards in northern South Africa throughout 2017. Vertical bars represent the standard error (SE).

From January to December 2017, 1,568 parasitized mealybugs (mummies) were found. Among the parasitized specimens, a total of 152 parasitoids were recovered and the number of adults emerged per mummy was one in all the cases. A high biodiversity of species was found in the sampled area: 126 of the recovered specimens were identified as primary parasitoids (82.89%), belonging to seven different species, and 23 were hyperparasitoids (15.13%) of two different species (Table 6.2.). Among the primary parasitoids identified, five belonged to the family Encyrtidae (*Anagyrus* sp. nov. 1, *Anagyrus* sp. nov. 2, *Anagyrus aurantifrons* Compere, *Anagyrus* sp. 3 and *Leptomastix dactylopii* Howard), one to Platygastridae (*Allotropa* sp. nov.) and one to Aphelinidae (*Thysanus* sp.). Two

species of hyperparasitoids were detected; one belonged to the family Pteromalidae (*Pachyneuron* sp.) and the other to Encyrtidae (*Procheiloneurus aegyptiacus* Mercet).

Table 6.2 Abundance of *D. aberiae* parasitoids recovered from samples taken from citrus orchards in northern Limpopo throughout 2017.

Family	Species	Biology	Number of parasitoids recovered from *D. aberiae* (n)		
			Females	Males	Total
Encyrtidae	*Anagyrus* sp. nov. 1	Primary parasitoid	32	40	72
Encyrtidae	*Anagyrus* sp. nov. 2	Primary parasitoid	8	0	8
Encyrtidae	*Anagyrus aurantifrons*	Primary parasitoid	2	0	2
Encyrtidae	*Anagyrus* sp. 3	Primary parasitoid	1	0	1
Platygastridae	*Allotropa* sp. nov.	Primary parasitoid	19	21	40
Encyrtidae	*Leptomastix dactylopii*	Primary parasitoid	1	1	2
Aphelinidae	*Thysanus* sp.	Primary parasitoid	1	0	1
Pteromalidae	*Pachyneuron* sp.	Secondary parasitoid	9	7	16
Encyrtidae	*Procheiloneurus aegyptiacus*	Secondary parasitoid	7	0	7
Other	Unknown	Unknown	2	1	3
					152

Among the complex of *Anagyrus* species (Hymenoptera: Encyrtidae), *Anagyrus* sp. nov. 1 represented 57.14% of the total of the parasitoids recovered and the set formed by the rest of *Anagyrus* accounted for 8.7%. *Anagyrus sp*. nov. 1 was the most abundant species recovered in the months of January, February and March (Fig. 6.3.). Besides, the number of *Anagyrus* sp. nov. 1 recovered from mummies of adult and gravid females was similar but significantly higher than the number of parasitoids recovered from second and third instars ($F = 3.04$, df = 3, 36, $P = 0.03$).

Apart from this complex, the species *Allotropa* sp. nov. (Hymenoptera: Platygastridae) accounted for 31.75% of the

parasitoids recovered (Table 6.2). *Allotropa* sp. nov. was the most numerous species in April, May, June and December (Fig. 6.3.). This species emerged similarly from mummies of second and third instars, being this values higher than the parasitoids recovered from mummies of adult and gravid females (F = 3.88, df = 3, 36, P = 0.02). The other primary parasitoids found, *L. dactylopii* and *Thysanus* sp., represented 1.59% and 0.79% of the total emerged species respectively.

The hyperparasitoid *Pachyneuron sp*. was the most abundant one (69.57%), followed by *P. aegyptiacus* (30.43%) (Table 6.2.). Hyperparasitoids were detected from January to May and the highest percentage of hyperparasitism was reached in February, representing almost a 30% of the total number of parasitoids recovered that month (Fig. 6.3.). In April, May and June the hyperparasitism rates were similar and January was the month with the lowest percentage of hyperparsitism (Fig. 6.3.).

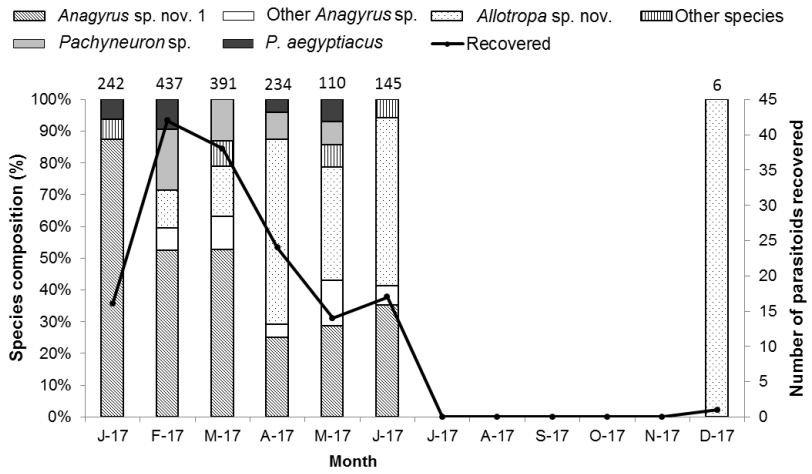

Fig. 6.3. *D. aberiae* parasitoid complex composition and number of parasitoids recovered. Results are based on samples taken from citrus orchards in northern South Africa throughout 2017. Value above each bar represents the total number of mealybugs parasitized (recovered parasitoids + not recovered parasitoids).

6.4. Discussion

6.4.1 *D. aberiae* seasonal phenology

Results reveal that *D. aberiae* population density starts to increase in spring, reaching a maximum in summer. Afterwards, populations decrease and remain at very low levels in autumn and winter (Fig. 6.1.). A similar trend may be observed in eastern Spain. However, there, *D. aberiae* density in spring is much higher than in northern South Africa (Martínez-Blay *et al.* 2018b). Different abiotic and biotic factors may affect the abundance of mealybugs in the field in spring, such as climate, the quality of the feeding substrate, the application of chemical treatments or the action of natural enemies or (Bartlett and Clancy 1972, Furness 1976, Franco 1994, Goolsby *et al.* 2002, Haviland *et al.* 2012, Beltrà *et al.* 2013a, Wunderlich *et al.* 2013).

The average temperature in spring (September to December in South Africa and March to June in Spain), from 2013 to 2017, was warmer in northern South Africa (22.03 ºC) (Tzaneen data, TuTiempo Weather Net) (TuTiempo Network 2018), than in the studied area by Martínez-Blay *et al.* (2018b) in eastern Spain (17.91 ºC) (Benavites data, SIAR's Weather Net) (IVIA 2011), suggesting that weather conditions are not limiting the development of the mealybug during this period. On the other hand, flowering and fruit set (September-October in South Africa) occur during this period. Thus, the quality of the feeding substrate does not seem to be a limiting factor because the new growing tissues, especially fruit in development, give very good food quality conditions for the development of mealybugs (Franco 1994, Geiger and Daane 2001, Franco *et al.* 2004a, Haviland *et al.* 2012). The action of parasitism in autumn that leads to lower winter population levels of the pest in South Africa than in Spain may result in a slowly population increase in spring. Finally, in recent years in South Africa the control of several pests and diseases is increasingly

dependent on the use of harmful chemical products to maintain quarantine pests at low enough levels to satisfy the increasing export market restrictions. Moreover, the application of chemical treatments is especially high during flowering and fruit set (September-October in South Africa) (Moore and Hattingh 2012, Grout 2015). Therefore, heavy chemical treatments in citrus in South Africa seem to be not only limiting *D. aberiae* population levels in spring but also would not allow for the development of natural enemies. Therefore, lower population levels in spring in northern South Africa are probably due to a combination of the action of natural enemies in autumn and the effect of heavy chemical treatments during flowering and fruit set period.

Besides, several overlapping generations of *D. aberiae* may be observed in northern South Africa (Fig. 6.1.), without distinctly peaks. In contrast, two generations are clearly defined in Spain (Martínez-Blay *et al.* 2018b). The first important generation observed in summer in South Africa probably coincides with the second peak observed in Spain, as well in summer (Martínez-Blay *et al.* 2018b). However, the summer generation in South Africa overlaps with the following ones and there is a mix of all developmental stages (Fig. 6.1.), while in Spain first instars predominate (Martínez-Blay et al., 2017). Similar studies carried out with other mealybug pests affecting agronomic and ornamental plants, such as *Paracoccus burnerae* (Brain), *Phenacoccus madeirensis* Green, *P. peruvianus*, *Planococcus citri* (Risso) or *Pseudococcus viburni* (Signoret), have shown a similar pattern with several, usually overlapping, generations throughout the year and high population densities in spring and summer (Panis 1986, Longo *et al.* 1995a, Martínez-Ferrer *et al.* 2003, Johnson and Giliomee 2012, Beltrà *et al.* 2013a).

6.4.2 Complex of natural enemies

Parasitoids identified varied in abundance seasonally (Fig. 6.3.), being total parasitism rates always below 30% (Fig. 6.1). These values are low in comparison to other similar studies (Roltsch *et al.* 2006, Reddy *et al.* 2009, Beltrà *et al.* 2013d). However, parasitism rates found in this study, in the native area *D. aberiae*, are much higher than those existing in Spain where some parasitoid species are able to parasitize *D. aberiae* under controlled conditions but suffer high encapsulation rates in the field. Subsequently, to date, there is a lack of effective parasitoids against *D. aberiae* in eastern Spain (Tena *et al.* 2017a, Tena *et al.* 2017b). Besides, this parasitism rates in South Africa were reached even under a high number of heavy chemical treatments in the area (Grout 2015), being expected to increase under controlled conditions and better field conditions. Variations in parasitoids abundance and parasitism rates may result from different factors, such as climate, different response to insecticides, the availability of suitable hosts or the particular behavioral characteristics of each species (Sun *et al.* 2004).

The present study carried out an extensive survey of natural enemies of *D. aberiae* in the native area of this pest (Limpopo, northern South Africa). Results showed a high biodiversity in the region, with a total of 9 different species of primary parasitois and hyperparasitoids (Table 6.2.). As not any other similar studies had been previously done in the area it was expected to find a significant number of different species. Besides, South Africa is a country with a high arthropod biodiversity and high host plant specificity (Procheş and Cowling 2006, 2007), being usual to find many undescribed species in this kind of studies. Among all the identified species, the complex of *Anagyrus* spp. and the species *Allotropa* sp. nov. should be considered of special interest due to their abundance.

The complex of *Anagyrus* spp. is formed by four different species, based on morphological and, when possible, molecular data. *Anagyrus* sp. nov. 1, *Anagyrus* sp. nov. 2 and *Anagyrus* sp. 3 are undescribed species. At the moment, the former two are in process of description as a new species by experts, whereas the latter is not being characterized by now due to the fact that only one specimen has been recovered (Table 6.2.). Among them, *Anagyrus* sp. nov. 1 has been found as the most abundant species parasitizing *D. aberiae*. Herein, we carried out a survey throughout an entire year in the native area of the Spanish invasive populations of *D. aberiae*. Encyrtid parasitoids (Hymenoptera: Encyrtidae) are considered one of the most important and diverse group of natural enemies to control mealybugs (Noyes and Hayat 1994, Prinsloo 1998). They usually establish host-specific interactions with mealybugs and their coevolution plays an important role on their ability to overcome defensive strategies of their hosts (Charles and Allan 2002, Charles 2011, Bugila *et al.* 2015). Within this family, parasitoids of the Anagyrini tribe, which contains the genus *Anagyrus*, have been widely studied and used as primary parasitoids for biological control of mealybugs, such as *Planococcus citri* (Risso) or *Planococcus ficus* Signoret (Noyes and Hayat 1994, Franco *et al.* 2004a, Bugila *et al.* 2015). Due to this fact of coevolution there is a high biodiversity of the genus *Anagyrus* with species adapted to certain hosts and geographic areas. Besides, there is a lack of identification keys of this genus for most regions of the world and the existing ones do not solve the problems to interpret great taxonomic variability in some structural characters, such as coloration (Timberlake 1924, Compere 1939b, Prinsloo 1998). Within this context, it is not surprising that the four species of *Anagyrus* are different among them and even from the *Anagyrus* sp. previously recorded by Beltrà *et al.* (2015).

The second most abundant parasitoid was *Allotropa* sp. nov. This species has been confirmed as a new undescribed species of this genus and is in process of description by Peter Buhl, who has previously described a number of *Allotropa* species. The genus *Allotropa* Förster is in the family Platygastridae and some parasitoids of this genus are known to be primary endoparasitoids of various mealybug species (Masner and Huggert 1989, Vlug 1995). Several *Allotropa* species have been used in biological control programs against mealybugs in different parts of the world. *Allotropa burrelli* Muesebeck is known to be a specialist parasitoid of *Pseusococcus comstocki* Kuwana and has been selected as a good candidate for classical biological control of *P. comstocki* in France (Clancy 1944, Malausa *et al.* 2016, Quaglietti *et al.* 2017a). *Allotropa citri* Muesebeck can parasitize all developmental stages of *Pseudococcus cryptus* Hempel (Arai and Mishiro 2004). Buhl (2005) recorded *Allotropa musae* Buhl from *Dysmicoccus grasii* (Lonardi) in banana (*Musa* sp.) in the Canary Islands. *Allotropa oracellae* Masner is host-specific on *Oracella acuta* (Lobdell), a mealybug affecting different pine species, and controls this pest in the United States (Clarke *et al.* 1990, Masner *et al.* 2004, Sun *et al.* 2004). Besides, this species have been introduced as a part of a classical biological control against *O. acuta* in China (Clarke *et al.* 2010). *Allotropa phenacocca* Chen, Liu & Xu has been reported parasitizing *Phenacoccus solenopsis* Tinsley on *Hibiscus rosa-sinensis* L. in Japan (Chen *et al.* 2011). *Allotropa suasaardi* Sarkar & Polaszek is a parasitoid of *Phenacoccus manihoti* Matile-Ferrero on cassava in Thailand (Sarkar *et al.* 2014, Sarkar *et al.* 2015) and *Allotropa* sp. near *mecrida* (Walker) is a parasitoid of *M. hirsutus* that was introduced into California against this pest (Roltsch *et al.* 2006, Roltsch *et al.* 2007, Reddy *et al.* 2009). Regarding behavioral characteristics, several *Allotropa* species, for example, exhibit gregarious parasitism (Clancy 1944, Löhr *et al.* 1991, Sun *et al.* 2004, Quaglietti *et al.* 2017a, Quaglietti *et al.* 2017b). This is not the case as herein only one

parasitoid was recovered from each mummy. This could be a characteristic of the *Allotropa* sp. nov. found in this study or could be due to the fact of mostly parasitizing *D. aberiae* second and third instars. Indeed, the species *A. oracellae* shows mainly gregarious parasitism when parasitizing females but presents solitary behavior when recovered from second and third instars (Sun *et al.* 2004).

Regarding hyperparasitoids, *Pachyneuron* sp. has been the species most recovered. It has been identified by Dr. Polaszek as very resembling to *Pachyneuron muscarum* (L.). However, this species has not been recorded before in sub-Saharan Africa and we are waiting for further confirmation by a specialist in this genus. This genus has been commonly report as hyperparasitoids in other studies of natural enemies of mealybugs (Beltrà *et al.* 2013d, Beltrà *et al.* 2015)

Finally, interspecific competition may occur among parasitoids which share the same host (Bográn *et al.* 2002, Beltrà *et al.* 2013d). In this study, both parasitoid species behaved differently as *Allotropa* sp. nov. parasitizes mostly small instars and *Anagyrus* sp. nov. 1 prefers the bigger ones. Further research is needed to confirm if these two species are able to co-exist or if they might compete between them.

As a conclusion, data on the complex of natural enemies of *D. aberiae* is scarce. Our results represent an important contribution for biological control of this mealybug. Both parasitoids *Anagyrus* sp. nov. 1 and *Allotropa* sp. nov. may have a significant role in a classical biological control program against *D. aberiae* in Spain. *Anagyrus* sp. nov. 1 is the most promising candidate species, for now, because it has shown higher parasitism rates. Further information is needed and our current studies are focused on laboratory assays to assess the host specificity of the aforementioned species. At the same time experts are working on the detailed taxonomical description of the species.

6.5. Aknowledgements

The authors thank the company Biobee SA for their help in the search of the sampled orchards and for allowing us to use their installations, especially Ndivhudzannyi Netshithuthuni. We are also grateful to Dr. Andy Polaszek, from the British National History Museum, for his assistance with the morphological identification of some parasitoids and to Peter Buhl (Denmark) for the identification of the *Allotropa* species. This research was supported by a predoctoral grant (FPU to V. Martínez-Blay) from the Spanish Ministry of Education, Culture and Sport, a project provided by The Generalitat Valenciana (Program 714.80 of Direcció General D'Agricultura, Ramaderia i Pesca) and by the European Union Seventh Framework Programme (FP7-IRSES #612566 'Biomodic'). The authors also thank Debra Westall (UPV) for English corrections.

CHAPTER 7.
General discussion

Chapter 7. General discussion

During recent decades, the increase in the number of problems associated with certain mealybugs, and the introduction of several new invasive species, has led to a growing interest in this group of insects in Spain (Beltrà and Soto 2012). Within this context, *D. aberiae*, a mealybug of Southern African origin, arrived to citrus orchards in eastern Spain in 2009 (García-Marí 2012, Beltrà *et al.* 2013c), probably through international trade of citrus plants or fruits, which is the main pathway of dispersion of scale insects in Europe (Pellizzari and Germain 2010). This was the first report of *D. aberiae* causing significant damage in citrus out of its native area.

When an invasive species arrives for the first time to a new region, it is necessary to carry out basic studies about the biology, behavior and control possibilities for the new pest. Usually, there are no effective natural enemies against new invasive pests in the invaded area. However, the latest European Directive on sustainable use of pesticides (2009/128/EC) stipulates the reduction in chemical applications which interfere with natural enemies and pollinators (European Parliament and Council 2009), compelling us to develop additional management strategies. Among them, classical biological control programs, based on the search for effective natural enemies in the original area of the pest, are one of the most effective management approaches used against other invasive insects in Spanish citrus orchards (Soto *et al.* 1999, Garcia-Marí *et al.* 2004, Vercher *et al.* 2005, Jacas and Urbaneja 2010, García-Marí 2012). Herein we discuss the results of these basic studies focussing on *D. aberiae*.

Biology and seasonal trend

The behavior of *D. aberiae* in citrus is described first in this doctoral thesis. Results showed that *D. aberiae* density increases in spring and reached a maximum between May and June. At the end of

August, populations decreased and remain at very low levels for the rest of the year. A similar trend was observed in northern South Africa. However, *D. aberiae* density in South Africa's spring was lower than in eastern Spain, afterwards it increased considerably in summer and remained at very low levels in autumn and winter. The lower population levels in spring in northern South Africa are probably due to a combination of two factors. The first one would be the action of parasitism in autum that leads to lower winter population levels of the pest in South Africa than in Spain and may result in a slowly population increase in spring. The second one would be the application of heavy chemical treatments during flowering and fruit set period in the area (Grout 2015). The rapid decrease at the end of the summer observed in both countries, Spain and South Africa, may be a consequence of the combination of different biotic and abiotic factors, such as climate, the action of natural enemies or the quality of the feeding substrate (Bartlett and Clancy 1972, Furness 1976, Franco 1994, Soto *et al.* 2016a). Besides, *D. aberiae* completed several generations in eastern Spain. Two of those generations were clearly defined and resulted in high population levels. In agreement with these results, several overlapping generations may also be observed in northern South Africa, none but is clearly defined. Similar studies carried out with other mealybug pests affecting agronomic and ornamental plants, such as *Paracoccus burnerae*, *Phenacoccus madeirensis*, *Phenacoccus peruvianus*, *Planococcus citri* or *Pseudococcus viburni*, show a similar pattern with several, usually overlapping, generations throughout the year and high population densities in spring and summer (Panis 1986, Longo *et al.* 1995a, Martínez-Ferrer *et al.* 2003, Johnson and Giliomee 2012, Beltrà *et al.* 2013a).

Seasonal distribution

Herein we also studied the seasonal distribution of *D. aberiae* in citrus. Our results showed that this mealybug is mostly found in the tree canopy. Besides, within the canopy, the feeding organ of *D. aberiae* changed seasonally, with a preference for the developing fruit. Many mealybug species are phloem feeders and follow the movement of plant nutrients (McKenzie 1967), tending to migrate to the different strata of their hosts (Browning 1959, Furness 1976, Franco 1994, Geiger and Daane 2001, Grasswitz and James 2008, Cid *et al.* 2010, Haviland *et al.* 2012, Wunderlich *et al.* 2013). The developing citrus fruit is a strong carbohydrate sink (Agustí 2003, Iglesias *et al.* 2007), being the preferred feeding organ of many mealybug species because it provides very good food quality conditions for their development (Franco 1994, Haviland *et al.* 2012). Here we demonstrate that *D. aberiae* is no exception.

Apart from searching for food, mealybugs might migrate between organs to find protection against bad weather conditions and natural enemies (Gutierrez *et al.* 2008, Daane *et al.* 2012, Mani and Shivaraju 2016). From February to September *D. aberiae* was also present and active in the trunk and soil. Some studies mention that mealybugs might migrate and overwinter in the soil (Bodenheimer 1951, Rotundo *et al.* 1979, Franco *et al.* 2000). Our results show that *D. aberiae* is present and active in soil in spring and summer, not spending the cold months protected in this stratum. Furthermore, most mealybugs in the soil were found within a distance of 0 to 15 cm, horizontally from the base of the trunk, showing that *D. aberiae* is in this stratum because nymphs and adult females move upwards and downwards the trunk intentionally depending on the phenology of the plant and the climatic conditions (Browning 1959, Bartlett and Clancy 1972, Furness 1976, Franco *et al.* 2000, Martínez-Ferrer *et al.* 2003, Beltrà *et al.* 2013a).

Sampling techniques

Direct sampling of mealybugs involves the visual examination of plant material by searching and counting live insects in different plant strata. This methodology is quite laborious and time-consuming (Grimes and Cone 1985, Geiger and Daane 2001, Haviland *et al.* 2012, Beltrà *et al.* 2013a, Wunderlich *et al.* 2013, Shah *et al.* 2015). Thus, in recent years there is an increasing interest in developing alternative indirect sampling techniques, based mainly on the use of different trap designs (Goolsby *et al.* 2002, Millar *et al.* 2002, Walton *et al.* 2004, Roltsch *et al.* 2006, Cid *et al.* 2010, Waterworth *et al.* 2011, Bahder *et al.* 2013).

In this thesis, direct and indirect sampling techniques were studied and compared to determine the seasonal trend of *D. aberiae.* Our results showed that corrugated cardboard band traps and sticky traps may be considered as promising and feasible simple techniques to monitor *D. aberiae*. Corrugated traps were able to detect immature male instars and gravid females, provided a quantitative measurement of *D. aberiae* density and can be recommended to monitor population levels. Sticky traps baited with virgin females seemed to be effective to determine male flight periods. Therefore, identification and synthesis of the female sex pheromone seems to be a good strategy not only to monitor *D. aberiae* but also as a possible control approach to be tested. For now, our results are useful to improve the management of *D. aberiae*, as previously seen in other mealybug pests (Geiger and Daane 2001, Walton *et al.* 2004, Martínez-Ferrer *et al.* 2006, Mudavanhu *et al.* 2011, Waterworth *et al.* 2011, Bahder *et al.* 2013, Beltrà *et al.* 2013a, Flores *et al.* 2015).

Characterization and damage period to fruit

Regarding the characterization of damage, *D. aberiae* can cause different types of direct fruit distortions, mainly protuberances around

fruit calyx or size reduction. A large percentage of the distorted fruit loses its commercial value completely. Frequently, these distortions are related with toxic compounds present in the insect's saliva, being this the case of the mealybug *N. viridis* (Hattingh *et al.* 1998, Nechols 2003, Thomas and Leppla 2008, Abdul-Rassoul 2014). However, we cannot confirm if *D. aberiae* injects a toxic substance on its host, and further research is needed to determine this possibility.

For now, this research has shown that citrus fruit distortions appeared only when *D. aberiae* feeds on the ovary of the flower or on small tender fruits. For fruit exceeding 15-20 mm in diameter, the percentage of damaged fruit decreased considerably and no distortions were observed if the mealybug attacked fruit surpassing 25 or 30 mm in diameter (varieties 'Ortanique' and 'Clemenules', respectively). Growth and development of a citrus fruit follows a sigmoid curve, divided into three stages: phase I is characterized by cell division and slow growth, phase II by a huge increase in fruit size, due to cell enlargement, and phase III corresponds with the maturation period (Agustí 2003, Iglesias *et al.* 2007). Our results indicate that damage from *D. aberiae* is caused during phase I. Therefore, *D. aberiae* is able to interfere with the cell division process, distorting the affected area while the rest of the fruit continues growing normally. This finding is quite relevant to improve the moment of chemical applications and avoid unnecessary spraying until more sustainable management methods can be implemented.

Biological control

To date, no effective natural enemies against *D. aberiae* have been found in eastern Spain. Recent studies (Tena *et al.* 2017a, Tena *et al.* 2017b) have shown that native and naturalized parasitoids present in the Mediterranean Basin fail to control this mealybug. Besides, the existing predators, mainly *C. montrouzieri*, appear too late, that is to say when the damage to the fruit has already been done

(Soto *et al.* 2016a). Thus, the implementation of a classical biological control program involving the introduction of a natural enemy known to be effective against *D. aberiae* in its native area (northern South Africa) is the only sustainable option to control this mealybug.

Herein we described several primary parasitoids as natural enemies of *D. aberiae* on its native area (Limpopo, northern South Africa). Among these, *Anagyrus* sp. nov. 1 (Hymenoptera: Encyrtidae) was found as the most abundant species parasitizing *D. aberiae* in the citrus orchards. Besides, the specific interactions established between encyrtid parasitoids and mealybugs (Charles and Allan 2002, Charles 2011, Beltrà *et al.* 2013b, Bugila *et al.* 2015) lead us to consider this species as a good biological control agent to be introduced into Spain. On the other hand, the second most abundant parasitoid found was *Allotropa* sp. nov. Several *Allotropa* species have also been used in biological control programs against mealybugs in different parts of the world (Arai and Mishiro 2004, Masner *et al.* 2004, Sun *et al.* 2004, Roltsch *et al.* 2006, Roltsch *et al.* 2007, Reddy *et al.* 2009, Clarke *et al.* 2010, Malausa *et al.* 2016, Quaglietti *et al.* 2017a). Thus, both parasitoids, *Anagyrus* sp. nov. 1 and *Allotropa* sp. nov., may have a significant role in a classical biological control program against *D. aberiae* in Spain. For now, *Anagyrus* sp. nov. 1 should be considered as the most promising candidate species, as it shows higher parasitism rates in Limpopo citrus orchards (native area of the pest).

Finally, the overlap of *D. aberiae* developmental stages found in this research has relevant implications for *D. aberiae* management. Host stage can influence the efficacy of natural enemies, especially parasitoids, and should be taken into account when designing biological control strategies for *D. aberiae* in Spain (Islam and Copland 1997, Jervis *et al.* 2005, Beltrà *et al.* 2013a).

Overall discussion

Currently, as a newly invasive and practically unknown pest, the management of *D. aberiae* depends on the use of the available insecticides against mealybugs in Spain. Given the newest European Directive on the sustainable use of pesticides (2009/128/EC), the development of alternative sustainable management strategies for *D. aberiae* is needed. Herein we provide the first description in citrus of the biology, seasonal trend and distribution of *D. aberiae*. We also develop sampling techniques for this mealybug and provide information on the damage period to fruit. A better understanding of these factors is crucial to develop alternative management strategies, especially those based on biological control.

The biological, behavioral and control aspects analyzed in this thesis will allow for the establishment of an IPM program against *D. aberiae* in Spain, based on an early detection of the pest and the minimization of chemical applications. These factors will serve as the base for the application of a classical biological control program against *D. aberiae*. This information will be beneficial not only to improve the management of this mealybug in citrus in eastern Spain but also for other citrus production areas where the pest could be found in the future.

CHAPTER 8.
Conclusions

Chapter 8. Conclusions

Density and phenology of the invasive mealybug *Delottococcus aberiae* on citrus: implications for integrated pest management

i. *D. aberiae* density was high in spring and summer, peaked between May and June and remained at very low levels in autumn and winter.
ii. Different sampling methods showed that *D. aberiae* completes multiple generations each year, two of them being clearly defined and resulting in high population levels.
iii. Corrugated cardboard band traps provide a quantitative measurement of *D. aberiae* density and are recommended to monitor population levels.
iv. Sticky pheromone traps can be used to determine male flight periods.
v. These results are the first description of *D. aberiae* seasonal trends in citrus.

Seasonal movement and distribution of the invasive mealybug *Delottococcus aberiae* (Hemiptera: Pseudococcidae) in citrus: implications for its integrated management

i. Within the sampled strata (canopy, trunk, soil), *D. aberiae* was present mostly in the tree canopy.
ii. Within the tree canopy, the preferred feeding organ of *D. aberiae* changed throughout the year, showing a significant preference for the developing fruit.
iii. From February to September some mealybugs were found in the trunk and soil, moving upwards and downwards depending on the phenology of the plant and the climatic conditions.
iv. Mealybugs in soil were located within a distance of 0 to 15 cm horizontally from the base of the trunk.

v. *D. aberiae* does not overwinter in the soil but rather it is found dispersed on different organs of the tree canopy, mainly on twigs.
vi. Results may be used to facilitate an early detection of the pest and to adapt management strategies throughout the year.

Characterization and damage period to fruits caused by the invasive pest *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae)

i. *D. aberiae* causes different types of direct fruit damage, mainly protuberances around fruit calyx or size reduction. A large percentage of the distorted fruit loses its commercial value completely.
ii. *D. aberiae* can distort citrus fruit shape and/or size only when it feeds on the ovary of the flower or on small tender fruits.
iii. When the cell division stage finishes, within citrus fruit development, the fruit practically stops being susceptible to *D. aberiae* direct damage.
iv. No damage is observed when *D. aberiae* attacks fruits exceeding 25 mm in diameter for the variety 'Ortanique' and 30 mm for 'Clemenules'.
v. Knowledge of these results may help to determine the most appropiated moment for chemical applications until more sustainable management methods can be implemented.

Application of classical biological control to manage the new invasive citrus mealybug *Delottococcus aberiae* (Hemiptera: Pseudococcidae)

i. *D. aberiae* populations in its native area, South Africa, peaked in February (summer season there).

ii. The maximum percentage of parasitism was reached in June (autumn season in South Africa).

iii. A complex of parasitoids, with a high biodiversity of species, was found parasitizing *D. aberiae* in Limpopo (northern South Africa). Among the primary parasitoids identified, five belonged to the family Encyrtidae (*Anagyrus* sp. nov. 1, *Anagyrus* sp. nov. 2, *Anagyrus aurantifrons* Compere, *Anagyrus* sp. 3 and *Leptomastix dactylopii* Howard), one to Platygastridae (*Allotropa* sp. nov.) and one to *Aphelinidae* (*Thysanus* sp.). Two hyperparasitoids were detected; one belonged to the family Pteromalidae (*Pachyneuron* sp.) and the other to Encyrtidae (*Procheiloneurus aegyptiacus* Mercet).

iv. *Anagyrus* sp. nov. 1 (Hymenoptera: Encyrtidae) was the most abundant primary parasitoid of *D. aberiae*, followed by *Allotropa* sp. nov. (Hymenoptera: Platygastridae).

v. Both parasitoids, *Anagyrus* sp. nov. 1 and *Allotropa* sp. nov., may have a significant role in a classical biological control program against *D. aberiae* in Spain. For now, *Anagyrus* sp. nov. 1 is the most promising candidate as it showed higher parasitism rates.

CHAPTER 9.
References

Chapter 9. References

Abdul-Rassoul, M. S. 2014. Host Plants of the Mealybug *Nipaecoccus viridis* (Newstead, 1894) (Homoptera, Pseudococcidae) in Iraq with Detection of New Hosts. Adv Biores 5: 3-6.

Afifi, S. A. 1968. Morphology and taxonomy of the adult males of the families Pseudococcidae and Eriococcidae: (Homoptera: Coccoidea), Bull Br Mus (Nat Hist) Entomol, Suppl 13, London.

Agricola, U., Agounké, D., Fischer, H. U., and Moore, D. 2009. The control of *Rastrococcus invadens* Williams (Hemiptera: Pseudococcidae) in Togo by the introduction of *Gyranusoidea tebygi* Noyes (Hymenoptera: Encyrtidae). Bull Entomol Res 79: 671-678.

Agustí, M. 2003. Citricultura, Mundi-Prensa, Madrid, Spain.

Ahmed, M. Z., He, R.-R., Wu, M.-T., Gu, Y.-J., Ren, J.-M., Liang, F., Li, H.-L., Hu, X.-N., Qiu, B.-L., Mannion, C. M., and Ma, J. 2015. First Report of the Papaya Mealybug, *Paracoccus marginatus* (Hemiptera: Pseudococcidae), in China and Genetic Record for Its Recent Invasion in Asia and Africa. Fla Entomol 98: 1157-1162.

Amarasekare, K. G., Mannion, C. M., and Epsky, N. D. 2009. Efficiency and establishment of three introduced parasitoids of the mealybug *Paracoccus marginatus* (Hemiptera: Pseudococcidae). Biol Control 51: 91-95.

Amarasekare, K. G., Chong, J.-H., Epsky, N. D., and Mannion, C. M. 2008. Effect of Temperature on the Life History of the Mealybug *Paracoccus marginatus* (Hemiptera: Pseudococcidae). J Econ Entomol 101: 1798-1804.

Arai, T., and Mishiro, K. 2004. Development of *Allotropa citri* Muesebeck (Hymenoptera: Platygastridae) and *Anagyrus subalbipes* Ishii (Hymenoptera: Encyrtidae) on *Pseudococcus cryptus* Hempel (Homoptera: Pseudococcidae). Appl Entomol Zool 39: 505-510.

Bahder, B. W., Naidu, R. A., Daane, K. M., Millar, J. G., and Walsh, D. B. 2013. Pheromone-Based Monitoring of

Pseudococcus maritimus (Hemiptera: Pseudococcidae) Populations in Concord Grape Vineyards. J Econ Entomol 106: 482-490.

Bar-Zakay, I., Peleg, B. A., and Chen, C. 1987. Spherical mealybug infesting citrus in Israel. Alon Hanotea 41: 855-860.

Bartlett, B. R. 1978. Pseudococcidae, pp. 137-170. In C. P. Clausen (ed.), Introduced parasites and predators of arthropod pests and weeds: a world review. Agricultural Research Service, U.S. Dept. of Agriculture, Washington, D.C.

Bartlett, B. R., and Clancy, D. W. 1972. The Comstock Mealybug in California and Observations on Some of Its Natural Enemies. J Econ Entomol 65: 1329-1332.

Beardsley, J. W. 1960. A Preliminary Study of the Males of some Hawaiian Mealybugs (Homoptera: Pseudococcidae). Proc Hawaii Entomol Soc 16: 199-243.

Beltrà, A., and Soto, A. 2011. New records of mealybugs (Hemiptera: Pseudococcidae) from Spain. Phytoparasitica 39: 385-387.

Beltrà, A., and Soto, A. 2012. Pseudocóccidos de importancia agrícola y ornamental en España, Editorial Universitat Politècnica de València, Spain.

Beltrà, A., Soto, A., and Malausa, T. 2012. Molecular and morphological characterisation of Pseudococcidae surveyed on crops and ornamental plants in Spain. Bull Entomol Res 102: 165-172.

Beltrà, A., Garcia-Marí, F., and Soto, A. 2013a. Seasonal Phenology, Spatial Distribution, and Sampling Plan for the Invasive Mealybug *Phenacoccus peruvianus* (Hemiptera: Pseudococcidae). J Econ Entomol 106: 1486-1494.

Beltrà, A., Tena, A., and Soto, A. 2013b. Reproductive strategies and food sources used by *Acerophagus* n. sp. *near coccois*, a new successful parasitoid of the invasive mealybug *Phenacoccus peruvianus.* J Pest Sci 86: 253-259.

Beltrà, A., Garcia-Marí, F., and Soto, A. 2013c. El cotonet de Les Valls, *Delottococcus aberiae*, nueva plaga de los cítricos. Levante Agrícola 419: 348-352.

Beltrà, A., Tena, A., and Soto, A. 2013d. Fortuitous biological control of the invasive mealybug *Phenacoccus peruvianus* in Southern Europe. BioControl 58: 309-317.

Beltrà, A., Navarro-Campos, C., Calabuig, A., Estopa, L., Wackers, F. L., Pekas, A., and Soto, A. 2017. Association between ants (Hymenoptera: Formicidae) and the vine mealybug (Hemiptera: Pseudococcidae) in table-grape vineyards in Eastern Spain. Pest Manag Sci 73: 2473-2480.

Beltrà, A., Soto, A., Germain, J.-F., Matile-Ferrero, D., Mazzeo, G., Pellizzari, G., Russo, A., Franco, J. C., and Williams, D. J. 2010. The Bougainvillea mealybug *Phenacoccus peruvianus*, a rapid invader from South America to Europe. Entomol Hellenica 19: 137-143.

Beltrà, A., Addison, P., Ávalos, J. A., Crochard, D., Garcia-Marí, F., Guerrieri, E., Giliomee, J. H., Malausa, T., Navarro-Campos, C., Palero, F., and Soto, A. 2015. Guiding Classical Biological Control of an Invasive Mealybug Using Integrative Taxonomy. PLoS ONE 10: e0128685.

Bellard, C., Cassey, P., and Blackburn, T. M. 2016. Alien species as a driver of recent extinctions. Biol Lett 12: 20150623.

Ben-Dov, Y. 1994. A Systematic Catalogue of the Mealybugs of the World (Insecta: Homoptera: Coccoidea: Pseudococcidae and Putoidae), with Data on Geographical Distribution, Host Plants, Biology and Economic Importance, Intercept Limited, Andover, UK.

Ben-Dov, Y. 2005. Note: The Malvastrum Mealybug *Ferrisia malvastra* (Hemiptera: Coccoidea: Pseudococcidae): Distribution, Host Plants and Pest Status in Israel. Phytoparasitica 33: 154-156.

Berlinger, M. J., and Gol'Berg, A. M. 1978. The effect of the fruit sepals on the citrus mealybug population and on its parasite. Entomol Exp Appl 24: 38-43.

Binns, M. R., and Nyrop, J. P. 1992. Sampling insect populations for the purpose of IPM decision making. Annu Rev Entomol 37: 427-453.

Blumberg, D., Klein, M., and Mendel, Z. 1995. Response by encapsulation of four mealybug species (Homoptera: Pseudococcidae) to parasitization by *Anagyrus pseudococci*. Phytoparasitica 23: 157-163.

Blumberg, D., Ben-Dov, Y., and Mendel, Z. 1999. The citrulus mealybug, *Pseudococcus cryptus* Hempel, and its natural enemies in Israel: history and present situation. Entomologica 33: 141-152.

Boavida, C., Neuenschwander, P., and Schulthess, F. 1992. Spatial distribution of *Rastrococcus invaden*s Williams (Hom., Pseudococcidae) in mango trees. J Appl Entomol 114: 381-391.

Bodenheimer, F. S. 1951. Citrus Entomology in the Middle East: with special references to Egypt, Iran, Irak, Palestine, Syria, Turkey., Dr W. Junk, The Hague, Netherlands.

Bográn, C. E., Heinz, K. M., and Ciomperlik, M. A. 2002. Interspecific competition among insect parasitoids: field experiments with whiteflies as hosts in cotton. Ecology 83: 653-668.

Bokonon-Ganta, A. H., de Groote , H., and Neuenschwander, P. 2002. Socio-economic impact of biological control of mango mealybug in Benin. Agric Ecosyst Environ 93: 367-378.

Boyce, A. M., and Korsmeier, R. B. 1941. The Citrus Bud Mite, *Eriophyes sheldoni* Ewing. J Econ Entomol 34: 745-756.

Brain, C. K. 1915. The Coccidae of South Africa-I. Trans R Soc S Afr 5: 65-194.

Browning, T. O. 1959. The long-tailed mealybug, *Pseudocuccus aonidum* (L.), in South Australia. J Agric Res 10: 322-339.

Bugila, A. A. A., Branco, M., Silva, E. B. d., and Franco, J. C. 2014. Host selection behaviour and specificity of the solitary parasitoid of mealybugs *Anagyrus* sp. nr. *pseudococci* (Girault) (Hymenoptera, Encyrtidae). Biocontrol Sci Technol 24: 22-38.

Bugila, A. A. A., Franco, J. C., Silva, E. B. d., and Branco, M. 2015. Suitability of five mealybug species (Hemiptera, Pseudococcidae) as hosts for the solitary parasitoid *Anagyrus* sp. nr. *pseudococci* (Girault) (Hymenoptera: Encyrtidae). Biocontrol Sci Technol 25: 108-120.

Buhl, P. N. 2005. A new species of *Allotropa*, a parasitoid of Pseudococcidae (Hemiptera) in banana on the canary Islands (Hymenoptera, Platygastridae). Ent Meddr 73: 67-69.

Calatayud, P. A., Polanía, M. A., Seligmann, C. D., and Bellotti, A. C. 2002. Influence of water-stressed cassava on *Phenacoccus herreni* and three associated parasitoids. Entomol Exp Appl 102: 163-175.

Campos-Rivela, J. M. 2008. Estudios comparativos de algunos aspectos de la biología de dos parasitoides: *Anagyrus pseudococci* (Girault) y *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae), para la mejora de los cítricos *Planococcus citri* Risso (Hemiptera: Pseudococcidae). Dissertation, Universitat Jaume I Castellón, Spain.

Campos, J. M., and Martínez-Ferrer, M. T. 2003. Host and parasitoid densities influence on progeny and sex ratio of *Anagyrus pseudococci* (Girault) and *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae); two *Planococcus citri* (Risso) (Homoptera: Pseudococcidae) parasitoids. Integrated Control in Citrus Fruit Crops. IOBC-WPRS Bull 26: 139-147.

Campos, J. M., Martínez-Ferrer, M. T., and Forés, V. 2006. Parasitism disruption by ants of *Anagyrus pseudococci* (Girault) and *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae), two parasitoids of the citrus mealybug *Planococcus citri* (Risso) (Homoptera: Pseudococcidae). Integrated Control in Citrus Fruit Crops. IOBC-WPRS Bull 29: 33-47.

Carrera-Martínez, R., Aponte-Díaz, L., Ruiz-Arocho, J., and Jenkins, D. A. 2015. Symptomatology of Infestation by *Hypogeococcus pungens*: Contrasts between Host Species. Haseltonia 21: 14-18.

Cid, M., Pereira, S., Cabaleiro, C., and Segura, A. 2010. Citrus Mealybug (Hemiptera: Pseudococcidae) Movement and Population Dynamics in an Arbor-Trained Vineyard. J Econ Entomol 103: 619-630.

Cid, M., Pereira, S., Cabaleiro, C., Faoro, F., and Segura, A. 2007. Presence of Grapevine leafroll-associated virus 3 in primary salivary glands of the mealybug vector *Planococcus citri*

suggests a circulative transmission mechanism. Eur J Plant Pathol 118: 23-30.

Clancy, D. W. 1944. Biology of *Allotropa burrelli*, a gregarious parasite of *Pseudococcus comstocki*. J Agric Res 69: 159-167.

Clarke, S. R., Debarr, G. L., and Berisford, C. W. 1990. Life History of *Oracella acuta* (Homoptera: Pseudococcidae) in Loblolly Pine Seed Orchards in Georgia. Environ Entomol 19: 99-103.

Clarke, S. R., Yu, H.-B., Chen, M.-R., DeBarr, G. L., and Sun, J.-H. 2010. Classical biological control program for the mealybug *Oracella acuta* in Guangdong Province, China. Insect Sci 17: 129-139.

Clausen, C. P. 1915. Mealybugs of citrus trees. Bull Univ Calif Agric Exp Stn 258: 19-48.

Cocco, A., Lentini, A., and Serra, G. 2014. Mating disruption of *Planococcus ficus* (Hemiptera: Pseudococcidae) in vineyards using reservoir pheromone dispensers. J Insect Sci 14: 1-8.

Compere, H. 1939a. Mealybugs and Their Insect Enemies in South America. Univ Calif Publ Entomol 7: 57-73.

Compere, H. 1939b. A second report on some miscellaneous African Encyrtidae in the British Museum. Bull Br Mus (Nat Hist) Entomol 30.

Correa, M. C. G., Germain, J. F., Malausa, T., and Zaviezo, T. 2012. Molecular and morphological characterization of mealybugs (Hemiptera: Pseudococcidae) from Chilean vineyards. Bull Entomol Res 102: 524-530.

Cox, J. M. 1983. An experimental-study of morphological variation in mealybugs (Homoptera, Coccoidea, Pseudococcidae). Syst Entomol 8: 361-382.

Cox, J. M. 1987. Pseudococcidae (Insecta: Hemiptera). Fauna of New Zealand, vol. 11, Science Information Publishing Centre, DSIR, Wellington, New Zealand.

Cox, J. M., and Ben-Dov, Y. 1986. Planococcine mealybugs of economic importance from the Mediterranean Basin and their distinction from a new African genus (Hemiptera: Pseudococcidae). Bull Entomol Res 76: 481-489.

Culik, M. P., Fornazier, M. J., dos Santos Martins, D., Zanuncio J.S., Ventura, J. A., Peronti, A. L. B. G., and Zanuncio, J. C. 2013. The invasive mealybug *Maconellicoccus hirsutus*: lessons for its current range expansion in South America and invasive pest management in general. J Pest Sci 86: 387-398.

Charles, J. G. 2011. Using parasitoids to infer a native range for the obscure mealybug, *Pseudococcus viburni*, in South America. BioControl 56: 155-161.

Charles, J. G., and Allan, D. J. 2002. An ecological perspective to host-specificity testing of biocontrol agents. N Z Plant Prot 55: 37-41.

Charles, J. G., Froud, K. J., and Henderson, R. C. 2000. Morphological variation and mating compatibility within the mealybugs *Pseudococcus calceolariae* and *P. similans* (Hemiptera: Pseudococcidae), and a new synonymy. Syst Entomol 25: 285-294.

Chen, H. Y., Liu, J. X., and Xu, Z. F. 2011. Description of a new platygastrid parasitoid *Allotropa phenacocca* (Hymenoptera) on *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae). Orient Insects 45: 275-280.

Chong, J.-H., Roda, A. L., and Mannion, C. M. 2008. Life History of the Mealybug, *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae), at Constant Temperatures. Environ Entomol 37: 323-332.

Chong, J. H., and Oetting, R. D. 2006. Influence of Temperature, Nourishment, and Storage Period on the Longevity and Fecundity of the Mealybug Parasitoid, *Anagyrus* sp. nov. nr. *sinope* Noyes and Menezes (Hymenoptera: Encyrtidae). Environ Entomol 35: 1198-1207.

Chong, J. H., Aristizabal, L. F., and Arthurs, S. P. 2015. Biology and Management of *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae) on Ornamental Plants. J Integr Pest Manag 6: 1-14.

Daane, K. M., Bentley, W. J., Walton, V. M., Malakar-Kuenen, R., Millar, J. G., Ingels, C., Weber, E., and Gispert, C. 2006.

New controls investigated for vine mealybug. Calif Agric 60: 31-38.

Daane, K. M., Middleton, M. C., Sforza, R., Cooper, M. L., Walton, V. M., Walsh, D. B., Zaviezo, T., and Almeida, R. P. 2011. Development of a multiplex PCR for identification of vineyard mealybugs. Environ Entomol 40: 1595-1603.

Daane, K. M., Almeida, R. P. P., Bell, V. A., Walker, J. T. S., Botton, M., Fallahzadeh, M., Mani, M., Miano, J. L., Sforza, R., Walton, V. M., and Zaviezo, T. 2012. Biology and Management of Mealybugs in Vineyards, pp. 271-307. In N. J. Bostanian, C. Vincent and R. Isaacs (eds.), Arthropod Management in Vineyards: Pests, Approaches, and Future Directions. Springer Netherlands, Dordrecht.

Davies, A. P., Ceballo, F. A., and Walter, G. H. 2004. Is the potential of *Coccidoxenoides perminutus*, a mealybug parasitoid, limited by climatic or nutritional factors? Biol Control 31: 181-188.

De Lotto, G. 1961. New Pseudococcidae (Homoptera: Coccoidea) from Africa. Bull Br Mus (Nat Hist) Entomol 10: 211-238.

De Villiers, M., and Pringle, K. L. 2007. Seasonal occurrence of vine pests in commercially treated vineyards in the Hex River Valley in the Western Cape Province, South Africa. Afr Entomol 15: 241-260.

DeBach, P. 1949. Population studies of the long-tailed mealybug and its natural enemies on citrus trees in Southern California. Ecology 30: 14-25.

Douglas, A. E. 2009. Honeydew, pp. 461-463. In V. H. Resh and R. T. Cardé (eds.), Encyclopedia of Insects, 2nd ed. Elsevier, San Diego, CA, USA.

El-Sayed, A. M., Unelius, C. R., Twidle, A., Mitchell, V., Manning, L.-A., Cole, L., Suckling, D. M., Flores, M. F., Zaviezo, T., and Bergmann, J. 2010. Chrysanthemyl 2-acetoxy-3-methylbutanoate: the sex pheromone of the citrophilous mealybug, *Pseudococcus calceolariae*. Tetrahedron Lett 51: 1075-1078.

European Parliament and Council. 2009. Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. Official Journal of the European Union 309: 71-86.

Ezzat, Y. M., and McConnell, H. S. 1956. A Classification of the Mealybug Tribe Planococcini: (Pseudococcidae, Homoptera), vol. 84, College Park, Md, University of Maryland, Agricultural Experiment Station.

Flores, M. F., Romero, A., Oyarzun, M. S., Bergmann, J., and Zaviezo, T. 2015. Monitoring *Pseudococcus calceolariae* (Hemiptera: Pseudococcidae) in Fruit Crops Using Pheromone-Baited Traps. J Econ Entomol 108: 2397-2406.

Foldi, I. 2000. Diversité et modification des peuplements de cochenilles des Isles d'Hyères en milieux naturels et anthropisés (Hemiptera: Coccoidea). Ann Soc Entomol Fr 36: 75-94.

Franco, J. 1994. Citrus Phenology as a Basis to Study the Population Dynamics of the Citrus Mealybug Complex in Portugal, pp. 929-930. In E. Tribulato, A. Gentile and G. Reforgiato (eds.), Proc Int Soc Citric, vol. 3.

Franco, J. C., Silva, E. B., and Carvalho, J. P. 2000. Cochonilhas-algodão (Hemiptera, Pseudococcidae) associadas aos citrinos em Portugal, ISA Press, Lisboa, Portugal.

Franco, J. C., Zada, A., and Mendel, Z. 2009. Novel Approaches for the Management of Mealybug Pests, pp. 233-278. In I. Ishaaya and A. R. Horowitz (eds.), Biorational control of arthropod pests: application and resistance managements. Springer, Netherlands.

Franco, J. C., Russo, A., Suma, P., Silva, E., and Mendel, Z. 2001. Monitoring strategies of the citrus mealybug, *Planococcus citri*, in citrus groves. Boll Zool agr Bachic 33: 297-303.

Franco, J. C., Suma, P., Silva, E. B., Blumberg, D., and Mendel, Z. 2004a. Management Strategies of Mealybug Pests of Citrus in Mediterranean Countries. Phytoparasitica 32: 507-522.

Franco, J. C., Gross, S., Silva, E. B., Suma, P., Russo, A., and Mendel, Z. 2004b. Is mass-trapping a feasible management

tactic of the citrus mealybug in citrus orchards? An Inst Sup Agron 49: 353-367.

Franco, J. C., Silva, E. B., Cortegano, E., Campos, L., Branco, M., Zada, A., and Mendel, Z. 2008. Kairomonal response of the parasitoid *Anagyrus* spec. nov. near *pseudococci* to the sex pheromone of the vine mealybug. Entomol Exp Appl 126: 122-130.

Furness, G. 1976. The Dispersal, Age-structure and Natural Enemies of the Long-tailed Mealybug, *Pseudocccus longispinus* (Targioni-Tozzetti), in Relation to Sampling and Control. Aust J Zool 24: 237-247.

Garcia-Marí, F., Vercher, R., Costa-Comelles, J., Marzal, C., and Villalba, M. 2004. Establishment of *Citrostichus phyllocnistoides* (Hymenoptera: Eulophidae) as a biological control agent for the citrus leafminer *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) in Spain. Biol Control 29: 215-226.

García-Marí, F. 2012. Plagas de los cítricos. Gestión integrada en países de clima mediterráneo, Phytoma, Spain.

García-Morales, M., Denno, B., Miller, D., Miller, G., Ben-Dov, Y., and Hardy, N. 2016a. ScaleNet: A literature-based model of scale insect biology and systematics. Database. doi: 10.1093/database/bav118. http://scalenet.info. Accessed 12-01-18

García-Morales, M., Denno, B. D., Miller, D. R., Miller, G. L., Ben-Dov, Y., and Hardy, N. B. 2016b. ScaleNet: a literature-based model of scale insect biology and systematics. Database (Oxford) 2016: 1-5.

Geiger, C. A., and Daane, K. M. 2001. Seasonal Movement and Distribution of the Grape Mealybug (Homoptera: Pseudococcidae): Developing a Sampling Program for San Joaquin Valley Vineyards. J Econ Entomol 94: 291-301.

Goldasteh, S., Talebi, A. A., Fathipour, Y., Ostovan, H., Zamani, A., and Shoushtari, R. V. 2009. Effect of temperature on life history and population growth parameters of *Planococcus citri* (Homoptera, Pseudococcidae) on Coleus [*Solenostemon scutellarioides* (L.) Codd.]. Arch Biol Sci 61: 329-336.

Gómez-Clemente, F. 1928. Aclimatación en España del *Cryptolaemus montrouzieri* Muls., para combatir el cotonet del naranjo. Bol Patol Veg Ent Agr 18: 106-123.

González-Hernández, H., Pandey, R. R., and Johnson, M. W. 2005. Biological characteristics of adult *Anagyrus ananatis* Gahan (Hymenoptera: Encyrtidae), a parasitoid of *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae). Biol Control 35: 93-103.

Gonzalez, D. 1971. Sampling as a basis for pest management strategies., pp. 83-101. In E. V. Komarek (ed.), Proc Tall Timbers Conf Ecol Anim Control Habitat Manag, vol. 2.

Goolsby, J., Kirk, A., and Meyerdirk, D. E. 2002. Seasonal phenology and natural enemies of *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae) in Australia. Fla Entomol 85: 494-498.

Grasswitz, T. R., and James, D. G. 2008. Movement of grape mealybug, *Pseudococcus maritimus*, on and between host plants. Entomol Exp Appl 129: 268-275.

Grimes, E., and Cone, W. 1985. Life History, Sex Attraction, Mating and Natural Enemies of the Grape Mealybug, *Pseudococcus maritimus* (Homoptera: Pseudococcidae). Ann Entomol Soc Am 78: 554-558.

Grout, T. G. 2015. The status of citrus IPM in South Africa. Acta Hortic 1065: 1091-1095.

Grout, T. G., and Richards, G. I. 1991. Value of pheromone traps for predicting infectations of red scale *Aonidiella aurantii* (Maskell) (Hom., Diaspididae), limited by natural enemy activity and insecticides used to control citrus thrips, *Scirtothrips aurantii* Faure (Thys., Thripidae). J Appl Entomol 111: 20-27.

Gullan, P. J. 2000. Identification of the immature instars of mealybugs (Hemiptera: Pseudococcidae) found on citrus in Australia. Austr J Entomol 39: 160-166.

Gullan, P. J., and Kosztarab, M. 1997. Adaptations in scale insects. Annu Rev Entomol 42: 23-50.

Gullan, P. J., and Martin, J. 2009. Sternorrhyncha (jumping plant-lice, whiteflies, aphids, and scale insects), pp. 957-967. In V. H. Resh and R. T. Cardé (eds.), Encyclopedia of Insects, 2nd ed. Elsevier, San Diego, CA, USA.

Gurr, G. M., Wratten, S. D., Tylianakis, J., Kean, J., and Keller, M. 2005. Providing plant foods for natural enemies in farming systems: balancing practicalities and theory, pp. 326-347. In F. L. Wäckers, J. Bruin and P. C. J. van Rijn (eds.), Plant-Provided Food for Carnivorous Insects: A Protective Mutualism and its Applications. Cambridge University Press, Cambridge.

Gutierrez, A. P., Daane, K. M., Ponti, L., Walton, V. M., and Ellis, C. K. 2008. Prospective evaluation of the biological control of vine mealybug: refuge effects and climate. J Appl Ecol 45: 524-536.

Hall, D. G., Roda, A., Lapointe, S. L., and Hibbard, K. 2008. Phenology of *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae) in Florida based on attraction of adult males to pheromone traps. Fla Entomol 91: 305-310.

Han, S. H., Ndiaye, A. B., and Hemptinne, J. L. 2007. Host-plants and predators of *Rastrococcus invadens* Williams, 1986, newly introduced in Dakar area, Senegal (Hemiptera, Pseudococcidae). Bull Soc Entomol Fr 112: 121-125.

Hardy, N. B., Gullan, P. J., and Hodgson, C. J. 2008. A subfamily-level classification of mealybugs (Hemiptera: Pseudococcidae) based on integrated molecular and morphological data. Syst Entomol 33: 51-71.

Hattingh, V., Cilliers, C., and Bedford, E. 1998. Citrus mealybugs, pp. 112–120. In E. Bedford, M. Van den Berg and E. De Villiers (eds.), Citrus Pests in the Republic of South Africa. ARC - ITSC, South Africa.

Haviland, D. R., Beede, R. H., and Daane, K. M. 2012. Seasonal Phenology of *Ferrisia gilli* (Hemiptera: Pseudococcidae) in Commercial Pistachios. J Econ Entomol 105: 1681-1687.

Haviland, D. R., Beede, R. H., and Daane, K. M. 2015. Crop Loss Relationships and Economic Injury Levels for *Ferrisia gilli*

(Hemiptera: Pseudococcidae) Infesting Pistachio in California. J Econ Entomol 108: 2683-2690.

Hefetz, A., and Tauber, O. 1990. Male response to the synthetic sex pheromone of *Planococcus citri* (Risso) (Hom., Pseudococcidae) and its application for population monitoring. J Appl Entomol 109: 502-506.

Heimpel, G. E., and Jervis, M. A. 2005. Does floral nectar improve biological control by parasitoids?, pp. 267-304. In F. L. Wäckers, J. Bruin and P. C. J. van Rijn (eds.), Plant-Provided Food for Carnivorous Insects: A Protective Mutualism and its Applications. Cambridge University Press, Cambridge.

Herren, H. R., and Neuenschwander, P. 1991. Biological Control of Cassava Pests in Africa. Annu Rev Entomol 36: 257-283.

Hill, W. B., and Burts, E. C. 1982. Grape Mealybug (Homoptera: Pseudococcidae) on Pear in North-Central Washington. J Econ Entomol 75: 501-503.

Hodgson, C., Abbas, G., Arif, M. J., Saeed, S., and Karar, H. 2008. *Phenacoccus solenopsis* Tinsley (Sternorrhyncha: Coccoidea: Pseudococcidae), an invasive mealybug damaging cotton in Pakistan and India, with a discussion on seasonal morphological variation. Zootaxa 1913: 1-35.

Hogendorp, B. k., Cloyd, R. A., and Swiader, J. M. 2006. Effect of Nitrogen Fertility on Reproduction and Development of Citrus Mealybug, *Planococcus citri* Risso (Homoptera: Pseudococcidae), Feeding on Two Colors of Coleus, *Solenostemon scutellarioides* L. Codd. Environ Entomol 35: 201-211.

Hulme, P. E. 2009. Trade, transport and trouble: managing invasive species pathways in an era of globalization. J Appl Entomol 46: 10-18.

Hulme, P. E., Bacher, S., Kenis, M., Klotz, S., Kühn, I., Minchin, D., Nentwig, W., Olenin, S., Panov, V., Pergl, J., Pyšek, P., Roques, A., Sol, D., Solarz, W., and Vilà, M. 2008. Grasping at the routes of biological invasions: a framework for integrating pathways into policy. J Appl Ecol 45: 403-414.

Hwang, J. S., and Chu, Y. I. 1987. The development of sex pheromone traps for the citrus mealybug, *Planococcus citri* (Risso). Plant Prot Bull Taiwan 29: 297-305.

Iglesias, D. J., Cercós, M., Colmenero-Flores, J. M., Naranjo, M. A., Ríos, G., Carrera, E., Ruiz-Rivero, O., Lliso, I., Morillon, R., Tadeo, F. R., and Talon, M. 2007. Physiology of citrus fruiting. Braz J Plant Physiol 19: 333-362.

Iperti, G. 1999. Biodiversity of predaceous coccinellidae in relation to bioindication and economic importance. Agric Ecosyst Environ 74: 323-342.

Islam, K. S., and Copland, M. J. W. 1997. Host Preference and Progeny Sex Ratio in a Solitary Koinobiont Mealybug Endoparasitoid, *Anagyrus pseudococci* (Girault), in Response to its Host Stage. Biocontrol Sci Technol 7: 449-456.

IVIA, I. V. d. I. A. 2011. Riegos IVIA. http://riegos.ivia.es/datos-meteorologicos. Accessed 09-03-2018

Jacas, J.-A., Urbaneja, A., and Viñuela, E. 2006. History and Future of Introduction of Exotic Arthropod Biological Control Agents in Spain: A Dilemma? BioControl 51: 1-30.

Jacas, J. A., and Urbaneja, A. 2010. Biological Control in Citrus in Spain: From Classical to Conservation Biological Control, pp. 61-72. In A. Ciancio and K. G. Mukerji (eds.), Integrated Management of Arthropod Pests and Insect Borne Diseases. Springer Netherlands, Dordrecht.

Jervis, M. A., Copland, M. J. W., and Harvey, J. A. 2005. The lyfe-cycle, pp. 73-165. In M. A. Jervis (ed.), Insect as Natural Enemies: A Practical Perspective. Springer, Dordrecht, Netherlands.

Johnson, T., and Giliomee, J. H. 2012. Seasonal Phenology and Natural Enemies of the Oleander Mealybug, *Paracoccus burnerae* (Brain) (Hemiptera: Pseudococcidae), in South Africa. Afr Entomol 20: 1-7.

Kairo, M. T. K., Pollard, G. V., Peterkin, D. D., and Lopez, V. F. 2000. Biological Control of the Hibiscus Mealybug, *Maconellicoccus hirsutus* Green (Hemiptera: Pseudococcidae) in the Caribbean. Integrated Pest Manag Rev 5: 241-254.

Kasuya, E. 2004. Angular transformation – another effect of different sample sizes. Ecol Res 19: 165-167.

Katsoyannos, P. 1996. Integrated Insect Pest Management for Citrus in Northern Mediterranean Countries, Benaki Phytopathological Institute, Athens, Greece.

Kaydan, M. B., Kozár, F., and Hodgson, C. 2015. A review of the phylogeny of Palaearctic mealybugs (Hemiptera: Coccomorpha: Pseudococcidae). Arthropod Syst Phylogeny 73: 175-195.

Kenis, M., Auger-Rozenberg, M.-A., Roques, A., Timms, L., Péré, C., Cock, M. J. W., Settele, J., Augustin, S., and Lopez-Vaamonde, C. 2009. Ecological effects of invasive alien insects, pp. 21-45. In D. W. Langor and J. Sweeney (eds.), Ecological Impacts of Non-Native Invertebrates and Fungi on Terrestrial Ecosystems. Springer Netherlands.

Kogan, M. 1998. Integrated Pest Management: Historical Perspectives and Contemporary Developments. Annu Rev Entomol 43: 243-270.

Kosztarab, M., and Kozár, F. 1988. Scale Insects of Central Europe, Dr W. Junk Publishers, Dordrecht, Netherlands

Kozár, F. 1989. Microhabitat specialization and similarity of scale-insect assemblages on different fruit trees and in different countries. Ecol Entomol 14: 175-180.

Kukovinets, O. S., Zvereva, T. I., Kasradze, V. G., Galin, F. Z., Frolova, L. L., Kuchin, A. V., Spirikhin, L. V., and Abdullin, M. I. 2006. Novel synthesis of *Planococcus citri* pheromone. Chem Nat Compd 42: 216-218.

Kumar, R., Nagrare, V. S., Nitharwal, M., Swami, D., and Prasad, Y. G. 2014. Within-plant distribution of an invasive mealybug, *Phenacoccus solenopsis*, and associated losses in cotton. Phytoparasitica 42: 311-316.

Kumar, S., Sidhu, J. K., Hamm, J. C., Kular, J. S., and Mahal, M. S. 2013. Effects of Temperature and Relative Humidity on the Life Table of *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) on Cotton. Fla Entomol 96: 19-28.

Lacirignola, C., and D'Onghia, A. M. 2009. The Mediterranean citriculture: productions and perspectives, pp. 13-17. In A. M.

D'Onghia, K. Djelouah and C. N. Roistacher (eds.), Citrus tristeza virus and *Toxoptera citricidus*: a serious threat to the Mediterranean citrus industry, Bari: CIHEAM.

Landis, D. A., Wratten, S. D., and Gurr, G. M. 2000. Habitat management to conserve natural enemies of arthropod pests in agriculture. Annu Rev Entomol 45: 175-201.

Langone, D. J. 2013. Efficacy of pheromone mating disruption for vine mealybug control. Dissertation, Jordan College of Agricultural Sciences and Technology California State University, Fresno.

Le Rü, B., and Iziquel, Y. 1990. Étude expérimentale, à l'aide d'un simulateur de pluies, de l'effet mécanique de la chute des pluies sur les populations de la cochenille du manioc, *Phenacoccus manihoti*. Acta Ecol 11: 741-754.

Levi-Zada, A., Fefer, D., David, M., Eliyahu, M., Franco, J. C., Protasov, A., Dunkelblum, E., and Mendel, Z. 2014. Diel periodicity of pheromone release by females of *Planococcus citri* and *Planococcus ficus* and the temporal flight activity of their conspecific males. Die Naturwissenschaften 101: 671-678.

Löhr, B., Varela, A. M., and Santos, B. 1991. Lifetable of *Allotropa* sp. (Hym., Platygasteridae), parasitoid of the cassava mealybug, *Phenacoccus manihoti* (Hom.: Pseudococcidae). J Plant Dis Prot 98: 351-357.

Longo, S., Mazzeo, G., and Russo, A. 1995a. Biological observations on some scale insects (Homoptera: Coccoidea) in Sicily. Isr J Entomol 29: 219-222.

Longo, S., Marotta, S., Pellizzari, G., Russo, A., and A, T. 1995b. An annotated list of the scale insects (Homoptera: Coccoidea) of Italy. Isr J Entomol 29: 113-130.

Lucas, A. 2002. Comportamiento de melazo (*Pseudococcus citri* Risso) en uva de mesa en la región de Murcia: alternativas de control biológico. Phytoma 138: 28-36.

Llorens, J. M. 1994. Reflexiones acercade las cochinillas en el contexto del control biologico de los citricos. Phytoma 58: 73-77.

Lloyd, D. C. 1952. Parthenogenesis in the Mealybug, *Phenacoccus solani* Ferris. Can Entomol 84: 308-310.

MacDonald, G. K., Brauman, K. A., Sun, S., Carlson, K. M., Cassidy, E. S., Gerber, J. S., and West, P. C. 2015. Rethinking Agricultural Trade Relationships in an Era of Globalization. BioScience 65: 275-289.

Malausa, T., Fenis, A., Warot, S., Germain, J. F., Ris, N., Prado, E., Botton, M., Vanlerberghe-Masutti, F., Sforza, R., Cruaud, C., Couloux, A., and Kreiter, P. 2011. DNA markers to disentangle complexes of cryptic taxa in mealybugs (Hemiptera: Pseudococcidae). J Appl Ecol 135: 142-155.

Malausa, T., Delaunay, M., Fleisch, A., Groussier-Bout, G., Warot, S., Crochard, D., Guerrieri, E., Delvare, G., Pellizzari, G., Kaydan, M. B., Al-Khateeb, N., Germain, J. F., Brancaccio, L., Le Goff, I., Bessac, M., Ris, N., and Kreiter, P. 2016. Investigating Biological Control Agents for Controlling Invasive Populations of the Mealybug *Pseudococcus comstocki* in France. PloS oNE 11: e0157965.

Mani, M., and Shivaraju, C. 2016. Mealybugs and their Management in Agricultural and Horticultural crops, Springer, New Delhi.

Mansour, R., Suma, P., Mazzeo, G., Grissa Lebdi, K., and Russo, A. 2011. Evaluating side effects of newer insecticides on the vine mealybug parasitoid *Anagyrus* sp. *near pseudococc*i, with implications for integrated pest management in vineyards. Phytoparasitica 39: 369-376.

Mansour, R., Grissa-Lebdi, K., Suma, P., Mazzeo, G., and Russo, A. 2017a. Key scale insects (Hemiptera: Coccoidea) of high economic importance in a Mediterranean area: host plants, bio-ecological characteristics, natural enemies and pest management strategies – a review. Plant Prot Sci 53: 1-14.

Mansour, R., Grissa-Lebdi, K., Khemakhem, M., Chaari, I., Trabelsi, I., Sabri, A., and Marti, S. 2017b. Pheromone-mediated mating disruption of *Planococcus ficus* (Hemiptera: Pseudococcidae) in Tunisian vineyards: Effect on insect population dynamics. Biologia 72: 333-341.

Marras, P. M., Sanna, F., and Pantaleoni, R. A. 2008. Influence of ant-exclusion on *Planococcus citri* density in a citrus orchard. Integrated Control in Citrus Fruit Crops. IOBC-WPRS Bull 38: 104-110.

Martínez-Blay, V., Benito, M., and Soto, A. 2018a. Characterization and damage period to fruits caused by the invasive pest *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae). Integrated Control in Citrus Fruit Crops. IOBC-WPRS Bull (in press).

Martínez-Blay, V., Pérez-Rodríguez, J., Tena, A., and Soto, A. 2018b. Density and phenology of the invasive mealybug *Delottococcus aberiae* on citrus: implications for integrated pest management. J Pest Sci 91: 625-637.

Martínez-Ferrer, M. T., García-Marí, F., and Ripollés, J. L. 2003. Population dynamics of *Planococcus citri* (Risso) (Homoptera: Pseudococcidae) in citrus groves in Spain. Integrated Control in Citrus Fruit Crops. IOBC-WPRS Bull 26: 149-161.

Martínez-Ferrer, M. T., Ripollés, J. L., and Garcia-Marí, F. 2006. Enumerative and Binomial Sampling Plans for Citrus Mealybug (Homoptera: Pseudococcidae) in Citrus Groves. J Econ Entomol 99: 993-1001.

Martínez-Ferrer, M. T., Ripollés, J. L., and Garcia-Marí, F. 2008. Treatment thresholds for the Citrus Mealybug *Planococcus citri* (Hemiptera: Pseudococcidae) based on the relationship between male's abundance and fruit infestation. Integrated Control in Citrus Fruit Crops. IOBC-WPRS Bull 38: 117-123.

Masner, L., and Huggert, L. 1989. World Review and Keys to Genera of the Subfamily Inostemmatinae with Reassignment of the Taxa to the Platygastrinae and Sceliotrachelinae (Hymenoptera: Platygastridae). Mem Entomol Soc Can 121: 3-216.

Masner, L., Jianghua, S., Clarke, S. R., and Berisford, C. W. 2004. Description of *Allotropa oracellae* (Hymenoptera: Platygastridae), a parasitoid of *Oracella acuta* (Heteroptera: Pseudococcidae). Fla Entomol 87: 600-602.

Matile-Ferrero, D. 1983. *Allococcus euphorbiae* (Ezzat & McConnell) nouvel ennemi du gèranium dans le midi de la France. L'Entomologiste 39: 253-255.

Matile-Ferrero, D., Etienne, J., and Tiego, G. 2000. Introduction of two important pests to French Guiana: *Maconellicoccus hirsutus* and *Paracoccus marginatus* (Hem., Coccoidea, Pseudococcidae). Bull Soc Entomol Fr 105: 89-104.

Mazzeo, G., Russo, A., and Suma, P. 1999. *Phenacoccus solani* Ferris (Homoptera: Coccoidea) on ornamental plants in Italy. Boll Zool agr Bachic 31: 31-35.

Mazzeo, G., Longo, S., Pellizzari, G., Porcelli, F., Suma, P., and Russo, A. 2014. Exotic Scale Insects (Coccoidea) on Ornamental Plants in Italy: a Never-Ending Story. Acta Zool Bulg 6: 55-61.

McDonald, J. H. 2014. Handbook of bilogical statics, 3rd ed. Sparky House Publishing, Baltimore, Maryland, U.S.A.

McFadyen, R. E. 1979. The cactus mealybug *Hypogeococcus festerianus* [Hem. : Pseudococcidae] an agent for the biological control of *Eriocereus martinii* [Cactaceae] in Australia. Entomophaga 24: 281-287.

McKenzie, H. L. 1967. Mealybugs of California, with taxonomy, biology, and control of North American species (Homoptera: Coccoidea: Pseudococcidae). Cambridge University Press, Berkeley, CA, USA.

Mendel, Z., Watson, G. W., Protasov, A., and Spodek, M. 2016. First record of the papaya mealybug, *Paracoccus marginatus* Williams & Granara de Willink (Hemiptera: Coccomorpha: Pseudococcidae), in the Western Palaearctic. EPPO Bull 46: 580-582.

Mendel, Z., Gross, S., Steinberg, S., Cohen, M., and Blumberg, D. 1999. Trials for the control of the citrus mealybug in citrus orchards by augmentative release of two encyrtid parasitoids. Entomologica 33: 251-265.

Meyerdirk, D. E., and Newell, I. M. 1979. Seasonal Development and flight Activity of *Pseudocccus comstocki* in California. Ann Entomol Soc Am 72: 492-494.

Meyerdirk, D. E., Newell, I. M., and Warkentin, R. W. 1981. Biological Control of Comstock Mealybug. J Econ Entomol 74: 79-84.

Meyerdirk, D. E., Warkentin, R., Attavien, B., Gersabeck, E., Fracis, A., Adams, M., and Francis, G. 2001. Biological Control of Pink Hibiscus Mealybug Project Manual, pp. 194. United States Department of Agriculture (USDA), Washington, USA.

Meyerson, L. A., and Mooney, H. A. 2007. Invasive alien species in an era of globalization. Front Ecol Environ 5: 199-208.

Mgocheki, N., and Addison, P. 2009a. Interference of ants (Hymenoptera: Formicidae) with biological control of the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae). Biol Control 49: 180-185.

Mgocheki, N., and Addison, P. 2009b. Effect of Contact Pesticides on Vine Mealybug Parasitoids, *Anagyrus* sp. near *pseudococci* (Girault) and *Coccidoxenoides perminutus* (Timberlake) (Hymenoptera: Encyrtidae). S Afr J Enol Vitic 30: 110-116.

Mgocheki, N., and Addison, P. 2010. Spatial distribution of ants (Hymenoptera: Formicidae), vine mealybugs and mealybug parasitoids in vineyards. J Appl Entomol 134: 285-295.

Mgocheki, N., and Addison, P. 2015. The Sublethal Effects of a Systemic Insecticide on the Vine Mealybug Parasitoids *Anagyrus* sp. near *pseudococci* (Girault) and *Coccidoxenoides perminutus* (Timberlake) (Hymenoptera: Encyrtidae). S Afr J Enol Vitic 36: 175-179.

Millar, J. G., and Midland, S. L. 2007. Synthesis of the sex pheromone of the obscure mealybug, the first example of a new class of monoterpenoids. Tetrahedron Lett 48: 6377-6379.

Millar, J. G., Moreira, J. A., McElfresh, J. S., Daane, K. M., and Freund, A. S. 2009. Sex Pheromone of the Longtailed Mealybug: A New Class of Monoterpene Structure. Org Lett 11: 2683-2685.

Millar, J. G., Daane, K. M., McElfresh, J. S., Moreira, J. A., Malakar-Kuenen, R., Guillén, M., and Bentley, W. J. 2002. Development and Optimization of Methods for Using Sex

Pheromone for Monitoring the Mealybug *Planococcus ficus* (Homoptera: Pseudococcidae) in California Vineyards. J Econ Entomol 95: 706-771.

Miller, D. R. 2005. Selected scale insect groups (Hemiptera: Coccoidea) in the southern region of the United States. Fla Entomol 88: 482-501.

Miller, D. R., and Kosztarab, M. 1979. Recent Advances in the Study of Scale Insects. Annu Rev Entomol 24: 1-27.

Miller, D. R., and Giliomee, J. H. 2011. Systematic revision of the mealybug genus *Delottococcus* Cox & Ben-Dov (Hemiptera: Pseudococcidae). Afr Entomol 19: 614-640.

Miller, D. R., Miller, G. L., and Watson, G. W. 2002. Invasive species of mealybugs (Hemiptera: Pseudococcidae) and their threat to U.S. agriculture. Proc Entomol Soc Wash 104: 825-836.

Moore, D. 1988. Agents used for biological control of mealybugs (Pseudococcidae). Biocontrol News and Information 9: 209-225.

Moore, S. D., and Hattingh, V. 2012. Mealybugs. In V. Hattingh (ed.), Integrated Production Guidelines, vol. 3. Citrus Research International (CRI), South Africa. http://www.citrusres.com/downloads/production-guidelines/integrated-production-guidelines-vol-3/integrated-pest-and-disease Accessed 03-03-18.

Moreno-Salmerón, J. 2011. Prospección e identificación de cochinillas algodonosas (Hemiptera: Pseudococcidae) y búsqueda de parasitoides asociados en cultivos hortícolas protegidos del poniente almeriense. Dissertation, Universidad de Almería, Spain.

Moreno, D. S., Fargerlund, J., and Ewart, W. H. 1984. Citrus Mealybug (Homoptera: Pseudococcidae): Behavior of Males in Response to Sex Pheromone in Laboratory and Field. Ann Entomol Soc Am 77: 32-38.

Moreno, D. S., Reed, D. K., Shaw, J. G., and Newell, I. M. 1972. Sex lure survey trap for comstock mealybug. Citograph 58: 43,68.

Mudavanhu, P. 2009. An investigation into the integrated pest management of the obscure mealybug, *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae), in pome fruit orchards in the Western Cape Province, South Africa. Dissertation, University of Stellenbosch South Africa.

Mudavanhu, P., Addison, P., and Pringle Ken, L. 2011. Monitoring and action threshold determination for the obscure mealybug *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae) using pheromone-baited traps. Crop Prot 30: 919-924.

Muniappan, R., Meyerdirk, D. E., Sengebau, F. M., Berringer, D. D., and Reddy, G. V. P. 2006. Classical Biological Control of the Papaya Mealybug, *Paracoccus marginatus* (Hemiptera: Pseudococcidae) in the Republic of Palau. Fla Entomol 89: 212-217.

Muniappan, R., Shepard, B. M., Watson, G. W., Carner, G. R., Sartiami, D., Rauf, A., and Hammig, M. D. 2008. First Report of the Papaya Mealybug, *Paracoccus marginatus* (Hemiptera: Pseudococcidae), in Indonesia and India. J Agr Urban Entomol 25: 37-40.

Nakahata, T., Itagaki, N., Arai, T., Sugie, H., and Kuwahara, S. 2003. Synthesis of the sex pheromone of the citrus mealybug, *Pseudococcus cryptus*. Biosci Biotechnol Biochem. 67: 2627-2631.

Nechols, J. R. 2003. Biological control of the spherical mealybug on guam and in the northern Marianas islands: a classic example of fortuitous biological control, 1st International Symposium on Biological Control of Arthropods, USA.

Nechols, J. R., and Seibert, T. F. 1985. Biological Control of the Spherical Mealybug, *Nipaecoccus vastator* (Homoptera: Pseudococcidae): Assesment by Ant Exclusion. Environ Entomol 14: 45-47.

Nestel, D., Cohen, H., Saphir, N., Klein, M., and Mendel, Z. 1995. Spatial Distribution of Scale Insects: Comparative Study Using Taylor's Power Law. Environ Entomol 24: 506-512.

Neuenschwander, P. 2001. Biological Control of the Cassava Mealybug in Africa: A Review. Biol Control 21: 214-229.

Neuenschwander, P., Boavida, C., Bokonon-ganta, A., Gado, A., and Herren, H. R. 1994. Establishment and spread of *Gyranusoidea tebygi* Noyes and *Anagyrus mangicola* Noyes (Hymenoptera: Encyrtidae), two biological control agents released against the mango mealybug *Rastrococcus invadens* Williams (Homoptera: Pseudococcidae) in Africa. Biocontrol Sci Technol 4: 61-69.

Noyes, J. S. 1982. Collecting and preserving chalcid wasps (Hymenoptera: Chalcidoidea). J Nat Hist 16: 315-334.

Noyes, J. S., and Hayat, M. 1994. Oriental mealybug parasitoids of the Anagyrini (Hymenoptera: Encyrtidae): with a world review of Encyrtidae used in classical biological control and an index of encyrtid parasitoids of mealybugs (Homoptera: Pseudococcidae), CAB International on behalf of The Natural History Museum.

OECD. 2010. International Standards Fruit and Vegetables. Citrus. http://www.oecd-ilibrary.org/agriculture-and-food/citrus-fruits_9789264083745-en-fr. Accessed 25-08-17

Pacheco da Silva, V. C., Kaydan, M. B., Malausa, T., Germain, J. F., Palero, F., and Botton, M. 2017. Integrative taxonomy methods reveal high mealybug (Hemiptera: Pseudococcidae) diversity in southern Brazilian fruit crops. Sci Rep 7: 15741. doi: 15710.11038/s41598-15017-15983-15745.

Pacheco da Silva, V. C., Bertin, A., Blin, A., Germain, J. F., Bernardi, D., Rignol, G., Botton, M., and Malausa, T. 2014. Molecular and morphological identification of mealybug species (Hemiptera: Pseudococcidae) in Brazilian vineyards. PloS ONE 9: e103267.

Paini, D. R., Sheppard, A. W., Cook, D. C., De Barro, P. J., Worner, S. P., and Thomas, M. B. 2016. Global threat to agriculture from invasive species. Proceedings of the National Academy of Sciences of the United States of America 113: 7575-7579.

Panis, A. 1986. Biological features of *Pseudococcus affinis* (Mask.) (Homoptera, Pseudococcidae) as guidelines of its control in water-sprinkled citrus orchards, pp. 59-65. In R. Cavalloro and

E. Di Martino (eds.), Integrated Pest Control in Citrus groves. Proceedings of the expert's meetings.

Park, D.-S., Leem, Y. J., Hahn, K.-W., Suh, S.-J., Hong, K.-J., and Oh, H.-W. 2010. Molecular Identification of Mealybugs (Hemiptera: Pseudococcidae) Found on Korean Pears. J Econ Entomol 103: 25-33.

Park, D. S., Suh, S. J., Hebert, P. D. N., Oh, H. W., and Hong, K. J. 2011. DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). Bull Entomol Res 101: 429-434.

Parsa, S., Kondo, T., and Winotai, A. 2012. The cassava mealybug (*Phenacoccus manihoti*) in Asia: first records, potential distribution, and an identification key. PloS ONE 7: e47675.

Pellizzari, G. 2005. Cocciniglie nuove o poco note potenzialmente dannose per l'Italia: Fiorinia pinicola Maskell, *Pseudococcus comstocki* (Kuwana), *Peliococcus turanicus* (Kiritshenko). Inf Fitopatol 55: 20-25.

Pellizzari, G., and Germain, J.-F. 2010. Scales (Hemiptera, Superfamily Coccoidea), Chapter 9.3. BioRisk 4: 475-510.

Pellizzari, G., and Porcelli, F. 2014. Alien scale insects (Hemiptera Coccoidea) in European and Mediterranean countries: the fate of new and old introductions. Phytoparasitica 42: 713-721.

Pérez-Hedo, M., Catalán, J., Garcia-Martinez, O., Beitia, F. J., and Urbaneja, A. 2018. Gestión Integrada de Plagas de Caqui. http://gipcaqui.ivia.es/area/plagas-principales. Accessed 04-01-18

Pérez-Rodríguez, J., Martínez-Blay, V., Soto, A., Selfa, J., Monzó, C., Urbaneja, A., and Tena, A. 2017. Aggregation Patterns, Sampling Plan, and Economic Injury Levels for the New Citrus Pest Delottococcus aberiae (Hemiptera: Pseudococcidae). J Econ Entomol 110: 2699-2706.

Phillips, P. A., and Sherk, C. J. 1991. To control mealybugs, stop honeydew-seeking ants. Calif Agric 45: 26-28.

Phillips, P. A., and Walker, G. P. 1997. Increase in Flower and Young Fruit Abscission Caused by Citrus Bud Mite (Acari:

Eriophyidae) Feeding in the Axillary Buds of Lemon. J Econ Entomol 90: 1273-1282.

Pieterse, W., Muller, D. L., and Vuuren, B. J. v. 2010. A Molecular Identification Approach for Five Species of Mealybug (Hemiptera: Pseudococcidae) on Citrus Fruit Exported from South Africa. Afr Entomol 18: 23-28.

Pimentel, D., Zuniga, R., and Morrison, D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol Econ 52: 273-288.

Pimentel, D., Lach, L., Zuniga, R., and Morrison, D. 2000. Environmental and economic costs of nonindigenous species in the United States. BioScience 50: 53-65.

Pimentel, D., McNair, S., Janecka, J., Wightman, J., Simmonds, C., O'Connell, C., Wong, E., Russel, L., Zern, J., Aquino, T., and Tsomondo, T. 2001. Economic and environmental threats of alien plant, animal, and microbe invasions. Agric Ecosyst Environ 84: 1-20.

Prasad, Y. G., Prabhakar, M., Sreedevi, G., Ramachandra Rao, G., and Venkateswarlu, B. 2012. Effect of temperature on development, survival and reproduction of the mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) on cotton. Crop Prot 39: 81-88.

Prinsloo, G. L. 1998. Poorly known and newly recorded species of mealybug parasitoids of the genus *Anagyrus* Howard (Hymenoptera: Encyrtidae) from South Africa. Afr Plant Prot 4: 81-90.

Procheş, Ş., and Cowling, R. M. 2006. Insect diversity in Cape fynbos and neighbouring South African vegetation. Glob Ecol Biogeogr 15: 445-451.

Procheş, Ş., and Cowling, R. M. 2007. Do insect distributions fit our biomes? S Afr J Sci 103: 258-261.

Quaglietti, B., Gautier, P., Groussier, G., Fleisch, A., Kreiter, P., Ris, N., and Malausa, T. 2017a. Pre-release host range determination of the parasitoid *Allotropa burrelli* for the biocontrol of *Pseudococcus comstocki* in France. J Appl Entomol 141: 665-668.

Quaglietti, B., Tamisier, L., Groussier, G., Fleisch, A., Le Goff, I., Ris, N., Kreiter, P., Fauvergue, X., and Malausa, T. 2017b. No inbreeding depression in laboratory-reared individuals of the parasitoid wasp *Allotropa burrelli*. Ecol Evol 7: 964-973.

Rahmouni, R., and Chermiti, B. 2013. Efficiency of *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) to control *Planococcus citri* Risso (Hemiptera: Pseudococcidae) in citrus orchards in Tunisia. Integrated Control in Citrus Fruit Crops. IOBC-WPRS Bull 95: 141-145.

Reddy, G. V. P., Muniappan, R., Cruz, Z. T., Naz, F., Bamba, J. P., and Tenorio, J. 2009. Present Status of *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae) in the Mariana Islands and Its Control by Two Fortuitously Introduced Natural Enemies. J Econ Entomol 102: 1431-1439.

Roltsch, W. J., Ertle, L. R., and Meyerdirk, D. E. 2007. No-choice host range tests for *Allotropa* sp. near *mecrida*, a parasitoid of the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae). Biocontrol Sci Technol 17: 977-981.

Roltsch, W. J., Meyerdirk, D. E., Warkentin, R., Andress, E. R., and Carrera, K. 2006. Classical biological control of the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green), in southern California. Biol Control 37: 155-166.

Roques, A., Rabitsch, W., Rasplus, J.-Y., Lopez-Vaamonde, C., Nentwig, W., and Kenis, M. 2009. Alien Terrestrial Invertebrates of Europe, pp. 63-79. In DAISIE (ed.), Handbook of Alien Species in Europe, vol. 3. Springer Netherlands.

Rotundo, G., and Tremblay, E. 1975. Sull'attrattività delle femmine vergini di due specie di Pseudococcidi (Homoptera Coccoidea) per un Imenottero parassita (Hymenoptera Chalcidoidea). Boll Lab Entomol Agr Portici 32: 172-179.

Rotundo, G., Tremblay, E., and Giacometti, R. 1979. Final results of mass captures of the citrophilous mealybug males (Pseudococcus calceolariae Mask.) (Homoptera Coccoidea) in a citrus grove. Boll Lab Entomol Agr Portici 36: 266-274.

Roy, H. E., Peyton, J., Aldridge, D. C., Bantock, T., Blackburn, T. M., Britton, R., Clark, P., Cook, E., Dehnen-Schmutz, K., Dines, T., Dobson, M., Edwards, F., Harrower, C., Harvey, M. C., Minchin, D., Noble, D. G., Parrott, D., Pocock, M. J., Preston, C. D., Roy, S., Salisbury, A., Schonrogge, K., Sewell, J., Shaw, R. H., Stebbing, P., Stewart, A. J., and Walker, K. J. 2014. Horizon scanning for invasive alien species with the potential to threaten biodiversity in Great Britain. Glob Chang Biol 20: 3859-3871.

Rung, A., Miller, D. R., and Scheffer, S. J. 2009. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Method to Distinguish Three Mealybug Groups Within the *Planococcus citri–P. minor* Species Complex (Hemiptera: Coccoidea: Pseudococcidae). J Econ Entomol 102: 8-12.

Saccaggi, D. L., Kruger, K., and Pietersen, G. 2008. A multiplex PCR assay for the simultaneous identification of three mealybug species (Hemiptera: Pseudococcidae). Bull Entomol Res 98: 27-33.

Sagarra, L. A., Vincent, C., and Stewart, R. K. 2000. Fecundity and survival of *Anagyrus kamali* (Hymenoptera: Encyrtidae) under different feeding and storage temperature conditions. Eur J Entomol 97: 177-181.

Samways, M. J. 1988. Comparative monitoring of red scale *Aonidiella aurantii* (Mask.) (Hom., Diaspididae) and its *Aphytis* spp. (Hym., Aphelinidae) parasitoids. J Appl Entomol 105: 483-489.

Sandanayaka, W. R. M., Charles, J. G., and Allan, D. J. 2009. Aspects of the reproductive biology of *Pseudaphycus maculipennis* (Hym: Encyrtidae), a parasitoid of obscure mealybug, *Pseudococcus viburni* (Hem: Pseudococcidae). Biol Control 48: 30-35.

Santorini, A. 1977. Etude de quelques caractères biologiques de *Planococcus citri* (Risso) en Grèce (Homoptera, Coccoidea, Pseudococcidae). Fruits 32: 611-612.

Sarkar, M. A., Wiwat, S.-a., and Sopon, U. 2014. Suitability of different mealybug species (Hemiptera: Pseudococcidae) as

hosts for the newly identified parasitoid *Allotropa suasaardi* Sarkar & Polaszek (Hymenoptera: Platygasteridae). Kasetsart J (Nat Sci) 48: 17-27.

Sarkar, M. A., Suasa-ard, W., and Uraichuen, S. 2015. Host stage preference and suitability of *Allotropa suasaardi* Sarkar & Polaszek (Hymenoptera: Platygasteridae), a newly identified parasitoid of pink cassava mealybug, *Phenacoccus manihoti* (Homoptera: Pseudococcidae). Songklanakarin J Sci Technol 37: 381-387.

Serrano, M. S., Lapointe, S. L., and Meyerdirk, D. E. 2001. Attraction of Males by Virgin Females of the Mealybug *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae). Popul Ecol 30: 339-345.

Sforza, R., Boudon-Padieu, E., and Greif, C. 2003. New mealybug species vectoring Grapevine leafroll-associated viruses-1 and -3 (GLRaV-1 and -3). Eur J Plant Pathol 109: 975-981.

Shah, T. N., Ahmed, A. M., and Memon, N. 2015. Population dynamics of cotton mealybug, *Phenacoccus solepnosis* Tinsely in three talukas of district Sanghar (Sindh). J Entomol Zool Stud 3: 162-167.

Sharaf, N. S., and Meyerdirk, D. E. 1987. A review on the biology, ecology and control of *Nipaecoccus viridis* (Homoptera: Pseudococcidae). Misc Publ Entomol Soc Am. 66: 1-18.

Sharon, R., Zahavi, T., Sokolsky, T., Sofer-Arad, C., Tomer, M., Kedoshim, R., and Harari, A. R. 2016. Mating disruption method against the vine mealybug, *Planococcus ficus*: effect of sequential treatment on infested vines. Entomol Exp Appl 161: 65-69.

Soler, J. M., and García-Marí, F. 2016. Resultados de la Red de Monitoreo Online de cítricos: plagas y sus enemigos naturales. Phytoma 284: 128-134.

Sorribas, J., van Baaren, J., and Garcia-Marí, F. 2012. Effects of climate on the introduction, distribution and biotic potential of parasitoids: Applications to biological control of California red scale. Biol Control 62: 103-112.

Soto, A., Ohlenschläger, F., and Garcia-Marí, F. 1999. Situación del control biológico de las moscas blancas de cítricos *Aleurothrixus floccosus*, *Parabemisia myricae* y *Dialeurodes citri* en la Comunidad Valenciana. Levante Agrícola 349: 475-484.

Soto, A., Martínez-Blay, V., Benito, M., and Beltrà, A. 2016a. *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae): viabilidad de su control biológico. Phytoma 284: 85-87.

Soto, A., Martínez-Blay, V., Beltrà, A., Pérez-Rodríguez, J., and Tena, A. 2016b. *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae), comportamiento de la plaga en parcelas de cítricos valencianos. Phytoma 277: 49-53.

Stern, V. M. 1973. Economic Thresholds. Annu Rev Entomol 18: 259-280.

Stocks, I. 2014. *Delottococcus confusus* (De Lotto) (Hemiptera: Pseudococcidae), a mealybug pest of cut-flower protea (Proteaceae: Protea spp.). Pest Alert. Florida Department of Agriculture and Consumer Services Division of Plant Industry. doi: 10.13140/rg.2.2.21810.81609.

Suckling, D. M. 2000. Issues afecting the use of pheromones and other semiochemicals in orchards. Crop Prot 19: 677-683.

Suckling, D. M., Stringer, L. D., Kean, J. M., Lo, P. L., Bell, V., Walker, J., Twidle, A. M., Jimenez-Perez, A., and El-Sayed, A. M. 2015. Spatial analysis of mass trapping: how close is close enough? Pest Manag Sci 71: 1452-1461.

Sujay, Y. H., Sattagi, H. N., and Patil, R. K. 2010. Invasive alien insects and their impact on agroecosystem. Karnatka J Agric Sci 23: 26-34.

Suma, P., Mazzeo, G., La Pergola, A., Nucifora, S., and Russo, A. 2015. Establishment of the pineapple mealybug *Dysmicoccus brevipes* (Hemiptera: Pseudococcidae) in Italy. EPPO Bull 45: 218-220.

Suma, P., Mansour, R., Russo, A., La Torre, I., Bugila, A. A. A., and Franco, J. C. 2012. Encapsulation rates of the parasitoid *Anagyrus* sp. nr. *pseudococci*, by three mealybug species (Hemiptera: Pseudococcidae). Phytoparasitica 40: 11-16.

Sun, J.-H., Clarke, S. R., DeBarr, G. L., and Berisford, C. W. 2004. Parasitoid Complex of the Mealybug *Oracella acuta* (Lobdell) (Hemiptera: Pseudococcidae), in Georgia, USA. J Entomol Sci 39: 11-22.

Sun, J., Clarke, S. R., DeBarr, G. L., and Berisford, C. W. 2002. Yellow Sticky Traps for Monitoring Males and Two Parasitoids of *Oracella acuta* (Lobdell) (Homoptera: Pseudococcidae). J Entomol Sci 37: 177-181.

Symondson, W. O. C., Sunderland, K. D., and Greenstone, M. H. 2002. Can generalist predators be effective biocontrol agents? Annu Rev Entomol 47: 561-594.

Tena, A., and Garcia-Marí, F. 2011. Current situation of citrus pests and diseases in the Mediterranean basin. Integrated Control in Citrus Fruit Crops IOBC/WPRS Bull 62: 365-378.

Tena, A., and García-Marí, F. 2011. Current situation of citrus pests and diseases in the Mediterranean basin. Integrated Control in Citrus Fruit Crops. IOBC-WPRS Bull 62: 365-378.

Tena, A., García-Bellón, J., and Urbaneja, A. 2017a. Native and naturalized mealybug parasitoids fail to control the new citrus mealybug pest *Delottococcus aberiae*. J Pest Sci 90: 659-667.

Tena, A., Catalán, J., Bru, P., and Urbaneja, A. 2014. *Delottococcus aberiae*, nueva plaga de cítricos. Agricultura 978: 746-748.

Tena, A., Nieves, E., Herrero, J., and Urbaneja, A. 2017b. Defensive behaviors of the new mealybug citrus pest, Delottococcus aberiae (Hemiptera: Pseudococcidae), against three generalist parasitoids. J Econ Entomol. doi: 10.1093/jee/tox307.

Thomas, D. D., and Leppla, N. C. 2008. The Likelihood and Consequences of Introduction of the Spherical Mealybug, *Nipaecoccus viridis* (Newstead), into Florida, and Its Potential Effect on Citrus Production. Proc Fla State Hort Soc 121: 152-154.

Timberlake, P. H. 1924. The Parasites of *Pseudococcus maritimus* (Ehrhorn) in California (Hymenoptera, Chalcidoidea). Part I. Taxonomic Studies.

Tremblay, E., Tranfaglia, A., Rotundo, G., and Iaccarino, F. 1977. Osservazioni comparate su alcune specie di Pseudococcidi (Homoptera: Coccoidea). Boll Lab Entomol Agr Portici 34: 113-135.

Tsai, C. W., Rowhani, A., Golino, D. A., Daane, K. M., and Almeida, R. P. 2010. Mealybug transmission of Grapevine leafroll viruses: an analysis of virus-vector specificity. Phytopathology 100: 830-834.

TuTiempo Network, S. L. 2018. World Weather. https://www.tutiempo.net/clima/2013/ws-681880.html. Accessed 09-03-18.

Urbaneja, A., Catalán, J., Tena, A., and Jacas, J. A. 2017. Gestión Integrada de Plagas de Cítricos. http://gipcitricos.ivia.es/. Accessed 04-08-17.

Vacante, V., and Bonsignore, C. P. 2016. Interactions between the citrus bud mite *Aceria sheldoni* (Acari: Eriophyidae) and the lemon host tree in a Mediterranean area. Acarologia 56: 167-174.

van Lenteren, J. C. 2006. How not to evaluate augmentative biological control. Biol Control 39: 115-118.

Varikou, K., Birouraki, A., Bagis, N., and Kontodimas, D. C. 2010. Effect of Temperature on the Development and Longevity of *Planococcus ficus* (Hemiptera: Pseudococcidae). Ann Entomol Soc Am 103: 943-948.

Venkatesan, T., Jalali, S. K., Ramya, S. L., and Prathibha, M. 2016. Insecticide Resistance and Its Management in Mealybugs, pp. 223-229. In M. Mani and C. Shivaraju (eds.), Mealybugs and their Management in Agricultural and Horticultural crops. Springer India, New Delhi.

Vercher, R., Costa-Comelles, J., Marzal, C., and García-Marí, F. 2005. Recruitment of Native Parasitoid Species by the Invading Leafminer *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) on Citrus in Spain. Environ Entomol 34: 1129-1138.

Villalba, M., Villa, N., Garcia-Marí, F., and Marzal, C. 2006. Influencia en el control biológico del cotonet *Planococcus citri* (Hemiptera: Pseudococcidae) de la liberación inoculativa de

enemigos naturales y la eliminación de hormigas, en parcelas de cítricos. Bol San Veg Plagas 32: 203-213.

Vitullo, J. 2009. Ecological and behavioral factors associated with monitoring and managing pink hibiscus mealybug (hemiptera: pseudococcidae) in the southern US. Dissertation, Virginia Polytechnic Institute and State University USA.

Vitullo, J., Zhang, A., Mannion, C., and Bergh, J. C. 2009. Expression of Feeding Symptoms from Pink Hibiscus Mealybug (Hemiptera: Pseudococcidae) by Commercially Important Cultivars of Hibiscus. Fla Entomol 92: 248-254.

Vitullo, J., Wang, S., Zhang, A., Mannion, C., and Bergh, J. C. 2007. Comparison of Sex Pheromone Traps for Monitoring Pink Hibiscus Mealybug (Hemiptera: Pseudococcidae). J Econ Entomol 100: 405-410.

Vlug, H. J. 1995. Catalogue of the Platygastridae (Platygastroidea) of the world : (Insecta: Hymenoptera), SPB Academic Publishing, Amsterdam.

Wakgari, W. M., and Giliomee, J. H. 2005. Description of adult and immature females of six mealybug species (Hemiptera: Pseudococcidae) found on citrus in South Africa. Afr Entomol 13: 281-332.

Walker, G. P. 1985. Stylet penetration by the bayberry whitefly, as affected by leaf age in lemon, *Citrus limon.* Entomol Exp Appl 39: 115-121.

Walther, G. R., Roques, A., Hulme, P. E., Sykes, M. T., Pysek, P., Kuhn, I., Zobel, M., Bacher, S., Botta-Dukat, Z., Bugmann, H., Czucz, B., Dauber, J., Hickler, T., Jarosik, V., Kenis, M., Klotz, S., Minchin, D., Moora, M., Nentwig, W., Ott, J., Panov, V. E., Reineking, B., Robinet, C., Semenchenko, V., Solarz, W., Thuiller, W., Vila, M., Vohland, K., and Settele, J. 2009. Alien species in a warmer world: risks and opportunities. Trends Ecol Evol 24: 686-693.

Walton, V. M., and Pringle, K. L. 2004. Vine mealybug, *Planococcus ficus* (Signoret) (Hemiptera : Pseudococcidae), a Key Pest in South African vineyards. A Review. S Afr J Enol Vitic 25: 54-62.

Walton, V. M., Daane, K. M., and Pringle, K. L. 2004. Monitoring *Planococcus ficus* in South African vineyards with sex pheromone-baited traps. Crop Prot 23: 1089-1096.

Walton, V. M., Daane, K. M., Bentley, W. J., Millar, J. G., Larsen, T. E., and Malakar-Kuenen, R. 2006. Pheromone-Based Mating Disruption of *Planococcus ficus* (Hemiptera: Pseudococcidae) in California Vineyards. J Econ Entomol 99: 1280-1290.

Wang, Y., Watson, G. W., and Zhang, R. 2010. The potential distribution of an invasive mealybug *Phenacoccus solenopsis* and its threat to cotton in Asia. Agric Forest Entomol 12: 403-416.

Waterworth, R. A., Redak, R. A., and Millar, J. G. 2011. Pheromone-Baited Traps for Assessment of Seasonal Activity and Population Densities of Mealybug Species (Hemiptera: Pseudococcidae) in Nurseries Producing Ornamental Plants. J Econ Entomol 104: 555-565.

Watson, G. W. 2007. Hemiptera: Sternorrhyncha. New federal records. *Delottococcus* sp. In S. Gaimari (ed.), California Plant Pest & Disease Report, vol. 23. CPPDR, California, USA.

Watson, G. W., and Kubiriba, J. 2005. Identification of mealybugs (Hemiptera: Pseudococcidae) on banana and plantain in Africa. Afr Entomol 13: 35-47.

Way, M. J., and van Emden, H. F. 2000. Integrated pest management in practice - pathways towards successful application. Crop Prot 19: 81-103.

Webster, K. W., Cooper, P., and Mound, L. A. 2006. Studies on Kelly's citrus thrips, *Pezothrips kellyanus* (Bagnall) (Thysanoptera: Thripidae): sex attractants, host associations and country of origin. Austr J Entomol 45: 67-74.

Williams, D. J. 1958. The mealybugs (Pseudococcidae-Homoptera) described byW.J. Hall, F. Laing and A.H. Strickland from the Ethiopian region. Bull Br Mus (Nat Hist) Entomol 7: 1-37.

Williams, D. J., and Granara de Willink, M. C. 1992. Mealybugs of Central and South America, CAB International, Wallingford, UK.

Williams, D. J., Gullan, P. J., Miller, D. R., Matile-Ferrero, D., and Han, S. H. 2011. A study of the scale insect genera Puto Signoret (Hemiptera: Sternorrhyncha: Coccoidea: Putoidae) and Ceroputo Šulc (Pseudococcidae) with a comparison to *Phenacoccus* Cockerell (Pseudococcidae). Zootaxa 2802: 1-22.

Wunderlich, L. R., Cooper, M. L., and Daane, K. M. 2013. Seasonal Biology of *Ferrisia gilli* (Hemiptera: Pseudococcidae) in California Sierra Foothill Vineyards. J Econ Entomol 106: 1716-1725.

Zada, A., Dunkelblum, E., Harel, M., Assael, F., Gross, S., and Mendel, Z. 2004. Sex Pheromone of the Citrus Mealybug *Planococcus citri*: Synthesis and Optimization of Trap Parameters. J Econ Entomol 97: 361-368.

Zada, A., Dunkelblum, E., Assael, F., Franco, J. C., Silva, E. B. d., Protasov, A., and Mendel, Z. 2008. Attraction of *Planococcus* ficus males to racemic and chiral pheromone baits: flight activity and bait longevity. J Appl Entomol 132: 480-489.