UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Biology and management of the invasive mealybug *Phenacoccus peruvianus* (Hemiptera: Pseudococcidae) in urban landscapes.

DOCTORAL THESIS

Presented by: Aleixandre Beltrà Ivars

Directed by: Dr. Antonia Soto Sánchez and Dr. Ferran Garcia Marí

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Als meus pares

Agraïments

Sense la contribució directa i indirecta de moltes persones aquesta tesi mai s'haguera portat a terme i és per a elles el meu més sincer reconeixement. Especialment, vull agrair a tots els que han conseguit que gaudira de l'elaboració de la tesi ja que amb ells tinc un deute impagable.

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Resum

Phenacoccus peruvianus (Hemiptera: Pseudococcidae) és un cotonet invasor d'origen Neotropical que va ser observat per primera vegada en la Conca Mediterrània a Almeria (Espanya) en l'any 1999. Durant els següents anys la seua presència es va estendre per altres àrees del Mediterrani, detectant-se també a Portugal i França, a més de les illes de Sicília, Còrcega i Balears. *Phenacoccus peruvianus* és una espècie polífaga que ocasiona danys rellevants en plantes ornamentals d'alta importància econòmica. Degut al desconeixement d'aquesta espècie, durant els primers anys del procés d'invasió la seua gestió és va portar a terme mitjançant l'ús de tractaments químics amb matèries actives d'ample espectre. Amb tot, la nova directiva europea sobre l'ús sostenible de productes fitosanitaris ha qualificat les àrees verdes urbanes com zones d'ús reduït o nul de plaguicides i insta a la utilització d'estratègies de gestió més sostenibles com el control biològic. En aquesta tesi es presenten les bases per a la introducció del control biològic en un programa de gestió de *P. peruvianus* en àrees verdes urbanes, centrant-se en la seua caracterització, mostreig, biologia i control.

Amb l'objectiu de facilitar la identificació d'aquesta i altres especies de pseudocòccids, es van caracteritzar 33 poblacions de cotonets presents en cultius i plantes ornamentals a l'est d'Espanya mitjançant la combinació de tècniques morfològiques i moleculars. Aquesta caracterització va permetre el reconeixement de deu espècies de pseudocòccids, facilitant la seua futura identificació rutinària mitjançant la seqüenciació d'ADN o l'ús d'altres ferramentes moleculars. A més, les dades obtingudes contribueixen al coneixement filogenètic de la família Pseudococcidae i proveeixen informació sobre el procés d'invasió d'algunes d'aquestes especies com *P. peruvianus*.

L'abundància de les poblacions de *P. peruvianus* en plantes de buguenvíl·lia en àrees verdes urbanes va ser alta a primavera i estiu, disminuint fins a nivells quasi imperceptibles durant la tardor i l'hivern. Pel que fa a la distribució en la planta, el cotonet va mostrar preferència per situar-se en les bràctees i no es van observar importants migracions entre

els diferents òrgans. *Phenacoccus peruvianus* es va distribuir de manera agregada en bràctees, fulles i branques. Per a la seua gestió en àrees verdes urbanes es recomana un mostreig binomial de 200 fulles amb un llindar de tractament del 55% de fulles infestades.

Els enemics naturals de major rellevància associats a *P. peruvianus* van ser els parasitoides primaris *Acerophagus* n. sp. prox. *coccois* i *Leptomastix epona* Walker (Hymenoptera: Encyrtidae). A més s'identificaren diferents espècies de depredadors de les famílies *Anthocoridae*, *Coccinellidae*, *Chamaemyiidae* i *Chrysopidae*. Les poblacions de *P. peruvianus* van disminuir en gran mesura durant el segon i tercer any de l'estudi coincidint amb l'augment del parasitisme per part d'*Acerophagus sp.* Aquest parasitoide d'origen desconegut va desplaçar al natiu *L. epona*. Entre les raons d'aquest desplaçament, s'observaren diferències en el nombre de femelles en la descendència i l'anticipació en l'ús de recursos.

Per a ampliar el nostre coneixement sobre la biologia del nou parasitoide *Acerophagus* sp. es varen estudiar alguns caràcters de les seues estratègies reproductives i alimentàries. El nombre d'ous disponibles va ser màxim quan el parasitoide va assolir els 5 dies d'edat amb quasi 30 ous madurs. El segon i tercer estadi nimfal, així com l'estadi adult de *P. peruvianus* van ser adequats per al parasitisme, mostrant *Acerophagus* sp. una major preferència pels estadis de major edat. En tots aquests estadis es va observar un baix percentatge d'encapsulació eficient (10.76 ± 0.31 %). Per altra banda, el parasitoide, es va desenvolupar com a solitari en nimfes de segon estadi i com a gregari en estadis majors (2-4 parasitoides per hoste). A més, es va reproduir de manera partenogenètica amb la completa absència de mascles en la descendència. El temps de desenvolupament dels estadis immadurs del parasitoide fou de 20-22 dies a 25°C i 65% HR. Amb aquestes mateixes condicions, els adults van viure més de 20 dies quan s'alimentaren de mel, però menys de tres dies quan s'alimentaren de fonts de sucre presents en àrees vedes urbanes com melassa de *P. peruvianus* o flors de *Bougainvillea glabra*.

Resumen

Phenacoccus peruvianus (Hemiptera: Pseudococcidae) es un cotonet invasor de origen Neotropical que fue observado por primera vez en la Cuenca Mediterránea en Almería (España) en el año 1999. Durante los años siguientes la presencia del cotonet se extendió por otras áreas del Mediterráneo detectándose también en Portugal y Francia, así como en las islas de Sicilia, Córcega y Baleares. *Phenacoccus peruvianus* es una especie polífaga que ocasiona daños en plantas ornamentales de alta importancia económica. Debido al desconocimiento de esta especie, durante los primeros años de la invasión la gestión del cotonet se llevó a cabo mediante el uso de tratamientos químicos con materias activas de amplio espectro. A pesar de ello, la nueva directiva europea sobre el uso sostenible de productos fitosanitarios ha calificado las áreas verdes urbanas como zonas de uso reducido o nulo de plaguicidas, instando a la utilización de estrategias de gestión más sostenibles como el control biológico. En esta tesis se presentan los fundamentos necesarios para introducir el control biológico en un programa de gestión de *P. peruvianus* en áreas verdes urbanas, centrándose en su caracterización, muestreo, biología y control.

Con el objetivo de facilitar la identificación de esta y otras especies de pseudocóccidos, se caracterizaron 33 poblaciones de cotonet presentes en cultivos y plantas ornamentales en el este de España mediante la combinación de técnicas morfológicas y moleculares. Esta caracterización permitió el reconocimiento de diez especies de pseudocóccidos, facilitando su futura identificación rutinaria mediante la secuenciación de ADN o el uso de otras herramientas moleculares. Además, las secuencias obtenidas contribuyen al conocimiento filogenético de la familia Pseudococcidae y proveen importante información sobre el proceso de invasión de algunas de estas especies como *P. peruvianus.*

La abundancia de las poblaciones de *P. peruvianus* en plantas de buganvilla en áreas verdes urbanas fue alta en primavera y verano, disminuyendo hasta niveles casi imperceptibles durante otoño e invierno. En cuanto a su distribución en la planta, el cotonet mostró preferencia por situarse en las brácteas y no se observaron importantes migraciones entre

los diferentes órganos. *Phenacoccus peruvianus* se distribuyó de manera agregada en brácteas, hojas y ramas. Para su gestión en áreas verdes urbanas se recomienda un muestreo binomial de 200 hojas y un umbral de tratamiento del 55% de hojas infestadas.

Los enemigos naturales más importantes asociados a *P. peruvianus* fueron los parasitoides primarios *Acerophagus* n. sp. prox. *coccois* y *Leptomastix epona* Walker (Hymenoptera: Encyrtidae). Además, se identificaron diferentes especies de depredadores de las familias *Anthocoridae*, *Coccinellidae*, *Chamaemyiidae* y *Chrysopidae*. Las poblaciones de *P. peruvianus* disminuyeron en gran medida durante el segundo y tercer año del estudio coincidiendo con el aumento del parasitismo de *Acerophagus sp.* Este parasitoide de origen desconocido desplazó al nativo *L. epona*. Entre las razones del desplazamiento se observaron diferencias en el número de hembras en la descendencia y la anticipación en el uso de los recursos.

Para ampliar nuestro conocimiento de la biología del nuevo parasitoide *Acerophagus* sp. se estudiaron algunos aspectos de sus estrategias reproductivas y alimentarias. El número de huevos disponibles fue máximo cuando el parasitoide alcanzó los cinco días de edad con aproximadamente 30 huevos maduros. El segundo y tercer estadio ninfal, así como el estado adulto de *P. peruvianus* fueron adecuados para el parasitismo, mostrando *Acerophagus sp.* mayor preferencia por los estadios más adultos. En todos estos estadios se observó un bajo porcentaje de encapsulación eficiente (10.76 ± 0.31 %). Por otra parte, el parasitoide se desarrolló como solitario en las ninfas de segundo estadio y como gregario en los estadios más grandes (2-4 parasitoides por hospedero). Además se reprodujo de manera partenogenética con la completa ausencia de machos en la descendencia. El tiempo de desarrollo de los estadios inmaduros del parasitoide fue de 20-22 días a 25°C y 65% HR. Con estas mismas condiciones, los adultos vivieron más de 20 días cuando se alimentaron de miel, pero menos de 3 días cuando se alimentaron de fuentes de azúcar comunes en áreas verdes urbanas como melaza de *P. peruvianus* o flores de *Bougainvillea glabra.*

Abstract

Phenacoccus peruvianus (Hemiptera: Pseudococcidae) is an invasive mealybug of Neotropical origin, first reported in the Mediterranean Basin in Almeria (Spain) in 1999. In the following years the mealybug spread into other Mediterranean regions and has also been recorded in Portugal and France, as well as in Sicily, Corsica and the Balearic Islands. *Phenacoccus peruvianus* is a polyphagous species and damages economically important ornamental plants. Since this was a relatively unknown species, during the first years of invasion, the mealybug was managed by the application of chemical treatments with wide-spectrum pesticides. However, the latest European directive on pesticide use reduces or even forbids pesticide applications in a wide range of urban green areas, giving significant priority to biological control (European Parliament and Council 2009). This thesis sets the basis for introducing biological control into a *P. peruvianus* management program in urban landscapes, focusing on its characterization, sampling, biology and control.

In order to facilitate the identification of this and other mealybug species, we characterised 33 mealybug populations infesting crops and ornamental plants in Eastern Spain, using a combination of molecular and morphological techniques. This characterisation led to the identification of ten mealybug species and made routine identification possible through DNA sequencing or the use of derived species-specific molecular tools. The sequences obtained also add to the phylogenetic knowledge of the Pseudococcidae family and provide insight into the invasion history of some species.

Phenacoccus peruvianus populations were high in bougainvillea plants during spring and summer, declining to almost undetectable levels in autumn and winter. The mealybug was mainly found in bracts and there were no significant migrations between plant strata. *Phenacoccus peruvianus* showed a high aggregated distribution on bracts, leaves and twigs. We recommend a binomial sampling of 200 leaves and an action threshold of 55% infested leaves for IPM purposes in urban landscapes.

Its most abundant natural enemies were found to be the primary parasitoids *Acerophagus* n. sp. *near coccois* and *Leptomastix epona* Walker (Hymenoptera: Encyrtidae). We also identified several predator species from the *Anthocoridae*, *Coccinellidae*, *Chamaemyiidae*, and *Chrysopidae* families. *Phenacoccus peruvianus* populations were lower during the second and third year of the survey, coinciding with an increase in the parasitoid *Acerophagus* sp. populations, which displaced the native *L. epona*. Differential female offspring and resource preemption are discussed as the main reasons for this displacement.

To obtain further information on the biology of the new parasitoid *Acerophagus* sp. we determined some traits of its reproductive and feeding strategies. *Acerophagus* sp. egg load reached its maximum when it was 5 days old with almost 30 mature eggs*. Phenacoccus peruvianus* second and third nymphal instars and adults were suitable for parasitism and efficient encapsulation was low (10.76 ± 0.31 %). The parasitoid always preferred older instars when different host instars were available. *Acerophagus* sp. developed as a solitary parasitoid in the second instar and as a gregarious parasitoid in older instars (2–4 parasitoids per host). Moreover, it reproduced parthenogenetically and all the emerged offspring were females. Immature development lasted between 20 and 22 days at 25°C and 65% HR. Under these conditions, adults lived for longer than 20 days when fed on honey, but fewer than 3 days when fed on naturally occurring sugar sources (host honeydew and *Bougainvillea glabra* flowers).

1- INTRODUCTION AND OBJECTIVES

1.1 Pest management in urban landscapes

1.1.1 Urban landscapes

The urban population has grown considerably over the last 50 years. It is estimated that the global urban population surpassed that of rural areas for the first time in human history in 2008, reaching more than 3000 million people (UN 2008). This trend is still continuing and in 2030 six out ten people are expected to be living in cities (UN 2008). Urban landscapes are thus becoming the natural environment for most of the world's population and thus play a major role in the quality of life and health of city dwellers, providing ecosystem services such as air filtering, micro-climate regulation, rain-water drainage, sewage treatment and recreational and cultural values (Bolund and Hunhammar 1999; Tratalos *et al.* 2007; Liu *et al.* 2010). For these reasons, the conservation and protection of urban ecosystems have capital relevance beyond the aesthetics of urban arboriculture.

Urban ecosystems constitute an anthropic harsh environment to which plants are not adapted, so that urban vegetation is particularly susceptible to insect outbreaks (Herms *et al.* 1984). The ecological relationships between plants, insects and the urban environment are specific for these ecosystems and an understanding of them is crucial to designing effective management strategies (Frankie and Ehler 1978; Raupp *et al.* 2010). Among the biotic attributes that influence insect populations, we can highlight the diversity of plants and natural enemies and the evolutionary relationships between plant and insect pests (Tallamy 2004; Shrewsbury and Raupp 2006). Other abiotic factors have a significant role in these interactions, especially impervious surfaces, habitat fragmentation and thermal regimes (Frankie and Ehler 1978; Faeth *et al.* 2005). Finally, the human impact on pollution and plant management due to fertilizers, pesticides, etc. is particularly relevant (Herms 2002; Jones *et al.* 2004). These factors favor infestations of small insects with limited mobility and the ability to produce multiple generations in the same plant, including mites, scale insects, lacebugs, adelgids or dipterous leafminers, which are particularly abundant in urban areas (Raupp *et al.* 2010).

1.1.2 Urban pest management

Urban pest management has traditionally relied on chemical applications (Jetter and Paine 2004). However, the evolution of pesticide resistance in some insect species, the proliferation of secondary pests, and the growing social awareness to pesticide use has led to the introduction of integrated pest management (IPM) practices in urban landscapes (Olkowski *et al.* 1976; Olkowski *et al.* 1978; Nielsen 1989; Raupp *et al.* 1992; Jetter and Paine 2004). These practices start with the identification and establishment of key pests and systematize regular samplings of insects and tree damage. They thus provide relevant information that optimizes decision making and enables the introduction of more sustainable management tools (Raupp *et al.* 1985; Ball 1987; Dreistadt and Dahlsten 1988; Raupp *et al.* 1988; Raupp *et al.* 1992). The fundamentals of IPM in urban landscapes are close to those of agricultural or forest ecosystems, but give special attention to the concept of aesthetic qualities in decision making and use thresholds based on Aesthetic Injury Levels (AILs) which reflect the minimum infestation level that causes aesthetic damage (Olkowski *et al.* 1978; Raupp *et al.* 1989; Klingeman III *et al.* 2001; Sadof and Sclar 2002).

In recent years there has been an evolution towards more sustainable pest management systems such as IPM in European urban landscapes. However, the process is slow and the use of wide-spectrum toxic pesticides such as organophosphates and carbamates in urban areas is considered a usual practice. Recent studies report similar pesticide concentrations in the air of Spanish and French cities as in rural areas in which intensive agriculture is practiced (Coscollà *et al.* 2010; Hart *et al.* 2012; Coscollà *et al.* 2013). Therefore, European legislation has now become more restrictive to pesticide use in sensitive urban areas: public parks and gardens, sports and recreation grounds, school grounds and children's playgrounds, and in close vicinity to healthcare facilities (European Parliament and Council 2009). The 2009/128/EC Directive on the sustainable use of pesticides stipulates that insecticide applications in these areas should be minimized or even forbidden, and alternative measures such as biological control should first be considered (European Parliament and Council 2009). This regulation opens up new horizons for pest management, particularly for insects that cause mainly aesthetic damage, such as mealybugs.

1.2. Mealybugs

1.2.1. General characteristics

Mealybugs (Hemiptera: Pseudococcidae) are oval soft-bodied insects covered with a cottony wax secretion. They are members of the superfamily Coccoidea, and after armored scales, constitute the second most important family in number of species, with over 2000 belonging to 268 genera (Ben-Dov *et al.* 2013). They are widely distributed in different habitats in all the zoogeographical regions of the world (McKenzie 1967; Ben-Dov 1994). The Paleartic Region has the highest number of recorded species, followed by the Oriental and Australasian Regions (Ben-Dov *et al.* 2013).

Their host range is broad, including herbaceous plants, woody shrubs and trees. However, unlike other scale insect families such as diaspidids, mealybugs occur predominantly in herbaceous plants (Miller 2005). The most relevant hosts belong to the Poaceae, Compositae and Fabaceae families, followed by Cactaceae and Rosaceae (Fig. 1) (Ben-Dov 1994). Many mealybug pests, such as *Planococcuss citri* (Risso), *Pseudococcus viburni* (Signoret) and *Phenacoccus madeirensis* Green are polyphagous species which feed on over 100 different host families (Ben-Dov *et al.* 2013). However, there are also oligophagous species such as *Planococcus vovae* Nasonov, which feed exclusively on Cupressaceae (Francardi and Covassi 1992), and numerous mealybugs present in natural ecosystems are monophagous that feed on native plants (Ben-Dov *et al.* 2013).

These insects cause significant losses in the crops they infest and harm the aesthetic quality of ornamental plants (McKenzie 1967). In the Mediterranean Basin, they cause serious damage to fruit trees such as citrus and vines, horticultural Solanaceae and Cucurbitaceae crops, and a wide range of ornamental plant families (Panis 1977a; Panis 1977b; Godinho and Franco 2001; Ben-Dov 2005; Tsolakis and Ragusa 2008; Beltrà and Soto 2011; Moreno 2011; Tena and Garcia-Marí 2011). Their feeding reduces plant vigor and the honeydew secreted promotes the growth of a black sooty mold that interferes with photosynthesis and affects fruit quality (Woodside 1936; McKenzie 1967; Franco *et al.* 2009; Gullan and Martin 2009). High population densities may also cause leaf fall, fruit loss or even the death of the plant (Franco *et al.* 2000). Other

mealybug species can also transmit virus to commercial crops, causing serious damage even when their populations are low (Engelbrecht and Kasdorf 1990; Charles 1993; Cabaleiro and Segura 1997, Petersen and Charles 1997; Sforza *et al.* 2003). In addition, some species such as *Hypogeococcus pungens* Granara de Willink inject toxins that distort plant tissues (McFadyen 1979) (Fig. 2).

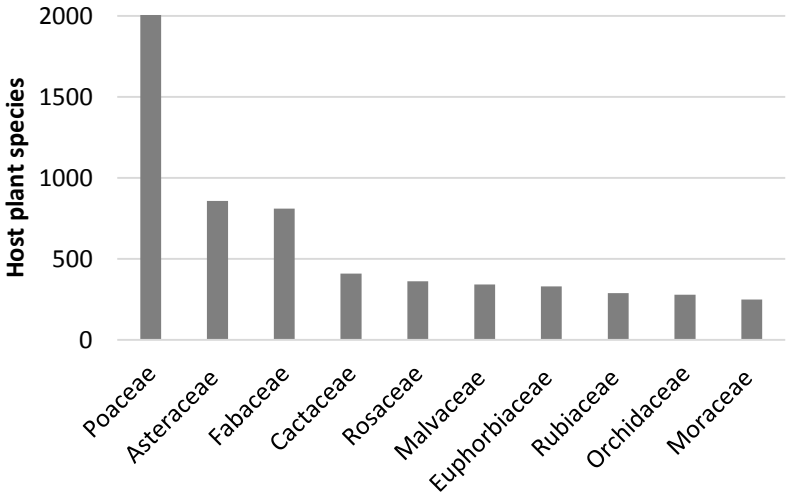

Figure 1. Most relevant host families for mealybugs (Ben-Dov *et al.* 2013).

Figure 2. Leave fall and organ deformation caused by mealybugs on ornamental plants.

After hatching, females go through three immature instars before reaching maturity, while males have four immature instars, including two pupae stages (McKenzie 1967; Gullan and Martin 2009). Population dynamics differs according to mealybug species and environmental conditions. In Central Europe, native species generally complete one to three generations per year (Kosztarab and Kozár 1988), while other species with an agricultural impact complete a higher number of overlapping generations, two to seven per year, in the Mediterranean Basin (Franco *et al.* 2000).

Mealybugs spread over almost all strata within a plant and can feed on leaves, herbaceous stems, fruits and roots. Due to their cryptic habits, they usually settle in small depressions or protected areas of plants such as *Planococcus ficus* (Signoret) under the bark of the vine or *P. citri* in the calyx of citrus fruits (Geiger *et al.* 2001; Martínez-Ferrer *et al.* 2003). Other species such as *Phenacoccus solani* Ferris or *Pseudococcus comstocki* (Kuwana) can be found inside sweet peppers and apples, respectively (Woodside 1936).

Although all female stages are mobile, these insects have sedentary habits. Crawlers show the greatest mobility, seeking for suitable feeding sites, but when conditions are favorable they settle in a plant close to their mothers, resulting in a clumped spatial distribution in colonies (Nestel *et al.* 1995; Gullan and Kosztarab 1997) (Fig. 3). They are also influenced by their host phenology moving to different plant strata for overwintering, feeding, mating, and ovipositing (McKenzie 1967; Geiger *et al.* 2001; Martínez-Ferrer *et al.* 2003; Franco *et al.* 2009; Cid *et al.* 2010; Haviland *et al.* 2012). Their dispersion over longer distances occurs mainly by human and wind action (Grasswitz and James 2008; Vitullo 2009).

Figure 3. Aggregated distribution of mealybugs in citrus fruit.

1.2.2. Mealybugs as invasive pests

Invasive species can pose a major risk to biodiversity and agricultural ecosystems, causing significant ecological and economic impacts (Williamson 1996; Pimentel *et al.* 2001; Kenis *et al.* 2009). The recent increase in the worldwide trade of horticultural and ornamental plants has facilitated the introduction and spread of several insect pests (Kenis *et al.* 2007; Roques *et al.* 2009). Within Europe, the Mediterranean Basin is especially susceptible to insect invasions, due to its climatic conditions being favorable for the establishment of tropical and subtropical non-native species (Roques *et al.* 2009; Walther *et al.* 2009).

Mealybugs are common invasive species, in that their small size and cryptic behavior allows them to pass quarantine controls unnoticed (Miller *et al.* 2002; Pellizzari and Germain 2010). They have been involved in serious pest outbreaks in tropical and subtropical regions, such as *Phenacoccus manihoti* Matile-Ferrero and *Rastrococcus invadens* Williams on cassava and fruit trees, respectively, throughout Africa (Herren and Neuenschwander 1991; Han *et al.* 2007); *Maconellicoccus hirsutus* (Green) on crops and ornamental plants in the Caribbean and South America (Matile-Ferrero *et al.* 2000; Culik *et al.* 2013) (Matile-Ferrero *et al.* 2000); *Paracoccus marginatus* Williams and Granara de Willink on several crops in America and Pacific region, and more recently in Southeast Asia (Matile-Ferrero *et al.* 2000; Muniappan *et al.* 2008); and *Phenacoccus solenopsis* Tinsley on cotton in India, Pakistan and China (Hodgson *et al.* 2008; Wang *et al.* 2010).

In their review of European alien scale insects, Pellizzari and Germain (2010) classified a quarter of the mealybug species recorded in Europe as exotic. They are thus among the families responsible for the highest number of infestations, after aphids and diaspidids. These authors found that the commonest pathway was the horticultural and ornamental trade, and most of these species are native to America. In mainland Spain, this proportion is even higher and forty percent of the known species are invasive pests in crops and ornamental plants (Ben-Dov *et al.* 2013). The rest, on the other hand, are native species, found mainly in natural ecosystems.

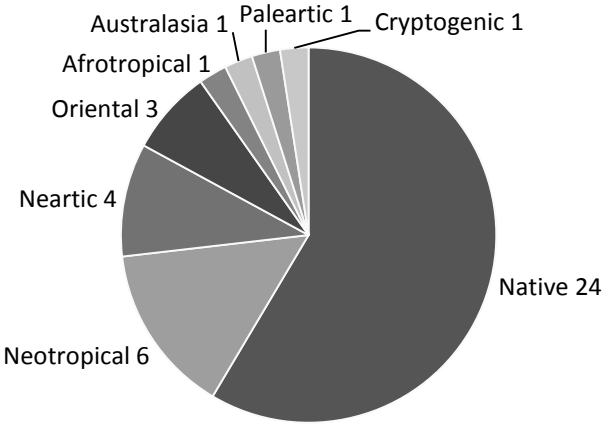

Figure 4. Zoogeographical origin of mealybugs in mainland Spain. (Pellizzari and Germain 2010; Ben-Dov *et al.* 2013)

1.2.3. Taxonomy

Systematics is essential to solve ecological and agricultural problems caused by insect pests. Taxonomy is required in the recognition of insect species by quarantine services and in the early detection of new invasions (Danks 1988). As pest management relies on accurate identification, the combination of ecological and systematic studies can facilitate the selection of appropriate control measures (Smith *et al.* 2011). Mealybug management is currently challenged by frequent species misidentification, which reduces the efficiency of crop protection methods and increases pesticide use. Accurate insect

identification is essential when applying biological control programs to mealybug species, as some of their most important natural enemies are host and habitat specific (Bartlett 1978; Neuenschwander 2001; Charles 2011).

Taxonomy and identification of the Pseudococcidae family has been traditionally achieved by comparing morphological characters of adult females (Miller and Kosztarab 1979). Very few attempts have been made to classify adult males and immature females (Afifi 1986; Gimpel and Miller 1996; Gullan 2000; Hodgson 2002; Wakgari and Giliomee 2005). However, morphological identification entails certain difficulties, as it can be a time-consuming process and needs to be applied by taxonomic specialists. Some environmental conditions can induce morphological variations in mealybugs, and sometimes it is impossible to differentiate between complexes of cryptic species (Cox 1983; Charles *et al.* 2000). All these difficulties have awakened an interest in applying molecular techniques to complement mealybug taxonomy (Beuning *et al.* 1999; Downie and Gullan 2004; Demontis *et al.* 2007; Cavalieri *et al.* 2008; Hardy *et al.* 2008; Rung *et al.* 2009; Park *et al.* 2010; Pieterse *et al.* 2010; Daane *et al.* 2011; Correa *et al.* 2012). Among their advantages are high accuracy and the feasibility of identifying nymphal and male stages, which have thus become very important in quarantine controls. DNA barcoding and multiplex PCR are currently the two most commonly used molecular techniques of mealybug identification (Daane *et al.* 2011; Malausa *et al.* 2011; Park *et al.* 2011).

DNA barcoding ensures accurate identification by employing short, standardized fragments of DNA (Jinbo *et al.* 2011). An unknown specimen can be identified by comparing its DNA with a barcode database library of reference sequences from previously known individuals. Different gene regions have been used for DNA barcoding, among which cytochrome c oxidase subunit 1 (COI) has been established as a standard region to barcode all living organisms (Hebert *et al.* 2003a; Hebert *et al.* 2003b). However, this region may not be suitable for barcoding families like mealybugs, and so other alternative genomic regions have been proposed (Malausa *et al.* 2011; Park *et al.* 2011). DNA barcoding also provides information on phylogenetic and population studies (Hajibabaei *et al.* 2007). Barcoded sequences can be used to create identification tools such as multiplex-PCR kits for systematic and rapid identification of certain species. Several kits have been created for the identification of mealybugs in vineyards in

the Mediterranean, South Africa and USA (Cavalieri *et al.* 2008; Saccaggi *et al.* 2008, Daane *et al.* 2011), citrus in South Africa (Pieterse *et al.* 2010) and pear trees in South Korea (Park *et al.* 2010).

The main contributions to mealybug fauna in Spain were carried out by Gómez-Menor (1937, 1946, 1960, 1965, 1968) and Martín-Mateo (1985), who provided a wide inventory of the native fauna and the first alien species, while the mealybug fauna specific to the Canary Islands was later inventoried by Carnero and Pérez-Guerra (1986). New invasive pest species have been discovered in recent years (Beltrà *et al.* 2010; Beltrà and Soto 2011). However, given the highly variable number of mealybug species officially registered in the Mediterranean countries, it is highly likely that several species remain unrecorded in many parts of this geographical area (Ben-Dov *et al.* 2013) (Table 1). This seems to be the case in Spain where so far less than 50 species have been identified. Due to the incomplete information on this fauna, field misidentifications are frequent and often lead to reduced efficiency in biological control and increased pesticide applications.

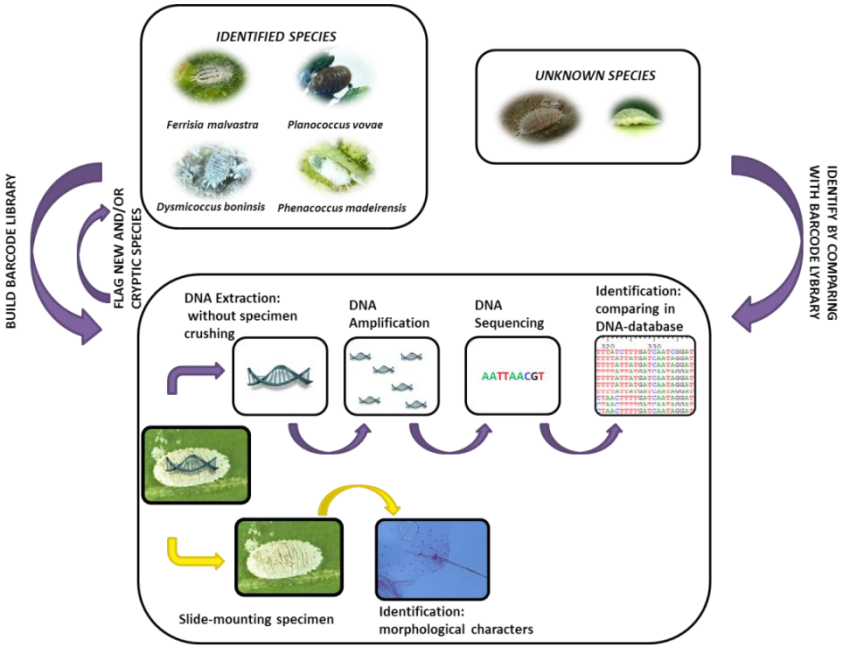

Figure 5. Mealybug DNA barcoding. Modified from Hajibabaei (2007).

Table 1. Mealybug species recorded in Mediterranean countries and islands (Ben-Dov *et al.* 2013).

Geographic area	Species identified
France	124
Italy	110
Turkey	77
Egypt	49
Sicily	48
Spain	41
Israel	39
Corsica	30
Crete	22
Balearic Islands	21
Algeria	19
Greece	18
Portugal	17
Morocco	16
Tunisia	13

1.2.4. Sampling

Sampling population dynamics is essential to understand the biology and ecology of arthropods and establish integrated pest management programs (Stern 1973; Binns and Nyrop 1992). Mealybug monitoring has been widely developed using direct and indirect sampling techniques. However, very few attempts have been made to establish thresholds for the management of these pests (Walton *et al.* 2004; Martínez-Ferrer *et al.* 2006; Martínez-Ferrer *et al.* 2008; Mudavanhu *et al.* 2011).

Direct sampling strategies are based on counting mealybug populations in different plant strata. Enumerative and binomial samplings have been broadly used in integrated pest management of species such as *P. citri*, *P. ficus, Pseudococcus maritimus* (Ehrhorn), *Pseudococcus longispinus* (Targioni-Tozzetti)*, Saccharicoccus sacchari* (Cockerell), and *M. hirsutus* in crops and ornamental plants (Furness 1976; Meyerdirk 1981; Allsopp 1991; Nestel *et al.* 1995; Geiger and Daane 2001; Martínez-Ferrer *et al.* 2006; Roltsch *et al.* 2006; Mgocheki and Addison 2009; Francis *et al.* 2012). In addition, the use of timed counts has also been carried out to study population dynamics in vineyards (Geiger and Daane 2001).

Among indirect monitoring strategies, mealybugs can be sampled with corrugated cardboard trunk bands (DeBach 1949; Browning 1959; Furness 1976) and sticky tapes (Hill and Burts 1982; Vitullo 2009; Cid *et al.*2010). The development of pheromones in recent years for several species such as *P. ficus, P. citri, P. viburni, M. hirsutus, P. longispinus* or *P. madeirensis* has simplified sampling (Millar *et al.* 2002; Zada *et al.* 2004; Francis *et al.* 2007; Vitullo *et al.* 2007; Martínez-Ferrer *et al.* 2008; Franco *et al.* 2009; Mudavanhu *et al.* 2011; Waterworth *et al.* 2011).

1.2.5. Management

Mealybug management has been traditionally carried out by chemical and biological methods. However, pheromone-based tactics such as mate disruption and mass trapping, which are currently in experimental development stage, are increasingly attracting interest (Franco *et al.* 2009).

Insecticides have been widely used to control mealybugs (Daane *et al.* 2006; Castle and Prahbaker 2011), particularly on species of unknown taxonomy and biology. However, insecticide efficacy can be reduced by some of this family's morphological and ecological characteristics. Their waxy cover and cryptic behavior protect them against contact insecticides, while systemic insecticides are not effective in stages in which the insects do not feed, such as eggs, adult males, and some gravid females (Moore 1988). Chemical control also has other drawbacks and the overuse of some active ingredients has led to resistance in some species (Charles *et al.* 1993). Pesticide use is also one of the most harmful practices for natural enemies and pollinators (Meyerdirk *et al.* 1982; Croft 1990; Anand and Ayub 2000; Campos and Martínez-Ferrer 2008; Szczepaniec *et al.*2011; Henry *et al.* 2012; Whitehorn *et al.* 2012). However, there is an increasing interest in the application of short-life insecticides respectful with natural enemies (Mgocheki and Addison 2009; Mansour *et al.* 2011). Restrained application of environmentally safe insecticides in mealybug hot-spots is also used so as not to disturb biological control in greenhouses (Protasov *et al.* 2010).

1.2.6. Biological control

The biological control of mealybugs has been widely studied since the early twentieth century, due to the economic importance and invasive habits of this family (McKenzie 1967). They have a wide variety of predators, including: Coccinellidae coleopterans, lacewings of the families Chrysopidae, Coniopterygidae and Hemerobiidae, flies of the families Cecidomyiidae and Chamaemyiidae, Anthocoridae bugs, Lycaenidae lepidopterans, and Phytoseiidae mites (Franco *et al.* 2000). Additionally, encyrtids are the most important mealybug parasitoids and species belonging to the genera *Anagyrus* Howard, *Leptomastix* Förster, *Leptomastidea* Mercet, *Gyranusoidea* Compere, *Coccidoxenoides* Girault or *Acerophagus* Smith are worldwide used in biological control (Moore *et al.* 1988).

Most of the predators are generalist and their relation with their prey is not density dependent. Although the efficiency of generalist predators against mealybugs remains essentially unknown (Franco *et al.* 2009), they may play an important role during the first infestations, when mealybug populations are low and specific natural enemies are not present (Symondson *et al.* 2002). On the other hand, some coccinellids show specificity for mealybugs and are commonly used in classical and inundative biological control (Bartlett 1978; Iperti 1999). Among them, *Cryptolaemus montrouzieri* Mulsant, of Australian origin, has been introduced in a large number of countries, including Spain, with the aim of controlling different mealybug species (Moore *et al.* 1988; Jacas *et al.* 2006) (Fig. 6). This coccinellid is also mass reared by several biological control companies and is widely used in augmentative biological control.

Encyrtids are very important natural enemies of mealybugs and have a major influence on their population dynamics. They establish host-specific relationships with mealybugs and consequently have been broadly used in classical biological control (Charles *et al.* 2011). Serious mealybug outbreaks have been solved by the introduction of encyrtid parasitoids from their area of origin, such as *P. manihoti* by *Epidinocarsis lopezi* (De Santis) (Neuenschwander 2001), *M. hirsutus* by introducing *Anagyrus kamali* (Roltsch *et al.* 2006) or *R. invadens* by introducing *Anagyrus mangicola* Noyes and *Gyranusoidea tebygi* Noyes (Agricola *et al.* 1989; Neuenschwander *et al.* 1994). Encyrtids are also used in augmentative

biological control of mealybugs. In Spain this is a relatively widespread practice and parasitoids are mass-released to control *P. citri* in citrus orchards and ornamental plants, *P. ficus* in vineyards, and *P. solani* in crop-protected ecosystems (Lucas 2002; Villalba *et al.* 2006; Campos 2009; Calvo and Belda 2011; Beltrà and Soto 2012).

Figure 6. ***Cryptolaemus montrouzieri*** **feeding on** ***Planococcus citri.***

Besides applying insecticides, the efficiency of mealybugs' natural enemies can be limited by other factors, such as food sources and the absence of alternative hosts (Moore 1988; Davies *et al.* 2004). Reducing pesticide applications or manipulating the environment to enhance the populations of natural enemies and increase their effectiveness can play an important role in mealybugs' management (Barbosa 1998; Landis *et al.* 2000). Predators and adult parasitoids require food sources other than their hosts, such as nectar, pollen or insect honeydew to increase their longevity and fertility (Landis *et al.* 2000; Gurr *et al.* 2005; Heimpel and Jervis 2005). Numerous laboratory experiences show that mealybug parasitoid longevity and offspring increase when they feed on sugar (Sagarra *et al.* 2000b; González-Hernández *et al.* 2005; Chong and Oetting 2006; Sandanayaka *et al.* 2009). Further research is still being carried out on the use of groundcovers in improving mealybug biological control (Addison and Samways 2006).

Mealybug and ant mutualism also have an important and complex role in biological control (Fig. 7). Ants feed on mealybug honeydew and in return provide

protection against predators and parasitoids, transporting them to new areas, and cleaning up excess honeydew, which can kill mealybug nymphs (McKenzie 1967; Franco *et al.* 2004). Several studies have shown that ants control or their exclusion by physical barriers increases the action of natural enemies and improves biological control (Nechols and Seibert 1985; Campos *et al.* 2006; Mgocheki and Addison 2010).

Figure 7. Ant attending *Phenacoccus peruvianus.*

1.2.7. Mealybugs in urban landscapes

Mealybugs use ornamental plants as a pathway of introduction in new areas, so that urban landscapes and nurseries are the first habitats in which they become established (Pellizzari and Germain 2010). In general, they are well adapted to different environments (McKenzie 1967) and some of their biological traits favor their adaptation to urban ecosystems: sucking mouthparts, limited mobility and multiple generations on the same plant (Raupp, 2010). They are mostly found in flowering herbaceous plants, grasses, bulbs, succulents and cacti, shrubs, palms, and trees (McKenzie 1967). Important polyphagous species, such as *P. citri*, *P. madeirensis, P. marginatus*, and *M. hirsutus* are key pests in nurseries and urban landscapes worldwide (Kairo *et al.* 2000; Miller and Miller 2002; Sadof *et al.* 2003; Chong *et al.* 2003; Laflin and Parrella 2004; Culik *et al.* 2013). They cause direct damage to ornamental plants by reducing plant growth and causing leaf fall and indirectly by their honeydew, which drips on urban furniture and

makes it unsightly (McKenzie 1967; Kairo *et al.* 2000; Dreistadt *et al.* 2004; Villalba 2005; Franco *et al.* 2009).

Although mealybugs are common in ornamental plants, the high complexity and number of hosts present in urban landscapes means that specific programs for their management are absent. On the other hand, general guidelines for monitoring, decision making and control are based mainly on previous experience in agricultural ecosystems (Buss and Turner 1993), and thus in urban landscapes they have traditionally been countered by chemical methods.

1.3. The Bougainvillea mealybug *Phenacoccus peruvianus*

1.3.1. Genus *Phenacoccus*

Phenacoccus Cockerell is one of the largest genera of the Pseudococcidae, accounting for 206 species (Ben-Dov *et al.* 2013). This genus is a member of the *Phenacoccinae* subfamily, which includes 69 genera such as *Geococcus* Green, *Rastrococcus* Ferris and *Rhizoecus* Kunckel d'Herculais (Hardy *et al.* 2008).

Phenacoccus species are widely distributed throughout the world's zoogeographical regions and most of its species originate from Paleartic, Nearctic and Neotropical areas (Danzig 2003; Downie and Gullan 2004; Granara de Willink and Szumik 2007). In the Mediterranean Basin 30 species have been recorded, of which five are American invaders: *Phenacoccus gossypii* Townsend and Cockerell*, Phenacoccus peruvianus* Granara de Willink, *P. madeirensis*, *P. solani*, and *P. solenopsis* (Beltrà *et al.* 2010; Kaydan *et al.* 2013). Some species of this genus have been involved in serious pest outbreaks: *Phenacoccus manihoti* devasted cassava crops in Africa, leading to a food shortage in some countries (Herren and Neuenschwander 1991), and more recently *P. solenopsis* spread through Asia to become a key pest of cotton (Wang *et al.* 2010). Likewise, the dispersion of other *Phenacoccus* species such as *P. solani* and *P. peruvianus* in the Mediterranean Basin is causing significant problems in crops and ornamental plants (Ben-Dov 2005; Beltrà *et al.* 2010; Beltrà and Soto 2011).

1.3.2. *Phenacoccus peruvianus*

The bougainvillea mealybug *P. peruvianus* is a species of Neotropical origin that was described by Granara de Willink in 2007 from different populations of Peru and Argentina.The author found small morphological differences between these populations but they were finally grouped in the same species (Granara de Willink and Szumik 2007).

The presence of *P. peruvianus* in Europe was reported for the first time in Almeria (Spain) in 1999 and it was subsequently observed in other European areas such as the Madeira Islands (2001), Sicily (2002), the UK and Corsica (2005), Portugal (2006), France and the Canary Islands (2008) and Majorca (2010) (Beltrà *et al.* 2010; Franco *et al.* 2011) (Fig. 8).

Figure 8. Invasion of *Phenacoccus peruvianus* in Europe (Beltrà *et al.* 2010).

The bougainvillea mealybug is a polyphagous species. In South America, where it is native, it has only been responsible for small infestations in ornamental plants and natural ecosystems (Granara de Willink personal communication). However, in Europe this insect has been observed in several host species, showing some preference for Solanaceae family plants (Beltrà *et al.* 2010) (Table 2). In a recent study, different important Mediterranean crops were tested for hosts of *P.*

peruvianus, but only tomato and tobacco were found to be suitable (A. B. *et al.* unpublished data). *Phenacoccus peruvianus* is an important pest of several ornamental plants, causing problems in nurseries and urban landscapes in the Mediterranean Basin. It settles on bougainvillea leaves, twigs and bracts, where it feeds on the sap (Fig. 9). Its feeding reduces plant growth and highly infested leaves and bracts become yellowish and fall off. The honeydew secreted promotes the growth of a black sooty mold that interferes with photosynthesis and spoils urban furniture.

Table 2. Host list of *Phenacoccus peruvianus* (Beltrà *et al.* 2010).

Family	Species
Acanthaceae	*Justicia suberecta*
Amaranthaceae	*Alternanthera sp.*
Asclepiadaceae	*Araujia sericifera*
Asclepiadaceae	*Eupatorium sp.*
Asteraceae	*Baccharis sp.*
Aucubaceae	*Aucuba japonica*
Juglandaceae	*Juglans jamaicensis*
Lamiaceae	*Solenostemon blumei*
Malvaceae	*Hibiscus rosa-sinensis*
Myoporaceae	*Myoporum laetum*
Myrtaceae	*Psidium guaiavita*
Nyctaginaceae	*Bouganvillia spp.*
Rubiaceae	*Coffea sp.*
Scrophulariaceae	*Buddleja sp.*
Solanaceae	*Cestrum sp.*
Solanaceae	*Lycopersicon esculentum*
Solanaceae	*Solanum vespertillo*
Verbenaceae	*Lantana camara*

Figure 9. Colony and damage of *Phenacoccus peruvianus* in bougainvillea plants.

1.4. Justification and objectives

The recent introduction of new species into the Mediterranean Basin has caused serious problems for crops and ornamental plants. Deficient information on the fauna in some areas of Southern Europe is the cause of species misidentification, limiting the application of efficient specific management strategies. Among the newly introduced mealybugs, *P. peruvianus* is spreading around Mediterranean countries damaging plants in urban landscapes and ornamental nurseries. No information on its biology, behavior, and natural enemies in Europe is currently available.

The management of this pest in the Mediterranean Basin, and specifically in Eastern Spain, has relied mainly on insecticides. However, the new European directive on pesticide use stipulates the reduction or even the prohibition of chemical control in urban landscapes, limiting the management strategies of *P. peruvianus*. The development of alternative management strategies requires a better understanding of the taxonomy, biology and ecology of this mealybug and its natural enemies.

Therefore, with the aim of integrating biological control into the management of *P. peruvianus* in urban landscapes in the Mediterranean Basin, we propose the following objectives:

i) Provide a multi-criterion characterization of mealybug populations in Eastern Spain to be used as a basis for their routine identification through DNA sequencing or the use of derived species-specific molecular tools.

ii) Study the seasonal phenology and spatial distribution of *P. peruvianus*.

iii) Establish a sampling plan for *P. peruvianus* in *Bougainvillea* plants.

iii) Study the natural enemy complex of *P. peruvianus* in Eastern Spain and its influence on mealybug populations.

iv) Evaluate biological and ecological traits of the most important biological control agents of *P. peruvianus* to optimize the biological control of this pest.

2- MOLECULAR AND MORPHOLOGICAL CHARACTERISATION OF PSEUDOCOCCIDAE SURVEYED ON CROPS AND ORNAMENTAL PLANTS IN SPAIN

Beltrà, A., Soto, A. and Malausa, T. (2013) **Molecular and morphological characterisation of Pseudococcidae surveyed on crops and ornamental plants in Spain.** Bulletin of Entomological Research, 102:165-172.

Abstract

Mealybugs (Hemiptera: Pseudococcidae) are common invasive pests in Europe, causing major problems on crops and ornamental plants. However, very few data are available concerning the mealybug fauna of Southern Europe. This lack of data and the difficulty of identifying mealybugs morphologically by traditional techniques currently limit the perspectives for efficient specific pest management. The aim of this study was to provide multi-criterion characterization of mealybugs surveyed in Eastern Spain in order to facilitate their routine identification through DNA sequencing or the use of derived species-specific molecular tools. We characterised 33 mealybug populations infesting crops and ornamental plants in Eastern Spain, using a combination of molecular and morphological techniques, including the sequencing of the universal barcode DNA region cytochrome c oxidase subunit I (COI). This characterisation has led to the identification of ten species and provides sequence data for three previously unsequenced species, contributing to the phylogenetic knowledge of the family Pseudococcidae. In addition, the intraspecific variations found in the populations of five mealybug species provide insight into their invasion history.

Keywords: DNA barcoding, molecular characterization, mealybug, species identification

2.1 Introduction

Invasive species constitute a major threat to biodiversity and agricultural ecosystems and may have a significant ecological and economic impact (Williamson 1996; Pimentel *et al.* 2001; Kenis *et al.* 2009). Scale insects are typical invasive pests; due to their small size and cryptic behaviour, they often remain undetected during quarantine inspections (Miller *et al.* 2005; Hulme *et al.* 2008; Pellizzari and Germain 2010). One particular group of scale insects, mealybugs (Hemiptera: Pseudococcidae), constitutes the third most common family of alien insects in Europe, with about 40 new established species (Roques *et al.* 2009; Pellizzari and Germain 2010). Mealybugs are common pests of a wide range of agricultural and ornamental plants (Ben-Dov 1994) and may cause serious problems if they become established in new environments lacking natural enemies (Miller *et al.* 2002). They damage the plant by sucking its sap and transmitting viruses. Furthermore, the honeydew they produce may also favour the development of mould fungi and decrease ornamental plant quality (Williams 1985; Kosztarab and Kozár 1988; Franco *et al.* 2000).

Mealybug management is currently challenged by frequent species misidentification that decreases the efficiency of crop protection methods and increases pesticide use. This situation can be explained by the lack of reliable surveys and characterisations of mealybug species, mainly because their identification has been difficult or even sometimes impossible until recently. Indeed, taxonomy and identification of members of the family Pseudococcidae have generally been based on comparisons of the morphological characters of adult females. However, there are several drawbacks to this method. Firstly, it is a time-consuming process requiring specialized taxonomic knowledge, which is not available on a daily basis for most practitioners. Secondly, some environmental conditions may induce morphological variation in mealybugs, making it impossible in some cases to differentiate between complexes of cryptic species (Cox 1983; Charles *et al.* 2000). Thirdly, mealybug morphological identification is generally impossible when specimens are collected at larval stage (a common situation in the field and of special concern in quarantine controls).

These difficulties can be dealt with by taking profit from the complementarities between morphological and molecular characterization to identify the species. Indeed, once a reference specimen is taxonomically identified by morphological examination and characterized by DNA sequencing, any new sample displaying the same DNA sequence can be identified quickly without the need of any competence in taxonomy. For taxonomists, such an approach also avoids repetitive identification of the most common species. This method is the basis of the so-called DNA barcoding international projects (Hebert *et al.* 2003a). The main barcode region used in international projects is a 648bp region of the cytochrome c oxidase subunit I (COI) (Hebert *et al.* 2003b). However, despite COI having been used in various taxonomic studies of mealybugs (Gullan *et al.* 2003, 2010; Demontis *et al.* 2007; Cavalieri *et al.* 2008; Rung *et al.* 2008, 2009; Saccaggi *et al.* 2008; Ashfaq *et al.* 2010; Pieterse *et al.* 2010; Park *et al.* 2011), the universal primers used to amplify this region do not work well in several species of this family (Malausa *et al.* 2011). Therefore, new primers for this region have been recently designed (Malausa *et al.* 2011; Park *et al.* 2011). Moreover, the use of combinations of different DNA markers as nuclear DNA, mitochondrial DNA and endosymbiont DNA (from Tremblaya Princeps) proved to be successful not only for DNA barcoding but also to better estimate the genetic distance between species and for disentangle complexes of cryptic taxa (Malausa *et al.* 2011).

In this study, we coupled the morphological examination of slide-mounted samples and their DNA sequencing at five markers to generate multi-criterion identification of 33 mealybug populations infesting crops and ornamental plants in Eastern Spain. This work provides a comprehensive characterisation of ten species found in Eastern Spain and will be used as basis for the routine identification of mealybugs in Spain and more generally in Southern Europe, by DNA sequencing or with molecular identification tools derived from DNA sequences.

2.2 Materials and methods

2.2.1 Sample collections

Thirty-three mealybug populations damaging crops and ornamental plants were sampled in Eastern Spain between the years of 2007 and 2009 (Table 1). An additional sample of *Phenacoccus peruvianus* Granara de Willink was collected from Southern France, for comparison of the populations of this new invasive species in the two countries. The samples consisted mostly of adult females and immature instars, which were just taken when adults were not available. Individuals were checked under a stereoscope and discarded if any parasitoids were detected. The collected insects were preserved in 70% ethanol and stored at 20°C for molecular analysis and morphological identification.

2.2.2 DNA extraction and amplification

DNA was extracted from 239 specimens by using the DNeasy Tissue Kit (QIAGEN). The extraction was performed without crushing the insect body, which enabled us to recover the specimen for its posterior morphological identification. Therefore, the process followed the manufacturer's guidelines with two small variations to improve DNA extraction: cell lysis was carried out over a period of six to eight hours and two elution steps (2×50µl of AE buffer) (Malausa *et al.* 2011).

DNA was amplified from five different loci, chosen for analysis on the basis of their suitability for DNA barcoding, population genetics and phylogenetic studies: two regions of mitochondrial mealybug DNA (the 2183–2568 and LCO regions of COI), two regions of nuclear DNA (28s-D2 and the entire ITS2 region) and one region of DNA from the bacterium Tremblaya princeps (leuA-16 s) (Malausa *et al.* 2011). PCR was performed with a 23µl reaction mixture and 2µl of diluted DNA (1–20 ng). The reagent concentrations were 1×Phusion HF buffer (Phusion High-Fidelity DNA polymerase 530 (FINNZYMES, Espoo, Finland)), 0.01Uµl1 Phusion enzyme, 200µM dNTPs and 0.5µM of each primer (Table 2).

Table 1. List of the material sampled: Population codes, geographic origin and host origin of the samples and number of individuals used for DNA extraction and morphological identification.

Pop #	Region	City	GPS coordenates	Host	Collection date	N	Identification
1	Comunitat Valenciana	Valencia	39.480998 N, 0.349395 W	*Coronilla sp.*	03/06/08	8	*Phenacoccus madeirensis*
2	Comunitat Valenciana	Ibi	38.622584 N, 0.575401 W	*Cupressus sempervirens*	10/09/08	6	*Planococcus vovae*
3	Comunitat Valenciana	Valencia	39.472218 N, 0.351524 W	*Cupressus sempervirens*	15/07/08	8	*Planococcus vovae*
4	Comunitat Valenciana	Valencia	39.476822 N, 0.386716 W	*Dyospiros duclouxii*	12/09/08	8	*Planococcus citri* *Phenacoccus madeirensis*
5	Comunitat Valenciana	Valencia	39.476822 N, 0.386716 W	*Erytrina bogotensis*	12/09/08	8	*Phenacoccus madeirensis*
6	Comunitat Valenciana	Altea	38.602324 N, 0.045092 W	*Lantana camara*	23/08/08	8	*Phenacoccus madeirensis*
7	Comunitat Valenciana	Altea	38.602324 N, 0.045092 W	*Unknown host*	23/08/08	8	*Phenacoccus madeirensis*
8	Comunitat Valenciana	Valencia	39.467628 N, 0.344121 W	*Lantana camara*	22/09/08	4	*Phenacoccus madeirensis*
9	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Saccharum officinarum*	02/03/08	8	*Dysmicoccus boninsis*
10	Comunitat Valenciana	Altea	38.608801 N, 0.041615 W	*Ceratonia siliqua*	23/08/08	6	*Planococcus citri*
11	Comunitat Valenciana	Algimia d'Alfara	39.753015 N, 0.360651 W	*Solanum lycopersicum*	21/07/08	8	*Planococcus citri*
12	Comunitat Valenciana	Valencia	39.485907 N, 0.362367 W	*Ocimum basilicum*	22/09/08	8	*Planococcus citri*
13	Comunitat Valenciana	Valencia	39.476822 N, 0.386716 W	*Cleistocactus strausii*	13/11/07	8	*Planococcus citri*
14	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Acalipha wilkesiana*	02/03/08	8	*Planococcus citri*
15	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Aucuba japonica*	18/04/08	8	*Phenacoccus peruvianus*
16	Catalunya	Blanes	41.676872 N, 2.801936 E	*Bougainvillea glabra*	24/09/08	8	*Phenacoccus peruvianus*
17	Catalunya	Blanes	41.676872 N, 2.801936 E	*Cordilyne stricta*	24/09/08	3	*Pseudococcus longispinus*
18	Comunitat Valenciana	Valencia	39.476364 N, 0.357291 W	*Unknown host*	10/06/08	5	*Phenacoccus peruvianus*
19	Comunitat Valenciana	Valencia	39.478956 N, 0.367683 W	*Myoporum sp.*	10/06/08	8	*Phenacoccus peruvianus*
20	Comunitat Valenciana	Altea	38.608801 N, 0.041615 W	*Malva parviflora*	23/08/08	8	*Phenacoccus madeirensis*
21	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Cereus peruvianus*	02/03/08	8	*Hypogeococcus pungens*
22	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Olla carnosa*	03/06/08	5	*Planococcus citri*
23	Comunitat Valenciana	Altea	38.602324 N, 0.045092 W	*Euonymus japonicus*	23/08/08	8	*Pseudococcus longispinus*
24	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Salvia sp.*	15/07/08	8	*Pseudococcus viburni*
25	Comunitat Valenciana	Altea	38.602324 N, 0.045092 W	*Pittosporum tobira*	23/08/08	8	*Pseudoccus longispinus*
26	Comunitat Valenciana	Altea	38.602782 N, 0.047820 W	*Hibiscus rosa-sinensis*	23/08/08	5	*Pseudoccus longispinus*
27	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Bougainvillea glabra*	12/03/08	7	*Phenacoccus peruvianus*
28	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Parietaria judaica*	12/12/08	8	*Planococcus citri*
29	Andalucia	El Ejido	36.719749 N, 2.789198 W	*Capsicum annum*	20/12/08	8	*Phenacoccus solani*
30	Catalunya	Deltebre	40.724924 N, 0.839764 W	*Myoporum laetum*	18/08/08	8	*Phenacoccus peruvianus*
31	Comunitat Valenciana	Valencia	39.482109 N, 0.343475 W	*Chamaedorea sp.*	30/01/08	8	*Pseudococcus longispinus*
32	Alps Maritimes	Antibes	43.575327 N, 7.125707 E	*Bougainvillea glabra*	25/09/08	8	*Phenacoccus peruvianus*
33	Comunitat Valenciana	Faura	39.732309 N, 0.269403 W	*Citrus reticulata*	19/09/09	4	*Delottococcus aberiae*
34	Illes Balears	Soller	39.764619 N, 2.709765 W	*Justicia suberecta*	22/10/09	4	*Phenacoccus peruvianus*

Table 2. Molecular markers and annealing temperatures used in the study.

Locus	Primer name	Primer sequence	Annealing temperature	PCR product length (bp)	Reference
28s	None(D2) None(D2)	(F) AGAGAGAGTTCAAGAGTACGTG (R) TTGGTCCGTGTTTCAAGACGGG	60°C	~ 320	Belshaw and Quicke (1997)
COI	LCO-M-2d-F LCO-M-2d-R	(F) ATAACTATACCTATYATTATTGGAAG (R) AATAAATGTTGATATAAAATTGG	50°C	491	Malausa *et al.* (2011)
COI	C1-J-2183 C1-N-2568	(F) CAACATTTATTTTGATTTTTTGG (R) GCWACWACRTAATAKGTATCATG	56°C	385	Gullan *et al.* (2003)
ITS2	ITS2-M-F ITS2-M-R	(F) CTCGTGACCAAAGAGTCCTG (R) TGCTTAAGTTCAGCGGGTAG	58°C	~ 800	Malausa *et al.* (2011)
rpS15-16ST	leuA U16S	(F) GTATCTAGAGGNATHCAYCARGAYGGNG (R) GCCGTMCGACTWGCATGTG	60°C	~ 1050	Baumann *et al.* (2002)

PCR was carried out as follows: initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10s, annealing for 15 s at a temperature of 48°C–60°C, depending on the primer (Table 2), and elongation at 72°C for 5 min. The final products were separated by electrophoresis in a 2% agarose gel, to check their quality. They were then sequenced in both directions, by capillary electrophoresis on an ABI 3130XL automatic sequencer (Applied Biosystems, Foster City, CA, USA) at Genoscreen (Lille, France). Consensus sequences were generated and analysed with Seqscape v2.5 (ABI), and alignments were manually edited with Bioedit (Hall 1999). When a sequence of a specimen displayed a genetic variation at one or more nucleotide(s), it was considered as a different haplotype. The analysed sequences were deposited in GenBank to ensure future access and use (accession numbers JF714157–JF714210).

2.2.3 Morphological identification

Mealybug populations were identified on the basis of morphological characters. All the individuals sequenced were recovered after the DNA extraction, preserved in 70% ethanol and stored at 20°C. Individuals were posteriorly mounted on slides as described by Williams and Granara de Willink (1992), with the modifications described by Malausa *et al.* (2011) and a few additional changes: a small ventral incision was made behind the back leg, with a micro scalpel (BioQuip Products Inc., Rancho Dominguez, CA, USA). The specimen was heated at 60°C in 10% KOH for 20 min and washed in distilled water for 20 min. It was then stained with a 1:1:1 acid fuchsin (1% solution), lactic acid and glycerol.

Specimens were then immersed in acetic acid for one hour and transferred to lavender oil for an additional one hour. Finally, the insects were mounted on a slide in Heinze Mounting Medium (Heinze 1952) and covered with a coverslip. Slides were then heated at 30°C for 48 h.

Specimens were identified principally with the keys of Williams and Granara de Willink (1992), Gimpel and Miller (1996), Williams (2004) and Granara de Willink and Szumik (2007). For nymph voucher specimen identification, some species for which immature instars have never been described were identified to genus level only. The slides are available from the Polytechnic University of Valencia (Valencia, Spain).

2.2.4 Phylogenetic analysis

Phylogenetic studies were performed by merging our populations with other samples for which the same loci had been sequenced by Malausa *et al.* (2011). Bayesian inference was carried out with Bayes Phylogenies (Pagel and Meade 2004). Interspecific variability was too high for the alignment of ITS2 sequences. Thus, for this region, we inferred the phylogenetic relationships from a mixture model based on the other four loci. Analyses were carried out with nQ+C mixture models, with n varying between one and six independent rate matrices (Qs). The best model was chosen by comparing Bayes factors. We also applied a general time-reversible model, as recommended by Pagel and Meade (2004). Four Markov chains were used for ten million iterations and a print frequency of 1000 iterations. The length of the burn-in period was determined by plotting likelihood across iterations. All iterations corresponding to the burn-in period (around one million iterations) were removed from the output of Bayes Phylogenies before subsequent analyses. We used the sump command of MrBayes (Ronquist and Huelsenbeck 2003) to obtain a summary of Bayes Phylogenies outputs and to calculate Bayes factors. Majority rule consensus trees were then drawn with PAUP 4.0b10 (Swofford 2003) ('contree/Majrule' command) from the output of the Bayes Phylogenies analysis (9000 trees) using the best model selected. *Phenacoccus* species was used as outgroups because they are the most divergent taxa of this study (Hardy *et al.* 2008).

2.3 Results

We surveyed a total of 33 mealybug populations from Eastern Spain and one from Southern France between 2007 and 2009. We identified 239 specimens from these samples morphologically, and DNA was sequenced, when possible, at five loci.

This resulted in the occurrence of 16 multi-locus haplotypes (Table 3), which corresponded to ten species in terms of taxonomic identification: *Delottococcus aberiae* (De Lotto), *Dysmicoccus boninsis* (Kuwana), *Hypogeococcus pungens* Granara de Willink, *Phenacoccus madeirensis* Green, *P. peruvianus*, *Phenacoccus solani* Ferris, *Planococcus citri* Risso, *Planococcus vovae* (Nasonov), *Pseudococcus longispinus* (Targioni Tozzetti) and *Pseudococcus viburni* (Signoret). DNA sequences from the species *D. aberiae*, *P. peruvianus* and *P. vovae* were obtained for the first time in this study, and the universal barcode region cytochrome c oxidase subunit I (COI) was also sequenced for the first time from *D. boninsis* and *P. madeirensis* (Table S1, supporting information). The genetic markers of the various DNA regions studied generated sequences that distinguished successfully between all the taxa studied.

Table 3. Summary of the mealybug species identified, populations sampled (see Table 1) and different haplotypes obtained for each genetic marker. Haplotype numbers are as in the paper by Malausa *et al.* (2011) and are based on Genbank accession number. The rpS 15-16ST is expected to fail in *Phenacoccus spp.* because *T. princeps* is absent from these species. Different haplotypes obtained for the same species are shown in bold.

Multilocus haplotypes	Species	Populations sampled	LCO COI	2183-2568 COI	28S-D2	ITS2	rpS15-16ST
1	*Delottococcus aberiae*	33	E014	A018	C013	D014	
2	*Dysmicoccus boninsis*	9	E015	A017	C001	D003	B003
3	*Hypogeococcus pungens*	21	E009	A015	C012		
4	*Phenacoccus madeirensis*	1,4,5,6,7,8,20	E012	A013	C004	D013	-
5	*Phenacoccus peruvianus*	15,16,18,19,27,30,32,34	E007	A010	C002	D008	-
6	*Phenacoccus solani*	29	E010	A011	C003	D009	-
7	*Planococcus vovae H1*	2	E011	**A008**	C006	**D010**	B002
8	*Planococcus vovae H2*	3	E011	**A009**	C006	**D011**	B002
9	*Planococcus citri H2*	10,11,28	**E003**	**A001**	C007	D012	B001
10	*Planococcus citri H5*	12	**E004**	**A003**	C007	D012	B001
11	*Planococcus citri H6*	13,22	**E001**	**A004**	C007	D012	B001
12	*Planococcus citri H7*	14	**E002**	**A002**	C007	D012	
13	*Pseudococcus longispinus H1*	17,25,26	E006	A005	C009	**D004**	B006
14	*Pseudococcus longispinus H2*	23	E006	A005	C009	**D005**	
15	*Pseudococcus longispinus H3*	31	E006	A005	C009	**D006**	B006
16	*Pseudococcus viburni H2*	24	E016	A012	C008	D001	B005

For five mealybug species, we could sequence more than one population. Among those five species, three species displayed intraspecific variations among or within populations. Four different multi-locus haplotypes were recovered from six populations of *P. citri*, three multi-locus haplotypes were recovered from five populations of *P. longispinus* and two multi-locus haplotypes were recovered from two populations of *P. vovae* (Table 3). The distribution of these different haplotypes did not follow any obvious geographic pattern (Fig. 1). Other species, such as *P. peruvianus* and *P. madeirensis*, displayed high levels of genetic homogeneity, even though several populations from different hosts and geographic regions were studied. Intraspecific differences were observed at the sequences obtained from both regions of COI and ITS2. The regions rpS15–16s and 28s-D2 displayed no intraspecific variation.

The phylogenetic tree revealed that the genera *Phenacoccus* and *Planococcus* formed monophyletic groups. On the contrary, the genus *Pseudococcus* appeared paraphyletic. Indeed, in the topology, the *Pseudococcus* species are found in two separate clusters, each containing several *Pseudococcus* species and one *Dysmicoccus* species (Fig. 2). In addition, the species for which no DNA sequence was previously available were positioned in the topology with good support: *P. peruvianus* was located close to *Phenacoccus parvus* Morrison populations of Neotropical origin. *Planococcus vovae* differed slightly from the other three species of the genus *Planococcus*. *Delottococcus aberiae* was found in a cluster with *Vryburgia rimariae* Tranfaglia located inside part of the tree corresponding to the tribe Pseudococcini.

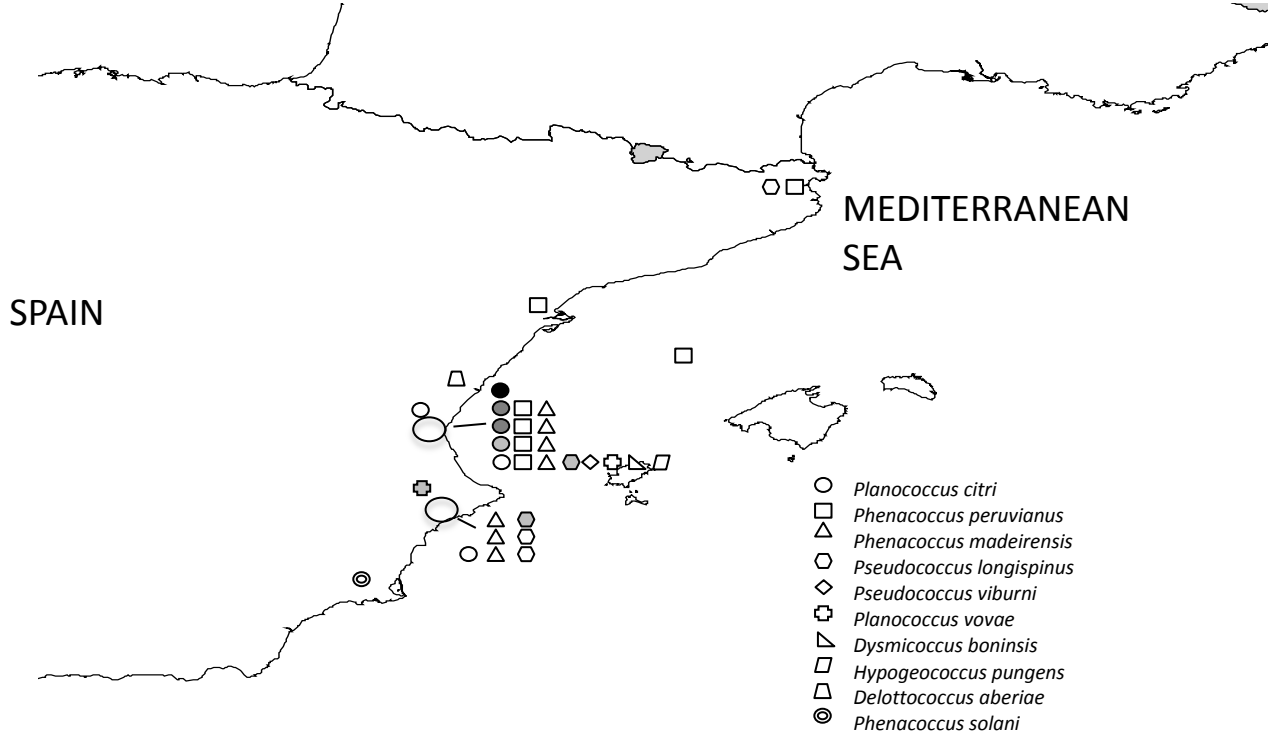

Fig 1. Distribution of the mealybug populations surveyed in Eastern Spain and France. The different symbols indicate the population species and colors denote the haplotypes.

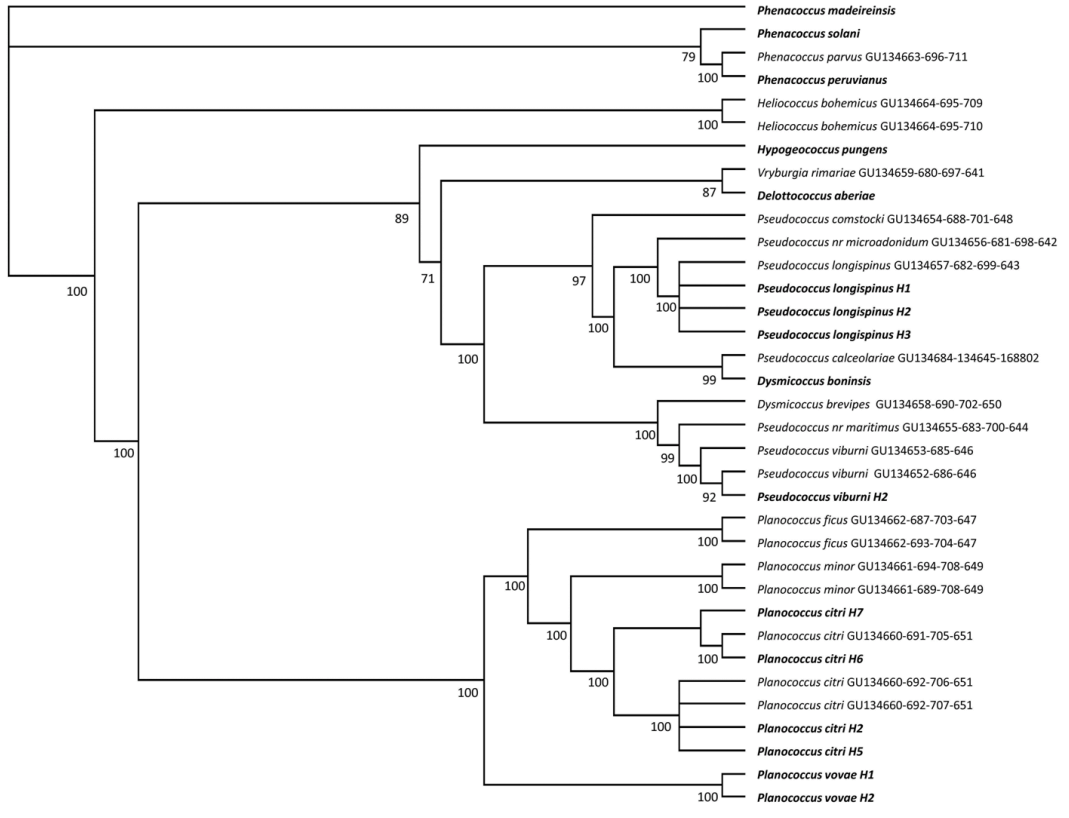

Fig 2. Bayesian phylogenetic tree of mealybug multilocus haplotypes generated by this study (shown in bold) or by Malausa *et al.* (2011) for multilocus haplotypes including information for at least three loci: 2183–2568 and LCO regions of COI, 28s-D2 and rpS15-16 s when possible. The majority-rule consensus tree was calculated from the Bayesian analysis, based on the best selected mixture model (three matrices). Bayesian posterior probabilities are represented beyond the nodes (9000 trees, values <70% not shown). Haplotypes are named according to Genbank accession numbers.

2.4 Discussion

The main interest of this study is probably to provide a solid basis for further works focusing on mealybug management. For researchers or practitioners with an access to DNA sequencing facilities, our data makes it possible to quickly identify taxa based on simple DNA sequence comparisons. This study also provides the raw data to design rapid identification kits based on the use of species-specific PCR: the large set of sequences available makes it possible to design species-specific PCR primers annealing to regions displaying variations among species but not among populations or individuals of the same species. One additional piece of information directly relevant for pest management is the occurrence of two species (*P. peruvianus* and *D. aberiae*) that represent two cases of recent introductions in Europe.

However, such a survey using multi-criteria sample characterisation also generates valuable data for researches on the evolutionary history of Pseudococcidae.

First, by generating DNA data for various species that had not been sequenced before (*P. peruvianus*, *P. vovae* and *D. aberiae*), this study gives insights into the phylogenetic relationships inside the family Pseudococcidae. *Phenacoccus peruvianus* appears more closely related to *P. parvus* than *P. solani* or *P. madeirensis*. This result is in conflict with the findings of Granara de Willink and Szumik (2007), whose morphological phylogenetic studies placed *P. peruvianus* closer to *P. madeirensis*. *Planococcus vovae* mapped close to the other species of the same genus, but did not come between *P. ficus* and the cryptic species *P. citri* and *P. minor* (Rung *et al.* 2008; Saccaggi *et al.* 2008). Moreover, the South African species *D. aberiae* was located close to *V. rimariae* on the phylogenetic tree, providing further evidence for the existence of a Southern African clade, as proposed by Hardy *et al.* (2008). In addition, the phylogenetic tree computed in this study confirms several trends observed in previous studies (Hardy *et al.* 2008; Malausa *et al.* 2011): (i) the genera *Phenacoccus* and *Planococcus* were found monophyletic, although few closely related species of other genera were in this study; (ii) the presence of two *Dysmicoccus*

species among the *Pseudococcus* species suggests that these two genera are paraphyletic, as proposed by Downie and Gullan (2004), Hardy *et al.* (2008) and Malausa *et al.* (2011).

Second, the contrasted patterns of intraspecific variability found in *P. vovae*, *P. citri*, *P. longispinus*, *P. peruvianus* and *P. madeirensis* may be explained by the species histories. Indeed, the extent of intraspecific variation observed in those species does not display any geographic pattern and may rather be accounted for by the time elapsed since these species first began their invasion of Europe. Substantial divergences were observed in the populations of the native species *P. vovae*, as well as in the exotic species *P. citri* and *P. longispinus*. These two exotic species have been present in the Mediterranean Basin for more than a century (Pellizzari and Germain 2010), long enough for population divergence to have occurred in the new area or for repeated introductions from different regions of the world (Thompson 1998; Dlugosch and Parker 2008). By contrast, the invasive species *P. peruvianus* and *P. madeirensis* displayed little or no DNA variability in the multilocus analysis. This suggests (i) that the populations experienced a genetic bottleneck, probably caused by their recent introduction into Europe and specifically in Spain (Marotta and Tranfaglia 1990; Beltrà *et al.* 2010; Beltrà and Soto 2011), and (ii) that the invasive populations came from the same geographic region or spread in Spain and France from a single introduced population.

In conclusion, this study provided a molecular characterisation at several DNA markers and a taxonomic identification for a set of 239 mealybug samples from 33 populations of Eastern Spain. Among them, ten different species were identified, and this study provided the first molecular data for three species. In addition, this multi-criteria characterization produced new data for the study of the Pseudococcidae phylogeny and revealed various patterns of intraspecific variations among populations of five mealybug species that may be related to their invasion histories.

2.5. Acknowledgements

We would like to thank Jean François Germain (ANSES Montpellier) for assistance with the morphological identification of some mealybug species. We would also like to thank Dr Douglass Miller and Dr Jan Giliomee for providing the key characters for identifying *Delottococcus aberiae* populations and for confirming species identification. This work was funded by the grants FP7-IRSES #269196 'Iprabio', FP7-KBBE 'PURE', Bibliothèque du Vivant and the French Agropolis Fondation (RTRA – Montpellier, BIOFIS project).

2.6. Supplementary material

Table S1. Number of identified mealybug species in Mediterranean countries and islands (Ben-Dov *et al.* 2011).

Geographic area	Mealybug species
France	130
Italy	114
Turkey	76
Sicily	49
Egypt	47
Israel	40
Spain	33
Corsica	31
Algeria	21
Morocco	18
Greece	18
Crete	15
Tunisia	14
Portugal	14

Table S2. Complete list of samples with corresponding haplotypes: code of individual, Genbank accession numbers for haplotypes, codes of voucher slide-mounted specimens, population code (see Table 1).

ind #	Genbank Accession numbers					Voucher slide #	Pop
	LCO	COI	28S	ITS2	16S		
001	JF714207	JF714167	JF714179	JF714196	-	465	1
002	-	JF714167	JF714179	-	-	466	
003	JF714207	JF714167	JF714179	JF714196	-	467	
004	-	-	JF714179	-	-	468	
005	-	-	-	JF714196	-	521	
006	-	JF714167	JF714179	-	-	522	
007	-	JF714167	-	JF714196	-	523	

008	-	JF714167	JF714179	-	-	524	
009		JF714162	JF714180			698	
010		JF714162	JF714180	JF714193	JF714172	699	
011		JF714162	JF714180	JF714194		700	2
012	-	JF714162	JF714180	-	-	701	
013	JF714206	JF714162	JF714180	JF714193	JF714172	702	
014	JF714206	JF714162	JF714180	JF714194	-	703	
015	JF714206	-	-	-	-	457	
016	JF714206	JF714163	JF714180	JF714194		458	
017	-	-	JF714180	-	-	459	
018	-	-	-	-	-	525	3
019	JF714206	JF714163	JF714180	JF714194	JF714172	526	
020	JF714206		JF714180	JF714194	JF714172	527	
021	-	-	JF714180	JF714193	-	528	
022	JF714206	JF714163	-	-	-	529	
023	JF714207	JF714167	JF714179	JF714196	-	657	
024	JF714200	JF714157	-	JF714195	JF714171	658	
025	JF714207	JF714167	JF714179	JF714196	-	659	
026	JF714200	JF714157	JF714181	JF714195	JF714171	660	4
027	JF714200	JF714157	JF714181	JF714195	JF714171	661	
028	-	JF714157	-	JF714195	JF714171	662	
029	-	-	JF714181	-	JF714171	663	
030	JF714200	-	-	-	JF714171	664	
031	-	-	-	-	-	469	
032	-	-	-	-	-	470	
033	-	JF714167	JF714179	-	-	471	
034	-	-	JF714179	-	-	472	5
035	JF714207	JF714167	JF714179	JF714196	-	530	
036	-	-	-	-	-	531	
037	-	JF714167	-	-	-	532	
038	-	-	-	-	-	533	
039	-	-	JF714179	-	-	473	
040	-	JF714167	-	-	-	474	
041	-	-	-	-	-	475	
042	-	-	-	-	-	476	6
043	JF714207	JF714167	JF714179	JF714196	-	537	
044	-	JF714167	JF714179	-	-	538	
045	-	-	JF714179	-	-	539	
046	-	-	-	-	-	540	
047	-	-	JF714179	-	-	581	
048	JF714207	JF714167	JF714179	JF714196	-	582	
049	-	-	JF714179	JF714196	-	583	
050	-	JF714167	JF714179	-	-	609	7
051	JF714207	JF714167	-	JF714196	-	610	
052	JF714207	JF714167	JF714179	JF714196	-	611	
053	JF714207	JF714167	JF714179	JF714196	-	612	
054	-	JF714167	-	-	-	613	

055	-	JF714167	JF714179	-	-	585	8
056	-	JF714167	JF714179	-	-	586	
057	-	JF714167	JF714179	-	-	587	
058	JF714207	JF714167	JF714179	-	-	588	
059	JF714209	JF714169	JF714176	JF714187		460	9
060	JF714209	JF714169	JF714176	JF714187	JF714173	461	
061	-	JF714169	-	-	-	462	
062	-	-	-	JF714187	-	463	
063		JF714167??	JF714176			534	
064	-	-	-	-	-	535	
065	JF714209	JF714169	JF714176	JF714187	JF714173	541	
066	-	JF714169	JF714176	-	-	542	
067	-	-	JF714181	JF714195	JF714171	589	10
068	JF714200	JF714157	JF714181	JF714195	JF714171	590	
069	-	-	JF714181	JF714195	JF714171	591	
070	JF714200		JF714181	JF714195	JF714171	592	
071	JF714200	-	-	JF714195	JF714171	614	
072	JF714200	JF714157	JF714181	JF714195	JF714171	615	
073	JF714200	JF714157	JF714181	JF714195		593	11
074	JF714200	JF714157	JF714181	JF714195	JF714171	594	
075	JF714200	JF714157	-	-	-	595	
076	-	-	-	-	-	596	
077	JF714200	JF714157	JF714181	-	-	617	
078	-	-	JF714181	-	-	619	
079	JF714200	-	JF714181	-	-	616	
080	JF714200	JF714160	JF714181	JF714195	JF714171	618	
081	JF714201	-	JF714181	JF714195	-	477	12
082	JF714201	JF714159	JF714181	JF714195	-	478	
083	JF714201	JF714159	JF714181	JF714195	-	479	
084	JF714201	JF714159	JF714181	JF714195	JF714171	480	
085	JF714201	JF714159	JF714181	JF714195	JF714171	543	
086	-	JF714157	-	JF714195	JF714171	544	
087	-	-	-	-	-	545	
088	-	-	JF714181	-	-	546	
089	JF714198	-	JF714181	JF714195	JF714171	597	13
090	JF714198	JF714164	JF714181	JF714195	JF714171	598	
091	JF714198	JF714160	JF714181	JF714195	JF714171	599	
092	JF714198	JF714160	JF714181	JF714195	JF714171	600	
093	JF714198	*JF714160*	JF714181	JF714195	JF714171	620	
094	-	-	JF714181	-	-	621	
095	-	JF714160	JF714181	-	-	622	
096	JF714198	JF714160	-	-	JF714171	623	
097	JF714199	JF714158	JF714181	JF714195	-	601	14
098	JF714199	JF714158	JF714181	JF714195	-	602	
099	-	JF714158	JF714181	-	-	603	
100	-	-	JF714181	-	-	604	
101	JF714199	JF714158	JF714181	JF714195		624	

102	JF714199	JF714158	JF714181	-	-	625	
103	JF714199	JF714158	-	JF714195	-	626	
104	JF714199	-	-	-	-	627	
105	-	-	-	-	-	481	15
106	-	-	JF714177	JF714191	-	482	
107	-	-	JF714177	-	-	483	
108	JF714203	JF714164	JF714177	JF714191	-	484	
109		JF714164	-	JF714191	-	547	
110	-	-	-	-	-	548	
111	JF714203	JF714164	JF714177	JF714191	-	549	
112	-	-	JF714182	JF714186	-	550	
113	-	-	JF714177	JF714191	-	485	16
114	JF714203	JF714164	JF714177	JF714191	-	486	
115	-	JF714164	-	JF714191	-	487	
116	-	-	JF714177	JF714191	-	551	
117	-	-	-	JF714191	-	552	
118	JF714203	JF714164	JF714177	JF714191	-	553	
119	JF714203	JF714164	JF714177	JF714191	-	554	
120	-	-	-	-	-	555	
121	-	-	-	JF714189	-	489	17
122	JF714202	JF714161	JF714183	JF714188		490	
123	JF714202	JF714161	JF714183	JF714188	JF714175	491	
124	-	JF714164	-	-	-	728	18
125	JF714203	JF714164	JF714177	JF714191	-	729	
126	JF714203	JF714164	JF714177	JF714191	-	730	
127	JF714203	JF714164	-	-	-	731	
128	JF714203	JF714164	JF714177	-	-	732	
129	JF714203	JF714164	JF714177	JF714191	-	492	19
130	JF714203	JF714164	JF714177	JF714191	-	493	
131	-	JF714164	JF714177	-	-	494	
132	-	JF714164	JF714177	JF714191	-	495	
133	-	JF714164	JF714177	JF714191	-	496	
134	JF714203	JF714164	JF714177	JF714191	-	605	
135	JF714203	-	-	JF714191	-	606	
136	JF714203	-	-	-	-	607	
137	-	-	-	-	-	497	20
138	-	-	JF714179	-	-	498	
139	-	JF714167	-	-	-	499	
140	-	JF714167	-	-	-	500	
141	JF714207	JF714167	JF714179	JF714196	-	556	
142	JF714207	JF714167	JF714179	JF714196	-	557	
143	-	JF714167	-	JF714196	-	558	
144	-	-	-	JF714196	-	559	
145		JF714168			-	501	21
146	-	-	-	-	-	502	
147	-	-	-	-	-	503	
148	-	-	-	-	-	504	

149	JF714204	JF714168	-	-	-	561	
150	JF714204	JF714168	JF714184	-	-	562	
151	-	-	-	-	-	563	
152	-	-	-	-	-	564	
153	JF714198	JF714160	JF714181	JF714195	JF714171	633	
154	JF714198	JF714160	JF714181		JF714171	634	
155	JF714198	JF714160	JF714181	JF714195	JF714171	635	22
156	JF714198	JF714160	JF714181	JF714195	JF714171	636	
157	JF714198	JF714160	JF714181	-	-	637	
158	JF714202	JF714161	JF714183	JF714189		628	
159	JF714202	JF714161	JF714183	-	-	629	
160	-	-	JF714183	-	-	630	
161	JF714202	-	-	JF714189	-	631	23
162	JF714202	JF714161	JF714183	JF714189		638	
163	-	JF714161	-	JF714189	-	639	
164	-	JF714161	-	-	-	640	
165	JF714202	JF714161	JF714183	JF714189		641	
166	-	-	-	-	-	642	
167	-	-	-	-	-	643	
168	-	-	-	-	-	644	
169	JF714210	JF714166	JF714182	JF714186	JF714174	645	24
170	-	-	JF714182	JF714186	-	646	
171	JF714210	JF714166	JF714182	JF714186	JF714174	647	
172	JF714210	JF714166	JF714182	JF714186	JF714174	648	
173	JF714210	JF714166	JF714182	JF714186	JF714174	649	
174	JF714202	JF714161	JF714183	JF714188		653	
175	JF714202	JF714161	JF714183	JF714189	JF714175	654	
176	-	JF714161	JF714183	-	-	655	
177	JF714202	JF714161	JF714183	JF714188	JF714175	665	25
178	-	JF714161	JF714183	-	-	666	
179	-	JF714161	JF714183	-	-	667	
180	-	-	-	-	-	668	
181	JF714202	-	-	-	-	669	
182	-	-	-	-	-	681	
183	JF714202		JF714183	JF714188	JF714175	682	
184	JF714202	JF714161	JF714183			683	26
185	JF714202	JF714161	JF714183	JF714188		684	
186	JF714202	JF714161	-	-	-	685	
187	JF714200	JF714157	JF714181	JF714195		690	
188	JF714203	-	-	-	-	505	
189	-	-	-	-	-	506	
190	JF714203	JF714164	JF714177	JF714191	-	507	
191	-	JF714164	-	-	-	508	27
192	JF714203	JF714164	JF714177	JF714191	-	565	
193	JF714203	JF714164	-	-	-	566	
194	-	JF714164	-	-	-	567	
195	JF714200	JF714157	JF714181	JF714195	-	686	28

196	JF714200	JF714157	JF714181	JF714195	JF714171	687	
197	JF714200	JF714157	JF714181	JF714195	JF714171	688	
198	JF714200	JF714157	JF714181	JF714195	JF714171	689	
199	-	JF714157	JF714181	JF714195	JF714171	691	
200	-	JF714157	JF714181	-	-	692	
201	-	JF714157	JF714181	JF714195	JF714171	693	
202	-	JF714165	-	-	-	509	29
203	JF714205	JF714165		JF714192	-	510	
204	-	-	-	-	-	511	
205	-	-	-	-	-	568	
206	JF714205	JF714165	JF714178	JF714192	-	569	
207	-	-	-	-	-	570	
208	-	-	-		-	571	
209	JF714205	JF714165	JF714178	JF714192	-	572	
210		JF714164	JF714177		-	515	30
211	-	-	-	-	-	516	
212		JF714164			-	573	
213	JF714203	JF714164	JF714177	JF714191	-	574	
214	JF714203	JF714164	JF714177	JF714191	-	575	
215	-	JF714164	JF714177	-	-	577	
216	-	-	JF714183	-	-	517	31
217	-	-	-	-	-	518	
218	JF714202	JF714161	JF714183	JF714190	JF714175	519	
219	JF714202	JF714161	JF714183	JF714190	JF714175	520	
220	JF714202	JF714161	-	-	JF714175	576	
221	-	JF714161	-	-	-	578	
222	-	JF714161	-	-	-	579	
223	-	-	-	-	-	580	
224	JF714203	JF714164	-	-	-	670	32
225	-	-	-	-	-	671	
226	-	-	-	-	-	672	
227	-	JF714164	-	-	-	673	
228	-	JF714164	-	-	-	674	
228		JF714164	JF714177		-	675	
229		JF714164			-	676	
230		JF714164			-	677	
231	JF714208	JF714170	-	JF714197	-	1647	33
232	JF714208	JF714170	-	JF714197	-	1648	
233	JF714208	JF714170	JF714185	JF714197	-	1649	
234	JF714208	JF714170	JF714185		-	1650	
235	JF714203	JF714164	JF714177	JF714191	-	1651	34
236	JF714203	JF714164	JF714177	JF714191	-	1652	
237		JF714164	-	JF714191	-	1653	
238	JF714203	JF714164	JF714177	JF714191	-	1654	

3- SEASONAL PHENOLOGY, SPATIAL DISTRIBUTION, AND SAMPLING PLAN FOR THE INVASIVE MEALYBUG *PHENACOCCUS PERUVIANUS* (HEMIPTERA: PSEUDOCOCCIDAE)

Beltrà, A., Garcia-Marí, F., and Soto, A. (2013) **Seasonal phenology, spatial distribution, and sampling plan for the invasive mealybug *Phenacoccus peruvianus* (Hemiptera: Pseudococcidae).** Journal of Economic Entomology, 106:1486-1494.

Abstract

Phenacoccus peruvianus Granara de Willink (Hemiptera: Pseudococcidae) is an invasive mealybug of Neotropical origin. In recent years it has invaded the Mediterranean Basin causing significant damages in bougainvillea and other ornamental plants. This article examines its phenology, location on the plant and spatial distribution, and presents a sampling plan to determine *P. peruvianus* population density for the management of this mealybug in Southern Europe. Six urban green spaces with bougainvillea plants were periodically surveyed between March 2008 and September 2010 in Eastern Spain, sampling bracts, leaves, and twigs. Our results show that *P. peruvianus* abundance was high in spring and summer, declining to almost undetectable levels in autumn and winter. The mealybugs showed a preference for settling on bracts and there were no significant migrations between plant organs. *Phenacoccus peruvianus* showed a highly aggregated distribution on bracts, leaves, and twigs. We recommend a binomial sampling of 200 leaves and an action threshold of 55% infested leaves for integrated pest management purposes in urban landscapes and enumerative sampling for ornamental nursery management and additional biological studies.

Keywords: *Phenacoccus peruvianus*, sampling, phenology, spatial distribution, urban landscape

3.1 Introduction

Invasive pests are an important threat to landscape and agricultural ecosystems and can cause significant ecological and economical losses (Williamson 1996; Pimentel *et al.* 2001; Kenis *et al.* 2009). Mealybugs are typical invasive pests and because of their small size and cryptic behavior, they are easily introduced into new areas through international horticultural and ornamental trade (Miller *et al.* 2002; Hulme *et al.* 2008). In Europe they represent the third most numerous family of alien insect species after aphids and armored scale insects, accounting for 40 newly established species (Roques *et al.* 2009; Pellizzari and Germain 2010). Mealybug species such as *Phenacoccus solani* Ferris (Mazzeo *et al.* 1999), *Pseudococcus comstocki* (Kuwana) (Pellizzari 2005), *Phenacoccus solenopsis* (Tinsley) (EPPO 2011), and *Phenacoccus peruvianus* Granara de Willink (Beltrà *et al.* 2010) have recently been recorded as new invaders in the Mediterranean Basin causing significant damage to crops and ornamental plants.

Phenacoccus peruvianus is a mealybug of Neotropical origin, first described in Peru and Argentina (Granara de Willink and Szumik 2007). The first time it was found in Europe was in 1999 in Almeria (Spain), and in the following years it was also reported in other areas such as Sicily, France, United Kingdom, Portugal, the Canary Islands, and Corsica (Beltrà *et al.* 2010). *Phenacoccus peruvianus* is a polyphagous species that has been reported on several ornamental plants such as *Aucuba japonica* Thunb. (Aucubaceae), *Myoporum laetum* G. Forst. (Myoporaceae), *Lantana camara* L. (Verbenaceae), and particularly on *Bougainvillea* spp. (Nyctaginaceae) (Granara de Willink 2007; Beltrà *et al.* 2010). On *Bougainvillea* spp. the mealybug settles on leaves, bracts, and soft plant tissue where it feeds on sap, reducing plant growth and causing leaves to fall when high populations are present. Moreover, the honeydew excreted promotes black sooty mold that interferes with plant photosynthesis, reduces its esthetic quality and disturbs urban landscape users (Dreistadt *et al.* 2004).

Broad-spectrum chemicals have traditionally been relied on for the management of this mealybug in urban landscapes and commercial

nurseries. However, the new European directive on pesticide use requires the reduction or even prohibits pesticides in a wide range of urban green areas, giving significant priority to biological control (European Parliament and Council 2009). The establishment of integrated pest management (IPM) programs in urban landscapes requires a better understanding of the biology and ecology of the target pests as well as efficient sampling techniques and management action thresholds (Binns and Nyrop 1992; Raupp *et al.* 1992). The development of sampling techniques for assessing mealybug populations has led to an improvement in their control in agricultural ecosystems and ornamental plants (Geiger and Daane 2001; Walton *et al.* 2004; Martínez-Ferrer *et al.* 2006; Mudavanhu *et al.* 2011; Waterworth *et al.* 2011). Thus, in this work we studied 1) the seasonal phenology, 2) location on the plant, 3) spatial distribution, and finally 4) defined a sampling methodology for *P. peruvianus* on *Bougainvillea* spp., as first steps toward developing a reliable IPM program for this mealybug in Southern Europe.

3.2 Materials and methods

3.2.1 Survey sites and sampling protocol

Six urban green spaces (UGS) located on the city of Valencia (Eastern Spain) were sampled from March 2008 to September 2010. The UGS were located on the Polytechnic University of Valencia Campus (39.481536 N, 0.343685 W), Aiora Park (39.467340 N, 0.344363 W), Vivers Park (39.478889 N, 0.367822 W), Lluis Vives College (39.468063 N, 0.377609 W), University of Valencia Campus (39.476423 N, 0.339589 W), and Ramon Llull College (39.476054 N,75 0.346413 W) covering a total area of 5.200.000 m^2. Each sampling site averaged a surface area of one ha with at least 15 mature bougainvillea plants. Two of the sites included *Bougainvillea glabra* Choisy and four had a mixed group of *Bougainvillea glabra* and the hybrid *Bougainvillea x buttiana* Holttum and Standl plants. As three of the sampling sites were sprayed with pesticides in July 2008, they were replaced by new unsprayed UGS with similar pest population densities located within a distance of 500 m. The periodicity of the sampling depended on the developmental biology of the mealybug: weekly or twice

a month in the months of most rapid development (March-November) and monthly during the rest of the year.

One 10-cm long tender twig, which was less than 1 year old, together with its leaves; and two bracts were taken per plant from 10 randomly selected plants for each sampling site. Twigs and bracts were taken from heights ranging between 0.3 and 2 m above the ground. In the four sites with mixed bougainvillea plant species, each half of the sample belonged to one of the two species found. Bougainvillea blooming is not continuous throughout the year depending on various factors such as temperature and humidity (Schoellhorn and Alvarez 2002); thus, bracts can be scarce in the winter. In these cases, bracts were only collected when >10 units were available in the sampling site. Samples were bagged and immediately transported to the laboratory inside a portable cooler. The material was then deposited in climatic chambers at 10°C and 50% relative humidity (RH) and processed within the next 24 h.

3.2.2 Mealybug phenology and distribution on the plants

For each sample, we counted the mealybugs present in 20 bracts, 10 twigs, and 50 leaves (five leaves randomly selected from each twig). The sex and instar of each mealybug were also recorded. Female instars of *P. peruvianus* can only be distinguished by their morphological characteristics after mounting each specimen on the slides. However, as body length appears to be well correlated with developmental stages (A.B., unpublished data), the following body length ranges (obtained by previously measuring 30 mealybugs of each instar on the slide mounts) were used to separate instars: first nymphal instar (0-0.5 mm), second nymphal instar (0.5-0.9 mm), third nymphal instar (0.9-1.4 mm), and adult females (>1.4 mm). Then, for routine samplings, the different mealybugs were separated by measuring them with a dissecting microscope fitted with an ocular micrometer. For the first and second nymphal instars males and females were pooled together as sex cannot be distinguished at these stages (Gullan and Martin 2009).

One-way analysis of variance (ANOVA) was used to check for differences in mealybug abundance between 1) the 3 yr of the study (2008-2010), 2) bougainvillea species (*B. glabra* and the hybrid *B. x buttiana*), and 3) plant strata (bracts, leaves, or twigs). When analyzing the effect of mealybug abundance on bougainvillea species we compared the data from the four UGS that contained mixed bougainvillea populations. Data were log (x + 1) transformed before analysis to normalize the distribution. A two-way ANOVA was used to compare the proportion of infested sampling units between plant strata and years. The proportions were arc-sine square-root transformed before analysis. Means were compared using Fisher least significant difference (LSD) test with significance level set at $\alpha = 0.05$. All these analyses were performed using the statistical software Statgraphics Centurion XVI (Statpoint Technologies 2009).

3.2.3 Measure of dispersion

The spatial patterns of the mealybug populations were analyzed using Taylor's power law that relates the mean density of all counts and the variance: $s^2 = am^b$ (Taylor 1961). After applying logarithms, Taylor's parameters *a* and *b* can be estimated by a simple regression analysis. The first parameter *a* is a function of the sample size unit while *b* is the index of aggregation and it is specific and constant for the species. According to the value of *b,* populations are classified as regular ($b < 1$), random ($b = 1$) or aggregated ($b > 1$) (Taylor 1984). When no insects were found in a sample, the related data were omitted from analysis. We used a Student *t*-test with n-2 degrees of freedom to test if the slope *b* differed significantly from unity. To contrast the index of aggregation between instars, seasons, plant parts, and bougainvillea species, a 95% CI based on the *t*-distribution was used and significant differences were considered when the intervals did not overlap.

3.2.4 Enumerative and binomial sampling

The enumerative minimum sample size was obtained by applying Green's formula: $n = a * m^{(b-2)} / E^2$, where *n* is the sample size, *a* and *b* are the Taylor's parameters, *m* the sampling mean and *E* the desired ratio of

the standard error to the mean (Green 1970). For our purposes a value of E = 0.25 was applied, which allows the detection of doubling or halving the sample means. This ratio is considered adequate for extensive sampling to carry out pest management decisions (Southwood 1978).

Binomial sampling can be applied if the mean m of insects per sampling unit and the proportion p of infested units in each sample are correlated. We compared mean densities and the proportion of infested leaves, bracts, or twigs through the use of the method of Wilson and Room (1983) based on the negative binomial distribution and the empirical model of Kono and Sugino (1958). The Wilson and Room's equation is:

$$p = 1 - \exp(- m\ [\ln (a*m^{(b-1)} / a*m^{(b-1)} - 1)]$$

where a and b are the coefficients of Taylor power law. The empirical model of Kono and Sugino (1958) was also proposed independently by Gerrard and Chaing (1970) and Nachman (1984):

$$p = 1 - \exp(- a' m^{b'})$$

where a and b are constants that can be obtained from the regression:

$$\ln (m) = a' + b' \ln (- \ln [1 - p]).$$

To test if these models were conducive with the data obtained in the field, a simple regression analysis was carried out between the infestation levels observed and the levels predicted by the models. Finally, sample size in the presence-absence sampling was calculated by applying the formula proposed by Kuno (1986):

$$n = E^{-2} (1 - P_0)\ P_0^{-(2/k) - 1}\ [k\ (P_0^{-1/k} - 1)]^{-2}$$

where P_0 is the probability of empty samples, and k is a parameter characteristic of the negative binomial distribution. Wilson and Room (1983) related k to the mean and Taylor parameter's a and b:

$$k = m^2 / (am^b - m).$$

To prepare a practical and useful sampling plan for IPM programs, we selected leaves as the plant strata to determine optimum sample size because bracts are not present throughout the entire plant cycle and twigs are less abundant and more difficult to observe than leaves. First nymphal instars were omitted from the analysis because in practice they are difficult to detect and to count. The rest of the instars were then plotted together, as they cannot be separated without measuring them using a micrometer.

3.2.5 Sampling plan validation

To assess the reliability of the sampling plan for *P. peruvianus* we used the resampling software for analysis and validation of enumerative and binomial sampling plans (RSVP) (Naranjo and Hutchinson 1997). Fourteen independent datasets were used to validate the sampling plan. Datasets represented a range of 0.1-12.34 mealybugs per leaf and were taken in different months from 2008 to 2009 in five independent urban green spaces. Each dataset consisted on 50 leaves collected from bougainvillea plants following the methods described above. Five hundred simulations were conducted with replacement and a minimum sampling size of 10, for a prefixed precision level of 0.25.

3.3 Results

3.3.1 Mealybug phenology and distribution on the plants

The phenology of *P. peruvianus* showed a similar trend over the 3 years of the study. The mealybug completed multiple overlapping generations within a year with intense fluctuations in abundance. Mealybug density increased in spring and reached its peak at the end of this season or in early summer (June and July) (Fig. 1). Afterwards, populations decreased and the presence of the insect was almost undetectable in autumn and winter, except for 2008 when medium densities of mealybugs were observed in autumn. *Phenacoccus peruvianus* populations were more abundant in 2008 (mean ± SE: 6.08 ± 0.74 mealybugs per sampling unit) than in 2009 (1.42 ± 0.80) or 2010 (1.20 ± 0.80) (F = 13.86; df = 2, 161; P < 0.0001). Significant differences were also found in mealybug abundance

depending on the bougainvillea species, with higher populations on *B. glabra* (4.88 ± 0.67 mealybugs per sampling unit) than on the hybrid *B. x buttiana* (2.69 ± 0.67) (F = 4.79; df = 1, 223; P = 0.03). *Phenacoccus peruvianus* was irregularly distributed on plant strata; higher population densities were present on bracts (7.45 ± 0.59 individuals per sampling unit) than on twigs (2.77 ± 0.59) or leaves (2.02 ± 0.59) (F = 32.52; df = 2, 723; P < 0.0001) (Fig. 2).

The percentage of infested sampling units was also influenced by plant strata (F = 37.76; df = 2, 723; P < 0.0001) and year (F = 74.82; df = 2, 723; P < 0.0001) and there was no significant interaction between these two factors (F = 1.74; df = 4, 723; P = 0.14). Over the 3 years of the study the percentage of infestation was higher on bracts (36.81 ± 1.85%) than on leaves (20.78 ± 1.85%) or twigs (16.64 ± 1.85%) (F = 37.76; df = 2, 723; P < 0.0001) (Fig. 3). Bract infestation reached its maximum in June with 71.21 ± 6.22% bracts occupied in the first year of the study, 53.53 ± 6.57% in the second year and 35.83 ± 7.81% in the third year. The monthly average percentage of infested leaves also peaked in June reaching 47.68 ± 6.19%, 25.17 ± 6.55%, and 15.17 ± 7.80% from 2008, 2009, and 2010, respectively. The proportion of infested sampling units decreased during the winter months always remaining below 10%.

3.3.2 Measure of dispersion

Taylor's power law was used to study the distribution patterns of *P. peruvianus* in *Bougainvillea* spp. Comparing the logarithm of the mealybug mean abundance and its associated variance, significant regressions with high determination coefficients were obtained for all the mealybug instars in the three plant organs sampled, leaves, bracts, or twigs (Fig. 4). The values of the slope were significantly > 1(t-test) in all cases, indicating that there was a clumped distribution of the mealybug on leaves, bracts, and twigs (Table 1).

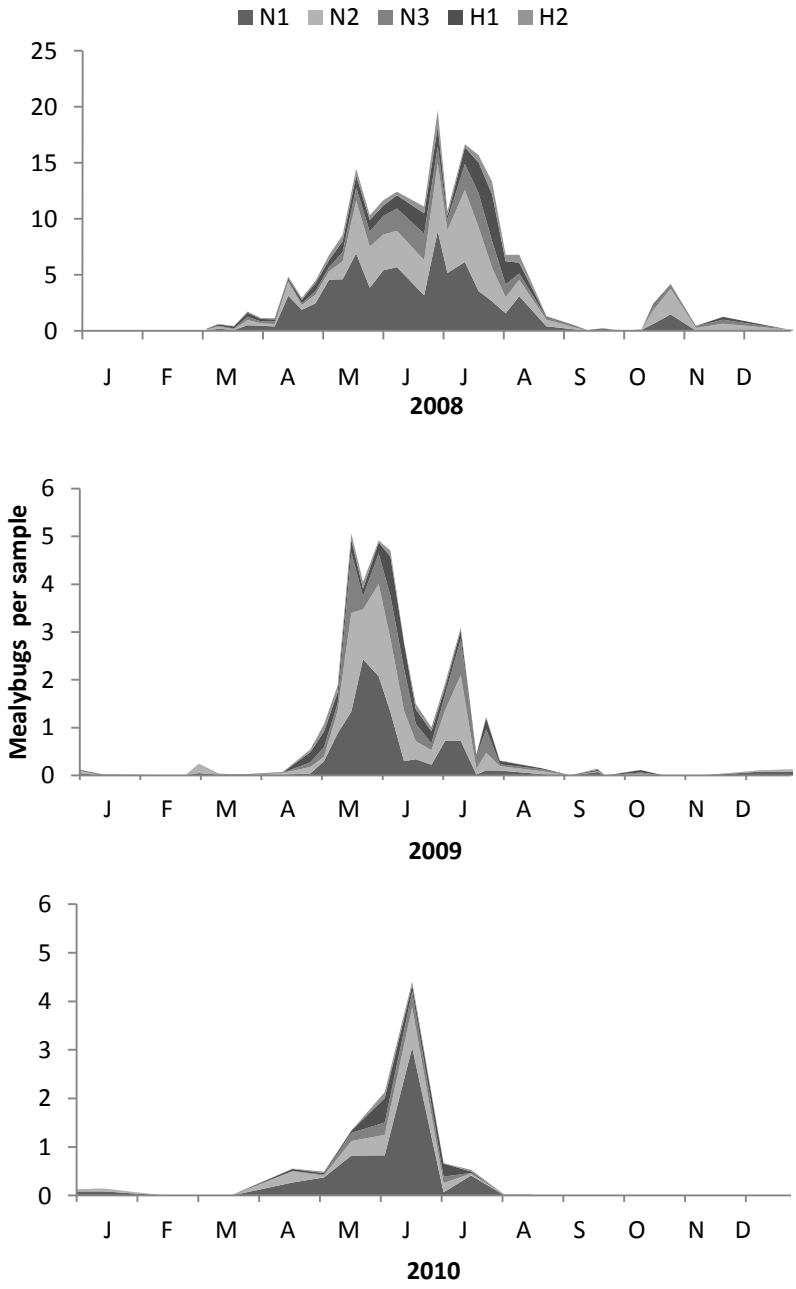

Figure 1. Seasonal phenology of *Phenacoccus peruvianus* in *Bougainvillea* spp. in six urban green spaces of Valencia. Mean number of mealybugs collected per sample unit. (N1= first nymphal instar (females and males); N2 = second nymphal instar (females and males); N3 third nymphal instar (females); H1 young female; H2 gravid female). Note that y-axis scales are different for 2008 and 2009-2010.

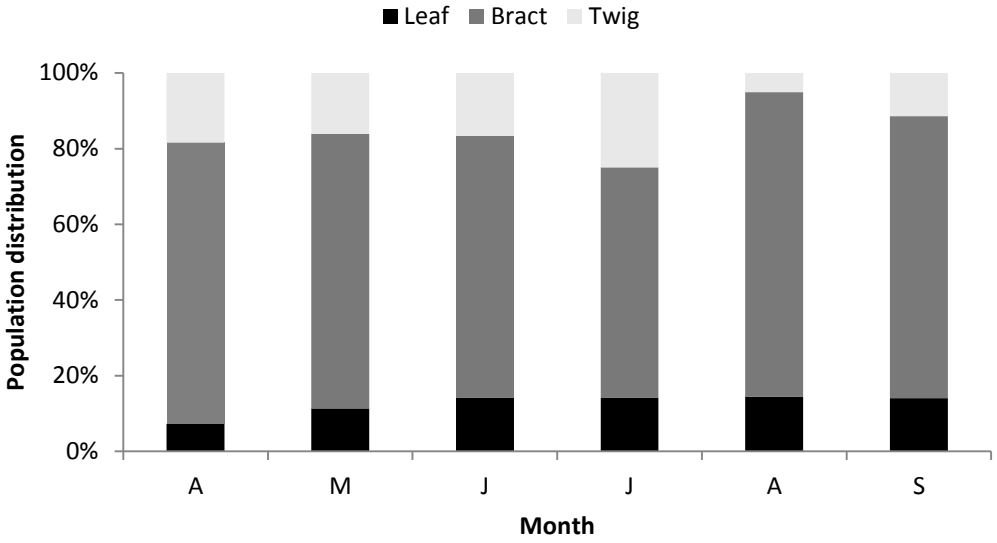

Figure 2. Percentage of *Phenacoccus peruvianus* individuals distributed on leaves, bracts and twigs of *Bougainvillea* spp. plants. Data from six urban areas in Valencia (Eastern Spain) sampled in 2008, 2009, and 2010 from April to September.

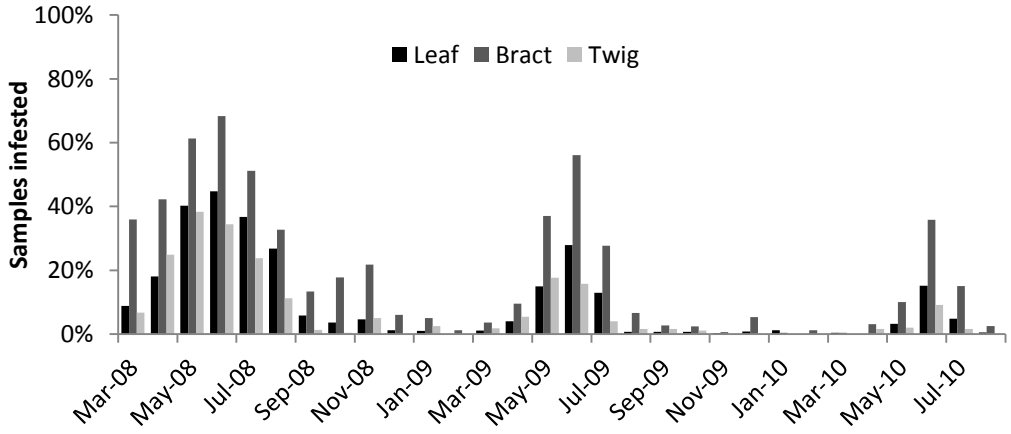

Figure 3. Percentage of leaves, bracts, and twigs of *Bougainvillea* spp. plants infested by *Phenacoccus peruvianus*. Data from six urban areas in Valencia (Eastern Spain) sampled in 2008, 2009, and 2010.

No significant differences were found in the aggregation parameter *b* between bougainvillea species [*B. glabra* (1.62-1.72, 95% CI), *B. x buttiana* (1.47-1.64, 95% CI) ($P > 0.05$)] nor between seasons [spring (1.59-1.69, 95% CI), summer (1.63-1.71, 95% CI), autumn (1.51-1.72, 95% CI), and winter (1.28-1.68, 95% CI) ($P > 0.05$)]. Conversely, the aggregation decreased with the age of the mealybugs, being higher in first (1.61-1.68, 95% CI) and second (1.62-1.68, 95% CI) nymphal instars than in third nymphal instars (1.48-1.55, 95% CI), young females (1.47-1.53, 95% CI), and gravid females (1.39-1.48, 95% CI) ($P < 0.05$). Clump size was also higher on bracts and twigs than on leaves ($P < 0.05$) (95% CI; Table 1).

3.3.3 Relationship between insect density and percent infestation

When mealybugs were located on leaves, the percent infestation levels observed in the field were similar to the values estimated by both Wilson and Room's model ($F = 1324$; df = 1, 223; $P < 0.0001$) ($R^2 = 0.86$) and Kono and Sugino's model ($F = 1029$; df = 1, 223; $P < 0.0001$) ($R^2 = 0.82$). This correlation was smaller on bracts: Wilson and Room's model ($F = 606$; df =1, 195; $P < 0.0001$) ($R^2 = 0.76$) and Kono and Sugino's model ($F = 350$; df = 1, 190; $P < 0.0001$) ($R^2 = 0.65$); and twigs: Wilson and Room's model ($F = 265$; df = 1, 125; $P < 0.0001$) ($R^2 = 0.67$) and Kono and Sugino's model ($F = 174$; df = 1, 124; $P < 0.0001$) ($R^2 = 0.58$) (Fig. 5). In all cases, Wilson and Room's (1983) negative binomial model adjusted better to our data than Kono and Sugino's (1958) empirical model.

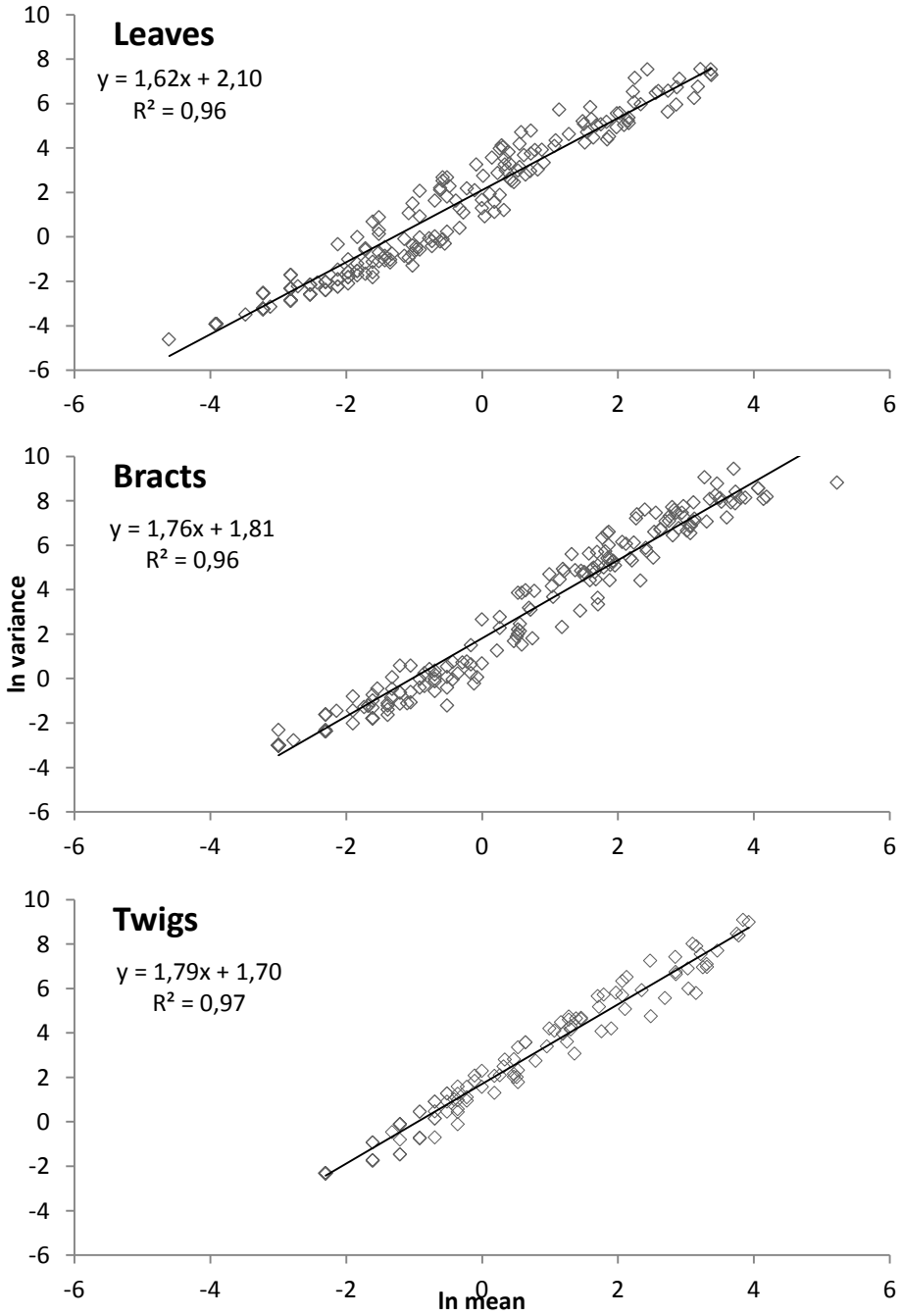

Figure 4. Relationship between mean mealybug density and variance on leaves, bracts and twigs of *Bougainvillea* spp. Data from six urban areas in Valencia (Eastern Spain) sampled in 2008, 2009, and 2010.

Table 1. Taylor's power law parameters for the different instars of *Phenacoccus peruvianus* on *Bougainvillea* spp. (N1= first nymphal instar (females and males); N2 = second nymphal instar (females and males); N3 = third nymphal instar (females); Females = all instars).

Plant substrate	Life stage	Samples n	Taylor Power law					Test for slope (b ≠ 1)		Confidence interval
			a	*b*	SE(*b*)	r^2	*P*	*t*	*P*	CI (*b*)
Bracts	N1	138	7.34	1.72	0.03	95.38	< 0.001	22.22	< 0.001	1.66-1.79
	N2	148	6.67	1.73	0.03	96.63	< 0.001	27.35	< 0.001	1.68-1.78
	N3	153	4.79	1.62	0.03	94.25	< 0.001	19.04	< 0.001	1.56-1.68
	Adult young female	162	4.68	1.59	0.03	95.78	< 0.001	22.31	< 0.001	1.54-1.64
	Adult gravid female	115	4.04	1.52	0.04	93.96	< 0.001	14.28	< 0.001	1.44-1.59
	Females	200	6.10	1.76	0.02	96.38	< 0.001	31.32	< 0.001	1.71-1.81
	N2-N3-Adult female	196	5.65	1.72	0.02	96.51	< 0.001	30.77	< 0.001	1.68-1.77
	N3-Adult female	207	4.83	1.66	0.03	95.51	< 0.001	24.27	< 0.001	1.56-1.66
Leaves	N1	164	8.84	1.61	0.03	96.19	< 0.001	22.08	< 0.001	1.55-1.66
	N2	168	8.17	1.61	0.03	95.34	< 0.001	18.32	< 0.001	1.41-1.51
	N3	160	5.19	1.46	0.03	95.56	< 0.001	14.80	< 0.001	1.34-1.44
	Adult young female	164	4.30	1.39	0.03	94.49	< 0.001	16.28	< 0.001	1.36-1.46
	Adult gravid female	107	4.73	1.41	0.03	96.72	< 0.001	11.12	< 0.001	1.51-1.73
	Females	238	8.21	1.62	0.02	95.91	< 0.001	24.95	< 0.001	1.53-1.62
	N2-N3-Adult female	226	7.07	1.57	0.02	95.44	< 0.001	24.02	< 0.001	1.69-1.81
	N3-Adult female	188	5.34	1.48	0.02	95.62	< 0.001	24.02	< 0.001	1.69-1.82
Twigs	N1	83	5.54	1.75	0.03	97.48	< 0.001	15.76	< 0.001	1.60-1.77
	N2	87	5.81	1.75	0.03	97.35	< 0.001	18.25	< 0.001	1.66-1.82
	N3	84	4.42	1.69	0.04	94.82	< 0.001	14.12	< 0.001	1.61-1.82
	Adult young female	101	4.95	1.74	0.04	94.92	< 0.001	31.06	< 0.001	1.89-2.04
	Adult gravid female	31	5.17	1.72	0.05	97.53	< 0.001	32.24	< 0.001	1.74-1.84
	Females	140	5.46	1.79	0.02	97.47	< 0.001	25.00	< 0.001	1.61-1.71
	N2-N3-Adult female	128	5.32	1.78	0.03	97.08	< 0.001	21.56	< 0.001	1.43-1.52
	N3-Adult female	120	5.05	1.75	0.03	96.29	< 0.001	23.68	< 0.001	1.68-1.81

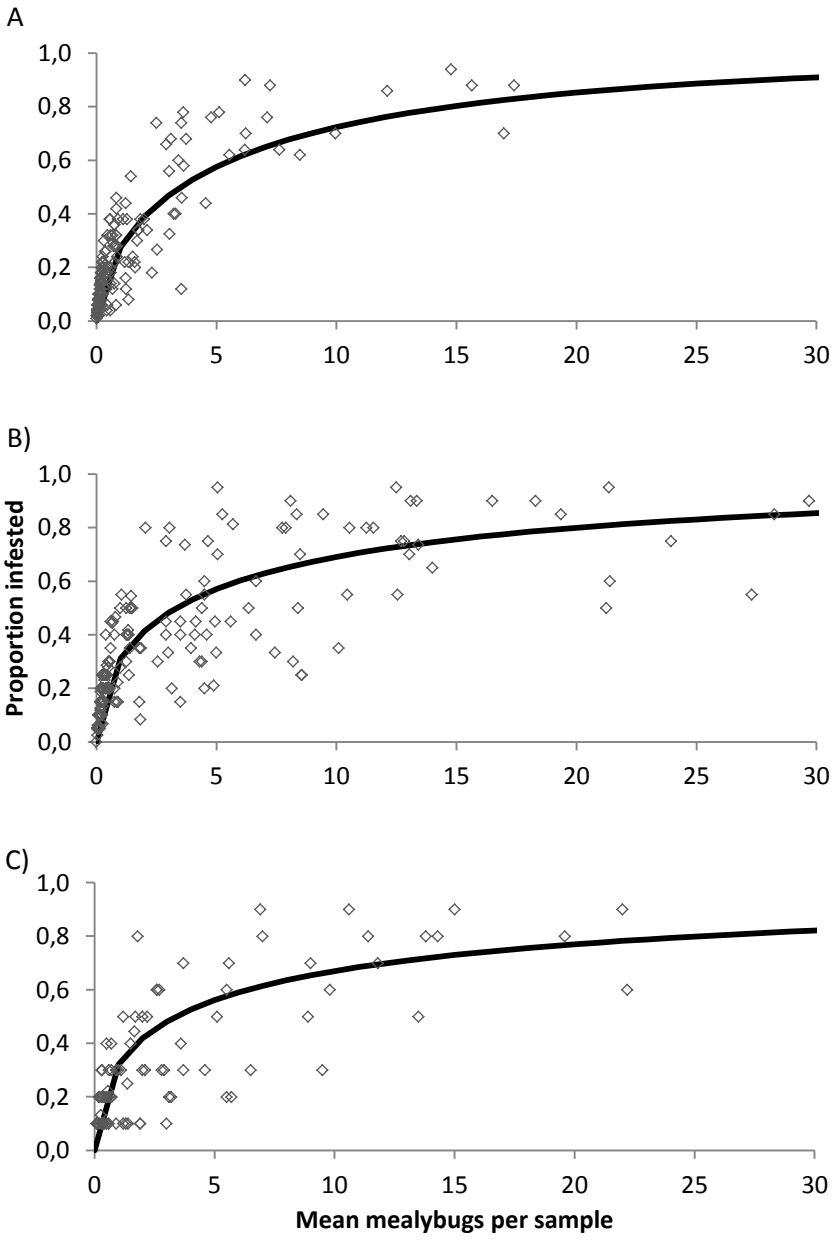

Figure 5. Relationship between the proportion of leaves occupied by *Phenacoccus peruvianus* and the mean population density per (A) leaf, (B) bract, or (C) twig. Data predicted using the equations of Wilson and Room's (1983) model: p = 1 exp(m [ln (a*m(b1)/a*m(b1) 1)].

3.3.4 Enumerative and binomial sampling

The number of leaves per sample required to estimate *P. peruvianus* populations was calculated using the values of the coefficients of Taylor's power law for second and third nymphal instars and adults. Optimal sample size was always higher for binomial than enumerative sampling for a precision of 0.25 (Fig. 6). According to our sampling plan, for population densities from 5 to 30 mealybugs per leaf, 25-55 leaves should be monitored in enumerative samplings and 120-200 leaves in binomial samplings.

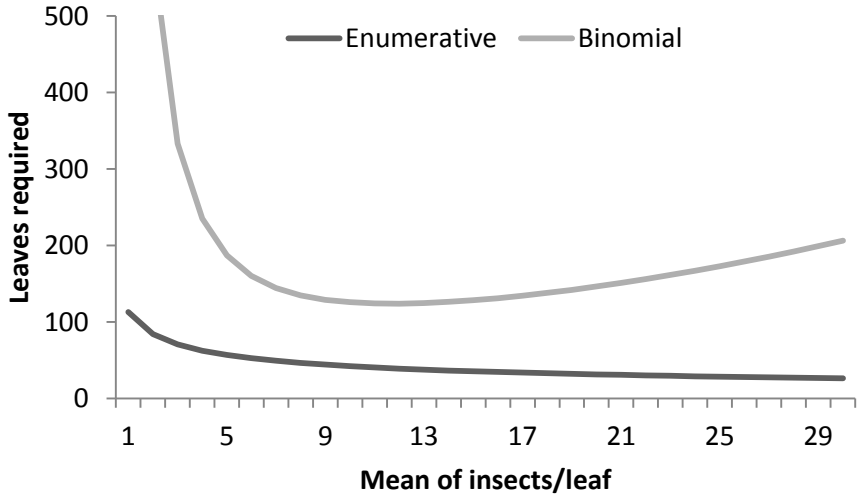

Figure 6. Optimum sample size to estimate different mealybug densities by using enumerative and binomial sampling plans for *Phenacoccus peruvianus* on *Bougainvillea* spp. leaves. Prestablished relative variation level: *E* = 0.25. Data for mealybugs from second nymphal instar to adult.

3.3.5 Sampling plan validation

The validation of the sampling plan with a desired precision of 0.25 resulted to a sample number that ranged from 39 to 309 (Table 2). Actual mean precision levels varied from 0.13 to 0.44 and averaged the desired precision of 0.25.

Table 2. Resampling simulation results used to validate Green's fixed precision sequential sampling plan for *P. peruvianus* in bougainvillea plants with desired precision levels adjusted to 0.25 with replacement (Taylor's *a* = 7.07 and *b* = 1.57).

Validation data set	Observed mean density	Avg statistics for 500 sequential sampling simulations						
		Density Mean	Precision (*D*)			Average sample number		
			Mean	Max.	Min.	Mean	Max.	Min.
1	0.10	0.10	0.26	0.32	0.20	309	435	200
2	0.18	0.18	0.16	0.18	0.13	238	294	198
3	0.22	0.22	0.15	0.19	0.13	218	270	181
4	0.36	0.36	0.14	0.18	0.12	177	220	149
5	0.58	0.58	0.13	0.16	0.11	144	168	124
6	0.60	0.62	0.30	0.40	0.16	145	225	101
7	0.82	0.87	0.44	0.69	0.30	130	239	72
8	1.34	1.41	0.34	0.46	0.26	103	165	69
9	2.12	2.24	0.32	0.41	0.21	84	124	53
10	2.44	2.64	0.36	0.47	0.25	79	118	49
11	6.22	6.25	0.20	0.25	0.16	53	68	39
12	7.12	7.30	0.24	0.32	0.16	50	68	36
13	8.48	8.63	0.28	0.35	0.20	47	69	34
14	12.34	12.71	0.22	0.28	0.17	39	55	29
Overall	3.06	3.15	0.25	0.33	0.18	129.71	179.86	95.29

3.4 Discussion

The purpose of the current study was to determine the phenology and spatial distribution of the invasive species *P. peruvianus* in Eastern Spain. Subsequently, these data were used to design a sampling plan for this pest which will be helpful to establish an IPM program in urban landscapes in accordance with the new European legislation. Our results indicated a similar trend in mealybug phenology over the 3 years of the study; population densities increased in spring with peaks occurring during June and July. Mealybug populations then decreased in August and remained at very low levels for the rest of the year. This fast decrease might be a consequence of biotic and abiotic factors such as plant phenology, climate, and the action of natural enemies. In Eastern Spain, bougainvillea

plants have a vegetative growth period in early spring followed by various continuous blooming periods. The high temperatures and low humidity that frequently occur in the Mediterranean summer may cause high mortality in mealybugs, especially in first instars (Browning 1959; Bartlett and Clancy 1972). Interestingly, studies carried out under laboratory conditions showed high mortality rates in *P. peruvianus* crawlers at 30°C and 65 % H.R. (A.B. *et al.* unpublished data). Furthermore, a companion study (Beltrà *et al.* 2013b) demonstrated that parasitism reaches its peak in August, and thereby contributes to the decline of mealybug populations. In the same study it was found that parasitism was higher in 2009 and 2010 than in 2008; this might explain the differences in the mealybug abundance observed over the years.

In our study, *P. peruvianus* completed several overlapping generations during the year, with all development stages present throughout this period. Similar studies carried out in the Mediterranean Basin with other mealybug species of agronomic importance such as *Phenacoccus madeirensis* Green (Longo *et al.* 1995), *Planococcus citri* (Risso) (Panis 1969; Santorini 1977; Martínez-Ferrer *et al.* 2003) or *Pseudococcus viburni* (Signoret) (Panis 1986) showed a similar pattern. The continuous overlap of development stages has relevant implications for the mealybug management. Host stage can influence natural enemies' efficiency and must be taken into account when designing biological control strategies (Jervis *et al.* 2007). Parasitism of scale insects that complete a small number of generations in Eastern Spain is strongly influenced by host instar/size (Tena *et al.* 2008; Pekas *et al.* 2010; Beltrà *et al.* 2011). In this study, all *P. peruvianus* instars are present all year round and therefore, host stage availability should not be a limiting factor for efficient biological control by its parasitoids. Conversely, the low mealybug populations found in long periods of the year such as autumn and winter might limit the action of insect parasitoids that commonly show host delayed density dependence (Kidd and Jervis 2007; Van Driesche *et al.* 2008). Moreover, chemical control may also be hampered by the constant presence of adult mealybugs that are more resistant to contact chemical applications because of their hydrophobic wax cover (McKenzie 1967; Moore 1988; Franco *et al.* 2009). If chemical control is required to manage occasional population outbreaks,

we suggest start monitoring in the spring, before mealybug populations grow and reach higher densities. At that time, selective insecticides should be used to allow the future establishment of biological control agents.

Scale insects show a sedentary behavior and crawlers tend to settle near their mothers when conditions are favorable (Greathead 1997). However, some mealybug species migrate to different strata of the plant host, adapting to the plant phenology (Browning 1959; Furness 1976; Geiger and Daane 2001; Grasswitz and James 2008; Ben-Dov *et al.* 2009; Cid *et al.* 2010; Haviland *et al.* 2012). When *P. peruvianus* feeds on bougainvillea plants it is primarily found on bracts, leaves, and green twigs. Contrary to the expectations, our results showed no seasonal migrations between plant strata and the location of the mealybugs remained stable along the spring and summer. A possible explanation for this might be the fact that the plant phenology is rather constant, with continuous flower blooming during this period. The small densities of the mealybug in autumn and winter did not allow us to detect whether there is an overwintering migration. *Phenacoccus peruvianus* shows a preference for settling on bracts. This distribution could play a significant role in mealybug survival, because bracts offer good protection against climatic fluctuations and the action of contact pesticides.

The mealybug showed a strongly clumped distribution on bracts, twigs and leaves. This distribution is typical of scale insects (Nestel *et al.* 1995) and has also been reported for other mealybug species such as *Rastrococcus invadens* Williams on mango leaves (Boavida *et al.* 1992), *P. citri* on orange fruits (Martínez-Ferrer *et al.* 2006), grapefruit (Nestel *et al.* 1995), *Saccharicoccus sacchari* (Cockerell) on sugarcane stalks (Allsopp 1991), and *Pseudococcus maritimus* (Ehrhorn) in vines (Geiger and Daane 2001). Differences in aggregation between instars were also found, with the clumped distribution decreasing as the mealybugs aged. This finding agrees with the observations of Martínez-Ferrer *et al.* (2006) with *P. citri.* The differences may be a consequence of density dependent natural mortality on younger mealybug instars because of parasitism or food sources and dispersion resulting from spatial limitation (Martínez-Ferrer *et al.* 2006).

The elevated indices of aggregation lead to high optimum sample sizes in both enumerative and binomial sampling. Our field observations showed that *P. peruvianus* densities lower than five mealybugs per leaf did not cause important esthetic damage to plants. This corresponds to 57.5% of infested leaves. In our experience an enumerative sampling of 50 leaves takes more time than a binomial sampling of 200 leaves that requires ≈10-15 min. Therefore, for IPM purposes in urban landscapes we recommend a feasible binomial sampling of 200 leaves and a management decision threshold of 55% infested leaves. However, for ornamental nursery management, which requires lower action thresholds (Sadof and Sclar 2002), or for further biological studies, we recommend enumerative sampling to accurately estimate lower or higher mealybug populations. In these cases the sample size required in binomial sampling increases considerably and the sampling becomes a time-consuming work (Fig. 6).

3.5 Acknowledgments

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4- FORTUITOUS BIOLOGICAL CONTROL OF THE INVASIVE MEALYBUG *PHENACOCCUS PERUVIANUS* IN SOUTHERN EUROPE.

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

Abstract

Phenacoccus peruvianus Granara de Willink (Hemiptera: Pseudococcidae) is a Neotropical invasive mealybug that has rapidly spread throughout Mediterranean Basin. It has established itself as the principal pest of several ornamental plants, causing considerable problems in nurseries and urban landscapes. The aim of this study was to determine the natural enemy complex of this pest and report the feasibility of its biological control. Six urban green spaces were surveyed in Eastern Spain from 2008 to 2010. The most abundant natural enemies of *P. peruvianus* were found to be the primary parasitoids *Acerophagus* n. sp. *near coccois* and *Leptomastix epona* Walker (Hymenoptera: Encyrtidae). *Phenacoccus peruvianus* populations were lower during the second and third year of the survey, coinciding with an increase of the parasitoid *Acerophagus* sp., which displaced the native *L. epona*. Differential female offspring and resource preemption are discussed as the main reasons for this displacement.

Keywords: Pseudococcidae, *Acerophagus*, *Leptomastix epona*, Parasitism, Sex ratio, Host density dependence.

4.1 Introduction

An increase in international commercial traffic in recent decades has led to a concomitant increase in the number of exotic insects entering Europe (Roques *et al.* 2009). Mealybugs (Hemiptera: Pseudococcidae) have entered Europe at a high rate, surpassed only by members of the Aphididae and Diaspididae families, and account for 37 alien species, which constitute one fourth of the mealybug fauna currently present in Europe (Pellizzari and Germain 2010). They are typical invasive pests due to their small size and cryptic behavior (Miller *et al.* 2002; Pellizzari and Germain 2010). The invasive nature and the difficulties presented by the chemical control of Pseudococcidae have made them a principal target of classical biological control programs (Moore 1988; Miller *et al.* 2002).

The classical biological control strategy for managing insect pests, namely, the introduction and release of exotic natural enemies, has met with some success in the control of mealybug species, such as *Phenacoccus manihoti* Matile-Ferrero (Neuenschwander 2001), *Maconellicoccus hirsutus* (Green) (Kairo *et al.* 2000), *Rastrococcus invadens* Williams (Agricola *et al.* 1989; Neuenschwander *et al.* 1994) and, more recently, *Paracoccus marginatus* Williams and Granara de Willink (Muniappan *et al.* 2006; Amarasekare *et al.* 2009). Mealybug invasions have also been controlled by the accidental importation of natural enemies. At least six cases of biological control by fortuitous introductions of parasitoids and predators have been reported in mealybugs (Cox 1940; Beardsley 1985; Anonymous 2000; Nechols 2003; Muniappan *et al.* 2006; Gautam *et al.* 2009).

Most of the successful biological control programs involving the Pseudococcidae family have involved insect parasitoids (Moore 1988). Of these, encyrtids (Hymenoptera: Encyrtidae) are the most abundant and widely distributed natural enemies of mealybugs, and various classical biological control programs have demonstrated that encyrtids often establish host-specific interactions with mealybugs (Moore 1988; Charles and Allan 2002; Charles 2011). Apart from parasitoids, the coccinellid *Cryptolaemus montrouzieri* Mulsant is also a well-known and successful

example of a predator-based biological control program (Bartlett 1978; Moore 1988).

Phenacoccus peruvianus Granara de Willink (Hemiptera: Pseudococcidae) is a mealybug of Neotropical origin (Granara de Willink and Szumik 2007) that has been recently recorded in Europe. This mealybug was first reported in Southern Spain in 1999 and, subsequently, in other areas such as Sicily (2002), the UK and Corsica (2005), Portugal (2006), France (2008), and Majorca Island (2010) (Beltrà *et al.* 2010). It is a polyphagous pest that attacks a wide variety of plants, including several ornamental species. Among their hosts it shows a preference for members of the Solanaceae; a recent study shows that it settles and feeds on tomato and tobacco plants, which are major members of this family (Beltrà *et al.* unpublished data). At the present time, this mealybug has established itself as a key pest of the ornamental plants of genus Bougainvillea (Nyctaginaceae) in nurseries and urban landscapes of Southern Europe (Beltrà *et al.* 2010).

Biological control of *P. peruvianus* has not been investigated, primarily because its natural enemy complex has never been determined in Europe or South America, where it is considered to be a native species (Granara de Willink and Szumik 2007). In this study, we examine the natural enemy complex of *P. peruvianus* and report on its potential for the biological control of this pest. In particular, in this study we determine (1) the natural enemy species complex of *P. peruvianus*, (2) its seasonal abundance, (3) its effect on host populations, (4) the brood size and sex ratio of its main parasitoids in the field.

4.2 Materials and methods

4.2.1 Survey sites and sampling protocol

Six urban green spaces (UGS) located in the city of Valencia (Eastern Spain) were sampled from March 2008 until September 2010. *Phenacoccus peruvianus* was first reported in this area in 2005 (authors' personal observation). The UGS were located on the Polytechnic University of

Valencia Campus (39.481536 N, 0.343685 W), Aiora Park (39.467340 N, 0.344363 W), Vivers Park (39.478889 N, 0.367822 W), Lluis Vives College (39.468063 N, 0.377609 W), University of Valencia Campus (39.476423 N, 0.339589 W), and Ramon Llull College (39.476054 N, 0.346413 W). Each sampling site had an average surface area of 1 ha with more than 15 mature climbing *Bougainvillea glabra* and/or hybrid *B. glabra x buttiana* plants. Because of the high population levels of *P. peruvianus*, three sampling sites were sprayed with pesticides in July 2008. These were replaced by new unsprayed UGS with similar pest population densities, located within a distance of 500 m from the old ones. Sampling periodicity depended on *P. peruvianus* density: weekly or twice a month when medium and high densities, respectively, were found per sampling (March - November) and monthly when fewer than ten individuals were found per sampling (rest of the year).

One 10-cm softwood twig, <1 year old, was removed, with its leaves and two bracts, from ten randomly selected plants at each site. Twigs and bracts were taken from heights ranging between 0.3 and 2 m above the ground. During the winter, Bougainvillea plants have fewer bracts, and therefore <20 bracts were collected per site. Samples were bagged and immediately transported to the laboratory inside a cooler where the plant material was deposited in climate chambers set to a temperature of 10 °C and 50 % relative humidity (RH). The samples were then processed according to the procedures described below over the next 24 h.

Occasionally, a variable quantity of highly infested leaves, bracts, and twigs were also taken from the sampling sites. These samples were used to complement data on the sex ratio and brood size of the main parasitoids.

4.2.2 Mealybug density and parasitism abundance

For each sampling, we counted all of the *P. peruvianus* individuals susceptible to parasitism (second and third instars and adults) present in 20 bracts, the softwood of ten twigs, and 50 leaves (five leaves randomly selected from each twig). All the mealybugs were morphologically checked

for parasitism. We considered an individual to be parasitized when it was mummified or when it showed the first signs of mummification (body deformation and cuticle sclerotization). When a mummy was found, the length was first measured to the nearest 0.01 mm, and then the mummy was gently separated from the plant with a wet camel hair brush and placed into 3.0 x 0.8 cm glass vial (1 mummy per vial). The vials were covered with a cotton plug and stored at 23 ± 5 °C and the natural outdoor photoperiod and checked twice a week for parasitoid emergence. Upon emergence, each vial was placed in a freezer to kill the adult parasitoids, which were then identified. The number and sex of parasitoids that emerged per mealybug were recorded. Parasitism rates, active parasitism, were estimated as the proportion of mummified mealybugs (those that contained parasitoids) to the total number of mealybugs susceptible to parasitism (alive and mummified mealybugs). To obtain parasitism rates, the data from the ten twigs, 50 leaves, and 20 bracts from each sampling were pooled. The proportion of parasitized mealybugs on twigs and leaves per plant and date was also used to determine the parasitoid patch aggregation.

Parasitoids were mounted using conventional techniques (Noyes 1982) and identified using different Chalcidoidea keys (García-Mercet 1917; Graham 1969; Rosen 1981; Trjapitzin 2008). The identity of some parasitoid species was confirmed by Dr. Andy Polaszek at the British National History Museum. Additionally, each sample was inspected for foraging predators within *P. peruvianus* colonies. Predators found preying on the colonies during field sampling or below the ovisacs were captured and counted in the laboratory. Where necessary these were removed, identified, and recorded along with the preyed-upon mealybug instars. Predators were identified following various taxonomic keys (Díaz-Aranda and Monserrat 1990; Ferragut and González-Zamora 1994; Oosterbroek 2007).

4.2.3 Statistical analysis

We used a generalized mixed model with repeated measurements to analyze the dynamics of parasitism from May to September (Bolker *et al.* 2009). Year was considered as a fixed factor and month as a random one.

We assumed binomial variance for parasitism ratio and fitted the parameters by Laplace approximation. Moreover, linear models were applied assuming normal error variance to determine the relationship between parasitism and the number of mealybugs per plant. We examined all possible regressions using linear, power, log, exponential, and polynomial functions and selected the model with the highest coefficient of determination.

Brood size and secondary sex ratio were analyzed by using generalized linear modeling (GLM) techniques (Wilson and Hardy 2002; Crawley 2007). A Poisson error variance was initially assumed for the analysis of brood size, and binomial error variance was assumed for sex ratio analysis. If over- or underdispersion was detected, the significance of the explanatory variables was re-evaluated using an F test after re-scaling the statistical model by a Pearson's χ^2 divided by the residual degrees of freedom (Crawley 2007). The statistical software 'R' (http://www.R-project.org) and its package lme4 (Bates *et al.* 2012) were used in our analyses.

4.3 Results

4.3.1 Phenacoccus peruvianus *density and parasitism*

Over the three-year study period, *P. peruvianus* populations were found to increase during the spring months in Bougainvillea plants, reaching a maximum between June and July (Fig. 1). In 2008, up to 470 specimens of *P. peruvianus* were collected per sample. This number was approximately threefold lower in 2009 and 2010 than in 2008. In contrast, parasitism rates augmented during the course of the study and were higher in 2009 than in 2008 (GLMM based on binomial distribution: χ^2 = 904.07; *df* = 1; *P* < 0.0001), and in 2010 compared to 2009 (GLMM based on binomial distribution: χ^2 = 71.64; *df* = 1; *P* < 0.0001).

4.3.2 Parasitoid species complex and seasonal trend

A total of 2,147 parasitoid specimens were obtained over the three-year study period. Three species of primary parasitoids and three

hyperparasitoids were reared from *P. peruvianus* (Table 1). *Acerophagus* n. sp. *near coccois* Smith (Hymenoptera: Encyrtidae) was identified as the main primary parasitoid, accounting for 91.6 % of the parasitoids recovered. The other primary parasitoids, *Leptomastix epona* Walker (Hymenoptera: Encyrtidae) (3.8 %) and *Anagyrus pseudococci* Girault (Hymenoptera: Encyrtidae) (0.1 %), were much less abundant. The most important hyperparasitoid was *Pachyneuron* sp. (Hymenoptera: Pteromalidae) (3.5 %), while specimens of *Chartocerus* sp. (Hymenoptera: Signiphoridae) (0.9 %) and *Prochiloneurus bolivari* Mercet (Hymenoptera: Pteromalidae) (0.1 %) were recovered only sporadically. *Acerophagus* sp. was recovered from all of the sites sampled, while *L. epona* and *Pachyneuron* sp. were recovered from five of the six sampled sites.

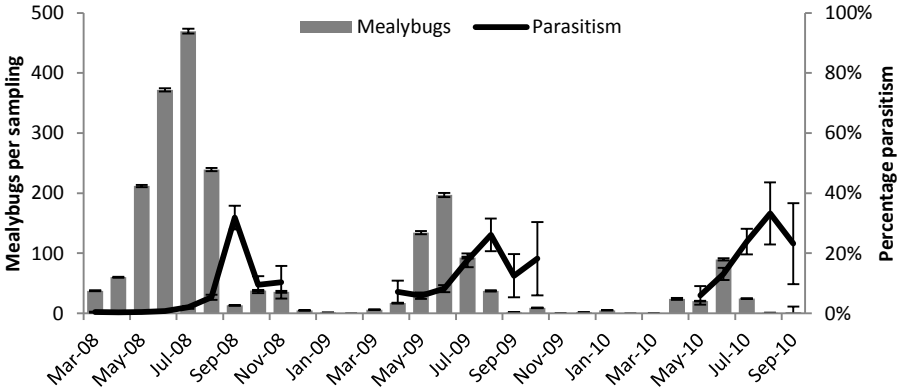

Figure. 1 Seasonal correlation between the population density of *Phenacoccus peruvianus*, which was suitable for parasitism, and percentage parasitism, based on samples taken from plants of *Bougainvillea spp.* in six urban green spaces of Valencia (Eastern Spain) during the period 2008–2010. Vertical bars Standard error (SE).

Table 1. Abundance of *Phenacoccus peruvianus* parasitoids recovered from six urban green spaces of Valencia (Eastern Spain) from March 2008 to September 2010.

Family	Species	Biology	Abundance of *P. peruvianus* parasitoids (*n*)			Presence at urban green space (*n* = 6 sample sites)
			2008	2009	2010	
Encyrtidae	*Acerophagus* sp.	Primary parasitoid	83	1275	609	6/6
Encyrtidae	*Leptomastix epona*	Primary parasitoid	49	29	3	5/6
Encyrtidae	*Anagyrus pseudococci*	Primary parasitoid	0	2	0	2/6
Pteromalidae	*Pachyneuron* sp.	Secondary parasitoid	40	29	6	5/6
Pteromalidae	*Prochiloneurus bolivari*	Secondary parasitoid	2	0	0	1/6
Signiphoridae	*Chartocerus* sp.	Secondary parasitoid	0	16	4	1/6

The abundance of the parasitoid complex of *P. peruvianus* varied throughout the three-year study period (Fig. 2). In 2008, the native *L. epona* comprised 28.2 % of the emerged parasitoids and *Acerophagus* sp. was recovered in 47.7 % of the cases. In 2009 and 2010, the percentages of *L. epona* decreased to 2.1 and 0.5 %, respectively, while the abundance of *Acerophagus* sp. specimens increased to 94.4 % and 97.9 %, respectively. The abundance of the hyperparasitoid *Pachyneuron* sp. also fell from 23.0 % in 2008 to 2.1 and 1.0 % of the parasitoid specimens in 2009 and 2010.

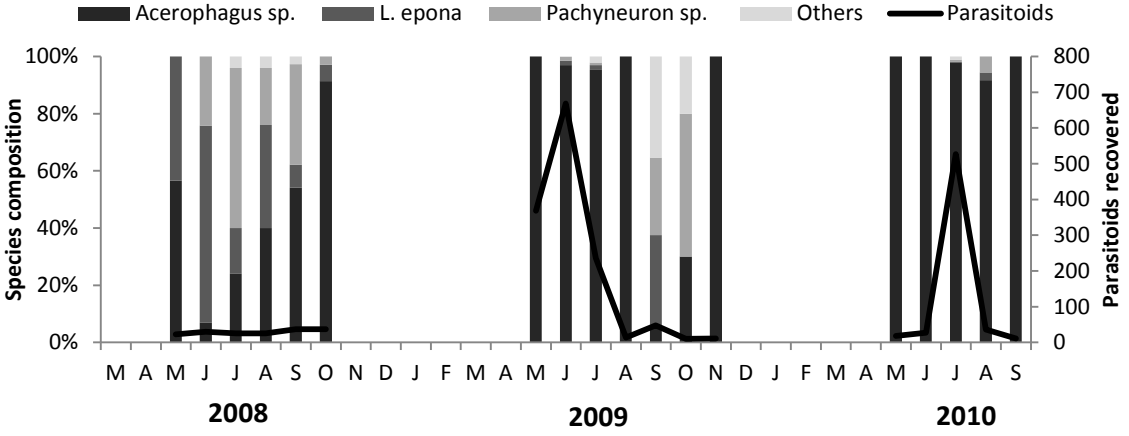

Fig. 2 *Phenacoccus peruvianus* parasitoid complex composition and number of parasitoids recovered from plants of *Bougainvillea spp.* in six urban green spaces in Valencia (Eastern Spain) during the period 2008–2010.

4.3.3 Host use: size preference, brood size, sex ratio, and spatial distribution

Acerophagus sp. mummy sizes ranged from 0.6 to 3.4 mm. This species developed as a facultative gregarious parasitoid, with brood sizes that ranged from one to 12 parasitoids with an average of 2.75 ± 0.10 parasitoids per host. There was a significant relationship between brood size of the parasitoid and mealybug mummy size, with larger brood sizes emerging from longer hosts {brood size = Exp [1.08 x (mealybug length) - 1.02]; GLM based on semi-Poisson distribution: F = 668.39; df = 1,485; P < 0.0001; 41.73 % deviance explained}. All of the *Acerophagus* sp. recovered were females. Percentage parasitism by *Acerophagus* sp. was negatively

correlated with the mealybug density. Parasitism decreased when the number of mealybugs per plant increased [parasitism = 1.10 x (number of mealybugs per sample)-0.77; R^2 = 0.78; F = 551.32; df = 1,152; P < 0.0001)] (Fig. 3).

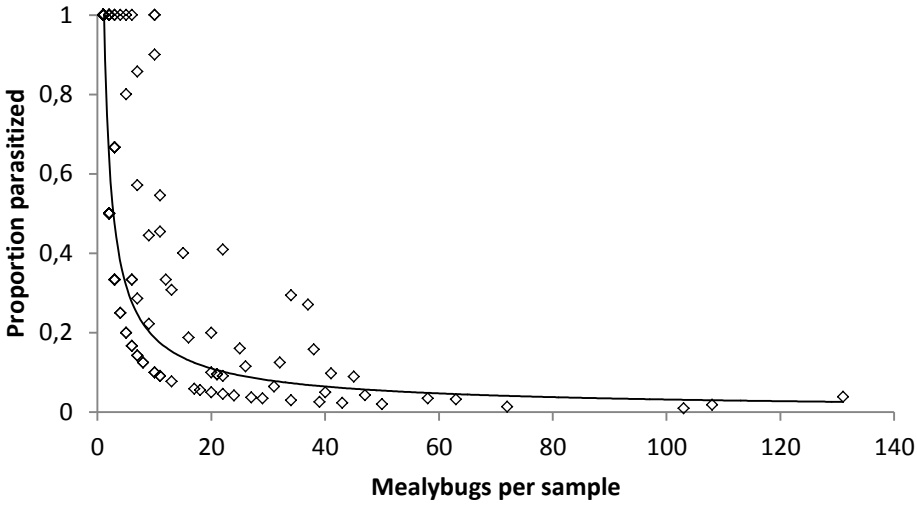

Fig. 3 Effect of *P. peruvianus* aggregation on *Acerophagus* sp. parasitism in samples of 10-cm softwood twigs and 5 leaves of *Bougainvillea spp.* in six urban green spaces of Valencia (Eastern Spain) during the period 2008–2010. Plotted curve fits to the power function [parasitism = 1.10 x (number of mealybugs per sample) $^{-0.77}$**; *df* = 1,152; *F* = 551.32; R^2 = 0.78; *P* < 0.0001].**

Leptomastix epona mummy sizes ranged from 1.0 to 2.5 mm. This species always developed as a solitary parasitoid. Its secondary sex ratio was negatively correlated with mealybug mummy size, and it became female biased in hosts larger than 1.8 mm (GLM of sex ratio based on binomial distribution: sex ratio = 1/ 1 + {1/[Exp (-1.30 x mealybug length) + 1.75]}; χ_1^2 = 8.34; P = 0.004; 92.38 % deviance explained).

4.3.4 Predator abundance and feeding behavior

Several species of generalist predators were found preying on *P. peruvianus* eggs and nymphs in Bougainvillea plants. We recorded 105 *Orius laevigatus* Fieber (Heteroptera: Anthocoridae), 58 coccinellids

belonging to species *Cryptolaemus montrouzieri* Mulsant, *Propylea quatuordecimpunctata* L., *Oenopia lyncea* Olivier, and *Scymnus spp.* (Coleoptera: Coccinellidae), 43 *Leucopis* sp. (Diptera: Chamaemyiidae), and 22 *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae). *Orius laevigatus*, *C. carnea* larvae, and all the coccinellid species preyed on *P. peruvianus* nymphs.

Leucopis sp. larvae foraged below the ovisacs of adult *P. peruvianus* and preyed on the mealybug eggs. This predator was observed during the summer and was present in 5.51 % of the ovisacs counted in July.

4.4 Discussion

The *Phenacoccus peruvianus* populations described in this paper decreased gradually over the three-year study period. The high parasitism rates observed in 2009 and 2010 suggest that *P. peruvianus* populations may be naturally controlled by the parasitoid *Acerophagus* sp. under Mediterranean conditions. During the first year, *P. peruvianus* populations remained at >200 mealybugs per sample until *Acerophagus* sp. became the major parasitoid in September. The following years, *Acerophagus* sp. appeared and its populations remained high from as early as the spring, whereas the populations of its host did not increase.

Before this study, *Acerophagus* sp. had never been described in the Mediterranean Basin, yet the results obtained in this study show that during the study period this species became the most abundant natural enemy of *P. peruvianus* and contributed the most to its control. However, if this species has been introduced, its origin and date of introduction remain unknown. Genus *Acerophagus* Smith consists of 46 species, all of which are parasitoids of mealybugs (Charles 2011). Although it might be a non-monophyletic genus (Charles 2011), most of the species are of Nearctic and Neotropical origin, and some have been introduced in new areas to control invasive mealybugs: *Acerophagus papayae* Noyes and Schauff from Mexico to control *P. marginatus* in the Caribbean, USA, and Pacific Islands, *A. coccois* Smith from Venezuela and the USA States to control *Phenacoccus herreni* Cox and Williams in Brazil and *Oracella acuta* (Lobdell) in China, and

A. maculipennis (Mercet) from Australia and *A. flavidulus* (Brèthes) from Chile to control *Pseudococcus viburni* (Signoret) in New Zealand and the USA respectively (Bento *et al.* 1999; Charles *et al.* 2004; Muniappan *et al.* 2006; Daane *et al.* 2008a; Amarasekare *et al.* 2009; Clarke *et al.* 2010). The successful biological control of *P. peruvianus* by *Acerophagus* sp. during the last two years of the study and the common specificity of encyrtid parasitoids of mealybugs (Moore 1988; Charles and Allan 2002; Charles 2011) lead us to hypothesize that *Acerophagus* sp. could have been accidentally imported with the introduction of *P. peruvianus* or a few years later.

In our field samples, *Acerophagus sp.* was recovered from mealybug mummies that ranged in length from 0.6 to 3.4 mm. These results and those presented in a companion manuscript (Beltrà *et al.* 2013c) show that *Acerophagus* sp. parasitizes second and third nymphal instars as well as adults. Its brood size increased with host size, which is a common characteristic of gregarious encyrtids when they parasitize mealybugs and this is consistent with the findings reported for other species of this genus, such as *A. flavidulus*, *A. coccois* and *A. papayae* (Karamaouna and Copland 2000a; Amarasekare *et al.* 2009; Sandanayaka *et al.* 2009). More importantly, we did not recover any *Acerophagus* sp. males during the three-year study period, suggesting that reproduction might occur by way of thelytokous parthenogenesis. To our knowledge, strict parthenogenesis has never been reported for any species of this genus.

Acerophagus sp. showed a negative correlation between host density and parasitism rates in Bougainvillea plants. In other words, parasitism decreased as the number of mealybugs per plant increased. These results are not consistent with the empirical review carried out by Walde and Murdoch (1988), where small parasitoids of multivoltine hosts frequently present direct host density dependence in large spatial scales, such as trees. There are several possible explanations for our results. During our field observations, we observed the presence of ants tending mealybug colonies. Although *Acerophagus* sp. has a high egg load averaging 30 eggs when it is five days old (Beltrà *et al.* 2013c), a female usually needs more than 30 min to lay one egg (Beltrà *et al.* in preparation). This long process

could be disturbed by ants, if ant attendance is density dependent, causing a disruption in parasitism in patches with high mealybug densities (González-Hernández *et al.* 1999; Barzman and Daane 2001). Our findings may also be explained as a response to a high rate of catastrophic patch failure (Heimpel and Casas 2008). High mealybug infestations entail leaf fall (authors' personal observation), which could endanger the progeny of the parasitoid allocated to this patch.

The second most abundant parasitoid was the native species *Leptomastix epona*. It is a generalist parasitoid that has also been recovered from *Phenacoccus solani* Ferris, another new invasive species of Nearctic origin, in Southern Spain (Calvo and Belda, 2011). *Leptomastix epona* is a koinobiont parasitoid and was recovered from mealybug mummies that ranged in size from 1.00 to 2.46 mm, indicating that it might parasitize mostly third nymphal instars and adults. Its secondary sex ratio became female biased in mealybug mummies >1.8 mm. These findings are consistent with those of Karamaouna and Copland (2000a) who found that the parasitism rates of *L. epona* when parasitizing *P. viburni* were smaller in second instars than in higher mealybug stages and that the parasitoid sex ratio became female biased in mummies >1.83 mm under laboratory conditions.

During the first year of the study, *Acerophagus* sp. displaced *L. epona* as the principal parasitoid of *P. peruvianus*. This displacement took place at all the sampling sites. Interspecific competition commonly occurs among parasitoids which share the same host (Bogran *et al.* 2002). In this study, both parasitoid species behaved differently when they used *P. peruvianus* as host, and these differences might explain the displacement (Reitz and Trumble 2002). The first is the differential female offspring: *Acerophagus* sp. presents a high brood size of female offspring which leads to a rapid growth of its population densities, while *L. epona* is a solitary parasitoid with host size-dependent sexual reproduction. The second cause is resource preemption, as *Acerophagus* sp. exhibits a range in a scale size that is wider than *L. epona* which allows for parasitism of smaller mealybugs. Moreover, *P. peruvianus* encapsulates *Acerophagus* sp. eggs at low rates (<11 %) under laboratory conditions (Beltrà *et al.* 2013c). This

might also be an advantage for *Acerophagus* sp. if *P. peruvianus* encapsulates *L. epona* eggs. Although the influence of other important mechanisms of parasitoid competition was not the aim of this study, our findings seem to be consistent with other research and reflect the difficulties of mealybug parasitoid coexistence. In concert with other factors, the ability to produce females from smaller hosts has been known to bring about a shift in different mealybug parasitoids: *Anagyrus lopezi* (De Santis) displaced *Anagyrus diversicornis* (Howard) when introduced into Africa to control *P. manihoti* (Neuenschwander 2001); *Anagyrus antoninae* Timberlake was substituted by *Neodusmetia sangwani* (Subba Rao) as the main parasitoid of *Antonina graminis* in North America (Schuster and Dean 1976); *A. papayae* proved to be a better competitor than *Anagyrus loecki* Noyes and Menezes and *Pseudleptomastix mexicana* Noyes and Schauff in the classical biological control of *P. marginatus* (Amarasekare *et al.* 2009). However, resource preemption has not always led to parasitoid displacement. For example, *Gyranusoidea tebygi* Noyes uses smaller hosts than *Anagyrus mangicola* Noyes, but both parasitoids co-exist as parasitoids of *Rastrococcus invadens* in Africa. This may be due to the fact that *A. mangicola* is a better competitor in cases of multiparasitism (Bokonon-Ganta *et al.* 1996). In the present parasitoid complex, the complete exclusion of *L. epona* might be also discarded as it parasitizes other hosts, such as *Pseudococcus longispinus* and *Pseudococcus viburni*, which are widely spread in Eastern Spain. This also occurs with *A. diversicornis* and *A. lopezi*, which coexist in their native South America due to the presence of *Phenacoccus* herreni where *A. diversicornis* refuges and persists (Pijls and van Alphen 1996).

4.5 Acknowledgments

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5- REPRODUCTIVE STRATEGIES AND FOOD SOURCES USED BY *ACEROPHAGUS* N. SP. *NEAR COCCOIS*, A NEW SUCCESSFUL PARASITOID OF THE INVASIVE MEALYBUG *PHENACOCCUS PERUVIANUS*

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

Abstract

Phenacoccus peruvianus Granara de Willink (Hemiptera: Pseudococcidae) is a new invasive mealybug that causes important damages in ornamental plants in urban landscapes and nurseries in Southern Europe. Recently, a new species of genus *Acerophagus* Smith (Hymenoptera: Encyrtidae) has been recorded as the main parasitoid of *P. peruvianus* in Spain, displacing the native parasitoid *Leptomastix epona* Walker (Hymenoptera: Encyrtidae). In this work, we have determined some traits of the reproductive and feeding strategies of *Acerophagus* sp.: fecundity, immature developmental time, host instar suitability, and preference when parasitizing *P. peruvianus*, and the effect of natural occurring sugar sources on adult longevity. *Acerophagus* sp. egg load reached its maximum when it was 5 days old. Second and third nymphal instars and adults were suitable for parasitism and immature development (efficient encapsulation was low). Immature development lasted between 20 and 22 days. *Acerophagus* sp. developed as a solitary parasitoid in the second instar and as a gregarious parasitoid in older instars (2–4 parasitoids per host). All the emerged offspring were females. *Acerophagus* sp. always preferred older instars when different host instars were available. Finally, adults lived more than 20 days when fed on honey, but they lived fewer than 3 days when fed on naturally occurring sugar sources (host honeydew and host plant flowers, *Bougainvillea glabra*). The consequences of these characteristics on biological control and parasitoid rearing are discussed.

Keywords: Pseudococcidae, Encyrtidae, Egg load, Survivorship, Host suitability, Parasitism

5.1 Introduction

Mealybugs are important pests of agricultural and ornamental plants (McKenzie 1967; Ben-Dov 1994). In recent years, the invasion of new mealybug species and the substitution of broad-spectrum chemical applications by integrated pest management strategies have led to the emergence of mealybugs as key pests (Charles 1993; Hattingh *et al.* 1998; Miller *et al.* 2002; Daane *et al.* 2008b; Franco *et al.* 2009; Pellizzari and Germain 2010). *Phenacoccus peruvianus* Granara de Willink (Hemiptera: Pseudococcidae) is a mealybug of Neotropical origin that was first detected in the Mediterranean Basin, in Almeria in 1999 (Beltrà *et al.* 2010). Since then, it has extended its range throughout Europe: Portugal, France, Sicily, Majorca, Corsica, UK (under greenhouse conditions), and Canary Islands. It is a polyphagous species that feeds on several ornamental plants, including *Bougainvillea spp.* (Nyctaginaceae), *Hibiscus spp.* (Malvaceae), *Myoporum laetum* (Myoporaceae), *Lantana camara* (Verbenaceae), and *Aucuba japonica* (Aucubaceae) disturbing pest management in urban landscapes and ornamental plant nurseries in Southern Europe (Beltrà *et al.* 2010).

In a recent 3-year survey in Eastern Spain (2008-2010), a new parasitoid species of unknown origin, *Acerophagus* n. sp. *near coccois* (Hymenoptera: Encyrtidae), was the most abundant and effective parasitoid of *P. peruvianus* (Beltrà *et al.* 2013b). *Acerophagus* sp. was recorded at the end of the first year, replaced the native parasitoid *Leptomastix epona* Walker (Hymenoptera: Encyrtidae), controlling populations of *P. peruvianus* in the years that followed. Because of its unknown origin and its high efficacy, the authors considered it a case of fortuitous control. *Acerophagus* sp. behaved as a gregarious parasitoid and it probably displaced *L. epona* because of its higher female fecundity and resource preemption (Beltrà *et al.* 2013b).

One of the greatest challenges of biological control practitioners is to understand those factors which affect the efficiency of natural enemies, such as fecundity, sex ratio, progeny size, and host selection (King 1987; Ridley 1988). Moreover, some of these reproductive traits are closely related to the feeding resources that parasitoids can use (nectar,

honeydew, and host-feeding) and that are available (Jervis *et al.* 2008). Because of the new description of *Acerophagus* sp. as a biological control agent, most of these reproductive and feeding traits remain unknown. Thus, in this study we attempt to determine some of these parameters: (i) effect of parasitoid age and body size on egg load, (ii) gustatory acceptance and effect of diet on longevity, (iii) host instar suitability and selection, and (iv) immature developmental time. These results will provide essential information to mass rear *Acerophagus* sp. and improve the efficiency of the biological control of *P. peruvianus* in nurseries and urban landscapes. A high capacity of the parasitoid for increasing the population rapidly throughout a high brood size and fecundity may make its mass-rearing easy and provide a good efficacy in augmentative releases. Moreover, its potential adaptation to use natural occurring sugar sources to increase its longevity in the field may facilitate the conservation of the parasitoid and its efficacy as biological control agent.

5.2 Material and Methods

5.2.1 Mealybug and parasitoid rearing

The culture of *P. peruvianus* was established in the laboratory of the Polytechnic University of Valencia using specimens collected from *Bougainvillea glabra* plants on the university campus. The mealybugs were reared on organic sprouted potatoes inside plastic sandwich boxes (16.5 x 11 x 6 cm) with a 6.5-cm diameter aperture covered by muslin with a mesh of 0.2 x 0.2 mm for ventilation. The mealybug colony was maintained in darkness in a climatic chamber at 25 ± 2 ˚C and 65 ± 10 % HR.

The culture of *Acerophagus* sp. was started using specimens emerged from *P. peruvianus* mummies collected from *Bougainvillea glabra* plants on the university campus. *Acerophagus* sp. was reared on *P. peruvianus* from the laboratory culture in total darkness under the same conditions described above. To obtain newly emerged parasitoids for our experiments, mealybug mummies were gently transferred into 10 x 1.5 cm glass vials topped with a plastic lid with a central hole covered with muslin to allow ventilation and a streak of honey on the inner wall. These vials

were maintained in a climatic chamber at 25 ± 2 ˚C, 65 ± 10 % HR and a 14:10 D/N photoperiod and were checked daily from 8:00 to 10:00 for adult emergence. If gregarious parasitoids emerged from the same mummy, they were separated and placed in new vials. For our experiments, we used *Acerophagus* sp. unmated females because it reproduces parthenogenetically.

5.2.2 Effect of parasitoid age and body size on egg load

The egg load of *Acerophagus* sp. was determined at emergence and at days 1, 2, 3, 5, 7, and 11. At emergence, parasitoids were individually placed in a 10 x 1.5 cm glass vial as explained above. Parasitoids were then honey-fed by supplying a honey streak twice a week in the inner wall of the vial, and maintained in a climatic chamber at 25 ± 2 ˚C, 65 ± 10 % HR, and a 14:10 D/N photoperiod from emergence until they were dissected. Vials were placed in a freezer at (-20 ˚C) for 1 h to kill parasitoids. After that, specimens were placed in a droplet of fuchsine (1 %) on a microscope glass slide (Maple 1954). Using two fine dissection needles, the thorax was separated from the abdomen leaving the eggs in the solution droplet (Jervis *et al.* 2007). After the staining was completed (2–3 min), the eggs were counted using a compound microscope (40x magnification) and the length of the hind tibia was measured to the nearest 0.001 mm using the numeric image analysis software NIS-Elements D 64-bit 3.10 (Nikon) under a compound microscope (40x magnification). In total, 92 parasitoids were dissected with at least nine individuals per age.

5.2.3 Gustatory acceptance and effect of diet on longevity

To assess the influence of different diets on *Acerophagus* sp. longevity, adults were introduced into 10 x 1.5 cm clean glass vials at emergence, under the same conditions as described above, and fed on four diets: (i) honey, (ii) *P. peruvianus* honeydew, (iii) *Bougainvillea glabra* flowers, and (iv) water alone (control). Water was supplied daily for all treatments by spraying the vials through the muslin mesh. Honey was supplied by introducing a streak on the inner wall of the vials twice a week (Tena and Garcia-Marí 2009). Honeydew was collected over a period of 24 h

by placing a 3-cm diameter clip-cage around a colony of mealybugs settled on *Bougainvillea glabra x buttiana* plants in the climatic chambers of the laboratory of the Polytechnic University of Valencia. Once in the lab, the honeydew was collected using microcapillaries and transferred to the inner wall of the vials. Freshly collected honeydew was supplied daily to avoid crystallization (Hogervorst *et al.* 2007). *Bougainvillea glabra* flowers were taken from untreated plants from the university greenhouses and replaced daily to avoid wilting. Flowers were directly introduced inside the vials. After supplying the food for the first day, we observed if parasitoids were able to feed on the different diets for 30 min using a compound stereoscope or until the fed. Vials were checked daily from 8:00 to 10:00 to determine the number of surviving parasitoids. Accidental deaths (wasps stuck in honey) were not taken into account for the analysis.

5.2.4 Host instar suitability and selection

The suitability and preference of the parasitoid for the different host instars were evaluated under choice and no-choice tests. Experimental arenas consisted of a 5.3-cm diameter Petri dish with two, 1-cm diameter holes covered by a muslin mesh to permit ventilation. Inside, a leaf-disk (Ø 5 cm) of *Aucuba japonica* was introduced upside down over a layer of 8 g/l Bacteriological agar (Karamaouna and Copland 2000a). Mealybugs were gently transferred with a small wet camel brush from the mass culture to the arenas 24 h before the assays. Mealybug instars were estimated from their length: (i) second nymphal instar (0.5–0.9 mm), (ii) third nymphal instar (0.9–1.4), (iii) young adult (1.4–2 mm), and (iv) preovipositing adult (more than 2 mm) (Beltrà *et al.* 2013a). Arenas were placed inside a climatic chamber at 25 ± 1 ˚C and 65 ± 10 % HR and a 14:10 D/N photoperiod during the experiments.

To determine the mealybug instars susceptible to parasitism, we conducted a no-choice experiment. One 3–5-day old female parasitoid was introduced in an arena with 15 mealybugs of the same size during 24 h. Then, the female was removed with a manual vacuum device. Ten days later, mummified mealybugs were recovered and isolated in small vials 3.0 x 0.8 cm in diameter covered with a cotton plug. In addition, non-

parasitized mealybugs were dissected to check for encapsulated eggs and the percentage of efficient encapsulation (hosts with all parasitoid eggs encapsulated) was calculated. Parasitized mealybugs were checked daily until emergence. Then, the sex, brood size (number of parasitoids per mealybug), and immature developmental time were recorded. The hind tibia length (HTL) of 30 newly emerged parasitoids of each mealybug instar was also measured as mentioned above. At least 14 repetitions were carried out for each mealybug instar.

Two-at-a-time choice tests were performed to determine the parasitoid preference for each mealybug instar. One 3–5-day-old female parasitoid was introduced into one arena with 10 mealybugs (five of each instar) for 6 h. The female was then removed and mealybugs were separated according to their instar and placed on new disk leaves inside the climatic chamber (Amarasekare *et al.* 2010). Ten days later, we recorded the mummified mealybugs and dissected the rest of the specimens to check for encapsulated eggs. Encapsulated and successful parasitized mealybugs were pooled to calculate parasitism rates. The experiment was carried out by pairing all the different instars: second nymphal instar, third nymphal instar, young female, pre-oviposition female and was repeated at least 15 times for each combination.

5.2.5 Statistical analysis

The influence of the host instar on the number of parasitized mealybugs, parasitoid brood size, developmental time, encapsulation, and adult parasitoid size was compared by a one-way ANOVA. Data were normalized by logarithmic transformation when required. The influence of parasitoid age and body size (HTL) on the number of mature eggs (egg load) was evaluated using generalized linear models. Akaike's Information Criterion was used to select a log-linear analysis over a standard analysis (Akaike 1974). After that, a stepwise backward elimination was performed to find the minimum adequate model (Hardy and Field 1998). The effect of the feeding treatments on the longevity of adult parasitoids was represented by Kaplan–Meier survivorship curves and analyzed by a log-rank test. The preference of the parasitoid for the different host instars was

analyzed by a t test. All the analyses were carried out by means of STATGRAPHICS Centurion 16.1.11 (Statpoint Technologies 2009) software, except the egg load analyses which were performed by means of statistical software R (http://www.R-project.org).

5.3 Results

5.3.1 Effect of parasitoid age and body size on egg load

Acerophagus sp. egg load was significantly influenced by body size (HTL) and parasitoid age, and the minimum adequate model [Egg load = exp (7.06363 + 0.222949 * age - 0.0662735 * HTL - 0.0163974 * age^2 + 0.000241833 * HTL^2)] explained 55.42 % of the total deviance (n = 92; P < 0.001) (Fig. 1). Females averaged 12.17 ± 1.32 mature eggs at emergence (0–24 h-old) and their egg load increased until the fifth day, when they contained 29.50 ± 1.66 mature eggs. After the fifth day, the number of eggs per female started to decrease.

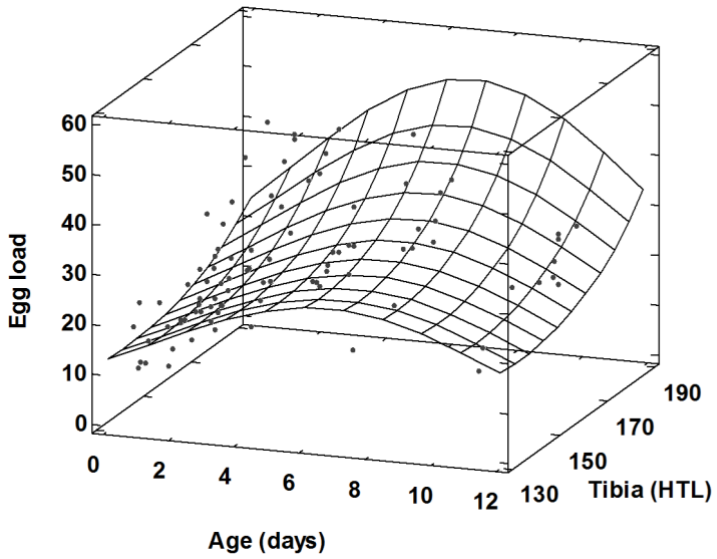

Figure 1. Effect of parasitoid age (days) and size (hind tibia length [µm]) on the egg load (mature eggs) of Acerophagus sp. [Egg load = exp (7.06363 + 0.222949 * age – 0.0662735 * HTL – 0.0163974 * age^2 + 0.000241833 * HTL^2)]. The model explained 55.42% of the total deviance.

5.3.2 Gustatory acceptance and effect of diet on longevity

All *Acerophagus* sp. parasitoids fed on honey and honeydew during our direct observations. However, none of the 20 parasitoids observed were able to feed on Bougainvillea nectar. The small size of the Bougainvillea flower impeded adult wasps from reaching the nectar.

Diet in form of water, honeydew, or honey significantly influenced the life span of *Acerophagus* sp. (Global Log-rank test: χ^2 = 137.32; *df* = 2; *P* < 0.001) (Fig. 2). Parasitoids lived longer when they fed on honey (21.85 ± 0.91 days; n = 55) than when fed on honeydew (2.51 ± 0.13; n = 49) (Log-rank test: χ^2 = 119.84; *df* = 1; *P* < 0.001). Parasitoid longevity was also slightly higher when they fed on honeydew than on water (1.71 ± 0.12; n = 14) (Log-rank test: χ^2 = 10.42; *df* = 1; *P* = 0.001).

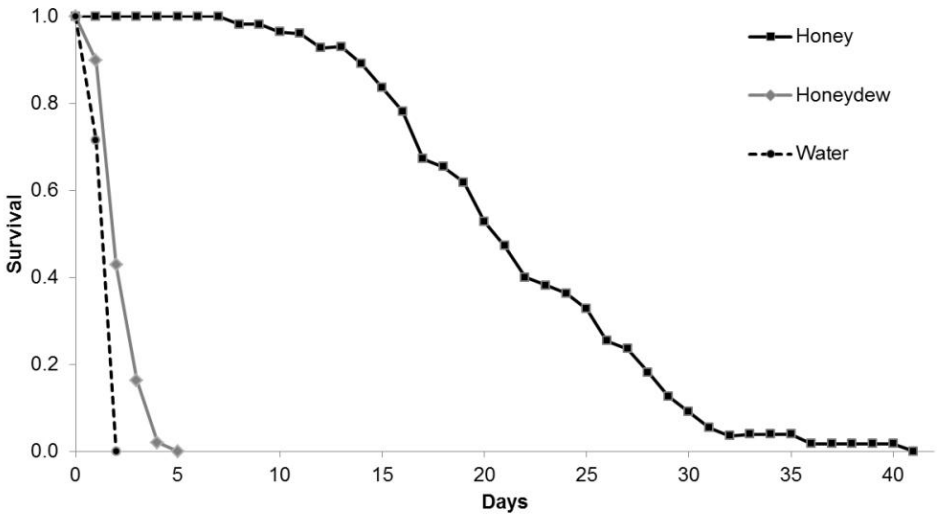

Fig. 2 Survivorship curves for *Acerophagus* sp. subjected to four diet treatments: honey, *P. peruvianus* honeydew when it fed on *B. glabra x buttiana*, and control (water) (Global Log-rank test: $\chi 2$ = 137.32; *df* = 2; *P* < 0.001).

5.3.3 Host instar suitability and selection

All the host instars offered in the non-choice experiments were parasitized by *Acerophagus* sp. The number of parasitized mealybugs was significantly higher on the third instar, young adults, and preovipositing adults (9.29 ± 0.41 parasitized mealybugs), than on the second instar (3.50 ± 0.80) (ANOVA: $F = 18.14$; $df = 3, 54$; $P < 0.001$) (Table 1).

Table 1. Effect of host instar on parasitism, efficient encapsulation, brood size, and immature developmental time (number of replicates, mean ± SE) of *Acerophagus* sp. when parasitizing *P. peruvianus.*

Host instar	Parasitized hosts		Efficient encapsulation (%)*		Brood size		Immature developmental time (days)	
N2 (0.5-0.9)	14	3.50 ± 0.80[b]	14	0.65 ± 3.26	43	1.09 ± 0.32[c]	33	21.85 ± 0.34[a]
N3 (0.9-1.4)	14	9.86 ± 0.80[a]	14	8.68 ± 2.89[a]	132	2.29 ± 0.18[b]	113	20.29 ± 0.18[b]
H1 (1.4-2)	15	8.60 ± 0.77[a]	15	14.31 ± 2.79[a]	100	4.09 ± 0.21[a]	100	19.59 ± 0.19[c]
H2 (> 2)	15	9.47 ± 0.77[a]	15	9.14 ± 2.79[a]	104	3.89 ± 0.20[a]	99	19.41 ± 0.19[c]

Different letters indicate significant differences between columns (one way ANOVA: $P < 0.05$)
N2 2nd nymphal instar; *N3* 3rd nymphal instar; *H1* young adult; *H2* preovipositing adult
* Second nymphal instar was excluded from the analysis because there was just one case of encapsulation

Overall, only 41 mealybugs (out of 458 parasitized mealybugs) successfully encapsulated all the eggs laid by *Acerophagus* sp. Only one out of the 49 parasitized second instars encapsulated the eggs. Among the other instars, the percentage of efficient encapsulation was similar (third instar, young adults, and preovipositing adults) and averaged 10.76 ± 0.31 % (ANOVA: $F = 1.00$; $df = 2, 41$; $P = 0.38$). *Acerophagus* sp. needed 19.98 ± 0.11 days to complete its immature development at 25°C. The developmental period differed among instars (ANOVA: $F = 15.57$; $df = 3, 341$; $P < 0.001$). It was slightly shorter in older hosts (Table 1). There were no significant differences among the sizes of the parasitoids that emerged from the different mealybug instars (ANOVA: $F = 2.16$; $df = 3, 117$; $P = 0.096$) (HTL averaged 163.42 ± 2.93 µm).

Acerophagus sp. behaved as a facultative gregarious parasitoid. Brood size was significantly influenced by scale instar (ANOVA: $F = 45.92$; $df = 3, 375$; $P < 0.001$) (Table 1). *Acerophagus* sp. behaved mainly as a solitary

parasitoid when it parasitized the second and third instars whereas more than one adult emerged from adult mealybugs. Brood size was nearly four parasitoids, reaching a maximum of 11 parasitoids per host.

Acerophagus sp. preferred the oldest instar in all the pair-way experiments (Table 2). The second instar was less parasitized when paired with third instar (t = -5.53; df = 28; P < 0.001), young females (t = -5.28; df = 28; P < 0.001), and pre-oviposition females (t = -7.59; df = 28; P < 0.001). Smaller, but significant, differences were found between the third instar and young females (t = -2.13; df = 28; P = 0.049) or between young females and gravid females (t = -2.47; df = 28; P = 0.026).

Table 2. Mean number of hosts (x ± SE) parasitized by *Acerophagus* sp. when combining different host instars under choice experiment.

Host instar combination		Mean parasitized hosts		*T* statistics		
Instar 1	Instar 2	Instar 1	Instar 2	*T*	df	*P*
N2	N3	0.87 ± 0.21	2.47 ± 0.23	-5.53	28	<0.0001
N2	H1	0.53 ± 0.19	2.33 ± 0.32	-5.28	28	<0.0001
N2	H2	0.88 ± 0.28	3.59 ± 0.27	-7.95	32	<0.0001
N3	H1	1.23 ± 0.22	2.00 ± 0.26	-2.13	28	0.049
N3	H2	1.26 ± 0.25	3.73 ± 0.30	-5.67	28	<0.0001
H1	H2	1.62 ± 0.27	2.75 ± 0.37	-2.47	28	0.026

N2 2nd nymphal instar; *N3* 3rd nymphal instar; *H1* young adult; *H2* preovipositing adult

5.4 Discussion

The results of this study show the potential of *Acerophagus* sp. as biological control agent of *P. peruvianus*. It parasitized a wide range of host instars and suffered low encapsulation rates in all the parasitized instars. These results corroborate the field observations by Beltrà *et al.* (2013b) and support the suitability of *Acerophagus* sp. as an effective biological control agent of *P. peruvianus*. Moreover, our research defines other biologic traits of this parasitoid such as ovigeny, immature developmental time, suitability of different natural occurring diets on adult longevity, and instar preference. The better understanding of these parasitoid traits will be useful to design an adequate biological control program against *P. peruvianus* (i.e., rearing protocols for its main parasitoid and conservation biological control).

Acerophagus sp. was able to parasitize all the mealybug instars assayed in this study. However, the number of parasitized mealybugs was lower in the second instar and it always preferred the oldest hosts, when given a choice among different mealybug instars. Even so, the suitability of second nymphal instars has important consequences on biological control, revealing host preemption as one of the possible mechanisms that can explain the displacement of the native *L. epona* in the field by this parasitoid. In fact, *L. epona* parasitizes third nymphal instars and adults of *P. peruvianus* and other mealybug species such as *Phenacoccus solani* (Green) and *P. viburni* (Karamaouna and Copland 2000a; Calvo and Belda 2011; Beltrà *et al.* 2013b). The wide range of host instars of *Acerophagus* sp. also simplifies its use in augmentative biological control because the parasitoid could be released independently of the scale instars present in the field. Our results differ from those obtained in no-choice experiments with other parasitoids of the same genus: the gregarious parasitoid *Acerophagus flavidulus* (Brèthes) uniformly parasitized all the instars of *Pseudococcus viburni* (Signoret) from the second instar to adult females (Karamaouna and Copland 2000a); in contrast, the solitary parasitoid *Acerophagus papayae* Noyes and Schauff showed higher parasitism rates in the smaller instars of *Paracoccus marginatus* Williams and Granara de Willink (Amarasekare *et al.* 2010). Our choice-test results agree with those of Sandanayaka *et al.* (2009) and Karamaouna and Copland (2000a) which showed that the gregarious parasitoids *A. maculipennis* and *A. flavidulus* preferred the oldest instars of *P. viburni*. Again, these results contrast the studies of Amarasekare *et al.* (2010) which indicate that the solitary parasitoid *A. papayae* preferred the youngest mealybug instars of *P. marginatus*.

Two of the most important reproductive traits of *Acerophagus* sp. are its high degree of gregariousness and its complete female brood. The brood size increased as the host aged and an average of four parasitoids developed in the adult mealybug stage. Moreover, some of the brood sizes were found to be higher, up to 11 parasitoids, and quite similar to the maximum found in the field, namely, 12 parasitoids (Beltrà *et al.* 2013b). Most species of genus *Acerophagus*, such as *A. maculipennis*, *Acerophagus coccois* Smith, A. *flavidulus*, and *Acerophagus angelicus* Howard, are also

facultatively gregarious and their brood size increase with host size (Maple 1954; Van Driesche *et al.* 1987; Karamaouna and Copland 2000a; Sandanayaka *et al.* 2009). No male parasitoid was recovered in the study, showing that *Acerophagus* sp. reproduces by thelytokous parthenogenesis. However, to our knowledge, thelytokous parthenogenesis has not been observed in other species of this genus, which show arrhenotokous parthenogenesis (Van Driesche *et al.* 1987; Karamaouna and Copland 2000a; Sandanayaka *et al.* 2009; Amarasekare *et al.* 2010). These attributes make *Acerophagus* sp. a better competitor than *L. epona*, which is a solitary parasitoid with a male-biased offspring when it parasitizes small hosts (Karamaouna and Copland 2000a; Beltrà *et al.* 2013b). Moreover, these results show that immature adult instar of *P. peruvianus* is the most suitable for mass-rearing *Acerophagus* sp. because of the higher brood size, low encapsulation rates, and faster immature development without reducing the size of the adult females that emerge from this instar.

Encapsulation is an important defense mechanism utilized by mealybugs against parasitoids (Bess 1939; Blumberg 1997; Blumberg and Van Driesche 2001). However, in our study, we found small rates of efficient encapsulation (i.e. hosts with all parasitoid eggs encapsulated) and, consequently, the biological control of *P. peruvianus* by *Acerophagus* sp. does not seem to be compromised by encapsulation. Among instars, the second instar was not able to avoid parasitism by encapsulating eggs and only 10 % of the older instars efficiently encapsulated eggs. This increase in encapsulation as age increases is common in mealybugs (Sagarra *et al.* 2000a; Karamaouna and Copland 2009) and soft scales (Hemiptera: Coccidae) (Blumberg 1997; Kapranas *et al.* 2012). For this reason, some parasitoid species tend to parasitize younger instars (Blumberg 1997; Sagarra *et al.* 2000a; Jervis *et al.* 2007).

The increase of the egg load in host-deprived females indicates that *Acerophagus* sp. is a synovigenic parasitoid (sensu Jervis *et al.* 2001). The parasitoid matured eggs after emergence and its egg load peaked on the fifth day, averaging 30 mature eggs. Beyond the fifth day, it started to resorb eggs (Jervis *et al.* 2007). Therefore, if *Acerophagus* sp. is augmentative released in biological control programs, females should be

ideally less than 5-days old. The number of mature eggs observed in this parasitoid is higher than that obtained in similar studies for parasitoids of the same genus. The egg load of *A. maculipennis* became constant 7 days after emergence, averaging 23 mature eggs (Sandanayaka *et al.* 2009), while A. *coccois* just contained 10 mature eggs 3 days after emergence (Dorn *et al.* 2001). In addition, this is the first time, to our knowledge, that egg resorption is reported for a parasitoid of genus *Acerophagus*.

Among synovigenic parasitoids, adult feeding is important for maintenance and reproduction (Olson *et al.* 2005; Jervis *et al.* 2008). Because *Acerophagus* sp. is a non-host-feeding species (Beltrà *et al.* in prep), the main food that is available for this parasitoid in the field is plant nectar and honeydew excreted by hemipterans, which are rich in carbohydrates. In our study, *Acerophagus* sp. increased its longevity when it fed on honey, living up to 22 days. However, the parasitoid died within 2 days when it fed on the sources which are commonly present in the field: honeydew from its main host and nectar from the host plant, Bougainvillea, which is located exclusively inside the flowers. In a similar study carried out by Sandanayaka *et al.* (2009), *A. maculipennis* had a similar lifespan when it fed on honey (nearly 16 days for females) or water (less than 4 days), but when it was fed on honeydew from *P. viburni* settled on potatoes its lifespan was found to be nearly 15 days. A possible explanation for the poor quality of *P. peruvianus* honeydew when it feeds on *Bougainvillea glabra x buttiana* could be the presence of secondary toxic compounds in this plant (Gupta *et al.* 2009). Although bougainvillea nectar could be expected to be a good food resource for *Acerophagus* sp., our observations revealed that the parasitoid was not able to enter the flowers due to their small size. Therefore, only the honeydew of other hemipterans such as aphids and soft scales, if they are suitable, may be available for *Acerophagus* sp. in bougainvillea plants. Thus, biological control of *P. peruvianus* could be enhanced by increasing the diversity of flowering plants in urban landscapes (Landis *et al.* 2000; Raupp *et al.* 2010). Conversely, sugar solutions could be provided in bougainvillea ornamental nurseries where plant diversity cannot be increased.

The results presented herein and in the previous paper by this group (Beltrà *et al.* 2013b) show that *Acerophagus* sp. is an efficient biological control agent for the invasive pest *P. peruvianus*. The conservation of this parasitoid in urban landscapes may act as a good control of *P. peruvianus* and its populations may be enhanced by increasing floral biodiversity or the availability of supplemental food. Moreover, its augmentative release may be especially interesting in bougainvillea nurseries or in urban landscapes where the parasitoid is not yet established and mealybug populations reach high levels during spring (Beltrà *et al.* 2013b). The introduction of the parasitoid into other European regions, where the mealybug constitutes a problem, should not be ruled out. *Acerophagus* sp. can be easily reared in *P. peruvianus* due to its wide host instar range and female-biased broods. Also, the adult instar is the most adequate instar for mass-rearing *Acerophagus* sp. Further research on this parasitoid should include its interaction with ants in the field and its capacity to parasitize other mealybug species.

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6- GENERAL DISCUSSION

General discussion

The problems associated with mealybugs in Spain have increased as a result of the recent legislative limits on pesticide use and the introduction of new invasive species. Several mealybug species are currently causing serious losses in important crops such as *Planococcus citri* and *Delottococcus aberiae* on citrus; *Pseudococcus viburni* on apples; *P. citri* and *Planococcus ficus* on vineyards; *Phenacoccus solani, Phenacoccus madeirensis* and *P. citri* on greenhouses crops; *P. madeirensis*, *P. peruvianus* and *P. citri* on ornamental plants; and *Dysmicoccus grassii* on banana trees (Beltrà and Soto 2012). These species are often misidentified leading to confusion and reducing the efficiency of pest control. In this work we carried out a multi-criterion identification of most of these species, combining morphological and molecular taxonomy. The DNA sequences obtained will facilitate routine identification by barcoding by any user with access to DNA sequencing. These sequences will also enable the further development of specific PCR kits and will make it possible to use molecular identification tools to track invasive species and detect quarantine pests during import and export controls. Constant reduction of sequencing costs and the emergence of Next-Generation Sequencing technologies is opening a new scenario for molecular biology, where we expect that DNA barcoding will play a major role in inscect taxonomy (Schuster 2008; Metzker 2010).

Our molecular studies in five different loci provide important insights into invasion biology. This work showed high genetic intraspecific variability in native and established mealybug populations, such as *P. citri*, *Planococcus vovae* and *Pseudococcus longispinus*, as compared with more recently-introduced species like *P. madeirensis* and *P. peruvianus.* The latter species is a relative newcomer to Europe and has rapidly spread around the Mediterranean countries, probably through the trade of ornamental plants, which is the main pathway of dispersion of scale insects in Europe (Pellizzari and Germain 2010). Its genetic homogeneity suggests that the different Spanish and French populations studied originated from the same geographical region or have spread from a single population, possibly from Almeria, where it was first observed.

Phenacoccus peruvianus' biology and behavior were first studied in Eastern Spain. Mealybug populations increased in early spring, reaching a peak in summer, and subsequently fell to their lowest levels during autumn and winter. All the mealybug instars overlapped during the whole year. This biology is similar to that of other mealybug species present in Mediterranean countries, such as *P. citri*, *P. viburni* and *P. madeirensis* (Panis 1986; Longo *et al.* 1995; Martinez-Ferrer *et al.* 2003). The presence of several overlapping generations limits chemical control in urban landscapes, due to the continuous presence of adult instars, which are more resistant to soft contact insecticides like mineral oils. However, this phenology may favor biological control agents that have instars available that are susceptible to parasitism or predation all the year round.

In order to improve the decision making process in the management of *P. peruvianus*, we studied its spatial distribution and developed a sampling plan in bougainvillea plants. The mealybug preferred bracts over leaves and twigs and showed a high clumped distribution typical of scale insects (Nestel *et al.* 1995). In view of our results, we propose a feasible binomial sampling of 200 leaves for urban pest management purposes, which can be done in 10-15 minutes. We also recommend a management threshold of five mealybugs per leaf, following aesthetic criteria. We expect that the simplicity of this methodology will facilitate its routine application in urban landscapes. We also provide an enumerative sampling method for cases in which monitoring is carried out for other purposes, such as ornamental nursery management or additional biological studies.

The action of natural enemies surprisingly resulted in a gradual reduction of mealybug populations between 2008 and 2010. Several parasitoids and predators were recorded feeding on *P. peruvianus.* Of these, a new parasitoid species, *Acerophagus* n. sp. *near coccois*, displaced the native parasitoid *Leptomastix epona* and provided a successful biological control of this pest. Fortuitous introductions of mealybug biological control agents resulting in pest control have also been recorded in other mealybug species in Spain and other countries (Nechols 2003; Jacas *et al.* 2006; Muniappan *et al.* 2006). Although the origin of this parasitoid

remains unknown, the specific interaction between encyrtids and mealybugs and the American origin of most *Acerophagus* species lead us to consider that the parasitoid might be of Neotropical origin and could have been introduced together with the pest or in successive introductions (Charles 2010).

Several insect displacement mechanisms have been detailed by Reitz and Trumble (2002): i) resource acquisition, ii) differential female fecundity, iii) searching ability, iv) resource preemption, v) resource degradation, vi) agnostic interference competition, vii) reproductive interference, and viii) intraguild predation. In the present study, the most likely causes of parasitoid displacement were differential fecundity and resource preemption. *Acerophagus* sp. is a gregarious and parthenogenetic species and therefore obtains higher female broods from the same resources than *L. epona,* which is a solitary parasitoid with sexual reproduction. Moreover, *Acerophagus* sp. can parasitize *P. peruvianus* from second nymphal instars anticipating its competence. On the other hand, *L. epona* may be a better competitor by resource degradation, because it is able to host-feed on small mealybug instars (Karamaouna and Copland 2000b). Further studies should be carried out to determine the effect of other competitive mechanisms, such as searching ability, resource acquisition when ants are present or interference by larval competition.

Besides the above mentioned characteristics, other biological traits studied, such as the high parasitoid egg-load and the small percentage of encapsulation when *Acerophagus* sp. parasitizes *P. peruvianus,* make this parasitoid a very effective biological control agent. Moreover, its parthenogenesis and gregariousness facilitate its mass rearing. The success of *Acerophagus* sp. raises the question whether this parasitoid can be used in other geographic areas and in a wider range of hosts. *Acerophagus* sp. has also been detected in other Mediterranean regions such as Catalonia and French Riviera (personal observations), and might be introduced in other European areas where biological control is not succesful. The suitability of other mealybug species for this parasitoid should be studied to determine whether it can also be used against other invasive species, such as *Phenacoccus solani* or *P. madeirensis.*

The results obtained in this study provide useful information for the management of *P. peruvianus* in urban landscapes. The biological control of this pest is successful when *Acerophagus* sp. is present. The conservation of this parasitoid and other natural enemies is therefore a key issue in mealybug management. The efficiency of *Acerophagus* sp. in urban landscapes could be improved by limiting the use of broad-spectrum chemicals, increasing plant biodiversity or applying sugars to ensure parasitoid nourishment, and controlling ants, if present. This last point is particularly relevant, as recent studies show that *Acerophagus* sp. oviposition is frequently disrupted by tending ants (Beltrà *et al.* unpublished data). Even so, if high mealybug infestations are present in urban landscapes or plant nurseries, inundation biological control could be considered. As noted above, *Acerophagus* sp. can be easily reared and could prove attractive to biocontrol companies. However, this parasitoid species is not commercially available at the present time and *L. epona* could be considered as a secondary option in inundation biological control. For those cases in which these strategies are unfeasible, mealybug management can also be carried out through chemical control. However, the use of insecticides in urban landscapes must be limited to active ingredients with low risk to human health and natural enemies such as soaps and mineral oils. In these cases, monitoring should be carried out during spring, before population outbreaks.

7- CONCLUSIONS

Molecular and morphological characterisation of Pseudococcidae surveyed on crops and ornamental plants in Spain

- Ten different mealybug species were identified and DNA sequenced at five loci, providing the first molecular data for *Delottococcus aberiae*, *Phenacoccus peruvianus* and *Planococcus vovae*.
- Genera *Phenacoccus* and *Planococcus* were found monophyletic, while both *Dysmicoccus* and *Pseudococcus* were found paraphyletic.
- Mealybug native species or those that have been present for over a century in the Mediterranean Basin showed substantial genetic intraspecific divergences, while the newly introduced species *P. peruvianus* and *P. madeirensis* showed no divergences.

Seasonal phenology, spatial distribution and sampling plan for the invasive mealybug *Phenacoccus peruvianus* (Hemiptera: Pseudococcidae)

- *Phenacoccus peruvianus* populations were high in spring and summer and decreased to almost undetectable levels in autumn and winter.
- The mealybug showed preference for bougainvillea bracts and there were no significant migrations between plant organs.
- We recommend a binomial sampling of 200 leaves and an action threshold of 55% of infested leaves for IPM purposes in urban landscapes.

Fortuitous biological control of the invasive mealybug *Phenacoccus peruvianus* in Southern Europe.

- Within the natural enemy complex of *P. peruvianus* we identified the following parasitoids: *Acerophagus* n. sp. *near coccois*, *Leptomastix epona*, *Anagyrus pseudococci*, *Pachyneuron sp.*, *Chartocerus sp*. and *Prochiloneurus bolivari*; and predators: *Orius laevigatus*, *Cryptolaemus montrouzieri*, *Propylea quatordecimpunctata*, *Oenopia lyncea*, *Scymnus spp.*, *Leucopis sp.*, and *Crysoperla carnea*.
- The high parasitism rates observed in 2009 and 2010 suggest that *P. peruvianus* populations may be naturally controlled by the parasitoid *Acerophagus sp.* under Mediterranean conditions.

- *Acerophagus sp.* displaced the native parasitoid *L. epona* during the three years of the study. Differential female offspring and resource preemption are exposed as the main causes for this displacement.

Reproductive strategies and food sources used by *Acerophagus* n. sp. *near coccois*, a new successful parasitoid of the invasive mealybug *Phenacoccus peruvianus*

- *Acerophagus sp.* egg load reached its maximum when it was 5 days old with nearly 30 mature eggs.
- Second and third nymphal instars and adults were suitable for parasitism.
- The parasitoid always preferred older instars when different host instars were available.
- Efficient encapsulation was low and should not compromise biological control.
- *Acerophagus* sp. developed as a solitary parasitoid in the second instar and as a gregarious parasitoid in older instars (2–4 parasitoids per host).
- The parasitoid reproduced parthenogenetically with a complete female offspring ratio.
- Immature development lasted from 20 to 22 days at 25ºC and 65% HR.
- Adult parasitoids lived more than 20 days when fed on honey, but fewer than 3 days when fed on naturally occurring sugar sources (host honeydew and host plant flowers, *Bougainvillea glabra*).

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