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Factors affecting the biological control of California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae) by *Aphytis* (Hymenoptera: Aphelinidae) in eastern Spain citrus: host size, ant activity, and adult parasitoid food sources

DOCTORAL THESIS

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Στη μητέρα μου

A Vero

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CERTIFICAN:

Que la presente memoria titulada: "Factors affecting the biological control of California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae) by *Aphytis* (Hymenoptera: Aphelinidae) in eastern Spain citrus: host size, ant activity, and adult parasitoid food sources", realizado bajo nuestra dirección por D. Apostolos Pekas, durante el periodo comprendido entre 2006 a 2010, constituye su Memoria de Tesis para optar al grado de Doctor, en el Departamento de Ecosistemas Agroforestales de la Universidad Politécnica de Valencia.

Para que así conste a todos los efectos oportunos, firman el presente certificado

Fdo: Dr. Ferran Garcia Marí

Dr. Alejandro Tena Barreda

Valencia, Julio 2010

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Resumen

El piojo rojo de California, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), es una de las plagas más importantes de los cítricos a nivel mundial. En España, los daños producidos por este diaspídido se detectaron por primera vez en 1986. Los principales agentes de control biológico de *A. aurantii* en esta zona son el parasitoide nativo *Aphytis chrysomphali* (Mercet) y el introducido *A. melinus* DeBach (ambos Hymenoptera: Aphelinidae). Sin embargo, el control de la plaga realizado por estos dos parasitoides es insuficiente. Con el fin de mejorar el control biológico de *A. aurantii*, se han estudiado varios factores bióticos que afectan la eficacia de *A. chrysomphali* y *A. melinus* en condiciones de campo. En concreto, hemos estudiado la variación espacial y temporal del tamaño de *A. aurantii*, los tamaños que usan como hospedador *A. chrysomphali* y *A. melinus*, así como el efecto del tamaño del hospedador sobre varias características de dichos parasitoides. Además, se estudió el impacto de las especies de hormigas nativas en el Mediterráneo sobre las poblaciones de *A. aurantii*, y finalmente, el estado nutricional y las fuentes de alimento que usan los adultos de *A. melinus* en los campos de cítricos.

En condiciones de campo, el tamaño del cuerpo de *A. aurantii* varió en función del sustrato vegetal sobre el cual el insecto se alimenta, de la zona geográfica, de la época del año, y probablemente del estado nutricional de la planta hospedadora. El sustrato vegetal influyó considerablemente en el tamaño de la cochinilla; los individuos desarrollados en los frutos fueron de mayor tamaño que los desarrollados en hojas o ramas. Asimismo, se registraron importantes diferencias en el tamaño de la cochinilla entre las diferentes parcelas muestreadas. La época del año influyó también en el tamaño de *A. aurantii*; las cochinillas desarrolladas durante el verano y otoño fueron más pequeñas posiblemente debido al efecto de la temperatura. Además, se detectó una asociación positiva entre el tamaño de *A. aurantii* y el contenido de las hojas en potasio. De los factores anteriormente mencionados, la variación estacional relacionada con la temperatura fue la que más influyó más en el tamaño de *A. aurantii*.

Aphytis chrysomphali y *A. melinus* utilizaron distintos tamaños de *A. aurantii* como hospedador en el campo. *Aphytis chrysomphali*, se recuperó principalmente de segundos estadíos (0.152-0.300 mm^2 en área de cuerpo de *A. aurantii*), pero parasitó más hembras jóvenes de tercer estadío, alcanzando una media de ~10% de parasitismo en cochinillas que median entre 0.80-0.85 mm^2. *Aphytis melinus*, se desarrolló y parasitó con más intensidad hembras jóvenes de tercer estadío. Alcanzó una media de ~30% de parasitismo en

cochinillas de tamaño entre 0.70-0.75 mm^2. Se encontró una asociación positiva entre el tamaño del hospedador y el comportamiento gregario y el tamaño de ambos parasitoides. De la misma manera, el tamaño del hospedador influyó en el sex ratio de *A. melinus*. En hospedadores de pequeño tamaño, *A. melinus* puso huevos que se desarrollarían en machos mientras que en hospedadores de tamaño grande puso huevos que darían lugar a hembras. El tamaño de *A. aurantii* a partir del cual se produjeron más hembras que machos de *A. melinus* fue alrededor de 0.40 mm^2. Este umbral para la producción de descendencia hembra se mantuvo constante, independientemente de la disposición de hospedadores de pequeño o grande tamaño. Dada la variabilidad estacional del tamaño de *A. aurantii*, se detectó una escasez de hospedadores de tamaño adecuado para la producción de hembras de *A. melinus* el período entre mayo y octubre. Consecuentemente, es de esperar, un descenso en las poblaciones del parasitoide durante este período, que probablemente resultará en un control de la plaga insuficiente. Para remediar dicho descenso poblacional de *A. melinus* sería recomendable que se realizaran sueltas masivas del parasitoide.

Las especies de hormigas (Hymenoptera: Formicidae) más abundantes en los cítricos Valencianos, las dominantes *Pheidole pallidula* (Nylander) y *Lasius grandis* Forel, se establecieron en territorios claramente separados dentro de la misma parcela, y muy raramente se encontraron sobre el mismo árbol. Al contrario, la especie subordinada *Plagiolepis schmitzii* Forel, se encontró habitualmente en el mismo árbol que una de las dos dominantes, principalmente *P. pallidula*. Esta distribución espacial es conocida como "mosaico" de hormigas. Las hormigas estuvieron activas en las copas de los árboles de cítricos desde abril hasta noviembre. La temperatura y las necesidades alimenticias de cada especie determinaron sus pautas de actividad estacional. En cuanto a sus pautas de actividad diaria, *L. grandis* y *P. pallidula* estuvieron activas durante las 24 horas del día, mientras *P. schmitzii* fue estrictamente diurna. El néctar de las flores de los cítricos y la depredación no representaron una fuente importante de alimento. La dieta de las hormigas en las copas de los árboles de los cítricos, consistió principalmente en melaza de hemípteros. Más del 60% de las colonias de hemípteros, y el 100% de las colonias de *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), estuvieron ocupadas por las hormigas en primavera y verano.

La exclusión de las hormigas de las copas de los árboles de los cítricos resultó en un descenso significativo en el número de escudos de *A. aurantii* en frutos en el momento de la cosecha comparado con los árboles control donde las hormigas tuvieron acceso a las copas. Además, el número de escudos de *A. aurantii* por fruto estuvo positivamente relacionado con el número de hormigas que ascendió a los árboles. Este estudio demuestra que las hormigas

nativas en el Mediterráneo pueden inducir incrementos poblacionales de *A. aurantii*. Dichos incrementos poblacionales de la plaga dependen de los niveles de actividad de las hormigas.

La comparación del contenido en azúcares totales y la relación glucosa-fructosa entre *A. melinus* recolectados en campo e individuos que recibieron un determinado tratamiento alimenticio en laboratorio, desveló que nueve de los once parasitoides recolectados en campo habían consumido hidratos de carbono recientemente. Los parasitoides que consumieron azúcares en el laboratorio no fueron capaces de sintetizar oligosacáridos. En cambio, los nueve parasitoides recolectados en campo contuvieron oligosacáridos, característicos de las melazas de los hemípteros, como melicitosa, rafinosa, melibiosa, o erlosa. Estos resultados demuestran que los adultos de *A. melinus* utilizan la melaza de los hemípteros como fuente de alimento en el campo.

Resum

El poll roig de California, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), és una de les plagues mes importants dels cítrics a nivell mundial. En Espanya, els primers danys produïts per aquest diaspídid es van detectar per primera volta l' any 1986. Els principals agents de control biològic d' *A. aurantii* en aquesta zona són el parasitoide natiu *Aphytis chrysomphali* (Mercet) i l' introduït *A. melinus* DeBach (ambdós Hymenoptera: Aphelinidae). No obstant això, el control de la plaga oferit pels dos parasitoides és insuficient. Amb la fi de millorar el control biològic d' *A. aurantii*, hem estudiat diversos factors biòtics que afecten l' eficàcia d' *A. chrysomphali* i *A. melinus.* Més concretament, s'ha estudiat la variació espacial i temporal de la grandària d' *A. aurantii*, les grandàries que utilitzen com hoste *A. chrysomphali* i *A. melinus,* així com l' efecte de la grandària sobre vàries característiques d' aquests parasitoides. A més a més, es va estudiar l' impacte que les espècies de formigues natives del Mediterrani exerceixen sobre les poblacions d' *A. aurantii*, i finalment, l' estat nutricional i les fonts d' alimentació que utilitzen els adults d' *A. melinus* als camps dels cítrics.

En condicions de camp, la grandària del cos d' *A. aurantii* va variar depenent del sostrat vegetal sobre el qual l' insecte s' alimenta, de la zona geogràfica, de l' època de l' any, i probablement de l' estat nutricional de la planta hoste. El sostrat vegetal va influir considerablement en la grandària del poll; els individus que estaven assentats sobre fruits van ser més grans que sobre fulles o branques. Així mateix, importants diferències en la grandària d' *A. aurantii* es van registrar entre les diferents parcel·les mostrejades. L' època de l' any va influir també en la grandària d' *A. aurantii*, sent de menor grandària els polls desenvolupats durant l' estiu i tardor, possiblement a causa de l' efecte de la temperatura. Per últim, es va detectar una associació positiva entre la grandària d' *A. aurantii* i el contingut de les fulles en potassi.

Aphytis chrysomphali i *A. melinus* van utilitzar distintes grandàries d' *A. aurantii* com hoste al camp. *Aphytis chrysomphali* es va recuperar principalment de segons estadis (0.152-0.300 mm^2 en area de cos d' *A. aurantii*) però va parasitar més femelles joves de tercer estadi, aconseguint una mitjana de ~10% de parasitisme en polls que mesuraven entre 0.80-0.85 mm^2. *Aphytis melinus*, es va desenvolupar i va parasitar amb més intensitat femelles joves de tercer estadi, aconseguint una mitjana de ~30% en polls quins mesuraven entre 0.70-0.75 mm^2. Es va trobar una associació positiva entre la grandària de l' hoste i el comportament gregari i grandària dels parasitoides. De la mateixa manera, la grandària de l' hoste va influir en la proporció de sexes d' *A. melinus*. En hostes menuts, *A. melinus* va posar ous que se desenvoluparen en mascles mentre que en hostes de grans va posar

ous que donarien lloc a femelles. La grandària d' *A. aurantii* a partir de la qual se van produir més femelles que mascles d' *A. melinus* va ser al voltant de 0.40 mm^2. Aquest límit per a la producció de descendència femella es va mantenir constant, independentment de si estigueren disponibles hostes menuts o grans. Donada la variabilitat estacional en la grandària d' *A. aurantii*, es pot donar una escassesa d' hostes de grandària adequada per a la producció de femelles d' *A. melinus* entre maig i octubre. En conseqüència, és d'esperar un descens en les poblacions del parasitoide durant aquest període, que probablement resultarà en un control insuficient de la plaga. Per a pal·liar eixe descens poblacional d' *A. melinus*, seria recomanable fer soltes massives d'este parasitoide.

Les espècies de formigues (Hymenoptera: Formicidae) més abundants als cítrics Valencians, les dominants *Pheidole pallidula* (Nylander) i *Lasius grandis* Forel, estaven establides en territoris clarament separats en la mateixa parcel·la, i molt rarament es van trobar sobre el mateix arbre. Pel contrari, l' espècie subordinada *Plagiolepis schmitzii* Forel, se va trobar habitualment en el mateix arbre amb una de les dos dominants, principalment amb *P. pallidula*. Aquesta distribució espacial se coneix com "mosaic" de formigues. Les formigues van estar actives en les copes dels arbres dels cítrics des d'abril fins a novembre. La temperatura i les necessitats alimentaries de cada espècie van determinar les seves pautes d'activitat estacional. Respecte a les seues pautes d' activitat diària, *L. grandis* i *P. pallidula* van estar actives durant les 24 hores del dia, mentre *P. schmitzii* va ser estrictament diürna. El nèctar de les flors dels cítrics i la depredació no va representar una font important d' aliment. La dieta de les formigues en les copes dels arbres dels cítrics consistia principalment de melassa de hemípters. Més del 60% de les colònies de hemípters, i el 100% de les colònies de *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), estaven ocupades per les formigues en primavera i estiu.

L' exclusió de les formigues de les copes dels arbres dels cítrics va resultar en un descens significatiu en el nombre d' escuts d' *A. aurantii* en fruits a l' hora de la collita comparat amb arbres control on les formigues tenien accés a les copes. A més, se va comprovar que el nombre dels escuts per fruit va estar positivament relacionat amb el nombre de les formigues que van ascendir als arbres. Aquest estudi demostra que les formigues natives del Mediterrani poden induir increments poblacionals d' *A. aurantii*. Els increments poblacionals de la plaga depenen dels nivells d' activitat de les formigues.

La comparació del contingut en sucres totals i la relació glucosa-fructosa entre individus d´ *A. melinus* recollits al camp i individus que van rebre un determinat tractament alimentari en laboratori, va desvetllar que nou dels onze parasitoids recollits al camp havien consumit hidrats de carboni

recentment. Els parasitoids que van consumir sucres al laboratori no van ser capaços de sintetitzar oligosacarids. En canvi, els nou parasitoids recollits al camp que estaven alimentats, contenien sucres característics de les melasses dels hemípters, com melicitosa, rafinosa, erlosa o melibiosa. Aquests resultats demostren que els adults d' *A. melinus* utilitzen la melassa dels hemípters com a font d' aliment al camp.

Summary

California red scale (CRS), *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), one of the most important pests of citrus worldwide, began to cause damages in eastern Spain in 1986. The main biological control agents of *A. aurantii* in this zone are the native parasitoid *A. chrysomphali* (Mercet) and the introduced *A. melinus* DeBach (both Hymenoptera: Aphelinidae). Nevertheless, the control they exert is insufficient. In order to improve the biological control of *A. aurantii* we studied several biotic factors that may affect the efficiency of *A. chrysomphali* and *A. melinus* in the field. More concretely, we studied the spatial and temporal variation in the scale size, the host sizes used by *A. chrysomphali* and *A. melinus* as well as the influence of host size on various parasitoid traits. Moreover, we studied the foraging ecology and the effect of the ants native to the Mediterranean on the populations of the scale and, finally, the nutritional state and food sources used by adult *A. melinus* in the field.

In the field, the body size of *A. aurantii* varied with plant substrate, locality, time of the year, and probably, with the nutritional state of the host plant. Plant substrate was found to substantially influence the body size of *A. aurantii* with scales being significantly larger on fruits that on leaves or twigs. Another important source of variation for *A. aurantii* size was geographic location since significant differences were found among orchards. Moreover, significant seasonal variation in the body size of *A. aurantii* was observed; body sizes were smaller during summer and autumn, apparently due to the effect of temperature. Finally, a positive relationship between the content of potassium in leaves and scale size was observed. From all the above factors, temperature related seasonal variation had the most profound effect on *A. aurantii* size.

Aphytis chrysomphali and *A. melinus* used different sizes of *A. aurantii* in the field. *Aphytis chrysomphali* was recovered mostly from second instars (0.152-0.300 mm^2 in *A. aurantii* body area), but parasitized more heavily third instars, reaching an average of ~10% parasitism on scales sized between 0.80-0.85 mm^2. *Aphytis melinus* developed mostly, and parasitized more heavily, third instars reaching an average of ~30% parasitism on scales sized between 0.70-0.75 mm^2. Gregariousness and parasitoid size were positively influenced by host size. Moreover, host size affected *A. melinus* sex ratio; male eggs were laid on small hosts and female eggs on large hosts. The host size at which the sex ratio of *A. melinus* turned female biased was found to be around 0.40 mm^2 and this threshold remained constant whether relatively small or large hosts were available. Given the seasonal variation in the size of *A. aurantii*, between May and October most scales were not suitable for production of female *A.*

melinus. Thus, a decrease of parasitoid populations is likely to be expected in this period of the year, which in turn may result in insufficient control of the scale. Augmentative releases of *A. melinus* should be carried during the period when hosts suitable for the production of females are scarce.

The most abundant ant species (Hymenoptera: Formicidae) in eastern Spain citrus, the dominants *Pheidole pallidula* (Nylander) and *Lasius grandis* Forel foraged in mutually exclusive territories within the same orchard but they both share their territory with the subordinate *Plagiolepis schmitzii* Forel forming a distribution pattern known as "ant mosaic". Ants were ascending to the canopies from April until November. Temperature and colony nutritional requirements shaped their seasonal foraging patterns. The daily activity pattern of *P. schmitzii* was strictly diurnal whereas *L. grandis* and *P. pallidula* were active during the 24 hours of the day. Citrus nectar and predation/scavenging did not represent important food sources. On the contrary, hemipteran honeydew was the principal food source for the ants on the canopies. More than 60% of the total honeydew sources, and 100% of the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) colonies, were tended by ants during spring and summer.

Ant-exclusion from the citrus canopies resulted in significantly lower scale densities on fruits at harvest when compared with the control treatment where ants had access to the canopies. Scale density on the fruits was positively correlated with the number of ants that climbed to the citrus canopy. This result suggests that the increase of *A. aurantii* densities induced by Mediterranean ants depends on the intensity of the ant-activity.

The comparison of the total sugar content and the glucose-fructose ratio between field-collected *A. melinus* and individuals that received a reference feeding treatment in the laboratory revealed that nine out of eleven field-collected *A. melinus* had recently consumed carbohydrates. The laboratory reared parasitoids did not synthesize oligosaccharides after sugar feeding. On the contrary, all the field-collected parasitoids characterized as "fed" contained oligosaccharides like melezitose, raffinose, erlose or melibiose, sugars typically present in different types of hemipteran-honeydew. These results suggest that adult *A. melinus* use hemipteran-honeydew as a food source in the field.

Chapter 1

Introduction

1.1. Systematic classification of *Aonidiella aurantii*

California red scale, *Aonidiella aurantii* (Maskell, 1879) is classified as follows:

Kingdom Animalia
Subkingdom Eumetazoa
Phylum Arthropoda
Subphylum Hexapoda
Class Insecta
Order Hemiptera
Suborder Sternorrhyncha
Superfamily Coccoidea
Family Diaspididae
Tribe Aspidiotini
Genus *Aonidiella*
Species *aurantii* (Maskell, 1879)

The following synonymies have been confirmed for this species: *Aspidiotus aurantii*, Maskell (1878), *Aspidiotus citri* Comstock (1881), *Aspidiotus coccineus* Gennadius (1881), *Aonidia aurantii* Targioni (1884), *Aonidiella aurantii* Berlesse (1886), *Chrysomphalus aurantii* Cockerell (1899), (Bodenheimer, 1951).

Common names for *Aonidiella aurantii* are “California red scale” in English, “piojo rojo de California” in Spanish, “poll roig de California” in Valencian, “cochenille jaune” in French, “cocciniglia rossa forte degli agrumi” in Italian, “escama roja de los citrus” in Portuguese (Gómez Menor, 1955-56; Ebeling, 1959; Llorens, 1990).

1.2. Origin and distribution

Aonidiella aurantii was originally described as *Aspidiotus aurantii* by W.W Maskell in 1878 in New Zealand (Quayle, 1911). Nevertheless, the presence in Australia and New Zealand was the result of introduction (Quayle, 1911). According to Bodenheimer (1951), the genus *Aonidiella* is native to the south-eastern Asia, an area between India and south-eastern China. Nowadays, *A. aurantii* is widely distributed worldwide, in all tropical and subtropical regions where citrus are cultivated. The pest has been recorded from the Mediterranean Basin, South Africa, the tropical and subtropical zone of South America, Australia, New Zealand, Pacific islands, Indian peninsula, Philippines, Middle East and Japan (Ebeling, 1959).

In Spain, *A. aurantii* is present since the beginning of the 20th century. It was cited for first time in Valencia by García Mercet in 1910. Also, Quayle (1911) confirms the presence of the scale in the Iberian Peninsula. A few years later, the scale was detected in Balearic and Canary islands (Carnero Hernández and Pérez Guerra, 1986; Blay Goicoechea, 1993; Pina, 2007). Nevertheless, the first serious damages produced by *A. aurantii* were recorded in 1986 in Valencia, in the municipality of Alzira (Rodrigo and Garcia-Marí, 1990, 1992). Currently, *A. aurantii* is present in all the citrus growing areas of Spain.

1.3. Host plants

Aonidiella aurantii is a polyphagous pest species, attacking a wide variety of plants belonging to at least 77 plant families (Borchsenius, 1966). It has been recorded on apple trees (*Malus domestica* Borkh), pear trees (*Pyrus communis* L.), olive trees (*Olea europaea* L.), pomegranate trees (*Punica granatum* L.), carob trees (*Ceratonia siliqua* L.), walnut trees (*Juglans regia* L.), mulberry trees (*Morus alba* L.), quince trees *Cydonia oblonga* L., laurel trees (*Laurus nobilis* L.), palm trees, ornamentals like the majority of the species belonging to the family Rosaceae, various *Solanum* species etc. (Ebeling, 1959; Crouzel et al., 1973; Beardsley and González, 1975; Miller and Davidson, 1990).

Nonetheless, *A. aurantii* attacks preferentially citrus and according to Talhouk (1975), it is the most important citrus pest worldwide. All citrus varieties are attacked by the scale, yet there are various levels of susceptibility. In order of descending susceptibility are reported lemon trees ((*Citrus limon* (L.)), grapefruit trees (*C. paradisi* Macf.), orange trees ((*C. sinensis* (L.) Osbeck)) and mandarin trees (*C. reticulata* Blanco and *C. unshiu* Markovitch) (Cameron et al., 1969, 1975; Habib et al., 1972; Bedford, 1998). Nevertheless, in young trees, the scale may cause severe damage to all varieties (Bodenheimer, 1951). According to Habib et al. (1972) susceptibility is associated with the number of oil glands present in leaves and fruits of the different varieties; higher number of oil glands results in higher resistance to the pest.

1.4. Damage and economic importance of *Aonidiella aurantii*

Aonidiella aurantii attacks all the above ground parts of the tree, fruits, leaves and wood (Beardsley and González, 1975). In fact, fruit is the most preferred plant substrate by *A. aurantii* followed by leaves while wood is the

least preferred substrate (Caroll and Luck, 1984; Hare et. al., 1990). The damage caused by *A. aurantii* may be direct, consequence of the feeding activity of the pest. Heavy infestations can cause leaf drop, twig dieback, loss of production even death of young trees (Ebeling, 1959; Crouzel et al., 1973; University of California, 1991; Smith et al., 1997; Bedford, 1998). Undoubtedly, the most important damage caused by *A. aurantii* is indirect associated with the presence of scales on fresh fruit (Crouzel et al., 1973; University of California, 1991; Smith et al., 1997; Bedford, 1998). As a result, *A. aurantii* devaluates fruits commercially causing important economic losses for growers. Additional economic costs are produced due to the difficult control of the pest associated with its complex morphology; susceptible instars alternate with instars invulnerable to chemical treatments since they are protected by the scale cover.

Aonidiella aurantii feeds principally in parenchyma cells. That is palisade and spongy mesophyll of leaves, cortex of twigs and flavedo of fruit (Washington and Walker, 1990). The same authors report that in general *A. aurantii* avoids penetrating vascular tissue especially in leaves; however, in some cases penetration of vascular tissue was observed in twigs. They suggest that the twig dieback observed under heavy *A. aurantii* infestations is the result of the destruction of cortex cells leaving the vascular cambium exposed to action of pathogens and dehydration. Washington and Walker (1990) also found a high frequency of empty stylet tracks in oil glands (stylet withdrawal) indicating unfavorable feeding sites. Moreover, they noticed that cells situated next to pierced ones were also damaged. They concluded that this was the result of the diffusion of toxic saliva by *A. aurantii* through intracellular space during the feeding process. In leaves, this damage is observed in form of chlorotic marks due to the chlorophyll destruction in mesophyll cells (Ebeling, 1959).

Aonidiella aurantii was the most serious pest in California (especially in the interior San Joaquin Valley) until the mid 1980´s when an Integrated Pest Management strategy, based on the reduction of insecticide treatments and the use of selective insecticides to allow natural and augmented populations of natural enemies to survive, managed to maintain the pest populations below economic damage (Haney et al., 1992; Luck et al., 1992, 1996). In Australia, California red scale is also considered a key pest and its control determines the entire pest management program (Smith et al., 1997; Papacek, 2006). In Uruguay, Asplanato and Garcia-Marí (2002) reported that *A. aurantii* is the most important citrus pest causing important economic damages every year.

In a recent survey of the current situation of citrus pests and diseases in the Mediterranean basin, *A. aurantii* was cited as the most important pest in the

majority of the countries (Tena and Garcia-Marí, 2010). In Spain, *A. aurantii* is the key pest and usually chemical applications are needed in order to keep infestations below economic thresholds. In the Community of Valencia, the scale is present throughout the citrus growing area, a zone approximately 400 km long from north to the south and 50 km wide, causing damages that vary depending on locality and year:
(http://www.agricultura.gva.es/rvfc/index.html).

1.5. Biology of *Aonidiella aurantii*

1.5.1. Morphology and life cycle

In diaspidids, the adults present a marked sexual dimorphism. Adult females have no wings or legs and are sessile. The body structure of the adult female is morphologically a nymph therefore it is considered neotenic (Takagi, 1990). The segmentation of the adult female body is obscured, since parts of the head and thorax are fused into a constricted area that is called *pygidium*. The *pygidium* bears the anus dorsally and the vagina ventrally. On the *pygidium* are also present wax pores and tubes that lead to wax glands that open on the dorsal and ventral surfaces of its posterior tip (Ebeling, 1959; Takagi, 1990). On the other hand, the adult diaspidid male has well-developed antennae, front wings and legs and are mobile (Giliomee, 1990).

Aonidiella aurantii, as the rest of the armored scales, is characterized by the presence dorsally of an "armor" or "scale" covering that protects the insect body from physical aggressions and adverse climatic conditions (Dickson, 1951; Ebeling, 1959; Foldi, 1990a). The scale cover is a product of the insect and not a part of it. Therefore, it can be removed without damaging the insect; however, without the scale cover the insect will die from desiccation (Foldi, 1990a). The cover consists of wax secreted by glands of the *pygidium* and exuviae that are incorporated during the molt (Dickson, 1951; Ebeling, 1959). There is also a ventral cover that is elaborated of secretions of ventral wax glands plus incorporated ventral exuvial residues. This cover is very thin and serves to separate the insect from the host plant (Foldi, 1990a).

In armored scales, the scale cover presents a marked sexual dimorphism. In *A. aurantii* the female cover is almost circular whereas it is elongate in males (Figure 1.1). The cover is of similar reddish-orange color in females and males. Although the cover of California red scale is thin and semitransparent, its physical properties, namely its hardness and impermeability constitute an effective barrier against chemical insecticides preventing them to reach the body underneath (Ebeling, 1959; Foldi, 1990a).

Fig. 1.1. Sexual dimorphism between the cover of female (left) and male instars (right) of *Aonidiella aurantii* (top view).

The developmental stages of *A. aurantii* differ between the two sexes. Females pass through three instars and males through five. Each instar is separated from the next one by a molt stage. During the instar stage the body has a yellow coloration and can be easily separated from the cover (in the case of adult females until insemination takes place). During the molt stage, the body becomes orange and cannot be separated from the cover (Figure 1.2). Great differences may be found in the average size of the scale cover depending on the instar of *A. aurantii*, plant substrate upon which the insect feeds, geographic location, time of the year and probably nutritional status of the host plant (Ebeling, 1959; Carroll and Luck, 1984; Luck and Podoler, 1985; Reeve, 1987; Yu, 1986; Walde et al., 1989; Hare et al., 1990; Hare and Luck, 1991, 1994; Hare and Morgan, 2000).

Like the majority of armored scales, California red scale reproduces sexually (Quayle, 1911; Flanders, 1953). It is ovoviviparous, i.e. the eggs develop inside the female body and for that reason are difficult to observe (Dickson, 1951; Ebeling, 1959).

Eggs hatch and develop in nymphs that stay under the gravid female cover a period that varies from a few hours until a couple of days depending on the climatic conditions, principally temperature and luminosity (Nel, 1933; Quayle, 1941; Bodenheimer, 1951; Ebeling, 1959; Tashiro and Moffitt, 1968; Willard, 1972; Koteja, 1990). California red scale first instar nymphs are mobile and they are called “crawlers”; they have eyes, antennae and legs and walk until they find a place to settle (Figure 1.3).

Fig. 1.2. Differences between instar (left) and molt (right) stages of *Aonidiella aurantii* (bottom view).

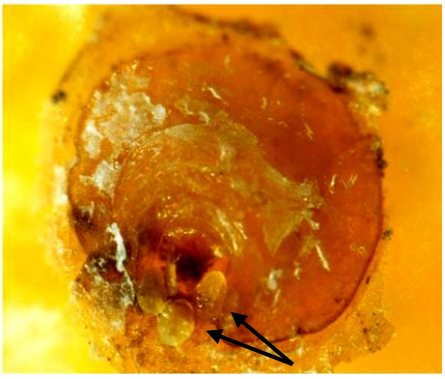

Fig. 1.3. "Crawlers" of *Aonidiella aurantii* beneath the body of a gravid female.

A few crawlers may wander for one or two days but the majority settles within one day (Willard, 1973). When the crawler finds an adequate site on a branch, leave or fruit, it settles down, tucks its legs and antennae beneath its body, inserts its stylets (*rostrum*) into the vegetal tissue and begins to feed (Dickson, 1951). From this stage onwards the insect remains fixed in its feeding site during the rest of its life. Mortality is higher during this stage than in any other (Ebeling, 1959).

Immediately after the insect starts feeding, waxy filaments secreted by the dorsal surface become felted together, envelop the body and extend down to the sides of the substrate. During a couple of days *pygidium* glands secret filaments while the nymph rotates around the point where it inserted its mouthparts. As a result, the cover becomes circular. This stage has a cotton-like appearance and is known as the "white cap" stage (Figure 1.4). The insect continues to feed and the waxy secretions become cemented with liquid from

the Malphigian tubules. After four to six days, the wax settles down further except from the top of the cover that stays like a distinct prominence like a nipple and then is said that the insect is in the “nipple stage” (Figure. 1.4).

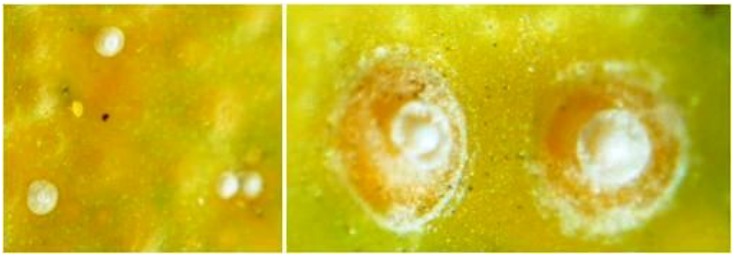

Fig. 1.4. White cap (left) and nipple stages (right) of *Aonidiella aurantii*.

The crawler, white cap and nipple stages are the first instar scale. Afterwards, the first molt takes place. The nymph detaches its mouthparts from the vegetal tissue; it stops feeding and undergoes morphogenetic changes. The antennae are reduced to short tubercles, legs are also completely reduced and the insect is sealed off inside its cover. This is the first molt (Figure 1.5).

Fig. 1.5. First molt of *Aonidiella aurantii* (body and cover are sealed together).

At the moment of the molt, the cast skin of the first instar splits and separates at the edge of the body. The dorsal part is pushed towards the middle of the scale cover whereas the ventral part remains under the body of the insect (Foldi, 1990b). The insect stays as first molt for approximately four days depending on the temperature (Yu and Luck, 1988). After the completion of the first molt, it transforms to second instar. During that stage, the insect becomes definitely apodous. The insect inserts a new feeding tube in the plant substrate and begins to feed. It rotates by means of body contractions and wax filaments produced by the *pygidium* are added to the outer margin of the scale covering that is circular (Foldi, 1990b). These new wax secretions produced by the insect during the second instar are visible as the grey part of the cover

surrounding the molt ring (Figure 1.6). The body continues to grow and the insect continuously adds filaments to the edge of the cover.

Male nymphs at first rotate while feeding, like females do, but later they cease completely to rotate (Koteja, 1990). As a result, the body and scale cover in males grow towards one direction only and therefore they become elongated (Ebeling, 1959; Koteja, 1990) (see Figure 1.1). Contrary to the females, male nymphs incorporate to the cover only the first exuviae. The exuviae of the second instar, prepupae and pupae males are pushed out or remain under the cover. Thus, the first molt and the cast skin become submarginal. The male scale cover at the second instar takes its definite shape and size, and serves as protection for the pupal instars and the newly-emerged males (Koteja, 1990).

Fig. 1.6. Cover and body of second instar female of *Aonidiella aurantii* (top view).

At the end of the second instar, the insect undergoes a metamorphosis that is different in males and females. For the female, it is the last metamorphosis and molt. Males develop eyes, buds of antennae, legs, wings and penial sheath while their feeding apparatus and integumental glands are reduced (Koteja, 1990) (Figure 1.7).

Fig. 1.7. Cover and body of second instar male of *Aonidiella aurantii* (top view).

After approximately five days, **males** begin to pupate. The morphological changes initiated at the end of the second instar continue to the third (prepupal) and fourth (pupal) male instars. Prepupae are characterized by the presence of a slight nub at the distal end indicating the development of genitalia (Figure 1.8). After a couple of days, the prepupal male transform into pupae. The sheaths of the appendages present in the end of the second instar become quite conspicuous (Figure 1.8) (Ebeling, 1959; Forster et al., 1995).

Fig. 1.8. Prepupa (left) and pupa (right) of male *Aonidiella aurantii*.

In about three days after pupation the adult males emerge. They are yellow-orange, approximately 0.6-0.8 mm long and have a pair of wings (the second pair has been replaced by a pair of halters), eyes, legs and a long style (Figure 1.10) (Ebeling, 1959). They are weak flyers, lack functional mouthparts and live usually from one to three days (Beardsley and González, 1975; Coteja, 1990).

Fig. 1.10. Adult male of *Aonidiella aurantii*.

After approximately 18 days (depending on temperature) as second instars, **females** molt for the second time (Ebeling, 1959; Beardsley and González, 1975). As in the first molt, body and cover are sealed together and cannot be separated. The insect detaches its mouthparts from the plant tissue and stops feeding. The cast skin is incorporated in the middle of the cover, under the first (Foldi, 1990b) (Figure 1.11).

Fig. 1.11. Second molt of *Aonidiella aurantii* (bottom view).

After the second molt, that lasts for approximately six days (Foldi, 1990b), females enter the third and ultimate instar; that of the adult female. The insect reinserts its mouthparts into the plant tissue and begins to feed. The scale cover can be easily detached. The body and the scale of the young adult female grow considerably. The thorax is expanded backwards forming a rounded lobe on each side of the *pygidium* giving the body a pear-like shape (Ebeling, 1959). The wax glands of the *pygidium* continue secreting filaments that are cemented gradually and incorporated to the cover. Like in the second instars, the new wax secretions produced by the insect are visible as the grey margin ("grey skirt") of the cover surrounding the first and second molt rings that remain constant in size (Figure 1.12). From the beginning of the formation of the grey margin onwards females become receptive to males. At this phase, non inseminated females are called "young" or "virgin" third instars. Third instars continue growing but they do not transform to mature females until mating occurs. Thus, third instars vary considerably regarding overall body and cover size (Forster et al., 1995). Appearance of young third instars and males is synchronized. Male prepupae and pupae instars coincide with the second female molt and adult males coincide with young third instar females.

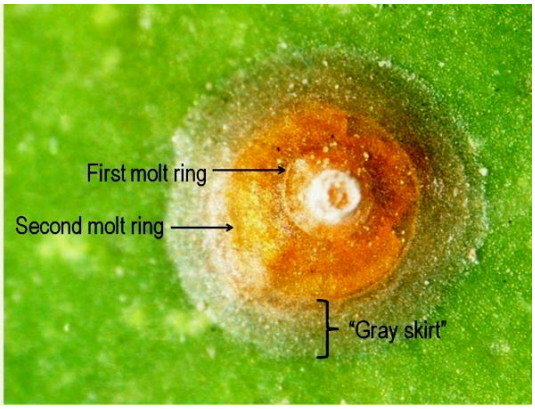

Fig. 1.12. Cover of third instar female showing molt rings and the newly incorporated "gray skirt".

Virgin third instar females release a pheromone that attracts the males. Pheromone emission begins with the formation of the grey skirt and may last until 84 days as long as they are not inseminated (Tashiro and Moffitt, 1968). For mating, the *pygidium* is extended until the edge of the grey skirt where is inseminated by the male. After insemination the *pygidium* is withdrawn past the thoracic lobes and mating can no longer occur (Tashiro and Moffitt, 1968) (Figure 1.13). This stage is called the mature or "gravid" female. From this point onwards, the scale cover cannot be detached from the body of the mature female. The insect stops feeding and is sealed inside the cover as occurs during the molts (Figure 1.13). Around two weeks later, the first crawlers will be produced and the circle will begin again. Carroll (1979) reported a range of 10-35 crawlers per female lifetime on the bark and foliage at various times of the year under field conditions. The complete life cycle of *A. aurantii* is represented in the Figure 1.14.

Fig. 1.13. Inverted cover and body of virgin third instar female (left) and body of mature third instar female (right, bottom view).

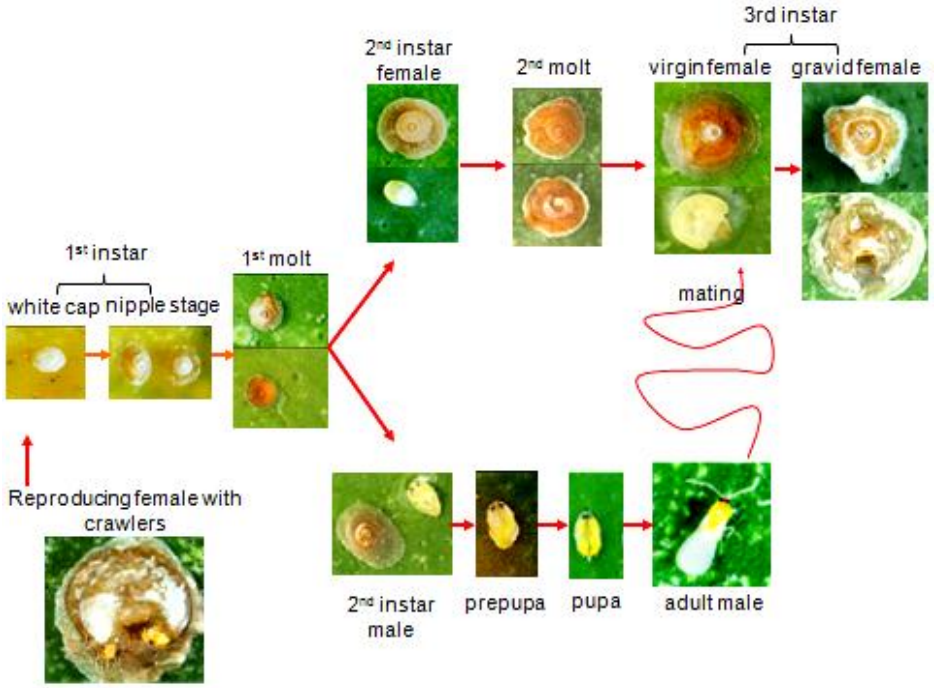

Fig. 1.14. Biological cycle of California red scale, *Aonidiella aurantii*.

1.6. Ecology of *Aonidiella aurantii*

Climate (principally temperature and humidity) is the principal abiotic factor affecting the ecology of *A. aurantii*, from the emergence of mobile nymphs until the establishment, reproduction and death of the adults. On the other hand, given that the scale remains fixed on the same site for almost its entire life, the vegetal tissue on which it develops is the main biotic factor that affects its ecology.

1.6.1. Influence of abiotic factors

Various studies have examined the developmental threshold and the thermal constant of *A. aurantii*. Bimboni (1970) on grapefruits and Willard (1972) using leaf disks floating on water determined the developmental threshold for female *A. aurantii* to be 11.6ºC. Yu and Luck (1988) on lemon fruits found a similar threshold (11.6ºC) for females and (12.6ºC) for males. The same authors reported the thermal constant to be 639.8 and 331 degree-days (DD) for females and males respectively. The higher thermal constants of 673 DD and 784 DD calculated by Bimboni (1970) and Willard (1972)

respectively are apparently due to the different substrates used to grow the scale (Yu and Luck, 1988). Under variable temperatures in the field, Kennett and Hoffman (1985) calculated a thermal constant of 615 DD using a threshold development of 11.7ºC. Rodrigo (1993) in orange groves, calculated a thermal constant 753 DD using 11.6ºC as threshold development.

Fisrt, second and third instars are the most resistant to low temperatures whereas females with crawlers are of intermediate resistance. On the other hand, molts and male pupae and prepuae are highly susceptible to low temperatures (Abdelrahman, 1974a). According to the same author, low temperatures are the most determinant factor for the abundance and distribution of the scale.

The duration of the life-cycle of *A. aurantii* increases under the influence of low temperatures. Willard (1972) found that females and males complete their development in 44.3 in 25.2 days respectively at 29ºC; whereas it takes 209 days for females and 149 for males at 15ºC. Interestingly, Yu and Luck (1988) found that developmental time was the same under constant and fluctuating temperatures in the field. Moreover, high summer temperatures cause a significant decrease in the body size of *A. aurantii* with serious implications for its biological control (Yu, 1986; Yu and Luck, 1988; Hare et al., 1990).

The population densities of *A. aurantii* depend on the temperature and relative humidity. Population increases are observed under conditions of low humidity when temperatures are below 30ºC and high humidity when temperatures are higher (McLaren, 1971). According to Bodenheimer (1951), optimum conditions for the development of the scale are temperatures between 23 and 27.5ºC and 70–80% de R.H. Nevertheless, Smith et al. (1997) reported that in Australia the scale develops under high temperatures 30-38ºC and even low relative humidity. Moreover, temperature affects fecundity of female *A. aurantii*. Willard (1972) obtained a maximum average of 267 of nymphs per female at 30 ºC and a minimum of 46 larvae at 15ºC. Similarly, Wentel (1979) found that females reared at 20 ºC produced on average 123 nymphs whereas for those reared at 30ºC productivity increased to 266 nymphs.

1.6.2. Influence of biotic factors

Aonidiella aurantii attacks all plant canopy substrates, wood, leaves and fruits. Nevertheless survival, fecundity and ultimate scale size, all depend on the substrate on which the scale grows.

Bodenheimer (1951) found that California red scale fecundity was higher on fruits than on leaves. Similarly, Carroll and Luck (1984) reported that fruits were the best substrate for the scale development, followed by leaves while wood was the least favorable substrate. Moreover, scales grown on fruits presented higher survival and fecundity than those grown on leaves or twigs (Bodenheimer, 1951; Willard, 1972; Atkinson, 1977; Carroll and Luck, 1984; Hare et al., 1990; Hare and Luck, 1991).

The ultimate (cover and body) size the scale attains also varies substantially among citrus cultivars and substrates within cultivars. Scales are largest when they grow on fruits, smallest when they grow on wood and of intermediate size when they grow on leaves (Carroll and Luck, 1984; Luck and Podoler, 1985; Hare et al., 1990; Hare and Luck, 1991). Similarly, scales are largest when reared on lemon and grapefruit cultivars compared to orange or mandarin cultivars (Hare et al., 1990; Hare and Luck, 1991, 1994). This size variability has important consequences for the biological control of the scale, since various fitness components of the adult *Aphytis* parasitoids (Hymenoptara: Aphelinidae), the main natural enemies of *A. aurantii*, depend on the size of their hosts.

1.6.3. Seasonal history

The number of generations per year of *A. aurantii* varies from two to six depending on the local climatic conditions, principally temperature and relative humidity (Beardsley and González, 1975). In general, higher number of generations is observed in zones with low humidity and relatively high temperatures (Bodenheimer, 1951). In California, *A. aurantii* completes two to three generations in the coastal area whereas in the interior San Joaquin Valley it completes three generations per year (Ebeling, 1959; Carroll and Luck, 1984; University of California, 1991; Luck, 1995). *Aonidiella aurantii* completes two to six generations in South Africa, (Bedford, 1998) and Australia (Smith et al., 1997). In Uruguay, Asplanato (2000) reports three annual generations for California red scale.

Similarly, in the Mediterranean basin the number of generations per year varies depending on the geographic area. In Israel, *A. aurantii* completes four to five generations (Avidov and Harpaz, 1969), between three and four in Italy (Sicily) (Tumminelli et al., 1996) and Egypt (Habib et al., 1972) and three generations per year in Morocco (Delucchi, 1965) and the isles of Crete (Alexandrakis, 1983) and Cyprus (Orphanides, 1984a).

In Spain, in the Valencia Community, *A. aurantii* completes three generations per year. The first peak of crawlers is observed around the end of May, the second at the end of August and the third around November depending on the climatic conditions (Ripollés, 1990; Rodrigo and García-Marí, 1990, 1992; Rodrigo, 1993).

1.7. Biological control of *Aonidiella aurantii*

1.7.1. Ectoparasitoids

All the known ectoparasitoids of *A. aurantii* belong to the genus *Aphytis* Howard (Hymenoptera: Aphelinidae) (Rosen and DeBach, 1979). *Aphytis* are very small (usually less than 1mm in length) yellow or grayish insects that develop exclusively as primary ectoparasitoids of diaspidid scales (Rosen and DeBach, 1979). The adult female *Aphytis* pierces with her ovipositor the scale cover and lays one or several eggs on the body of the scale. The larvae will feed by sucking the body fluids of their host eventually killing it. As natural enemies, *Aphytis* are more efficient compared with endoparasitoids and predators of *A. aurantii* and armored scales in general (DeBach and Rosen, 1976; Rosen and DeBach, 1990; Rosen, 1994).

Identification and separation of *Aphytis* species are extremely difficult. According to Rosen and DeBach (1979) this is due to their minute size, the lack of reliable taxonomic characters, the common occurrence of sibling species and the fact that in many species males are rare and thus, hybridization tests are impossible.

The genus *Aphytis* is classified as follows:

Kingdom Animalia
Subkingdom Eumetazoa
Phylum Arthropoda
Subphylum Hexapoda
Class Insecta
Order Hymenoptera
Suborder Apocrita
Superfamily Chalcidoidea
Family Aphelinidae

Many species of *Aphytis* have been introduced as part of classical biological control programs against *A. aurantii* in many parts of the world, including

California (DeBach, 1969; Rosen and De Bach, 1979), Argentina (De Santis and Crouzel, 1994), Australia (Smith et al., 1997), South Africa (Bedford, 1998), Greece (DeBach and Argyriou, 1967), Cyprus (Orphanides, 1984b), Sicily (Siscaro, 1999; Tumminelli et al., 1996), Morocco (Bénassy and Euverte, 1967), Turkey (Oztemiz et al., 2008). In general, the most efficient species against *A. aurantii* are *A. melinus* DeBach, *A. lingnanensis* Compere and to a lesser extend *A. chrysomphali* Mercet.

In Spain, the principal biological control agents of *A. aurantii* are the ectoparasitoids *A. chrysomphali* and *A. melinus* (Rodrigo and Garcia-Marí, 1990, 1992; Rodrigo et al., 1996; Pina et al., 2003; Pina, 2007; Pina and Verdú, 2007; Sorribas et al., 2008; Vanaclocha et al., 2009; Sorribas et al., 2010). *Aphytis chrysomphali* is thought to be native to the Mediterranean; it parasitized *Chrysomphalus dictyospermi* (Morgan) (Hemiptera: Diaspididae) before the introduction of California red scale (Rosen and DeBach, 1979). *Aphytis melinus* (origin Northen India) was successfully established in the Valencia (eastern-Spain) citrus growing area after its introduction in 1976 to control *C. dictyospermi* (Pina, 2007). In a recent study in Valencia citrus, both parasitoids were found to coexist and their abundance fluctuates along the year depending on weather conditions and geographic location (Sorribas et al., 2010). In 1999, another species, *A. lingnanensis* Compere was also introduced in Valencia citrus to control *A. aurantii*. However, several years later, in 2005, it was detected only in the northern part of the Valencia citrus growing area (Castellón) suggesting that it has not established (Verdú and Pina, 2002; Pina and Verdú, 2007).

1.7.1.1. Morphology and development

Aphytis are holometabolous and their development includes the following stages: egg, larvae, prepupae, pupae and adult. The following description of the morphology and developmental history of *Aphytis* is based on the works by Rosen and Eliraz (1978) and Rosen and DeBach (1979).

The adult female wasp inserts its ovipositor through the scale cover and deposits one or various eggs on the dorsal or ventral part of the scale insect body. The eggs are whitish, semitransparent with a teardrop-shape (Figure 1.15). The time it takes *Aphytis* eggs to hatch depends on temperature. Thus, at 20ºC it takes almost five days whereas at 26.7ºC eggs hatch in two days (Yu, 1986; Yu and Luck, 1988). *Aphytis melinus* passes around 18% of the total developmental time as egg (Yu and Luck, 1988).

Fig. 1.15. Egg of *Aphytis* deposited on a male prepupa of *Aonidiella aurantii*.

As in all aphelinids, *Aphytis* larvae pass through three instars. As larvae begin to feed they grow in size and the host depleted of body fluids gradually shrinks. Larval instars can be differentiated by their size and shape. First instar is ovoid in shape whereas second and third instars are considerably larger in size (Figure 1.16). *Aphytis* pass around 36% of their developmental time as larvae i.e. almost 11 days at 17°C, and four days at 26.7°C or 30°C (Yu and Luck, 1988).

As pointed out by Rosen and DeBach (1979), the prepupal stage cannot be considered a distinct instar since no apolysis neither ecdysis take place. The prepupa is white, with its caudal point distinctly pointed and more elongated compared with the larval stages. At this stage, feeding ceases completely and the hindgut becomes linked with the midgut. The larva turns around with its ventral part now facing the scale cover and excretes the gut feces in the form of brown or black meconian pellets (Figure 1.17). Then, it enters a resting period where it is rapidly transformed in pupa. *Aphytis* pass approximately 8% of their total developmental time as prepupae.

Fig. 1.16. Larva of *Aphytis* developing on a third instar female of *A. aurantii*.

At the beginning of the pupal stage, *Aphytis* have colorless eyes but after four or five days (at 26.7°C, and 60% R.H.) they develop eye pigmentation that changes as the pupa matures. Thus, the eye color passes from pink to red, then

transforms to red-brown and finally becomes green (Figure 1.18). Adult *Aphytis* emerge one day after the green-eyed pupae. In the dorsal aspect of the pupa the antennal and wing cases are also visible. At the pupa stage it is possible to differentiate sexes. Female pupae of *Aphytis* have a pair of minute sub-rectangular plates ventrally near the tip of the abdomen whereas a single sub-apical plate is present in male pupae and the tip of the abdomen is distinctly notched. *Aphytis* spent as pupae almost 38% of their total developmental time (Yu and Luck, 1988). In some cases the pupae coloration may be an important character that helps distinguish among *Aphytis* species e.g. differentiation between *A. melinus* and *A. chrysomphali* (Rodrigo, 1993; Sorribas et al., 2008).

The adult *Aphytis* emerges by pushing underneath the scale cover or by chewing on it an exit hole. Both males and females are minute, yellowish and difficult to distinguish without augmentation (Figure 1.19). The cephalic and thoracic exuvium remain recognizable after emergence whereas the abdominal exuvium is often fragmented. The exuvia together with the characteristic meconia and if present the exit hole are unequivocal signs that a scale has been parasitized by *Aphytis*. After emergence from the host, adult *Aphytis* rest for a while and then begins to preen itself. Adults mainly move by running and long distance dispersal is considered to occur by flying and is probably aided by low air movement (Rosen and DeBach, 1979).

Fig. 1.17. *Aphytis* prepupa with meconian pellets visible.

Fig. 1.18. Pupae of *Aphytis melinus* with red (left) and green (right) eye pigmentation.

Fig. 1.19. Adult *Aphytis melinus*.

1.7.1.2. Biology and ecology of *Aphytis*

The developmental period of *Aphytis* is usually short and depends on climatic conditions, principally temperature and humidity (Yu and Luck, 1988). For example, at 26.7°C, *A. melinus* completes its entire development in almost two weeks whereas it takes one month for complete development at 17°C. Most species are multivoltine; they develop continuously throughout the year. *Aphytis melinus* has been found to have two to three generations to one of its host *A. aurantii* (Yu and Luck, 1988).

The majority of *Aphytis* species are biparental and reproduce sexually. Females control the sex of their offspring at oviposition; unfertilized eggs produce sons whereas fertilized eggs produce daughters (Flanders, 1953; Rosen and DeBach, 1979). Female *Aphytis* are essentially monogamous. They mate only once and the sperm is stored in the spermatheca for egg fertilization. On the other hand, males are polygamous, capable of mating with several females (Rosen and DeBach, 1979).

Almost one quarter of the species are uniparental and exhibit thelytokous parthenogenesis, i.e. unfertilized eggs develop into females, as a consequence of infestation with *Wolbachia* symbiotic bacteria. In fact, *Wolbachia* has been detected in *A. chilensis*, *A. yanonensis*, *A. diaspidis*, in the uniparental line of *A. lingnanensis* and in the native to the Mediterranean *A. chrysomphali* (Zchori-Fein et al., 1994, 1995; Werren et al., 1995; Gottlieb et al., 1998; Pina, 2007). Males are very scarce in uniparental species, usually found at a rate of 1-5% (Rosen and DeBach, 1979).

Moreover, *Aphytis* are idiobionts. Before oviposition the female presumably paralyzes the host by inserting venom through her ovipositor (Rosen and DeBach, 1979; van Lenteren, 1994). Fischer (1952) transferred *Aphytis* eggs

from parasitized to unparasitized hosts. The unparasitized hosts continued developing and neither parasitoid eggs nor larvae were found later. Moreover, the same author observed that scales stopped rotating when parasitized. It is unknown the kind of substance the wasp injects into the scale body. As the host is paralyzed it stops growing and its size at the moment of oviposition represents the food available for the parasitoid offspring.

Most species of *Aphytis* are facultatively gregarious. The number of parasitoids per host is correlated with its size and also may be influenced by host and parasitoid density (Rosen and DeBach, 1979). Also, *Aphytis* female is synovigenic, i.e. emerges with zero or few eggs which develop and mature in the ovaries continuously through the wasp´s life (Rosen and DeBach, 1979; Opp and Luck, 1986; Collier 1995). Casas et al. (2000) showed that under field conditions, *A. melinus* produces about six mature eggs per day.

Adult *Aphytis* presumably feed on nectar and honeydew produced by Hemiptera in order to derive the carbohydrate bulk necessary mostly for locomotion (Bartlett, 1962; Avidov et al., 1970; Heimpel and Rosenheim 1995). Nevertheless, females require proteins for egg maturation which they obtain by predatory host-feeding (Rosen and DeBach, 1979; Heimpel et al., 1994; Collier, 1995). With a single host-feeding meal *A. melinus* matures approximately 3 eggs over a two day period (Heimpel et al., 1994; Collier, 1995). A scale upon which *Aphytis* has fed, soon develops necrotic spots and dies within few hours. *Aphytis* kill as many or even more scales by host feeding as by parasitism (DeBach et al., 1953; DeBach and White, 1960; DeBach and Sundby, 1963). However, mortality by host-feeding is rather difficult to quantify in the field because host die and dry up soon and thus they cannot be distinguished from hosts that have died due to other (abiotic) factors. Host-feeding occurs on host stages that are not used by *Aphytis* for oviposition, such as first instar or small sized second and third instars (Rosen and DeBach, 1979; Heimpel and Rosenheim, 1995). Moreover, hosts are used either for oviposition or host-feeding. When host-feeding and oviposition occurs concurrently on the same host *Aphytis* progeny do not develop to adults (Heimpel and Rosenheim, 1995).

Host location and selection

Armored scales exhibit aggregative distribution. Thus, when adult *Aphytis* emerge, hosts are probably available nearby (Rosen and DeBach, 1979). Nevertheless, it has been shown that *Aphytis* use learned, volatile cues from host plants as long-range attractants to potential habitats of their hosts (Smith, 1957; Morgan and Hare, 1998).

Once on the plant surface, the parasitoid forages for hosts in a random fashion by walking rapidly or occasionally by short flights. While searching for hosts, the wasp examines the plant surface with its antennae. According to Quednau and Hübsch (1964) *Aphytis* recognizes its host only by contact whereas DeBach and Sundby (1963) pointed out that it perceives its hosts from a distance of about 1cm. Hare et al. (1993) and Morgan and Hare (1997) demonstrated that *A. melinus* recognizes its host, *A. aurantii*, by the presence and quantity of the nonvolatile compound, O-caffeoyltyrosine a component of the scale covers. O-caffeoyltyrosine is a contact kairomone to which *A. melinus* respond innately. The highest concentrations of this kairomone are found in covers of third instar females (Hare et al., 1993; Hare and Luck, 1994).

After contacting the scale, the parasitoid may: i) reject it immediately without further examination, ii) examine the scale and then reject it after drumming it with its antennae, or iii) examine and accept the scale (van Lenteren, 1994; Casas et al., 2004). Acceptance consists of drumming, probing and ovipositing. The complete host inspection and oviposition sequence of *Aphytis* are described in Rosen and DeBach (1979), Luck et al. (1982) and van Lenteren (1994).

In brief, the parasitoid moves from the center to the periphery of the host cover tapping it with its antennae and mouthparts. Then, turns through about 30^{o} and continues moving (1-10 times) until the entire host surface has been explored. The parasitoid drills with the ovipositor the scale cover longitudinally, near the lateral edge. Drilling of the cover consists of rapid thrusts of the ovipositor at an angle of 45 degrees with the cover. There is considerable intra- and interspecific variation regarding the time required to penetrate the scale cover, presumably depending on the parasitoid size; for *A. lepidoshaphes* it takes between 30 seconds and approximately four minutes. After piercing the cover, the parasitoid inserts the ovipositor into the host´s body. The parasitoid probes internally the host by deliberate, repeated thrusts of the ovipositor. Following probing, the ovipositor is retracted and the parasitoid explores externally the host searching for an oviposition site. The ovipositor is held against the paralyzed scale and moved back and forth in a certain rhythm. This first period of vibration is followed by a short pause and then a second period of vibration at the end of which viscous droplets exude from the ovipositor´s tip. This substance precedes the egg and forms an adhesive pad on the surface of the host´s body. Then the egg balloons out of the ovipositor and is fixed on its side on the adhesive pad. Eggs are laid on the host´s body either dorsally (between the cover and the body) or ventrally (between the body and the plant substrate). The ovipositor then is withdrawn and the parasitoids preen their body. They may leave the sale or stay and lay another egg.

The total time of host inspection and oviposition events varies depending on *Aphytis* species and author. For example, van Lenteren (1994) reported that *A. melinus* needed more time to lay an egg than *A. coheni* or *A. lingnanensis* (288 and 184-180 seconds respectively using the oleander scale *Aspidiotus nerii* Bouché reared on lemons as host). Martinez-Ferrer et al. (2003) observed that on average *A. melinus* completed the oviposition process in 504 seconds whereas Casas et al. (2004) in 401 seconds (in both works third instars were used as hosts). Interestingly, *A. melinus* spend more time host-feeding than ovipositing. Martinez-Ferrer et al. (2003) registered a mean of 1,053 seconds per host (third instar) and Casas et al. (2004) 500 and 623 seconds per first and second instars respectively.

The pre-oviposition examination of the host by *Aphytis* also serves to prevent parasitizing an already parasitized host (superparasitism). Avoidance of superparasitism (host discrimination) is essential for host selection by *Aphytis*. According to Abdelrahman (1974b) females of *A. melinus* recognize a recently parasitized host by the "odor" (marking substance) left by the first wasp. Rejection is almost always based on external examination and the wasp needs only two to three seconds to discriminate if a host is parasitized (van Lenteren, 1994). Even if the external odor wears off, internal probing with the ovipositor can still determine if the host is parasitized (Rosen and DeBach, 1979). According to Van Lenteren and DeBach (1981) *Aphytis* parasitoids are also able to discriminate between unparasitized hosts and hosts parasitized by conspecifics.

Aphytis oviposit only in hosts whose body is not attached to the cover i.e. they avoid to parasitize molt stages. Likewise, mature females are not accepted as hosts because apart from having their body attached to the cover they also become heavily sclerotized (DeBach and White, 1960; Rosen and DeBach, 1979). As stated earlier, first instars and small-sized hosts usually are not parasitized; they are used for host-feeding instead.

1.7.1.3. Factors affecting *Aphytis* efficiency

The efficiency of *Aphytis* as biological control agents is intimately linked with physical and biotic factors that may limit distribution and affect parasitoid abundance. Physical factors include temperature, humidity, light, and the negative impact of pesticides and dust. Biotic factors that affect *Aphytis* efficiency are host scale, host plant, the availability of food for adults and ant activity (Rosen and DeBach, 1979).

Physical factors

As already pointed out, **temperature** affects the duration of development of *Aphytis* that in turn, influences their ability to regulate the pest´s populations. Moreover, extreme temperatures are considered the main factor of mortality for *Aphytis* in the field (Rosen and DeBach, 1979). According to these authors, low winter temperatures in inland California caused nearly 100% mortality of *A. chrysomphali* and *A. lingnanensis* pupae. Also, substantial rates of mortality were observed during the hot and dry months of July, August and September. Abdelrahman (1974c) reported that all *A. chrysomphali* stages are more adapted to cold and less to heat than *A. melinus*. The threshold of development was determined at 11ºC for *A. melinus* and at 8.5 ºC for *A. chrysomphali*. According to Kfir and Luck (1984) the thresholds were 6.77ºC and 5.91 ºC for *A. melinus* and *A. chrysomphali* respectively. Finally, Yu (1986) found the lower temperature threshold for *A. melinus* to be at 9.65 ºC. The different tolerance limits to temperature of *A. chrysomphali* and *A. melinus* were found to give a plausible explanation for their spatial and temporal distribution in the Valencia citrus growing area. The relative proportion of *A. melinus* was higher during the warm months and in the southern areas whereas *A. chrysomphali* was more abundant in winter and in the cooler northern areas (Sorribas et al., 2010).

Moreover, in biparental species, temperature has been found to affect the offspring sex ratio. At lower temperatures the sex ratio turns male biased. After one day of explosion at 15.6 ºC, 74% of the progeny of *A. lingnanensis* were males whereas at 26.7 ºC around 33% of the progeny are male (Rosen and DeBach, 1979). When female *A. lingnanensis* mated at 26.7 ºC, then exposed to -1.1 ºC for six hours and finally were allowed to oviposit at an optimal temperature all their progeny were male. According to Rosen and DeBach (1979) this result suggests that low temperature killed the sperm stored in the female spermatheca. Similar male biased sex-ratios under the influence of low temperatures were found for *A. melinus* (Kfir and Luck, 1984).

The effect of **humidity and light** are considered to be less important than temperature. However, high temperatures when combined with low humidity affect negatively adult *Aphytis* survival. *Aphytis chrysomphali* did not survive for more than one day at 32 ºC and 10% R.H., and more than three days at the same temperature and 40% R.H. (Kfir and Luck, 1984). Light is thought to affect flight initiation and searching (Rosen and DeBach, 1979). Wavelength is known to be important since *A. melinus* responded significantly more to yellow and green sticky traps than to white, blue, fluorescent yellow, black or red ones (Moreno et al., 1984).

The impact of **pesticides** is probably the most important abiotic factor affecting negatively *Aphytis* as well as the rest of the natural enemies. A broad

range of active substances including, organophosphates, carbamates and neonicotinoids used against California red scale and other pests, have been found to adversely affect the development and survival of immature and adult *Aphytis* (Rosen and DeBach, 1979 pp. 64 and references therein; Grafton-Cardwell et al., 2008). Nevertheless, Rill et al. (2008) when tested the effect of two insect growth regulators (pyriproxyfen and buprofezin) on immature stages and adults of *A. melinus* found no significant effects on survival or development when the parasitoid was treated at any of the egg, larval, or pupal stages. Thus, they concluded that the two insect growth regulators are compatible with augmentative releases of *A. melinus*. Nevertheless, insect growth regulators were found to disrupt the development of other natural enemies, namely coccinelid beetles (Grafton-Cardwell et al., 2006). Moreover, chlorpirifos, the most commonly used insecticide against *A. aurantii* in Valencia citrus, is known to reduce the number of female progeny of various parasitic Hymenoptera, including *A. melinus* (Desneaux et al., 2007).

Finally, airborne **dust** is another factor negatively affecting *Aphytis* efficiency. It is considered that dust particles abrade the integument of intersegmental membranes causing desiccation and eventually death of the parasitoid (Rosen and DeBach, 1979). Adult parasitoids that were exposed to dusty fruits were killed rapidly and as a consequence parasitism was markedly reduced.

Biotic factors

The host scale insect affects *Aphytis* abundance and efficiency in various ways. As already mentioned, not all developmental stages of the host scale are suitable for *Aphytis*, i.e. molts and gravid females are not accepted for parasitism because of their hard integument. In addition, the developmental stages which potentially can serve as hosts are not of the same quality. There is substantial variation in host size depending on the developmental stage (Yu, 1986; Reeve, 1987; Hare et al., 1990). Moreover, *Aphytis* are idiobionts, i.e. they paralyze their host and arrest its development at parasitism (Rosen and DeBach, 1979). Thus, host size represents the resources available for the developing parasitoid and is probably the most reliable cue of host quality for *Aphytis* (Hare and Luck, 1991).

Host size is known to have a strong influence on various fitness components of adult *Aphytis*. A positive correlation has been found between host size and the size of the adult males and females of *A. lingnanensis* and *A. melinus* (Opp and Luck, 1986; Yu, 1988; Reeve, 1987; Rosenheim and Rosen, 1992). Recently, Pina (2007) reported a similar positive relationship between the size of second instar males and third instars of California red scale reared

in lemons and adult *A. chrysomphali* size. This is particularly important because in the majority of parasitic Hymenoptera, adult size may influence fitness by affecting longevity, fecundity (females) or searching capacity (see Godfray, 1994). In fact, adult *A. chrysomphali* that emerged from large hosts (third instar females of *A. aurantii*) were significantly larger and lived almost twice compared with parasitoids that emerged from smaller hosts (second instar males) (parasitoids of both treatments had access to the same type of sugar source) (Pina, 2007). A similar positive relationship exists also between the number of mature eggs and adult size of *A. lingnanensis* and *A. melinus* (Opp and Luck, 1988) and *A. chrysomphali* (Pina, 2007). Host size also affects *Aphytis* fecundity; larger hosts yield more fecund *Aphytis*.

In biparental *Aphytis* species, sex ratio is also affected by host size. Females control the sex of their offspring at oviposition via haplodiploidy; unfertilized eggs produce sons whereas fertilized eggs produce daughters (Flanders, 1953; Rosen and DeBach, 1979). As already pointed out, the available hosts for *Aphytis* vary greatly in size due to developmental stage, plant substrate or time of the year (Ebeling, 1959; Carroll and Luck, 1984; Luck and Podoler, 1985; Reeve, 1987; Yu, 1988; Walde et al., 1989; Hare et al., 1990; Hare and Luck, 1991, 1994; Hare and Morgan, 2000). As a result, larger hosts are of higher quality because they are expected to give place to more "fit" parasitoids. Charnov et al. (1981) developed a model for solitary parasitoids suggesting that, under conditions of varying host size, females should allocate female eggs to large hosts and male eggs to small hosts. They were based on the assumption that the benefit from developing in large hosts would be greater for females than males. Additional assumptions were that there is a threshold above which only female eggs are laid and that this threshold is not absolute but depends on the distribution of the host sizes available. Under laboratory conditions, Luck and colleagues found that *A. melinus* allocated female eggs mostly to hosts larger than 0.39 mm^2 (in body area of *A. aurantii*) and *A. lingnanensis* to hosts larger than 0.55 mm^2 (Luck et al., 1982; Luck and Podoler, 1985). In a posterior field study, Yu (1986) confirmed that *A. melinus* laid female eggs mostly to hosts larger than 0.39 mm^2. This threshold is considered to be rather absolute than depending on the entire host size distribution (Hare and Luck, 1991; but see Luck and Nunney, 1999).

This sex allocation pattern of *A. melinus* has important implications for the biological control of *A. aurantii* as well as for the competitive interactions between *Aphytis* species. In biological control, production of female-biased sex ratios is desired because females are responsible for attacking the pest by ovipositing and/or host-feeding. Additionally, given that females build up parasitoid populations, the poorer the sex ratio, the poorer the rate of increase of the population. Evidently, the size of the parasitoid population is likely to

affect its efficiency to control the pest. As a result, host size is a key element for successful classical and augmentative biological control programs (Ode and Hardy, 2008). The importance of host size for the biological control of *A. aurantii* by *A. melinus* was confirmed in the interior California, in San Joaquin Valley. Scarcity of hosts suitable for the production of female *A. melinus* (larger than 0.39 mm^2) resulted in small *A. melinus* populations that provided poor biological control, especially in summer (Luck et al., 1996; Luck and Nunney, 1999). As a solution, augmentative releases of commercially produced *A. melinus* are used to suppress *A. aurantii* populations in that area (Moreno and Luck, 1992).

Furthermore, the host-size dependent sex allocation between *A. melinus* and *A. lingnanensis* explained the pattern of their competitive interactions (Luck and Podoler, 1985). In the San Joaquin Valley, BeBach and Sundby (1963) noticed that *A. melinus* was displacing *A. lingnanensis*. They considered the two species as ecological homologues i.e. that they used the same host stages to produce offspring. Moreover, they noticed that host stages were abundant for both parasitoids and attributed the displacement of *A. lingnanensis* by *A. melinus* to the fact that the latter was a more efficient searcher. However, later studies showed that *A. melinus* accepts smaller hosts for the production of female progeny than *A. lingnanensis* (0.39 mm^2 versus 0.55 mm^2 in body area of *A. aurantii*). Thus, *A. melinus* pre-empts the hosts before they reach the suitable size range for the production of female *A. lingnanensis* (Luck et al., 1982; Luck and Podoler, 1985; Luck and Nunney, 1999). As a result, *A. lingnanensis* cannot produce daughters to replace itself and inevitably is displaced by *A. melinus*.

The **host plant** may influence indirectly the efficacy of *Aphytis* as biological control agents by influencing the size of the host insect scale. *Aphytis melinus* reared on lemon leaves (*Citrus limon*) produced nearly twice of female progeny when compared with wasps reared on leaves of grapefruit (*Citrus paradisi*), orange (*Citrus cinensis*) or Satsuma mandarin (*Citrus unshiu*) (Hare and Luck, 1991). Aditionally, parasitoids from lemon leaves had higher initial eggload, followed by those reared from grapefruit, mandarin and orange.

Food for adults is a key element for the survival and efficiency of parasitoids as biological control agents. Their action of suppressing the activity and abundance of pests is performed by the parasitoid larvae that are carnivorous. However, adults require non-host food, primarily carbohydrates to cover their energetic requirements. Carbohydrate sources include plant-derived food like floral nectar, extrafloral nectar, pollen and food indirectly derived from plants, like honeydew, produced by hemiptera feeding on phloem sap (Wäckers, 2005). Food provided by plants can have a striking

impact on various life-history traits of natural enemies. It has been demonstrated that in the absence of an adequate plant food source longevity (Heimpel et al., 1997; Wäckers, 2001; Lee et al., 2004) and reproduction of natural enemies (Winkler et al., 2006) is seriously compromised. Moreover, the behavior of the natural enemies is also affected. Sugar deprived individuals show low overall activity levels (Takasu and Lewis, 1995) or shift their efforts to food search instead of host search (Wäckers, 1994). Therefore, availability of adequate food sources is expected to seriously affect the efficacy of natural enemies and consequently the outcome of biological control. There exists theoretical and empirical evidence that the provision of food supplements may have a strong impact on the population dynamics of parasitoid-host systems (Wäckers, 2003).

For *Aphytis*, availability of an adequate sugar source is crucial for adults. Already since the first mass rearing efforts it was noticed that most adults die within 24 hours unless honey, sugar-water or a similar carbohydrate source was provided (DeBach and White, 1960). Moreover, host-feeding alone cannot enhance longevity; it can do so only when the wasps have in addition access to a sugar source (Heimpel et al., 1997). The same authors observed that longevity of adult *A. melinus* that had not access to a sugar source did not exceed three days. Similarly, fecundity was also seriously compromised. Nevertheless, these results are based in lab experiments. No information is available regarding the food source use by adult *Aphytis* in the field. Presumably, nectar form citrus and other floral species as well as hemipteran honeydew are the main carbohydrate sources (Rosen and DeBach, 1979). Avidov et al. (1970) tested in the laboratory the effect of citrus nectar and various honeydew types on the longevity of *A. coheni* DeBach. They found that citrus nectar was an excellent food source for adults whereas the nutritional value of the honeydew varied markedly with its insect source. For instance, honeydew of the mealybug, *Pseudococci citriculus* Green (Hemiptera: Pseudococcidae) increased parasitoid longevity, while honeydew of *Toxoptera aurantii* (Boyer de Fonscolombe) (Hemiptera: Aphididae) yielded longevity below that of water, suggesting possible toxic effects.

Undoubtedly, it is usually difficult to determine the feeding sources of adult parasitoids in the field. The sole presence of nectar producing flowers or honeydew sources does not necessarily imply feeding by parasitoids. For example, parasitoids may be attracted to flowering plants for refugee, alternative food or mating sites (Wilkinson and Landis, 2005). Moreover, it has been demonstrated that flowers vary considerably in terms of attractiveness and nectar accessibility for parasitoids (Wäckers, 2004), since flower architecture may condition foraging performance (Patt et al., 1997). These difficulties can be subdued using high performance liquid

chromatography (HPLC). With this method, the overall carbohydrate levels as well as an ample range of carbohydrates can be quantified for individual parasitoids (Wäckers and Steppun, 2003; Heimpel et al., 2004; Steppuhn and Wäckers, 2004). Overall carbohydrate levels provide an accurate measure of parasitoid nutritional state (Fadamiro and Heimpel, 2001; Giron and Casas, 2003). Moreover, detection of specific sugars typical of hemipteran honeydews (di- and oligosaccharides) provides information about honeydew feeding by parasitoids (Wäckers and Steppun, 2003; Hogervorst et al., 2007).

Ant activity is ranked by Rosen and DeBach (1979) as the third most detrimental factor for *Aphytis* efficiency after extreme temperatures and pesticides. DeBach (1951) used ants as "biological check method" to evaluate the efficiency of Aphytis in biological control. By comparing pest densities in ant-free (parasitoids "undisturbed") and ant-infested trees (parasitoids were "disturbed") he evaluated the degree of the biological control achieved.

In agro-ecosystems it has long been known that ants are associated with the disruption of biological control of arthropod pest species (DeBach, 1951; Flanders, 1945; Bartlett, 1961; Way, 1963; Buckley, 1987). The most frequently documented situation involves the mutualism between ants and honeydew producing hemiptera. Many hemiptera of the order Sternorrhyncha produce a sugar-rich excretion called honeydew. Ants collect honeydew to cover a major part of their carbohydrate requirements (Figure 1.20). On the other hand, honeydew-producers benefit from ant-attendance in terms of protection from their natural enemies (Figure 1.21), higher growth rates, improved hygiene conditions, transport and dispersal (Way, 1963, Buckley, 1987, Stadler and Dixon, 2005).

Fig. 1.20. Worker of the ant *Linepithema humile* collecting honeydew produced by aphids.

In the citrus agro-ecosystem, ants represent a great part of the arthropod fauna (Haney, 1988). Given their abundance and feeding habits they affect the

composition of the rest of the arthropod community (Way, 1963). Although some species have been employed to suppress pest populations since ancient times (Way and Khoo, 1992), very often they have been associated with population outbreaks of honeydew-producing Hemiptera (Bartlett, 1961; Way, 1963; Itioka and Inoue, 1996). Nevertheless, *A. aurantii* does not produce any type of honeydew and consequently is not tended by ants. However, it has been found that ant activity may slightly (Murdoch et al., 1995) or considerably (DeBach, 1951; Steyn, 1954; Moreno, 1987; James et al., 1997) stimulate California red scale populations. It is assumed that ants disrupt or kill the California red scale parasitoids as an indirect consequence of ant-attendance to a coincident honeydew producer (Flanders, 1945; DeBach, 1951; Moreno, 1987; James et al., 1997). The main ant species implicated with the disruption of the biological control of the California red scale is the argentine ant *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) (DeBach, 1951; Moreno, 1987; Murdoch et al., 1995; James et al., 1997; Martinez-Ferrer et al., 2003). It is a highly invasive species that has spread in all ecosystems with Mediterranean climate causing serious problems in agriculture and natural ecosystems (Suarez et al., 2001; Carpintero et al., 2005). This species was first cited in Spanish citrus in 1923 associated with honeydew producing hemiptera (Font de Mora, 1923; Garcia Mercet, 1923). Nowadays, it is present in Valencia citrus but has not spread; it is present in citrus orchards close to anthropogenic activity (Espadaler and Gómez, 2003). In Spanish citrus, the most abundant ant species are the native to the Mediterranean *Lasius grandis* Forel and *Pheidole pallidula* (Nylander) (Alvis, 2003; Vanaclocha et al., 2005; Urbaneja et al., 2006; Cerdá et al., 2009) (Figures 1.22 and 1.23). Nevertheless, important aspects of the ecology of these species when they forage on the citrus canopies are poorly known. Other species also present are *Plagiolepis schmitzii* Forel, *P. pygmaea* (Latreille), *Tapinoma erraticum* (Latreille), *Camponotus sylvaticus* (Olivier), *Formica gerardi* Bondroit (Alvis, 2003; Vanaclocha et al., 2005). In Table 1, are shown the ant species reported in citrus in the Mediterranean Basin.

Fig. 1.21. Workers of the ant *Plagiolepis schmitzii* attacking the predatory ladybird *Coccinela septempunctata* L.

Fig. 1.22. Lateral and frontal view of a worker of the ant *Lasius grandis* (Foto: K. Gómez).

Fig. 1.23. Minor (up) and major (down) workers of the dimorphic ant *Pheidole pallidula* (Foto: K. Gómez).

Table 1. Ant species reported in citrus orchards in the Mediterranean Basin.

Subfamily	Ant species	Reference [a]
Dolichoderinae	*Linepithema humile* (Mayr)	2, 3, 4, 5, 6, 7, 8
	Tapinoma nigerrimum (Nylander)	2, 4, 6, 8
	Tapinoma erraticum (Latreille)	3, 5, 6, 7, 8
	Tapinoma israelis Forel	1
	Tapinoma simrothi phoenicium Emery	1
Formicinae	*Camponotus aethiops* (Latreille)	8
	Camponotus compressus thoracicus fellah Emery	1
	Camponotus foreli Emery	4, 5
	Camponotus lateralis (Olivier)	8
	Camponotus nylanderi Emery	3, 8
	Camponotus piceus (Leach)	8
	Camponotus pilicornis (Roger)	4
	Camponotus sylvaticus (Olivier)	5, 6, 7
	Formica cunicularia Latreille	4, 8
	Formica gerardii Bondroit	5
	Formica rufibarbis Fabricius	5, 7
	Lasius alienus (Foerste	8
	Lasius grandis Forel	5, 6, 7
	Lasius niger L.	4
	Paratrechina jaegerskioeldi (Mayr)	1
	Paratrechina longicornis (Latreille)	1
	Plagiolepis pygmaea (Latreille)	5, 6, 7, 8
	Plagiolepis schmitzii Forel	4, 6, 8
Myrmicinae	*Aphaenogaster pallida* (Nylander)	8
	Aphaenogaster semipolita (Nylander)	8
	Aphaenogaster senilis Mayr	4
	Cardiocondyla batesii Forel	5, 7
	Cardiocondyla bicolor Donisthorpe	1
	Cardiocondyla elegans Emery	7
	Cardiocondyla mauritanica Forel	5
	Cataglyphis gadeai De Haro & Collingwood	5, 7
	Crematogaster inermis Mayr	1
	Crematogaster jehovae Forel	1
	Crematogaster jehovae mosis Forel	1
	Crematogaster scutellaris Olivier	8
	Diplorhoptrum robusta Emery	4
	Messor barbarus (L.)	4, 5, 7
	Messor capitatus (Latreille)	8
	Messor structor (Latreille)	8
	Myrmica scabrinodis Nylander	4
	Pheidole pallidula Nylander	4, 5, 6, 7, 8
	Solenopsis fugax Latreille	8
	Temnothorax recedens Nylander	8
	Tetramorium caespitum (L.)	4, 8
	Tetramorium punicum (Smith)	1
	Tetramorium semilaeve André	5, 7, 8
Ponerinae	*Hypoponera eduardi* (Forel)	4, 5, 7, 8

[a] (1) Rosen (1967); (2) Panis (1981); (3) Tumminelli et al. (1996); (4) Palacios et al. (1999); (5) Vanaclocha et al. (2005); (6) Alvis and García-Marí (2006); (7) Urbaneja et al. (2006); (8) Pergola et al. (2008).

1.7.2. Endoparasitoids

Endoparasitoids are considered less effective biological control agents of *A. aurantii* when compared with *Aphytis*. *Comperiella bifasciata* Howard (Hymenoptera: Encyrtidae) and *Encarsia perniciosi* Tower (Hymenoptera: Aphelinidae) have been introduced in various regions worldwide to complement the biological control of *A. aurantii*.

Comperiella bifasciata is a solitary endoparasitoid of *Aonidiella* species. It is a shiny black wasp of approximately 1.5 mm long. It is arrhenotokous (reproduces sexually: unfertilized eggs develop into males; fertilized eggs into females) and in contrast with *Aphytis* does not engage in host-feeding. Moreover, *Comperiella* wasps are koinobionts i.e. they do not paralyze their hosts at oviposition and the latter continue to grow (Forster et al., 1995). Thus, it can parasitize almost all stages of *A. aurantii* including molts and gravid females (Forster et al., 1995). The most preferred host stages are third instars, followed by gravid females and second molts. It can also parasitize first instar, first molts and early second instars. The least preferred stages are males (Forster et al., 1995). *Comperiella* lays eggs inside the body of *A. aurantii* and parasitoid development is synchronized with that of its host (Smith et al., 1997). No matter the parasitized host stage, larvae begin their development when the host reaches gravid female stage eventually killing it. Each female lays approximately 50 eggs and lives for three-four weeks depending on food availability (Smith et al., 1997). The developmental time varies depending on the developmental stage of the host; it lasts from three to six weeks at 26 ^{o}C (Smith et al., 1997).

Comperiella bifasciata presents various advantages or disadvantages associated with aspects of its behavior and ecology when compared with *Aphytis*. Immature stages of *C. bifasciata* developing on the same host with *Aphytis* larvae are always consumed by the latter (Forster et al., 1995). Also, eggs and larvae by *C. bifasciata* may be encapsulated by *A. aurantii* (Blumberg and Luck, 1990). On the other hand, *C. bifasciata* is more resistant to extreme temperatures than *Aphytis*. In California, it coexists with *A. melinus* in all the citrus growing area but it is more common in the Central Valley and inland southern area than in the coast (Forster et al., 1995). Parasitism by *C. bifasciata* reduces *A. aurantii* populations during winter and midsummer, a period during which *Aphytis* are less efficient (Forster et al., 1995). Moreover, *C. bifasciata* attacks all *A. aurantii* stages in contrast with *Aphytis* that attacks only concrete stages. Finally, disruption of oviposition activity by ants has been found to be more detrimental for *A. melinus* than for *C. bifasciata* (Martinez-Ferrer et al., 2003).

Comperiella bifasciata is the most important endoparasitoid of *A. aurantii* in various regions of the world including California (Forster et al., 1995), Australia (Smith et al., 1997), South Africa (Bedford, 1998), Italy (Sicily) (Siscaro et al., 1999) and Turkey (Segonca et al., 1998). In Australia, Smith et al. (1997) reported that *C. bifasciata* parasitize up to 80% of mature females of *A. aurantii* eventually killing them. In Spain, *C. bifasciata* was initially described as *Habrolepistia cerapterocera* by Garcia Mercet (1921). Later, Gómez-Clemente and Planes (1950) introduced *C. bifasciata* parasitoids from California to control *Chrysomphalus dictyospermi* but without success. Apparently, the introduction involved the strain parasitizing *Aonidiella citrina* (Coquillet) a diaspidid not present in Spain (Pina, 2007). The *C. bifasciata* strain that attacks *A. aurantii* was introduced and released in Valencia citrus in 2001 (Pina and Verdú, 2001; Pina 2007). Nevertheless, subsequent samplings conducted during five years after the initial releases suggest that *C. bifasciata* has not been successfully established in this area (Pina, 2007).

Encarsia perniciosi is the second most important endoparasitoid attacking *A. aurantii*. The adult is yellow and brown, and considerably smaller than *C. bifasciata*. It is solitary, thelytokous (virtually produces only females) and does not engage in host feeding (Yu et al., 1990; Borer, 2002). It does not paralyze its host and can parasitize all *A. aurantii* stages. Moreover, because of its reduced size it needs less food than *Comperiella* and *Aphytis*. Thus, it prefers mostly first and second (males and females) instars (Yu et al., 1990; Forster et al., 1995). *Encarsia perniciosi* develops faster in second instars (approximately 19 days) than when it parasitizes gravid females (28 days). However, there is a trade-off between the host stage parasitized and fecundity; wasps emerging from second instars were about 80% as fecund as wasps emerging from gravid females (Yu et al., 1990).

Yu et al. (1990) argued that the preference of *Encarsia* for late first or second instars might be the result of interspecific competition with *Aphytis*. Immature *E. perniciosi* are predated by *Aphytis* larvae when they develop together on third instars (the most preffered by *Aphytis*). On the contrary, *E. perniciosi* developing in young instars avoid being killed by *Aphytis.* Another result of interspecific comptetition may be the fact that *E. perniciosi* is found parasitizing more scales on stems (Yu et al., 1990). *Aphytis* exploits more the scales on fruits and leaves (Yu, 1986; Walde et al., 1989) and additionally cannot distinguish between hosts unparasitized and parasitized by *Encarsia*. Therefore, the preference of *Encarsia* for scales on stems is likely to be the result of lower risk of intraguild predation by *Aphytis*. Finally, *E. perniciosi* is not well adapted to the high summer temperatures; it is more susceptible than *Aphytis* (Yu et al., 1990; Forster et al., 1995). It prefers milder climates and remains active at temperatures lower than 12.8 ^{o}C (Flanders, 1971).

Encarsia perniciosi has been reported from California (DeBach and Sundby 1963; Yu et al., 1990; Forster et al., 1995), Australia (Smith et al., 1997), Argentina (Crouzel et al., 1973) and Italy (Sicily) (Siscaro et al., 1999). In Australia, Smith et al. (1997) reported that percent parasitism of *A. aurantii* by *E. perniciosi* can exceed 20%. In Spain, individuals of *E. perniciosi* were introduced from the insectary of the University of California (Riverside) and released in various citrus orchards in Valencia in 2001 (Pina and Verdú, 2001; Pina, 2007). In samplings completed various years later, the parasitoid was not recovered, implying that it failed to establish (Pina, 2007). Nevertheless, its presence has been detected lately in the southern part of the Valencia citrus growing area (Alicante) (Sorribas et al., 2008; Sorribas and Garcia-Marí, 2010).

1.7.3. Predators

Predators can play an important role in the biological control of *A. aurantii* and complement the action of *Aphytis* (Rosen and DeBach, 1978). Predators include ladybird beetles (Coleoptera: Coccinelidae) (Drea and Gordon, 1990), Neuroptera (Drea, 1990), gall midges (Diptera: Cecidomyiidae) (Harris, 1990) and mites (Gerson, 1990).

There is a very ample list of **Coccinellidae** cited in the literature as *A. aurantii* predators. The most important are: *Chilocorus cacti* (L.) (DeBach and Rosen, 1976; Forster and Luck, 1996), *C. circundatus* (G.) (Smith et al., 1997), *C. bipustulatus* (L.) (Crouzel et al., 1973; DeBach and Rosen, 1976), *C. distigma* Klug (Bedford, 1998), *C. nigritus* (F.) (Bedford, 1998), *C. orbis* Casey (Forster and Luck, 1996), *Coccidophilus citricola* Brethes (Crouzel et al., 1973), *Halmus chalybeus (Bdvl) (=Orcus chalybeus)* Rosen and DeBach, 1978; Smith et al., 1997); *Exohomus quadripustulatus* (L.) (Argyriou, 1969); *Rhizobius lophanthae* (Blaisdell) (=*Lindorus lophanthae*) (Crouzel et al., 1973; Rosen and DeBach, 1978; Smith et al., 1997; Bedford, 1998).

In Spain (Valencia citrus growing area), principally *R. lophanthae* and to a lesser extend *C. bipustulatus* have been cited preying upon *A. aurantii* (Llorens, 1990; Pina 2007; Vanaclocha et al., 2009; Sorribas and Garcia-Marí, 2010). Interestingly, both species are more abundant in the southern and warmer area (Alicante) of the Valencia Community (Orts Fernádez, 2008). In a recent study, Vanaclocha et al., (2009) reported that the mortality of *A. aurantii* due to predation by *R. lophanthae* was higher in spring reaching values of approximately 20% of the live scales examined.

Various **Neuroptera** species are also known to attack *A. aurantii*. Drea (1990) cites *Chrysoperla plorabunda* (Fitch) and Bodenheimer (1951)

Conwentzia psociformis (Curtis) as predators of *A. aurantii*. In Valencia citrus, Sorribas and Garcia-Marí (2010) reported larvae of *C. carnea* and *Semidalis aleyrodiformis* preying upon "white caps" and third instars of *A. aurantii*.

The only species of **Diptera** known to be specialized predators of armored scales belong to the family Cecidomyiidae (Harris, 1990). Larvae of *Lestodiplosis aonidillae* Harris and *Cecidomyia coccidarum* (Cockerell) have been cited as predators of *A. aurantii* (Harris, 1990). Sorribas and Garcia-Marí (2010) found *L. aonidillae* to be the most abundant among the predators of *A. aurantii* in the Valencia citrus growing area.

Mites of the Hemisarcoptidae family have long been recognized as important generalist predators of armored scale insects (Gerson et al., 1990). Known predators of *A. aurantii* are the species *Hemisarcoptes malus* (Shimer) and *H. coccophagus* Meyer (Gerson et al., 1990; Luck et al., 1999). Sorribas and Garcia-Marí (2010) reported *H. coccophagus* feeding on *A. aurantii* in Valencia citrus. Moreover, **phytoseid** mites have been observed to feed upon crawlers of *A. aurantii* (Figure 1.22) (Samways, 1985; Gerson et al., 1990; personal observations). Juan-Blasco et al. (2008) found that the phytoseids *Typhlodromus phialatus* (Athias-Henriot) and *Amblyseius swirskii* (Athias-Henriot) completed their life cycles when fed exclusively upon crawlers of *A. aurantii*. Interestingly, the same authors demonstrated that in semi-field conditions preventive releases of *A. swirskii* significantly reduced the damage caused by *A. aurantii* compared with controls where no releases were carried out.

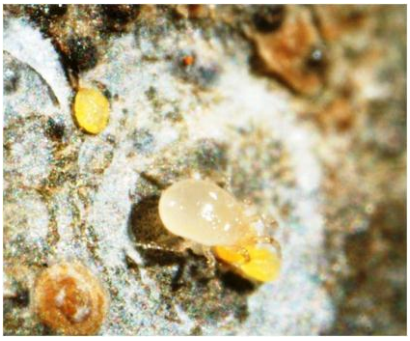

Fig. 1.24. Phytoseid mite preying upon a crawler of *Aonidiella aurantii*.

Finally, the **thrips** species *Aleurodothrips fasciapennis* (Franklin) has been cited as predator of *A. aurantii*. It is the most common predator of diaspidids in the tropics and subtropics (excluding Africa). There is however, very little information regarding its feeding habits and efficiency as predator (Palmer and Mound, 1990).

1.7.4. Entomopathogenic Fungi

The only group of pathogens capable of penetrating the scale cover of the diaspidids and invade the haemocoel are the fungi. Armored scales, particularly in the tropics, are subjected to devastating attacks by fungal pathogens. Nevertheless, humidity requirements for the action of the fungi are high and consequently control success depends on environmental factors (Evans and Prior, 1990). Entomopathogenic fungi attacking *A. aurantii* belong to the Subdivision Ascomycotina and the main species are (Evans and Prior, 1990):

- *Nectria aurantiicola Berk.* and *Br.* (sexual form) and *Fusarium larvarum* Fuckel (asexual form)
- *Nectria flammea* (Tul.) (sexual form) and *Fusarium coccophilum* (Desm.) (asexual form)
- *Myriangium duriaei* Mont. and Berk. (sexual form).

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Chapter 2

Rationale and Objectives

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California red scale, *Aonidiella aurantii* is the most important citrus pest in the Mediterranean Basin. Apart from direct damages, serious economic losses are produced due to the presence of scales on fresh fruit. In many parts of the world, the scale is successfully controlled by parasitoids of genus *Aphytis.* In eastern Spain (Valencia), the native parasitoid *A. chrysomphali* and the introduced *A. melinus* are the principal biological control agents of *A. aurantii*. However, their efficiency is unsatisfactory and usually applications of oil sprays and/or insecticides are necessary in order to keep scale infestations below economic thresholds. The size of *A. aurantii* is the most reliable cue for *Aphytis* parasitoids to asses host quality. Host size affects various fitness components of *Aphytis* and consequently their efficiency as biological control agents. Thus, as a first step, we have studied the relative importance of factors that might affect the size of *A. aurantii* under field conditions. We examined the size variation among plant substrates, among localities, the seasonal variation, as well as the influence of the nutritional state of the plant on the *A. aurantii* size.

In the field, *A. chrysomphali* and *A. melinus* encounter hosts that vary in size. Theoretical and empirical evidence suggest that under conditions of varying host size, female parasitoids lay female eggs on high-quality hosts and males eggs on low-quality hosts. This sex allocation pattern has important implications for biological control. Scarcity of high-quality hosts will lead to male-biased sex ratios that in turn will result in poorer biological control. No information is available regarding the host sizes used by *A. chrysomphali* and *A. melinus* in the Mediterranean. Therefore, we carried out a study to determine the *A. aurantii* instars and sizes used by *A. chrysomphali* and *A. melinus* in the field, the influence of scale size on percent parasitism by each parasitoid and, finally, the relationship between scale size and the brood size and sex ratio of each parasitoid.

Ants represent a great part of the arthropod fauna present in citrus ecosystem. By behaving as generalist predators, ants provide a positive service, namely they act as biological control agents. Nevertheless, their net role is controversial mostly because of their mutualism with honeydew-producing Hemiptera. Ants collect honeydew and in turn honeydew-producers benefit from ant-attendance in terms of protection from their natural enemies. Therefore, ant activity is associated with outbreak of honeydew producing pests. *Aonidiella aurantii* does not produce any type of honeydew and, consequently, is not tended by ants. However, it has been found that ant activity may result in increased *A. aurantii* populations. It has

been found that principally the argentine ant, *Linepithema humile*, disrupts the activity of *A. aurantii* parasitoids. In eastern-Spain, the most abundant ant species in citrus ecosystem are the native *Lasius grandis* and *Pheidole pallidula*. Nevertheless, important aspects of their ecology as well as their impact on *A. aurantii* populations are unknown. Thus, we firstly initiated a study where we sought to describe basic aspects of their ecology, more concretely: their daily and seasonal foraging patterns on citrus canopies, their temporal and spatial interspecific interactions and their main feeding sources. Secondly, we determined their effect on *A. aurantii* populations in citrus.

Food for adults is crucial for the survival and efficiency of *Aphytis* as biological control agents. Contrary to the carnivorous larvae, adults of *Aphytis* presumably rely on flower nectar as well as hemipteran honeydew for maintenance and locomotion. In the laboratory, most adults die within one or two days unless honey, sugar-water or a similar carbohydrate source is provided. Moreover, host-feeding alone cannot enhance *Aphytis* longevity. Despite the importance of *Aphytis* as biological control agents, no information is available regarding their feeding ecology in the field. Thus, we conducted a study to determine the nutritional state as well as the food sources used by adult *A. melinus* in the field.

Chapter 3

Factors affecting the size of California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae) under field conditions

Factors affecting the size of California red scale *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) under field conditions

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Abstract: The body size of California red scale (hereafter CRS) *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) affects the efficiency of its parasitoids and consequently the biological control of the scale. We measured the size of various developmental stages of CRS to determine the influencing factors and assess their relative importance under field conditions. Twelve citrus orchards were sampled periodically between February 2007 and February 2009 in eastern Spain. Scale size was compared among plant substrates (branches, leaves and fruits), localities and season of the year. Leaf analyses were also performed in order to relate scale size with plant nutrient content. Seasonal variation, due to temperature fluctuations along the year, was the most important factor affecting CRS size. Smaller body sizes were observed when temperatures were higher, during summer and autumn. For third instar females a reduction of approximately 50% in the body size was observed during summer. Among plant substrates, scales were larger on fruits than on leaves or branches. Significant variation in the scale body size was also registered depending on locality, although without a definite geographic pattern. The influence of the above factors was more evident for physiologically older scale stages, third instar females and gravid females, compared with second instar males and females. A positive correlation was registered between body size of some CRS developmental stages and leaf content in potassium.

3.1. Introduction

The California red scale (hereafter CRS) *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) is a major insect pest of citrus with worldwide distribution (Talhouk, 1974). It damages all aboveground parts of the citrus tree. In high densities the scale can deform fruits, cause defoliation or even kill branches of the tree (Bodenheimer, 1951; Ebeling, 1959). However, the main damage is cosmetic related with the presence of scales on fruits. Serious economic losses are caused since part of the production may be rejected due to the extremely low market tolerance for scale presence on fruits. In many parts of the world the scale is under biological control by ectoparasitoids of the genus *Aphytis* Howard. Mostly, *Aphytis melinus* DeBach, and to a lesser extend *A. lingnanensis* Compere and *A. chrysomphali* Mercet, are the most successful biocontrol agents in the majority of the cases (DeBach and Argyriou, 1967; DeBach, 1969; Rosen and DeBach, 1979; Orphanides, 1984; De Santis and Crouzel, 1994; Tumminelli et al., 1996; Smith et al., 1997; Bedford, 1998). The main CRS parasitoids present in eastern Spain citrus orchards are *A. chrysomphali* and *A. melinus* (Troncho, 1992; Rodrigo et al., 1996; Pina et al., 2003; Sorribas et al., 2010).

For *Aphytis* species, various fitness components of the adult parasitoids depend on the size of their hosts. For example, (Opp and Luck, 1986) demonstrated that the size of the parasitized host is directly correlated with *Aphytis* final size which in turn influences survival and fecundity of the parasitoid. Moreover, the sex-ratio of the offspring is influenced by the host size; typically, *Aphytis* females are able to manipulate the sex of their offspring and allocate daughters to large hosts and sons to small hosts (Luck et al., 1982). (Luck and Podoler, 1985) reported that there is a threshold in host size above which more female progeny is produced, that threshold being smaller for *A. melinus* (0.39 mm^2 in CRS body surface) than for *A. lingnanensis* (0.55 mm^2). Moreover, the concentration of the non-volatile compound O-caffeoyltyrosine, which is used by *A. melinus* as a kairomone for host recognition, is qualitative related with scale body size (Hare et al., 1993; Hare and Luck, 1994; Hare and Morgan, 2000). Therefore, California red scale size may affect *Aphytis* host selection, fecundity, longevity and sex ratio, and consequently may have important implications for the efficiency of the parasitoids. Thus, knowledge about the CRS size variation in the field may help understand and improve its biological control.

CRS size can be influenced by various factors. Luck and Podoler (1985) examined the size ranges of the third instar females CRS (the most preferred stage by *A. melinus*) on wood, leaves and fruits in three geographic locations, observing that scales are largest when they grow on fruits, smallest when they

grow on wood and of intermediate size when they grow on leaves. Besides, they found significant differences in the proportion of CRS sizes suitable for the production of female progeny for *A. melinus* between localities. Yu and Luck (1988), using six constant temperatures in the laboratory, and also under fluctuating temperatures in two citrus orchards, reported that that CRS size decreases with increasing temperatures. Also, Hare et al. (1990) found that CRS final size is larger when it grows on leaves than when it grows on bark. They also found significant differences in scales´ final size between four citrus cultivars. However, differences in the concentration of soluble nitrogen compounds between cultivars were not significantly correlated with the scales´ final size.

However, most studies have not been conducted on an area-wide and long-term basis under field conditions. Thus, the aim of the present study was establish the relative importance of factors influencing size variation in field populations of CRS. Moreover, previous studies have focused primarily on the variation in the body size of third instar females since it is the most preferred stage for parasitism by *A. melinus. Aphytis chrysomphali* in the field parasitizes mostly second instars (Pina, 2003). Thus, we determined scale sizes in a wider range of CRS developmental stages. We examined the size variation among plant substrates and among localities, as well as the seasonal variations, in Eastern Spain citrus area**.** We also sought to determine if differences in the body size of the scale among localities were related with differences in concentrations of nutrients in the plant substrate.

3.2. Materials and methods

CRS biology

A detailed description of CRS biology is given by Bodenheimer (1951). Gravid females release crawlers that disperse on the host plant. After a short time (usually less than 24 hours) the crawlers settle on a fruit, leave or branch. Once settled, a female remains immobile for the rest of its life. After settlement the scales insert their feeding stylets in the plant tissue, begin to feed and secrete a waxy covering over the body (first instar). The scales continue to develop for several days until they cut off the feeding tube and attach their body to the cover. This is the first molt which is a non-growing stage. After approximately four days as first molts, the scales insert a new feeding tube into the plant substrate and transform into second instars. As second instar the scale body separates from the cover and begins to grow. It is during the second instar when the two sexes begin to differentiate. The males become elongate, develop eyes and approximately five days later they transform into prepupae. After a day and a half the prepupae transform into pupae that

finally, about three days later, will transform into winged males. Meanwhile, the second instar females transform again into second molts. After about six days as second molts, the scales reinsert their feeding apparatus in the plant substrate and transform into third instar females. During this stage, scales continue to grow and they transform into mature females when they get inseminated. Third instar females vary greatly in size, but the first and second molts incorporated in their cover remain constant. After insemination, mature females stop feeding and their body becomes again attached to the scale cover. Gravid females are viviparous and begin to produce crawlers in approximately 12 days. Potential hosts for *Aphytis* parasitoids are second instar females and males, male prepupae and third instar virgin females (Forster et al., 1995). In the study area, Eastern Spain, CRS completes three generations annually (Rodrigo and Garcia-Marí, 1990, 1992).

Influence of the plant substrate and geographical variation in CRS size

The survey was conducted from February 2007 until January 2008. We collected data from 12 citrus orchards distributed all along the citrus-growing area of Valencia (Eastern Spain). The orchards were located in geographically separate zones in a citrus area 200 km long (north-south) and 50 km wide (east-west), in the following townships: Alzira, Bétera, Betxí, Chiva, La Vall d´Uixó, Massanassa, Montcada, Pobla Llarga, Real de Gandia, Riola, Sagunt and Tavernes de la Valldigna (see table 1 for agronomic characteristics of the orchards). Each orchard was sampled at least three times, with at least one month between sampling dates. The orchards were commercial plantations of sweet orange (*Citrus sinensis* (L.) Osbeck) and included organic orchards, orchards under Integrated Pest Management and orchards under chemical pest control. Organic orchards were not sprayed with chemicals for pest control while in IPM and chemical control orchards pesticides were applied at the end of spring for CRS control.

At each sampling date 40 branches (less than 10 mm in diameter) with at least ten leaves each from ten different trees infested with CRS were collected. Also, when available, 50 fruits from ten different trees infested with CRS were collected. The material was transferred to the laboratory where the three substrates, branches, leaves and fruits, were processed within the next 24 hours. For each substrate, 20 non-parasitized individuals for every stage of gravid females without crawlers, third instar females, second instar males and second instar females were processed using a stereomicroscope with a micrometer in the eyepiece. In addition to the bodies and covers of these four stages, we measured the second molt ring incorporated in the cover of the third instar females (second molt exuvia). All measures were made to the nearest 0.01 mm. The product of length and width of body or cover of these

different scale stages was used as an index of their size (Luck and Podoler, 1985).

Table 1. Agronomic characteristics of the citrus orchards sampled.

Locality	Variety	Rootstock	Irrigation Type	Block plantation	Age	Ground vegetation in Spring	Pest Managment
Alzira	Valencia Late	Volkameriana	Furrow-irrigated	6 x 5	10	Yes	IPM
Bétera	Navel Foios	Citrange carrizo	Drip-irrigated	6 x 4	5	No	Unsprayed
Betxí	Navel Lane Late	Citrange carrizo	Drip-irrigated	6 x 4	27	Yes	IPM
Chiva	Navelina	Troyer	Drip-irrigated	6 x 4	27	No	IPM
La Pobla Llarga	Navel Lane Late	Citrange carrizo	Furrow-irrigated	4 x 2	15	Yes	IPM
Massanassa	Valencia Late	Citrange carrizo	Furrow-irrigated	3 x 4	10	Yes	Unsprayed
Montcada	Navel Lane Late	Citrange carrizo	Drip-irrigated	4 x 4	7	No	Unsprayed
Real de Gandia	Valencia Late	Citrange carrizo	Furrow-irrigated	4 x 2	18	Yes	Unsprayed
Riola	Navelina	Citrange carrizo	Furrow-irrigated	4 x 4	20	Yes	Unsprayed
Sagunt	Valencia Late	Citrange carrizo	Drip-irrigated	3 x 4	6	Yes	Unsprayed
Tavernens de la Valldigna	Valencia Late	Citrange carrizo	Drip-irrigated	6 x 4	15	No	Unsprayed
Vall d´ Uixó	Navel Lane Late	Citrange carrizo	Drip-irrigated	3 x 4	10	Yes	Unsprayed

Leaf sampling and analyses

Two leaf nutrient analyses were carried out, in December of 2007 and December of 2008, on each of the 12 orchards sampled for the study of the geographic variation in CRS size. For the nutrient analyses, six to eight-month-old spring flush leaves were collected from 10 randomly selected trees. Each sample consisted of a minimum of 25 leaves taken randomly from each of the 10 trees. In the laboratory the leaves were washed in detergent solution and then dried at 70 C for 72 hours. Leaf analyses were realized following the protocols established by the Ministry of Agriculture, Fisheries and Food of Spain (M.A.P.A. 1992). Results were expressed as % of N, P, K, Ca, Na, Mg and mg/kg of Fe, Mn, Cu and Zn on dry material.

Seasonal variation in CRS size

The study was conducted from February 2008 until February 2009 in two citrus orchards, Alzira and Tavernes de la Valldigna, where high CRS populations were registered during the previous year. The sampling protocol was the same as described above with the difference that no fruits were collected. The orchards were sampled once a month in winter, twice a month

during spring and autumn, and three times a month during summer. Moreover, the population structure of the scale was registered on each sampling date. All live scales were systematically observed in a predefined area of the substrate (branches and/or leaves), changing this area when arriving to a maximum of 20 scales, and finishing the observation when arriving to a total of 200 live scales observed. The scales were classified as one of the following: gravid females with crawlers, gravid females, third instar females, second molts, second instar males, second instar females and first instars. Accumulation of degree days for CRS was estimated using 11.5 ^{o}C as developmental threshold (Yu and Luck, 1988).

Statistical analyses

We used regression analysis to determine the relationships between the scale bodies and cover sizes for the second instar females and males, and third instar females on each substrate. For each substrate per CRS life stage combination, individuals from the two years of observations were used. We compared the slopes of the regression lines between substrates for each CRS life stage to determine if the body cover/body relationship varied with substrate. Also, the relationships between the body sizes of second instar females, second instar males, third instar females and exuvia of the second molt were determined using correlation analysis. For each correlation, the individuals within each substrate, locality and sampling date combination were pooled. Finally, we evaluated the correlation between different developmental stages of the scale and average concentration of leaves in nutrients (data from the two leaf analyses pooled) to assess whether these factors were connected. Correlations were measured with Pearson´s correlation coefficient.

We used two-way analysis of variance (ANOVA) to analyze the variation in CRS body size among plant substrates (with plant substrate and sampling date as main factors) and among localities (with locality and sampling date as main factors). In both cases and for each CRS stage examined, we included in the analyses only samples in which at least ten individuals were measured within each substrate/locality and sampling date combination. For the comparison among localities, individuals measured on leaves and branches were pooled. Finally, a one-way ANOVA was used to test for differences in the body size among seasons. Differences among means were tested by the Tuckey's test at a 5% significance level. All analyses were performed using the Statgraphics Plus for Windows version 5.1 (Statgraphics 1994).

3.3. Results

Relationships of size between body and cover and among development stages

When all the scales for each of the three CRS life stages were examined, the size of each scale body ("*y*") was strongly correlated with the size of its cover ("*x*") (second instar females: $R^2 = 0.68$; $F = 4683$; d.f. = 1, 2174; $P < 0.0001$; $y = 0.075 + 0.218\ x$; second instar males : $R^2 = 0.52$; $F = 2341$; d.f. = 1, 2148; $P < 0.0001$; $y = 0.82 + 0.219\ x$; third instar females: $R^2 = 0.82$; $F = 13144$; d.f. = 1, 2873; $P < 0.0001$; $y = 0.199 + 0.24\ x$). However, within each CRS life stage, the slopes of the regression lines were significantly different at $P < 0.0001$ for the three substrates (branches, leaves or fruits) (Figure 1). Thus, for the same body size, scales can have different cover sizes depending upon the plant substrate they grow.

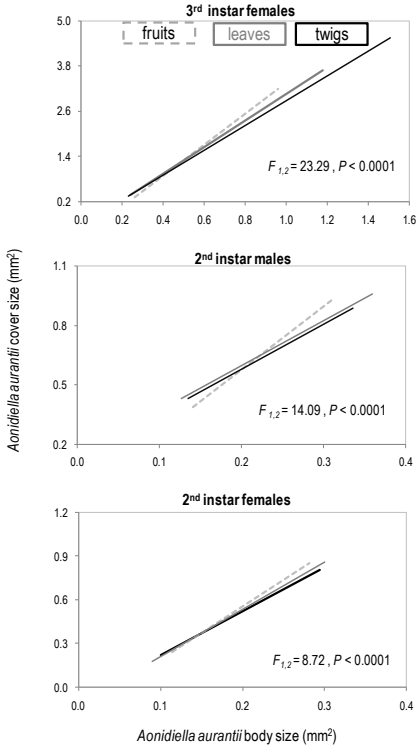

Fig 1. Regression lines between body and cover size for third instar females, second instar males and second instar females sampled on fruits, leaves and branches from field populations of *Aonidiella aurantii*. Within each stage the slopes of the regression lines were significantly different among the three substrates at $P < 0.0001$.

Also, significant correlations were found among the average scale sizes of different CRS developmental stages belonging to the same substrate and

sampling date (table 2). The most important correlations were found between the stage sizes which occur simultaneously or are contiguous in the process of development of the insect, i.e., second instar females and second instar males (Pearson's correlation coefficient r = 0.48; F = 41.47, d.f.= 1, 135; P < 0.001), second instar males and third instar females (r = 0.50; F = 45.43; d.f. = 1, 136; P < 0.001), third instar females and gravid females (r = 0.46; F = 39.48; d.f. = 1, 147; P < 0.001) and finally, between gravid females and second molt (r = 0.55; F = 60.07; d.f. = 1, 137; P < 0.001).

Influence of plant substrate and geographic location on CRS size

The body sizes of the different CRS life stages varied significantly among plant substrates (table 3). The older stages, second molt, third instar females and gravid females, were significantly larger on fruits than on leaves or branches (second molt: F = 3.55; d.f. = 2, 14; P = 0.029; third instar females: F = 12.76; d.f. = 2, 12; P < 0.0001; gravid females: F = 15.68; d.f. = 2, 15; P < 0.0001). A different pattern was found for the younger CRS stages. Second instar females were larger on fruits and branches, and smaller on leaves (F = 6.42; d.f. = 2, 8; P = 0.0017), whereas the body size of second instar males did not differ among substrates (F = 1.35; d.f. = 2, 11; P = 0.259). Differences in body size among substrates were greatest for third instar females (12% of increase between the highest size on fruits compared with the lowest size on branches).

Table 2. Correlation coefficients between size (in mm^2) of different *Aonidiella aurantii* developmental stages, comparing average values of 20 scales within the same substrate and sampling date. The product of length and width of the body was used as an index of *Aonidiella aurantii* size for all stages except for second molts where the product of length and width of the exuvia incorporated in the cover of the third instar females scale size was used. Data from 12 citrus orchards in eastern Spain sampled repetitively between February 2007 and February 2009. Values in parentheses represent the number of observations for each correlation. Significant correlations are denoted with (*). Significance levels: * P < 0.05; ** P < 0.001.

Second instar males	0.48** (137)			
Third instar females	0.15 (115)	0.50** (138)		
Gravid females	0.07 (100)	0.21** (122)	0.46** (149)	
Second molt	0.17* (140)	0.27** (141)	0.28** (156)	0.55** (139)
	Second instar females	**Second instar males**	**Third instar females**	**Gravid females**

Also the body sizes of the different CRS life stages varied significantly due to geographic location (table 4). Differences in body size between orchards were greater for the two older development stages, gravid females and third instar females (21 and 18% increase of the highest size orchard compared with the lowest size orchard, respectively). Conversely, in the youngest instar

measured (second instar females) differences among orchards were much lower, only 3%. Differences in second molt and second instar males were intermediate (12%).

Table 3. Size of different *Aonidiella aurantii* developmental stages (mean ± 1SE; in mm^2) on fruits, leaves or branches. Data from 12 citrus orchards in eastern Spain sampled repetitively between February 2007 and January 2008. Numbers in parenthesis represent the individuals measured for each life stage. Within each row means sharing the same letter do not differ significantly at $P > 0.05$ (two-way ANOVA followed by Tuckey´s test).

***Aonidiella aurantii* stage**	**Plant substrate**		
	Fruits	**Leaves**	**Branches**
Gravid females	1.512 ± 0.015 (277) a	1.401 ± 0.015 (289) b	1.414 ± 0.013 (345) b
Third instar females	0.574 ± 0.009 (267) a	0.534 ± 0.009 (226) b	0.512 ± 0.008 (304) b
Second molt	0.502 ± 0.003 (270) a	0.494 ± 0.003 (237) ab	0.489 ± 0.003 (343) b
Second instar males	0.231 ± 0.002 (274) a	0.235 ± 0.002 (280) a	0.232 ± 0.002 (166) a
Second instar females	0.180 ± 0.002 (202) a	0.169 ± 0.002 (203) b	0.182 ± 0.002 (197) a

Seasonal variation in CRS size

The body size of the different stages measured (gravid females, third instar females, and second instar males and females) differed significantly over the sampling period and this variation was consistent in the two orchards (Figure 2). Variation in the body size depended on the stage examined. Thus, the two older stages, gravid females and third instar females, were smaller during summer and autumn (one way ANOVA, gravid females: Tavernes de la Valldigna: $F = 19.66$; d.f. = 4, 501; $P < 0.0001$; Alzira: $F = 33.35$; d.f. = 4, 373; $P < 0.0001$; third instar females: Tavernes de la Valldigna: $F = 72.65$; d.f. = 4, 887; $P < 0.0001$; Alzira: $F = 24.46$; d.f. = 4, 478; $P < 0.0001$). On the other hand, the body size of second instar males and second instar females was smaller during the summer period (second instar males: Tavernes de la Valldigna: $F = 34.00$; d.f. = 4, 615; $P < 0.0001$; Alzira: $F = 78.26$; d.f. = 4, 448; $P < 0.0001$; second instar females: Tavernes de la Valdigna: $F = 6.83$; d.f. = 3, 543; $P = 0.0002$; Alzira: $F = 5.34$; d.f. = 4, 473; $P = 0.0003$). Differences in body size among seasons were greater for third instar females (57% and 46% between the smallest and largest size in Tavernes de Valldigna and Alzira, respectively) followed by second instar males (31 and 36%) and gravid females (20 and 25%). The lowest differences in body size among seasons were observed on second instar females (10 and 13%).

Table 4. Geographic variation in the size (mean ± 1SE; in mm2) of different *Aonidiella aurantii* developmental stages. Data from 12 citrus orchards in Valencia (eastern Spain) sampled from three to five times between February 2007 and January 2008. Means were based on pooled data from individuals of each stage measured on leaves and branches. Values in parentheses represent the numbers of individuals measured. Inside each column means followed by the same letter do not differ significantly at $P > 0.05$ (two-way ANOVA followed by Tuckey´s test).

	Stage size (mean ± 1SE; in mm^2)					
Orchard	**Gravid females**	**Third instar females**	**Second molt**	**Second instar males**	**Second instar females**
Bétxi	1.51 ± 0.02 (187) a	0.556 ± 0.01 (182) ab	0.501 ± 0.005 (182) abc	0.227 ± 0.003 (107) ab	0.169 ± 0.004 (105) a
Chiva	1.50 ± 0.02 (133) ab	0.575 ± 0.01 (148) a	0.507 ± 0.005 (148) ab	0.238 ± 0.004 (84) ab	0.170 ± 0.005 (77) a
Massamassa	1.44 ± 0.04 (50) abc	0.499 ± 0.02 (95) bcd	0.482 ± 0.007 (95) bcde	0.240 ± 0.004 (103) a	0.169 ± 0.004 (120) a
Vall d´ Uixó	1.44 ± 0.03 (85) abc	0.548 ± 0.02 (93) ab	0.485 ± 0.007 (93) bcde	0.229 ± 0.005 (62) ab	0.172 ± 0.005 (65) a
Real de Gandia	1.41 ± 0.04 (59) abc	0.488 ± 0.01 (59) cd	0.473 ± 0.008 (59) cde	0.231 ± 0.004 (106) ab	0.167 ± 0.005 (72) a
Tavernes de la Valldigna	1.36 ± 0.04 (60) bcd	0.507 ± 0.02 (96) abcd	0.481 ± 0.007 (96) bcde	0.233 ± 0.004 (106) ab	0.172 ± 0.004 (138) a
La Pobla Llarga	1.34 ± 0.03 (92) cd	0.527 ± 0.01 (135) abc	0.493 ± 0.006 (135) abcd	0.224 ± 0.003 (114) b	0.172 ± 0.004 (102) a
Sagunto	1.34 ± 0.03 (79) cd	0.540 ± 0.02 (74) abc	0.488 ± 0.007 (74) cde	0.238 ± 0.009 (34) ab	-
Alzira	1.33 ± 0.03 (130) cd	0.487 ± 0.01 (149) d	0.470 ± 0.005 (149) de	0.231 ± 0.003 (126) ab	0.171 ± 0.004 (115) a
Bétera	1.30 ± 0.02 (133) cd	0.547 ± 0.02 (112) abc	0.516 ± 0.006 (112) a	0.225 ± 0.004 (85) ab	0.167 ± 0.004 (86) a
Montcada	1.30 ± 0.03 (76) cd	0.526 ± 0.02 (72) abcd	0.485 ± 0.007 (72) bcde	0.214 ± 0.007 (31) b	0.168 ± 0.005 (63) a
Ribla	1.25 ± 0.02 (200) d	0.530 ± 0.01 (157) abc	0.460 ± 0.005 (157) e	0.228 ± 0.004 (126) ab	0.167 ± 0.005 (65) a

A more detailed approach regarding the seasonal variation of the CRS size reveals that it is apparently related with the three generations of the scale that occur along the year in the study area (Figures 3 and 4). For second instars, the smallest individuals observed along the year are those of the second generation. They appear between 1300 and 1600 degree-days in Tavernes de la Valldigna and between 1200 and 1400 degree-days in Alzira. These individuals continue their development and reach adult stage (approximately between 1600-1800 degree days in Tavernes de la Valldigna and between 1300-1500 degree-days in Alzira), giving the smallest third instar females and gravid females which develop along the year. All developmental stages measured are bigger when developing on the first or third annual generations.

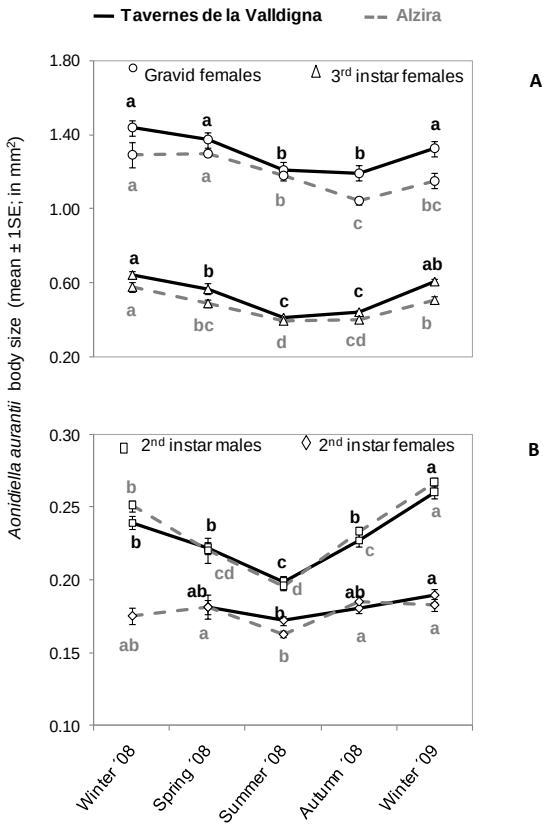

Fig. 2. Seasonal variation in body size (mean ± 1SE; in mm^2) of A) gravid females and third instar females and B) second instar males and females, of *Aonidiella aurantii*. Means based on pooled data from individuals measured on leaves and branches from two citrus orchards (Tavernes de la Valldigna and Alzira) in Valencia (eastern Spain) sampled two to three times a month between February 2008 and February 2009. For each orchard and developmental stage means followed by the same letter do not differ significantly at $P > 0.05$ (one-way ANOVA followed by Tuckey´s test).

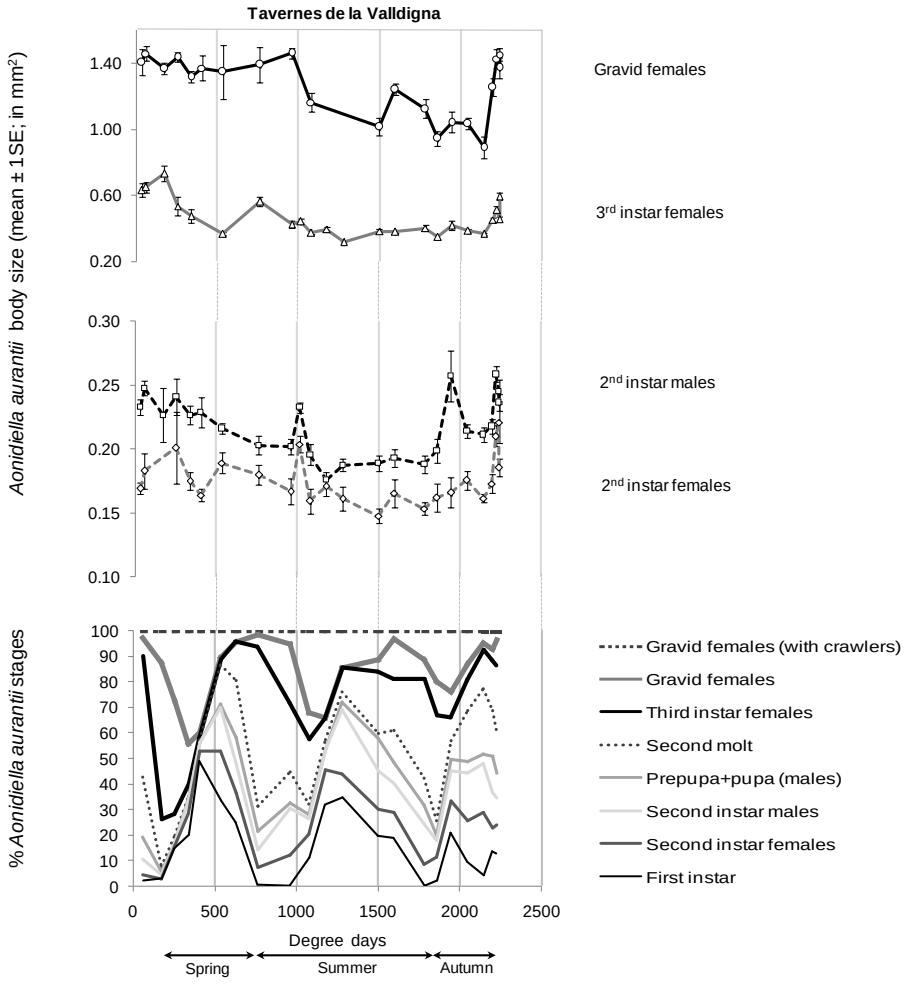

Fig. 3. Seasonal trend of the body size (mean ± 1SE; in mm^2) and relative seasonal abundance of different *Aonidiella aurantii* developmental stages in a citrus orchard (Tavernes de la Valldigna-eastern Spain) sampled two to three times a month between February 2008 and February 2009.

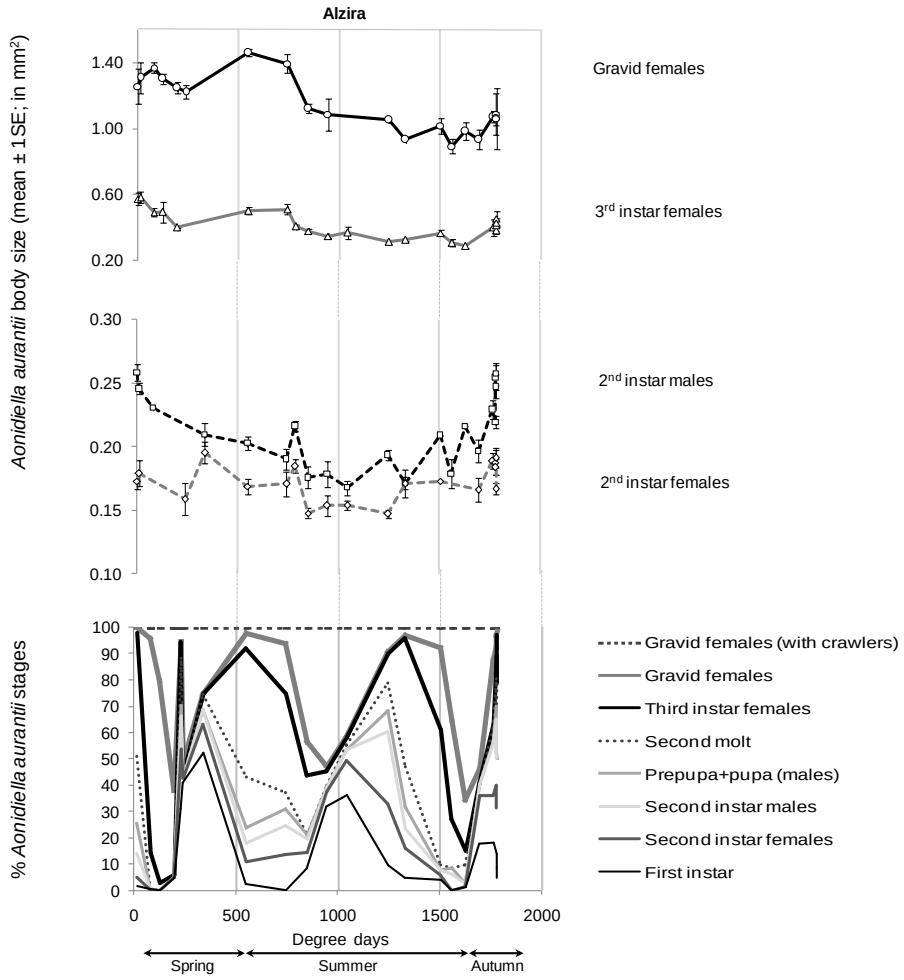

Fig. 4. Seasonal trend of the body size (mean ± 1SE; in mm^2) and relative seasonal abundance of different *Aonidiella aurantii* developmental stages in a citrus orchard (Alzira-eastern Spain) sampled two to three times a month between February 2008 and February 2009.

Nutrient influence

The correlation between the leaf content in nutrients and the size of CRS was in general very variable and non significant for most developmental scale stages and nutrients examined (table 5). There was a positive correlation (statistically significant for second instar females and gravid females) between CRS size and potassium leaf content. Moreover, a negative correlation between the leaf content in nitrogen and CRS size was apparently observed; it was not significant for any of the four stages measured but approached

statistical significance for second instar males, third instar females and gravid females.

Table 5. Correlation coefficients between size of different *Aonidiella aurantii* instars and leaf content in nutrients in nine citrus orchards. Scale size on each orchard was calculated as the average value from individuals measured on leaves and branches sampled from one to three times between February 2007 and January 2008. Leaf content in nutrients was calculated as average values from two analyses carried out in December 2007 and December 2008. Values of the correlation coefficient higher than 0.58, are statistically significant at $P < 0.05$.

***Aonidiella aurantii* instars**	**Nutrient**									
	N	**P**	**K**	**Na**	**Ca**	**Cu**	**Fe**	**Mg**	**Mn**	**Zn**
Gravid females	-0,51	0,27	0,61*	0,40	-0,05	0,44	0,05	0,21	-0,28	-0,02
Third instar females	-0,53	-0,19	0,40	0,72	-0,54	-0,12	0,14	0,31	-0,50	0,45
Second instar males	-0,49	-0,12	0,28	-0,39	-0,05	0,06	0,46	-0,47	-0,50	-0,56
Second instar females	-0,09	0,33	0,65*	-0,15	0,38	0,25	-0,46	-0,39	-0,43	-0,42

3.4. Discussion

The scale cover is significantly correlated with the body for the three instars measured; second instar females, males and third instar females. However, the same body size corresponds to different cover sizes depending on the substrate the insect is feeding on. These results suggest that the substrate upon which CRS develops must be taken into account if scale cover is used as a predictor of the insect body, e.g. when the body is consumed by *Aphytis* parasitoids. Our findings are in agreement with those reported by Hare and Luck (1994) and Hare and Morgan (2000).

Plant substrate was found to substantially influence the body size of CRS. However, the effect depended on the stage examined. Thus, the physiologically older stages, gravid females and third instar females, were significantly larger on fruits that on leaves or wood. These results agree with previous studies (Ebeling, 1959; Luck and Podoler, 1985). No differences among plant substrates were found in the body size of the second instar males, whereas second instar females were smaller on leaves. Differences in second instar females were lower than those found for older stages. Differences in nutritional quality among substrates might account for the differences in the body sizes observed. Bark is inferior to leaves and fruits for scale survival and growth (Morgan and Hare, 2000). Fruits seem to be the most favorable substrate for the scale, yet differences in the body sizes are manifested principally during the physiologically older life stages, probably because these stages are more demanding in terms of nutritional quality.

Apart from plant substrate, another important source of variation for the CRS body size is geographic location. Statistically significant differences were found among orchards. Again, the differences were more perceivable for the older stages. These differences are likely related with climatic and/or nutritional variation among orchards. Luck and Podoler (1985) also detected differences among localities and additionally they described a climate related pattern in the variation of the scale size; scales were smaller going inland from the coast. We did not detect a specific pattern in CRS size variation, probably because the Valencia citrus producing region is almost entirely located along the coast, in contrast with California were the citrus growing area includes coastal and inland areas with accentuated climatic differences.

The seasonal variation in the CRS body size observed is due to the effect of temperature (Yu and Luck, 1988; Hare et. al., 1990; Hare and Luck, 1994; Morgan and Hare, 2000). Smaller CRS body sizes are registered during the high summer temperatures for all the stages examined. Additionally, gravid females and third instar females remain relatively small during autumn as they originate from the second instar females of the second generation, the smallest along the year. The influence of season (temperature) was especially evident for third instar females. A reduction of approximately 50% in the body size was observed during summer in the two orchards sampled. The size range of the third instar females is the most preferred for parasitism by *A. melinus*, that additionally needs host sizes above a threshold (40 mm^2) for the production of female progeny (Luck and Podoler, 1985; Yu, 1986). Therefore, the reduction in the size of third instar females observed during summer and autumn might have implications for the production of female progeny by *A. melinus* in the study area (see chapter 4).

Our results regarding the influence of the nutrient content of the leaves on the size of different CRS stages are rather inconsistent; however rough conclusions might be drawn. Significantly larger CRS sizes were observed in orchards with higher leaf content in potassium. It is known that potassium affects the distribution and profile of primary metabolites in plant tissues which might have an impact on the growth of pests and pathogens (see Amtmann et al., 2008). Also, in animal cells, potassium is associated with the Na/K-ATPase, or sodium pump an enzyme that transports sodium and potassium across the plasma membrane by hydrolyzing ATP and controls among other things cell homeostasis and volume (Skou, 1988). It is likely that potassium serves a function related with body volume increase during CRS ontogeny. On the other hand nitrogen did not seem to increase CRS size since larger sizes were observed in orchards with low nitrogen concentrations in the leaves. Interestingly, Hare et al. (1990), when examining the influence of four cultivars on various parameters of CRS, reported that the scale performed

better on those cultivars with the lower concentrations in soluble nitrogen. It is known that CRS feeds principally in parenchyma cells: palisade and spongy mesophyll of leaves and cortex of twigs (Washington and Walker, 1990). Therefore nitrogen might not be the limiting nutrient component for CRS in contrast with other hemiptera species that feed in phloem sap (Raven, 1983).

In conclusion, CRS size in the field varies with plant substrate, geographic region, time of the year and probably with the nutritional state of the host plant. From the above factors, time of the year i.e. temperature, is likely to be the predominant cause of variation. Moreover, the influence of each factor affecting CRS size depends on the developmental stage; physiologically older stages are likely to be more affected. Given that scale size is the most reliable cue for *Aphytis* parasitoids to asses host quality (Hare and Morgan, 2000) the heterogeneity in CRS size observed may influence life-history traits of *Aphytis* parasitoids and the competitive interactions between them with apparent consequences for the biological control of the scale.

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Chapter 4

Influence of host size on parasitism by *Aphytis chrysomphali* and *A. melinus* (Hymenoptera: Aphelinidae) in Mediterranean populations of California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae)

Influence of host size on parasitism by *Aphytis chrysomphali* and *A. melinus* (Hymenoptera: Aphelinidae) in Mediterranean populations of California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae)

Pekas, A., A. Aguilar, A. Tena, F. Garcia-Marí. 2010. Influence of host size on parasitism by Aphytis chrysomphali and A. melinus (Hymenoptera: Aphelinidae) in Mediterranean populations of California red scale Aonidiella aurantii (Hemiptera: Diaspididae). Biological Control, 55: 132-140.

Abstract: The ectoparasitoids *Aphytis chrysomphali* Mercet and *A. melinus* DeBach (Hymenoptera: Aphelinidae) are the principal natural enemies of California red scale (CRS) *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) in the Mediterranean basin. In this paper, we determined the CRS sizes that the two parasitoids used as host in eastern-Spain citrus orchards over a two year period. *Aphytis chrysomphali* was recovered mostly from second instars (0.152-0.300 mm^2 in CRS body area), but parasitized more heavily third instars (larger than 0.325 mm^2). Within each CRS instar, percent parasitism by *A. chrysomphali* was positively related with host size, reaching an average of ~10% on scales sized between 0.80-0.85 mm^2. *Aphytis melinus* developed mostly, and parasitized more heavily, third instars. Percent parasitism was positively related with third instar size, reaching an average of ~30% on scales sized between 0.70-0.75 mm^2. Gregariousness and parasitoid size were positively influenced by host size. As expected, the sex ratio of the thelytokous *A. chrysomphali* was extremely female-biased. On the other hand, *A. melinus* laid male eggs on small hosts and female eggs on large hosts. The host size at which the sex ratio of *A. melinus* turned female biased remained constant (around 0.40 mm^2) whether relatively small or large hosts were available. Since the size of susceptible CRS instars varied significantly during the year, between May and October most scales are too small for production of female *A. melinus*. The implication of these results for a strategy of biological control of CRS using *Aphytis* is discussed.

4.1. Introduction

California red scale (CRS) *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) is a cosmopolitan and polyphagous pest species (Beardsley and González, 1975; Miller and Davidson, 1990). In citrus, it is one of the most important pests worldwide (Talhouk, 1975; Flint, 1991; Tena and Garcia-Marí, 2010). The main damage caused by CRS is cosmetic, since the presence of scales on fresh fruit reduces considerably their market value. Numerous IPM strategies have been developed to control CRS. These are mainly based on introductions and augmentative periodic releases of ectoparasitoids of genus *Aphytis* (Howard) (Hymenoptera: Diaspididae), in particular *A. melinus* DeBach in California (Moreno and Luck, 1992; Grafton-Cardwell and Reagan, 1995; Luck et al., 1996) and *A. lignanensis* Compere in Australia (Smith et al., 1997).

In eastern Spain (Valencia citrus growing area), CRS started to be a serious citrus pest since 1986 (Rodrigo and Garcia-Marí, 1990). Nowadays, it is the most important pest of the crop and it dictates the rest of the pest management program every year. The principal biological control agents of the scale in that area (as in the rest of the Mediterranean Basin) are the parasitoids *Aphytis chrysomphali* Mercet and *A. melinus* (DeBach and Argyriou, 1967; Orphanides, 1984; Rodrigo et al., 1996; Tumminelli et al., 1996; Pina et al., 2003; Pina, 2007; Pina and Verdú, 2007; Sorribas et al., 2008) since CRS mortality due to predation and endoparasitoids is always inferior to that caused by *Aphytis* (Pina, 2007; Vanaclocha et al., 2009). *Aphytis chrysomphali* is thought to be native to the Mediterranean, where it parasitized *Chrysomphalus dictyospermi* (Morgan) (Hemiptera: Diaspididae) before the introduction of CRS (Rosen and DeBach, 1979). *Aphytis melinus* (origin Northen India) was successfully established in the Valencia citrus growing area after its introduction in 1976 to control *C. dictyospermi* (Pina, 2007). In a recent study, both parasitoids were found to coexist in eastern Spain citrus and their abundance fluctuates along the year depending on weather conditions and geographic location (Sorribas et al., 2010). Nevertheless, the biological control of the scale is considered insufficient and frequent applications of oil sprays and/or insecticides are necessary to reduce fruit infestation and meet market requirements (Rodrigo et al., 2004).

Aphytis are facultatively gregarious (Rosen and DeBach, 1979), extremely synovigenic i.e. emerge with zero or few eggs (Opp and Luck, 1986; Collier, 1995) and also cause considerable mortality by probing and host-feeding (DeBach et al., 1969; Abdelrahman, 1974; Luck et al., 1982; Reeve, 1987) in order to obtain nutrients for egg maturation (Flanders, 1953; Collier, 1995; Heimpel and Rosenheim, 1995). The majority of *Aphytis* species are biparental

and reproduce sexually. Females control the sex of their offspring at oviposition; unfertilized eggs produce sons whereas fertilized eggs produce daughters (Flanders, 1953; Rosen and DeBach, 1979). Almost one quarter of the species are uniparental and exhibit thelytokous parthenogenesis, i.e. unfertilized eggs develop into females, as a consequence of infestation with *Wolbachia* symbiotic bacteria, as has been demonstrated for *A. chrysomphali* (Gottlieb et al., 1998; Pina, 2007). Moreover, *Aphytis* are idiobionts, i.e. they paralyze their host and arrest its development at parasitism (Rosen and DeBach, 1979). Thus, host size represents the resources available for the developing parasitoid and is probably the most reliable cue of host quality for *Aphytis* (Hare and Luck, 1991). Host size is known to have a strong influence on various fitness components of adult *Aphytis*. In laboratory, Pina (2007) reported that adult *A. chrysomphali* size was positively influenced by CRS size and interestingly, larger parasitoids showed increased potential fecundity and longevity. In field and lab studies with *A. melinus*, it has been found that host size affects the number of eggs laid per host, and the sex and size of emerging adult parasitoids (Abdelrahman, 1974; Luck and Podoler, 1985; Opp and Luck, 1986; Reeve, 1987; Walde et al., 1989; Hare and Luck, 1991). Although host size affects sex allocation decisions of individual parasitoids there are also consequences at the population level. A shift towards male-biased sex ratios (and consequently poorer biological control) is expected when high-quality hosts are scarce (Ode and Hardy, 2008 and references therein). In conclusion, the size of the scales from which they emerge is likely to have a strong impact on the efficiency of *A. chrysomphali* and *A. melinus* as biological control agents.

In the field, *A. chrysomphali* and *A. melinus* encounter CRS hosts that vary in size due to instar, age, plant substrate upon which they grow (branches, leaves or fruits), citrus cultivar, geographic region, time of the year and probably nutritional state of the plant (see chapter 3). Charnov et al. (1981) developed a model for solitary parasitoids suggesting that, under conditions of varying host size, females should allocate female eggs to large hosts and male eggs to small hosts since the benefit from developing in large hosts would be greater for females than males. Additionally, the model suggests that there is a threshold above which only female eggs are laid and that this threshold is not absolute but depends on the distribution of the host sizes available. Luck and Podoler (1985) and Yu (1986) found that *A. melinus* allocated male eggs mostly to hosts smaller than 0.39 mm^2 (in body area of CRS) and female eggs mostly to hosts larger than 0.39 mm^2. This threshold is considered to be rather absolute than depending on the entire host size distribution (Hare and Luck, 1991; but see Luck and Nunney, 1999). Important implications for the biological control of CRS derive from this sex allocation pattern of *A. melinus.* For example, the scarcity of hosts suitable for the production of daughters by *A. melinus* in San Joaquin Valley, especially in summer, has been proposed as

an explanation for the insufficient biological control of the scale in that area (Luck and Podoler, 1985; Luck and Nunney, 1999). Thus, augmentative releases of commercially produced *A. melinus* are used to suppress CRS population as part of the IPM strategy in that area (Luck et al., 1996).

The CRS size used by the *Aphytis* complex present and its possible influence on the biological control of the pest have never been studied in the Mediterranean Basin. Host size is likely to influence differently *A. chrysomphali* and *A. melinus.* For example, host size is not expected to affect sex allocation decisions for *A. chrysomphali* since nearly all progeny are female. Thus, determination of host size used by *A. chrysomphali* and *A. melinus* in the field may explain whether CRS size in eastern Spain affects parasitoid performance and consequently the biological control of the scale. Scale size and the sex allocation pattern of *Aphytis* might determine not only the parasitoid species to be used but also shape the biological control strategy (e.g. augmentative releases) within an Integrated Pest Management Programme.

Thus, the aims of this study were to determine: (i) CRS instars and sizes used by *A. chrysomphali* and *A. melinus* in the field; (ii) the influence of scale size on parasitism by each parasitoid and (iii) the relationship between scale size and brood size and sex ratio of each parasitoid. With these data and the seasonal trend of CRS size we suggest strategies to improve the biological control the pest under Mediterranean conditions.

4.2. Material and methods

Aphytis-CRS system

In brief, crawlers disperse and settle to a feeding site where they remain sessile for the rest of their lives. During their feeding, the insects secrete a waxy cover that protects the body beneath from physical factors and natural enemies. Female scales pass through three instars (growing periods) and two molts (non-growing periods) before they reach the adult stage. At the second instar, the two sexes begin to differentiate. Males pass through two additional instars, they pupate and finally emerge as winged adults. [Detailed information about the biology of CRS can be found in Bodenheimer (1951)]. In the study area, eastern Spain, CRS completes three generations per year (Rodrigo and Garcia-Marí, 1990; 1992). Male and female second instars and third instar female are the CRS instars that *Aphytis* mostly parasitize (Ablelrahman, 1974; Rosen and DeBach, 1979; Yu, 1986; Walde et al., 1989).

Study sites

We selected 12 commercial citrus groves (species: *Citrus sinensis* (L.) Osbeck) infested by CRS located in geographically separated townships in the Valencia citrus growing area which were either unsprayed or under IPM programs (Figure 1; also, see table 1 in chapter 3 for the agronomic characteristics of the orchards). No parasitoid releases were carried out in any of the groves.

Field samples were collected during two years from February 2007 until February 2009. During the first year (February 2007-January 2008), all the 12 groves were sampled and each grove was sampled at least three times per year, in different seasons. The second year we selected two citrus groves where dense CRS populations were registered during the previous year, Alzira and Tavernes de la Valldigna. These two groves were sampled once a month in winter, twice a month during spring and autumn, and three times a month during summer.

Fig. 1. Locations of the twelve citrus groves sampled in Valencia, eastern Spain. White circles denote unsprayed groves and black circles groves under Integrated Pest Management.

Field samples

On each sampling date, we collected 40 branches (less than 10 mm in diameter and bearing at least ten leaves), and 50 fruits when available, infested by CRS, from ten different trees. Samples were transferred to the

laboratory and were processed in the next 24 h using a stereomicroscope with a micrometer in the eyepiece. From each substrate, we measured the body and cover of 20 non-parasitized and 20 parasitized CRS individuals from each one of the three instars susceptible to *Aphytis*, i.e. second instar males, second instar females and third instar females. As an index of size, we used the product of length by width for bodies and covers (Luck and Podoler, 1985). All measures were made to the nearest 0.01 mm.

Scale instars and sizes parasitized by *A. chrysomphali* and *A. melinus*

Parasitism was indicated by the presence of *Aphytis* eggs, larvae, prepupae or pupae. For parasitized scales, only the cover was measured since the body was partially or entirely consumed by the parasitoid larva. Afterwards, cover sizes were converted in body sizes using the relationships between cover and body after adjusting for CRS instar and substrate (see chapter 3; Hare and Luck, 1994). For every parasitized scale, we annotated the species, sex and number of parasitoids developing per scale (hereafter we refer to it as brood size). Parasitoid species were identified according to their pupae coloration (Rosen and DeBach, 1979). During the first year of the study, the pupae were transferred to glass vials provisioned with a streak of honey and maintained at 22-25 ^{o}C, 60-70% RH and 16:8 L:D photoperiod for posterior adult emergence in order to determine the sex of the parasitoid. Then, adult parasitoids were mounted on microscope slides and sex was determined (Rosen and DeBach, 1979). However, this method was time consuming and additionally many pupae failed to emerge probably because of injuries caused during the transfer. Therefore, the second year, we determined the sex of *Aphytis* pupae directly, by the presence of two small rectangular plates ventrally in the abdomen tip of female pupae, as described in Rosen and Eliraz (1978) for *Aphytis chilensis* Howard. When we found parasitoid stages that could not be identified (eggs, larvae, prepupae) they were transferred to glass vials (3.0 by 0.8 cm) and maintained at the conditions described above for development to pupa. The vials were inspected every two days until the identification of the parasitoid species was possible.

Influence of host size on *A. chrysomphali* and *A. melinus* size

The influence of host size on the size of *A. chrysomphali* and *A. melinus* males and females was determined by measuring the length and width of the parasitoid pupae. Pupal area provides an accurate estimate of adult *A. chrysomphali* (Pina, 2007) and *A. melinus* size (Opp and Luck, 1986). The relationship between host size and parasitoid pupal area was examined for each host-instar/parasitoid species combination separately.

Influence of host size on brood size and sex ratio of *A. chrysomphali* and *A. melinus*

The influence of host size on *A. melinus* sex allocation pattern was examined for each CRS instar separately (not tested for the *A. chrysomphali* since it is thelytokous). To test whether the sex allocation pattern of *A. melinus* is realized on a basis of absolute or relative host size we pooled data from those samplings where small or large hosts were available. If *A. melinus* adjusts its oviposition decisions according to the entire host size distribution, the threshold for the production of female offspring would be lower when hosts are relatively small.

Statistical analyses

The number of parasitoids developing per host, parasitism and sex ratio of *A. melinus* (percentage of male progeny) were analyzed using generalized linear models. We assumed Poisson error variance for the number of parasitoids developing per host and binomial error variance for sex ratio and percent parasitism. The assumed error structures were assessed by a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If we detected an over- or under-dispersion, we re-evaluated the significance of the explanatory variables using an *F* test after rescaling the statistical model by a Pearson's chi-square divided by the residual degrees of freedom (see Mayhew and van Alphen, 1999; Crawley, 2007). We used one-way ANOVA followed by Tuckey´s post hoc test to check for differences in mean size of CRS used by the two parasitoids in the field, to compare pupal sizes among parasitoid species and sexes and to test for differences in the size of susceptible to parasitism CRS instars among months. Finally, we used linear regression to determine the relationship between host size and parasitoid (pupal area) size. Data were log-tranformed to meet normality assumptions when necessary. All statistical analyses were performed using the R freeware statistical package (R Development Core Team 2007).

4.3. Results

Scale instars and sizes parasitized by *A. chrysomphali* and *A. melinus*

A total of 2649 parasitized scales were found during the two years of the study. Of them, 1433 yielded parasitoids of three species (Table 1). The parasitoids in the remaining 1216 scales failed to develop. The most abundant parasitoid was *A. melinus*, accounting for 73.3% of the parasitoids. *Aphytis chrysomphali* represented 26.4%. These two species coexisted in 11 out of the

12 citrus groves sampled. We also recovered 3 specimens of *A. hispanicus* Mercet.

Table 1. Species/stage and number of parasitoids developing one, two or three per host.

Parasitoid species/stage recovered	Brood type		
	One	Two	Three
Aphytis melinus	971	76	4
Aphytis chrysomphali	367	12	0
Aphytis hispanicus	2	0	1
Eggs, larvae, prepupae	1117	82	17

In total, 8634 CRS were measured during the two years (Figure 2a). Second instar females body sizes, representing 30% of the non-parasitized individuals, ranged in size from 0.101 mm^2 to 0.302 mm^2 (mean ± 1SE; 0.175 ± 0.001 mm^2). Second instar males, accounting for 30% of the total, ranged in body sizes from 0.136 to 0.371 mm^2 (0.226 ± 0.001 mm^2). The remaining 40% corresponded to third instar females whose body sizes ranged from 0.230 mm^2 to 1.280 mm^2 (0.508 ± 0.002 mm^2).

We found significant differences in the mean size of CRS used as host by *A. chrysomphali* and *A. melinus* in the field (data log-transformed: one-way ANOVA: $F = 21.59$; d.f = 1, 1429; $P < 0.0001$). *Aphytis chrysomphali* parasitized hosts ranging from 0.152 mm^2 to 1.039 mm^2 (mean ± 1SE: 0.374 ± 0.011). This species was recovered mostly (66%) from small hosts (0.152-0.300 mm^2) whose sizes corresponded principally to second instar males (45%) and second instar females (21%) (Figure 2b). The remaining 34% was recovered from hosts bigger than 0.4 mm^2 which were entirely third instar females. Interestingly, small third instars (0.30-0.35 mm^2) were not parasitized by *A. chrysomphali.*

Aphytis melinus parasitized hosts ranging from 0.158 mm^2 to 1.047 mm^2 (mean ± 1SE: 0.433 ± 0.007). It developed mostly on third instar females (57%) with body sizes larger than 0.325 mm^2 (Figure 2c). Small third instars, around 0.30 mm^2, were not parasitized by *A. melinus.* The rest of *A. melinus* was found developing in small hosts (from 0.15 mm^2 to 0.3 mm^2) corresponding to second instar males (23%) and second instar females (20%).

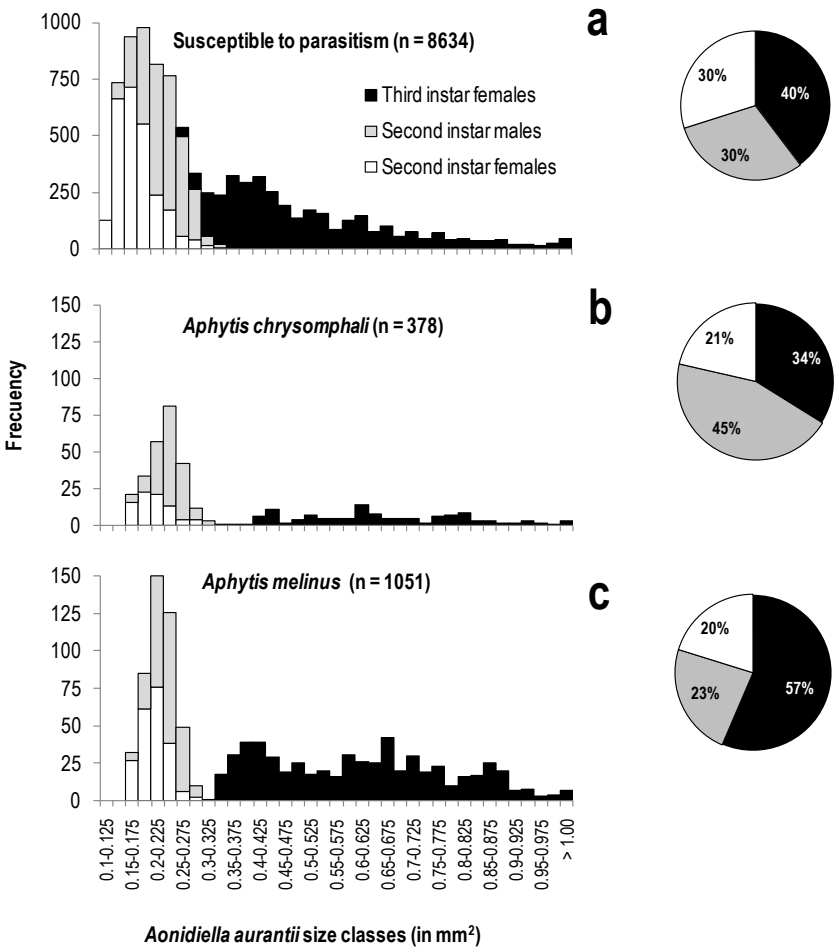

Fig. 2. Size distribution and overall percentage of *Aonidiella aurantii* instars susceptible to parasitism (a) and yielding the parasitoids *Aphytis chrysomphali* (b) and *A. melinus* (c).

Influence of CRS instar and size on parasitism

The relationship between percent parasitism and scale size varied according to parasitoid species and CRS instar (Figures 3a and b).

Aphytis chrysomphali parasitized more heavily third instar females than second instar females and males (Figure 3a). Percent parasitism by *A. chrysomphali* was positively related with the size of second and third instars, reaching an average of ~8% (scales between 0.300-0.350 mm^2) and ~10% (scales between 0.800-0.850 mm^2), respectively. A similar positive pattern,

though lower, was apparently observed when it parasitized second instar males.

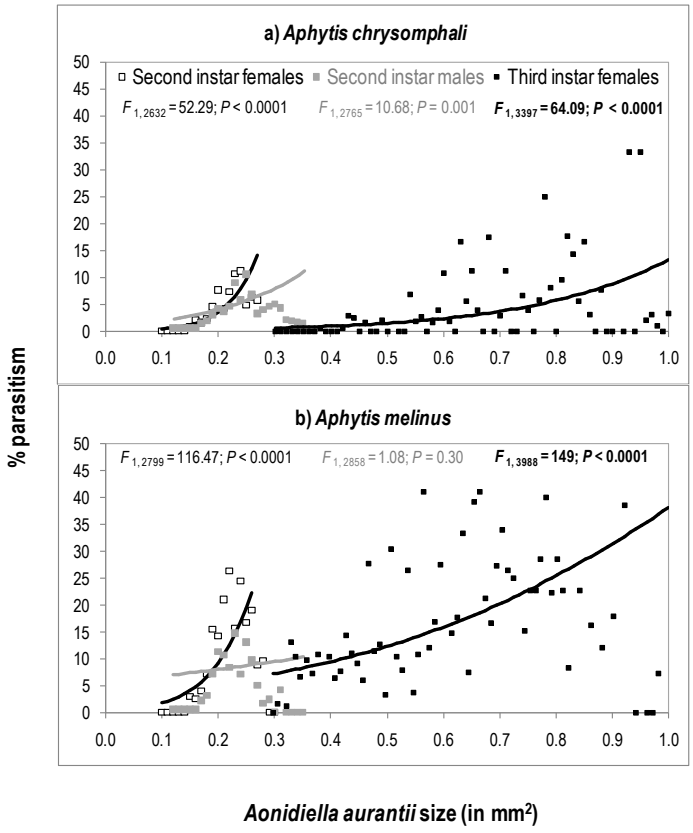

Fig. 3. Host size dependent parasitism into each *Aonidiella aurantii* instar susceptible to parasitism by *Aphytis chrysomphali* (a) and *A. melinus* (b). Each point represents mean percent parasitism at intervals of 0.01mm^2 of host size. Into each interval, percent parasitism was calculated as (number of parasitized scales) X 100 / (number of parasitized scales + number of unparasitized scales).

Aphytis melinus was also found parasitizing more heavily third instar scales and percent parasitism was positively related with third instar size, reaching average values of ~30% for scales sized between 0.700-0.750 mm^2 (Figure 3b). Also, high percent parasitism was registered in the size class of 0.20-0.25 mm^2 that corresponds to second instar females (~21%) and males (~12%). However, the relationship between parasitism and host size was not significant when *A. melinus* parasitized second instar males.

Influence of host size on brood size and sex ratio of *A. chrysomphali* and *A. melinus*

Both *A. chrysomphali* and *A. melinus* behaved as facultative gregarious parasitoids. The number of offspring developing per host ranged from one to three and the most common brood was one (96.8% for *A. chrysomphali*; 92.3% for *A. melinus*) (table 1). All the sizes on which both parasitoids expressed gregariousness corresponded to third instar females. The gregariousness expressed by both parasitoids depended on host size (*A. chrysomphali*; GLM: n = 379; F = 1.02; P < 0.0001; 88.41% deviance explained) [(Parasitoids developing per host) = exp (0.2205* (Scale size) – 0.0528)]; (*A. melinus*; GLM: n = 1051; F = 6.86; P < 0.0001; 87.56% deviance explained) [(Parasitoids developing per host) = exp (0.3699* (Scale size) – 0.0912)]. The minimum scale size in which two parasitoids developed per host was 0.498 mm^2 for *A. chrysomphali* and 0.480 mm^2 for *A. melinus*. In four cases, in hosts bigger than 0.809 mm^2, we observed three *A. melinus* pupae developing per host.

As expected, the brood sex ratio of *A. chrysomphali* was extremely female biased; we detected only one male out of 367 parasitoids recovered (0.3%). The brood sex ratio of *A. melinus* (percentage of male progeny) was negatively influenced by host size into each host instar examined ((GLM: Third instar females: n= 379 (116: 264, M:F), F = 5.03, P = 0.027; Second instar males: n = 180 (160:20), F = 7.46, P = 0.006; Second instar females: n = 159 (146:13), F = 3.93, P = 0.047)). Consequently, into each CRS instar, the probability of a host to receive a male egg decreased with increasing host size. Thus, the brood sex ratio of *A. melinus* when it behaves as solitary parasitoid was apparently determined by host size [host sizes of the three instars pooled into a single size distribution: GLM: n = 718; F = 136.47; P < 0.0001; 81.93% deviance explained, sex ratio = 1/(1+ (1/exp((-4.79*scale size) + 2.23)))]. The brood sex ratio turned female biased on hosts greater than 0.4 mm^2 (Figure 4). In fact, male *A. melinus* were allocated to small hosts and female to large hosts. The 85% of the female offspring were allocated to hosts greater than 0.4 mm^2. On the contrary, 77.4% of male eggs were allocated to hosts smaller than 0.4 mm^2.

This sex allocation pattern exhibited by *A. melinus* was apparently not influenced by changes in host size distribution. The change from male biased to female biased sex ratio remained at the same host size, around 0.4 mm^2, whether small or large hosts were available (Figure 5).

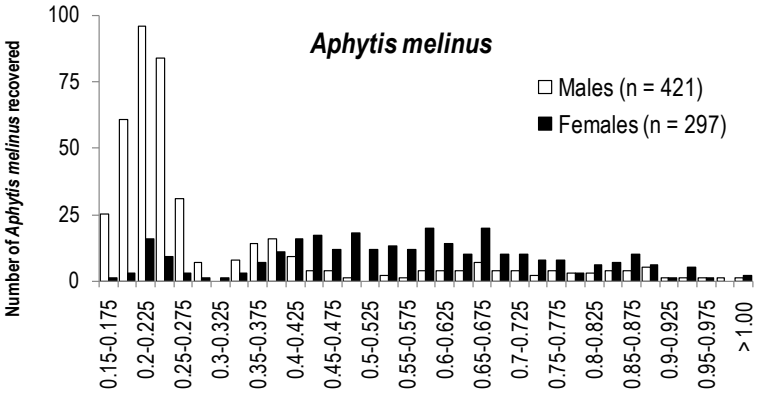

Fig. 4. Influence of *Aonidiella aurantii* body size on the sex ratio of *Aphytis melinus* when it develops as solitary parasitoid in eastern Spain citrus.

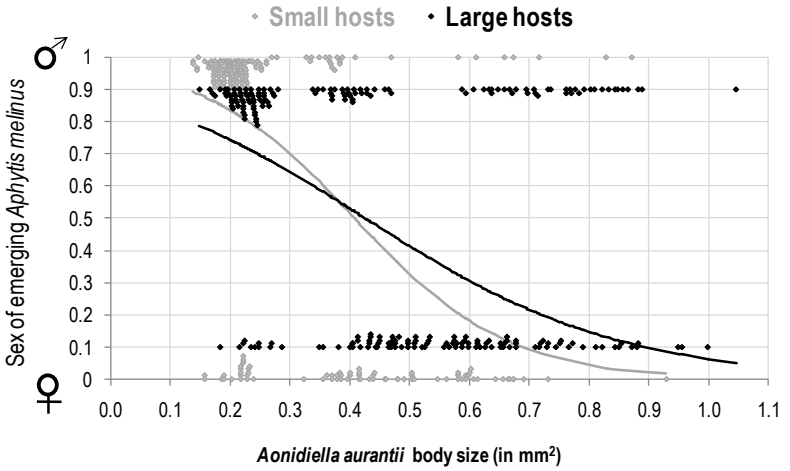

Fig. 5. Sex ratio of *Aphytis melinus* in response to host sizes available. Sex ratio turns female-biased around 0.4 mm^2 whether small or large hosts are available. Data for small hosts based on 24 sampling dates (n = 231; mean ± 1SE: 0.306 ± 0.01 mm^2; range: 0.138-0.929 mm^2); data for large hosts based on 21 sampling dates (n = 273, 0.481 ± 0.014; range: 0.148-1.096). Data are presented slightly displaced from their originally binary positions in order to better represent sample size.

Influence of host size on *A. chrysomphali* and *A. melinus* size

The size of *A. chrysomphali* and *A. melinus* pupae was affected by host instar (Figure 6). Pupae of *A. chrysomphali* developing on third instar females were significantly larger than those developing on second instars (data log-transformed: $F = 87.78$; d.f. = 2, 254; $P < 0.0001$). Similarly, female and male pupae of *A. melinus* were larger when developing on third instar females

(males: F = 29.27; d.f. = 2, 382; P < 0.0001; females: F = 26.51; d.f. = 2, 263; P < 0.0001).

The size (pupal area) of solitary *A. chrysomphali* increased with host size when it developed on second instar females (Figure 6a). Also, a slight but positive influence of host size on pupal area was found when *A. chrysomphali* developed on second instar males (Figure 6b). The size of *A. chrysomphali* pupae was not influenced by host size when developing on third instar females (Figure 6b).

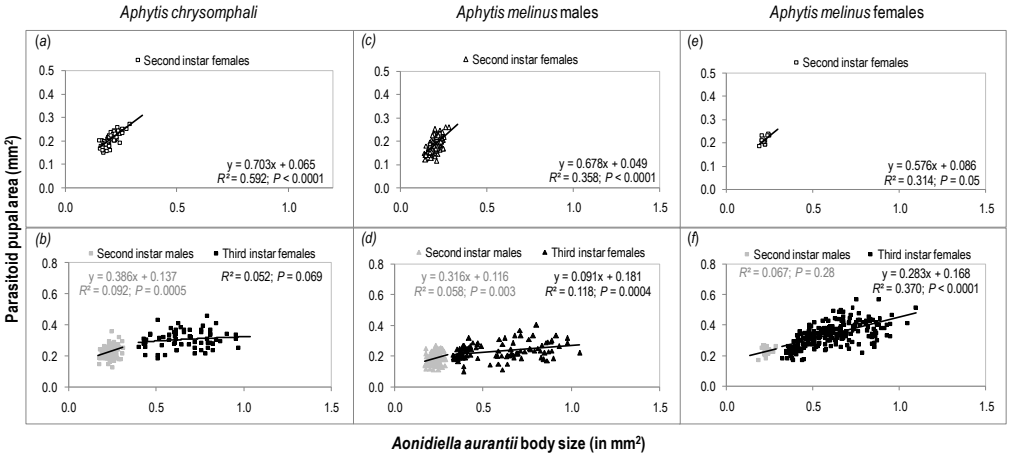

Fig. 6. Influence of *Aonidiella aurantii* body size (length by width; in mm^2) and instar on solitary parasitoid size (pupal length by width; in mm^2) of *A. chrysomphali* (all females) (a, b) and *Aphytis melinus* males (c, d) and females (e, f).

The size of male *A. melinus* pupae was influenced by host size in all host instars examined (Figure 6c and d). The size of female *A. melinus* pupae increased with host size when developing on second and third instar females but not when developing on second instar males (Figure 6e and f). However, the slopes of the regression lines did not differ significantly between host instars (F = 0.11; d.f. = 1,2; P = 0.89) suggesting that all host instars have qualitatively similar effects regarding female *A. melinus* size. Therefore, the size of female *A. melinus* is apparently determined by host size rather than host instar.

Moreover, significant differences were found among pupal sizes (data log-transformed: one-way ANOVA: F = 325.96; d.f. = 2, 896; P < 0.0001). Female *A. melinus* pupae were the largest (mean ± 1SE: 0.327 ± 0.005 mm^2), female *A. chrysomphali* pupae were of intermediate size (0.245 ± 0.003 mm^2) and, finally, *A. melinus* male pupae were the smallest (0.201 ± 0.002 mm^2).

Field size variation of CRS instars susceptible to parasitism

The mean body size of CRS instars susceptible to parasitism by *A. chrysomphali* and *A. melinus* varied considerably along the year following a similar pattern in both groves (data log-tranformed, one way-ANOVA: Tavernes de la Valldigna: F = 35.68; d.f. = 12, 2073; P < 0.0001; Alzira: F = 27.28; d.f. = 12, 1397; P < 0.0001) (Figures 7a and b).

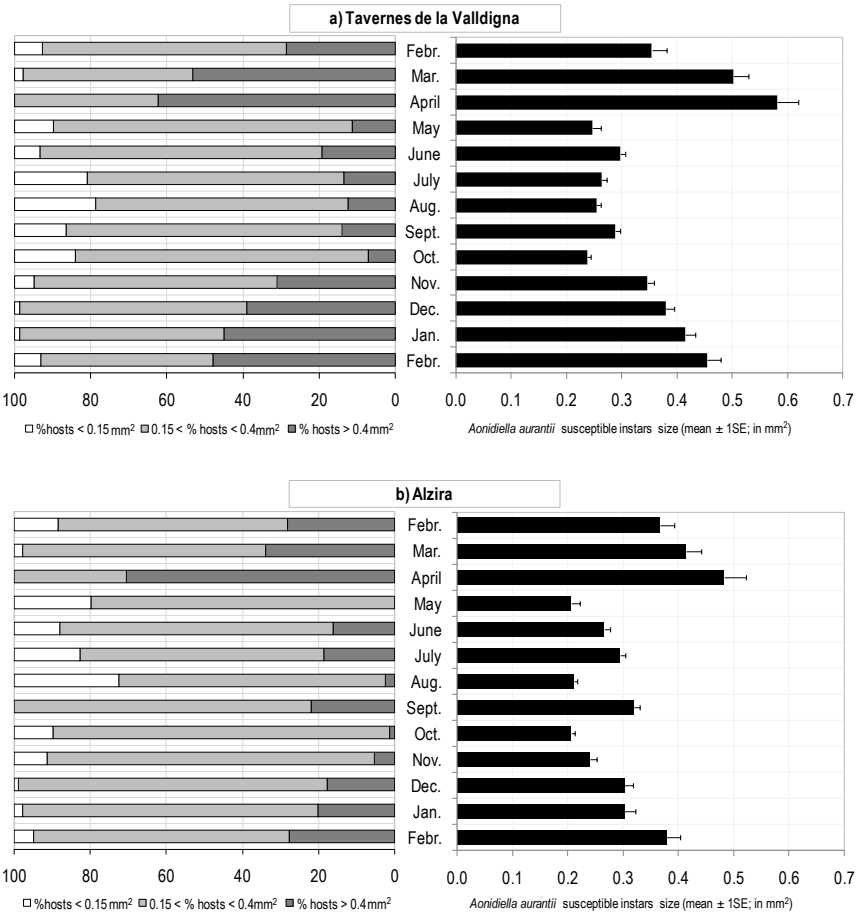

Fig. 7. Seasonal variation in the size (mean ± 1SE; in mm2) of Aonidiella aurantii instars susceptible to parasitism (right), percentage of scales susceptible to parasitism by Aphytis chrysomphali and A. melinus (bigger than 0.152 mm2, see text), and percentage of scales suitable for the production of female A. melinus (bigger than 0.4 mm2) (left), in (a) Tavernes de la Valldigna and (b) Alzira (eastern Spain). Pooled data from individuals measured on branches and leaves sampled from February 2008 to February 2009.

Larger body sizes were registered in April and May whereas the smallest were observed during summer and autumn. In November, the average size of susceptible instars increased again. Susceptible scales for the production of *A.*

chrysomphali females and *A. melinus* males (bigger than 0.152 mm^2) were available all year round. On the contrary, in both groves, there existed long periods during which the percentage of hosts available for the production of *A. melinus* females (bigger that 0.4 mm^2) was very low (Figures 7a and b). For example, from May to October, the average percentage of hosts available for the production of female *A. melinus* was 13% in Tavernes de la Valldigna and 10% in Alzira.

4.4. Discussion

In this study, we have determined the influence of CRS size on its two main parasitoids in Mediterranean Basin, the native *A. chrysomphali* and the introduced *A. melinus*. Overall, our data show that the impact of *A. chrysomphali* on CRS populations seems very low. And, more important, CRS size is a serious impediment to the natural biological control of the scale by *A. melinus*.

Aphytis chrysomphali was able to parasitize scales that ranged from 0.152 mm^2 to 1.039 mm^2 whose sizes corresponded to second instar males and females as well as third instar females. Scales susceptible for *A. chrysomphali* were very abundant along the year; they represented more than 80% of the population of CRS in branches and leaves. Despite this high availability of hosts, the average percentage parasitism by *A. chrysomphali* was usually lower than 10% and it only exceeded this percent on scales larger than 0.800 mm^2, the less common scales in the field. In general, we found a positive relationship between host size and parasitism into each CRS instar examined for this species. This is consistent with the finding that *A. chrysomphali* size (pupal area) increases with increasing host size, since larger *A. chrysomphali* gain more in fitness by means of increased potential fecundity and longevity (Pina, 2007). The fact that we did not detect a significant effect of host size on *A. chrysomphali* size when the later developed on third instars may be due to the small overall size this species attains. Apparently, that there is a host size above which no gain is achieved by *A. chrysomphali*, it is constrained genetically. A similar pattern was observed for the male *A. melinus* pupae that are also of small size.

However, despite the obvious benefits deriving from the exploitation of high quality hosts, *A. chrysomphali* in the field was recovered mostly from low quality hosts as in previous field studies (Rodrigo et al., 1996; Pina et al., 2003; 2007). The explanation for this contradiction is likely to be due to exploitative competition by *A. melinus* (Pekas et al., in preparation). Both parasitoids were found coexisting in all but one the studied sites. *Aphytis melinus* is considered to posses superior biological characteristics and also is thought to be more

efficient searcher (DeBach and Sisojevic, 1960; Rosen and DeBach, 1979). Thus, it is likely to encounter and exploit high quality hosts earlier than *A. chrysomphali*. As a result, *A. chrysomphali* is probably conformed by exploiting the remaining of the high quality hosts and/or the best of the bad hosts i.e. large second instars. Similar competitive interactions based on different host size-instar exploitation strategies have been proposed as the underlying mechanism for the displacement between *Aphytis* species observed in California citrus (see Luck and Podoler, 1985; Murdoch et al., 1996). Finally, despite the low impact on CRS populations, we believe that the role of natural occurring *A. chrysomphali* should not be overlooked because this species is native to Mediterranean and due to the infestation by *Wolbachia* bacteria it requires no specific host sizes for the production of females. Hence, it is ecologically more flexible than *A. melinus,* which needs hosts above a critical size for the production of female progeny.

The drop in parasitism observed in large second males for both parasitoid species may be due to the transitional stage of the scale i.e. males transform to prepupae. For *A. melinus*, it has been found that host recognition is mediated by a contact kairomone, the nonvolatile compound O-caffeoyltyrosine (Hare et al., 1993). During transformation to prepupae, the cover ceases to grow and in turn, the quantity of the kairomone may be also reduced (Hare et al., 1993; Hare and Morgan, 2000). This reduction in the quantity of the substance acting as kairomone could give a plausible explanation for the low parasitism levels observed in the largest second instar males.

The size variation of CRS along the year in Western Mediterranean citrus may explain the lack of biological control by the naturally occurring *A. melinus*. The impact of *A. melinus* on CRS populations was much higher than that of *A. chrysomphali*. The parasitism levels exhibited by *A. melinus* averaged ~30% on the largest size classes of third instars. However, the scarcity of suitable hosts for the production of females in summer and fall may result in a decrease of parasitoid populations that will exert insufficient control of the scale. This phenomenon has been previously described in San Joaquin Valley (Luck et al., 1996).

According to our data, *A. melinus* allocated 77.4% of the male progeny to small hosts (less than 0.4 mm^2) and 85% of females to large hosts (larger than 0.4 mm^2). These results agree with previously reported results for *A. melinus* in the lab (Luck and Podoler, 1985) and field (Yu, 1986). In fact, these authors found that the sex ratio of *A. melinus* turned female biased when the size of CRS was larger than 0.39 mm^2. Additionally, Luck and Podoler (1985) excluded the possibility that the observed pattern is due to differential mortality of the two sexes on different sized hosts by transferring young *A.*

melinus larvae to a larger host, the oleander scale *Aspidiotus nerii* Bouché (Hemiptera: Diaspididae). The sex allocation pattern of *A. melinus* is frequent in many solitary parasitoids and the explanation is based on the assumption that host size has a stronger impact on female than male fitness (Charnov et al., 1981). This assumption has not been proved for *A. melinus*. Nevertheless, it is considered that females are more severely penalized than males from being small because *Aphytis* has large eggs. Consequently, small females will be less fecund (Collier, 1995). Alternatively, small males suffer less compared to large males because sperm is probably unlimited (Nadel and Luck, 1985), although later studies report that sperm for males is never unlimited; in fact it can be a limiting factor in some species (see Boivin et al., 2005). In our case, even though we found a positive relationship between host size and male *A. melinus* size probably male fitness is not strictly correlated with parasitoid size (Reeve, 1987). As a result, *A. melinus* perhaps is not so selective in terms of host size when it parasitizes small hosts to allocate male eggs. On the contrary, a highly selective behavior at large size classes where females are allocated seems to be of advantage, resulting in positive size-dependent parasitism in these size classes.

In addition to the allocation of females to high quality hosts and males to low quality hosts and to the existence of a threshold for the production of females, Charnov´s et al. (1981) model makes a further prediction. The model suggests that the threshold for the production of females is not absolute but relative to the distribution of host sizes available. If sex allocation was on a relative basis, then *A. melinus* would lower the threshold for the production of females when relatively small hosts are available. Under these conditions, we would expect the shift towards female biased sex ratios to be produced in smaller size classes. Our results suggest that this is not the case, since no differences in sex ratios were detected when the size distributions of the hosts available differed substantially. Therefore, apparently *A. melinus* allocates progeny on a basis of absolute host size. Hare and Luck (1991) reached the same conclusion after observing significant differences in the sex ratios of *A. melinus* recovered from four citrus cultivars. These differences in sex ratios followed differences in CRS size among cultivars; almost twice the percentage of female *A. melinus* were produced in lemon cultivars where CRS was of bigger size. Additional indirect evidence supporting the absolute threshold argument is provided by the almost identical host size at which sex ratio turns female biased between this (0.4 mm^2) and other studies (0.39 mm^2) (Luck and Podoler, 1985; Yu, 1986) that apparently were conducted under very different conditions of host quality.

Finally, *A. chrysomphali* (96.8% of the scales observed) and *A. melinus* (93.2%) behaved mostly as solitary parasitoids when parasitizing CRS in the

field. These results agree with Abdelrahman (1974) who found the proportion of single eggs higher for *A. chrysomphali* than for *A. melinus* when comparing the oviposition behavior of the two parasitoids in the laboratory. Both species, behaved as gregarious depending on host size. According to our data, two *A. chrysomphali* emerged from hosts bigger than 0.498 mm^2. To our knowledge, no other study has previously linked gregariousness and host size use by *A. chrysomphali* in the field. For *A. melinus* gregariousness was expressed in hosts bigger than 0.480 mm^2. These results corroborated those previously reported for this species in lab and field (Luck et al., 1982; Yu, 1986; Luck and Podoler, 1985). However, Luck and Nunney (1999) reported that 10 to 15% of the hosts parasitized in California by *A. melinus* received more than one eggs. This is almost twice as much as in our study. Given that gregariousness increases with increasing host size, these results suggest that the scale is bigger in California citrus.

Overall, our results show that the size of CRS explains, at least in part, the lack of biological control by the naturally occurring *A. melinus* in Western Mediterranean citrus. Moreover, they confirm that *A. chrysomphali* is less efficient than *A. melinus* under Mediterranean conditions where the former is native. The importance of *A. melinus* for the control of CRS has been thoroughly documented in numerous studies when the parasitoid occurs naturally and when used in augmentative releases (Moreno and Luck, 1992; Luck et al., 1996; Grafton-Gardwell and Reagan, 1995). Thus, we suggest that augmentative releases of this parasitoid should be carried out to control the scale in Western Mediterranean citrus. According to our data, innoculative releases of *A. melinus* should begin in early spring and continue periodically until the end of May, a period in which suitable host sizes for the production of female *A. melinus* are available. Moreover, reduction of scale populations during that period will result in fewer crawlers, potential invaders of the fruits in July. Afterwards, during summer, CRS size, especially host sizes suitable for the production of female *A. melinus* (third instars), decrease dramatically, resulting in lower parasitism levels. Therefore, additional innundative releases of *A. melinus* in summer should be made again since August, when fruit infestation becomes evident, and continue in fall depending on fruit infestation and phenology. For example, for varieties that are harvested late in the season, there is time for augmentative releases of *A. melinus* until late fall. During that period, CRS size increases again and parasitoid efficiency may improve resulting in lower fruit infestation at harvest. Augmentative releases of *A. melinus* together with other environmental friendly strategies such as mating disruption (Vacas et al., 2009) and measures aiming to enhance parasitoid efficiency, like control of ant activity (Pekas et. al., 2010) should be considered as components of a wider strategy to control CRS.

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Chapter 5

Spatio-temporal patterns and interactions with honeydew-producing hemiptera of ants in a Mediterranean citrus orchard

Spatio-temporal patterns and interactions with honeydew-producing hemiptera of ants in a Mediterranean citrus orchard

Pekas, A., A. Tena, A. Aguilar, F. Garcia-Marí. 2010. Spatio-temporal patterns and interactions with honeydew-producing hemiptera of ants in a Mediterranean citrus orchard. Agricultural and Forest Entomology (DOI: 10.1111/j.1461-9563.2010.00501.x).

Abstract: The role of ants (Hymenoptera: Formicidae) in the citrus agro-ecosystem is controversial and understanding their ecology may help to clarify their function. In the present study, we determined the daily and seasonal foraging patterns, the spatial distribution, the feeding sources and the associations with honeydew producing hemiptera of three ant species that forage on citrus canopies. The dominants *Pheidole pallidula* (Nylander) and *Lasius grandis* Forel foraged in mutually exclusive territories within the field, but they both share their territory with the subordinate *Plagiolepis schmitzii* Forel, a distribution pattern known as "ant mosaic". The observed mean overlap for the spatial distribution was significantly lower than the generated by null models providing strong evidence of spatial interspecific competition especially between the two dominants. Ants ascended to the canopies from April until November. Colony nutritional requirements and temperature are likely to shape their seasonal foraging patterns. The daily activity pattern of *P. schmitzii* was strictly diurnal whereas *L. grandis* and *P. pallidula* were active during the entire day. Ants' diet on the canopies consisted principally of hemipteran honeydew while citrus nectar and predation/scavenging did not represent important food sources. More than 60% of the total honeydew sources, and 100% of the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) colonies, were tended by ants during spring and summer.

5.1. Introduction

Many hemiptera of the order Sternorrhyncha produce a sugar-rich excretion called honeydew. The majority of the ant species tend hemiptera to some extend (Hölldobler and Wilson, 1990). Ants collect honeydew to cover a major part of their carbohydrate requirements, when alternative sources e.g. extrafloral nectaries are not present (Gray and Oliveira, 2007). On the other hand, honeydew-producers benefit from ant-attendance in terms of protection from their natural enemies, higher growth rates, improved hygiene conditions, transport and dispersal (Way, 1963; Buckley, 1987; Stadler and Dixon, 2005). However, ant–hemipteran interactions may have broader ecological consequences (reviewed by Styrsky and Eubanks, 2007). For example, ant-attendance may have a negative or positive effect on plant health. Negative effects include the transmission of plant pathogens (Buckley, 1987) whereas positive effects for the plant health occur as a result of increased ant predation of other herbivorous insects (Skinner and Whittaker, 1981). The ant-hemiptera interaction may also influence the abundance and distribution of other herbivores (Flanders, 1945; Haney et al., 1987), and modify the population dynamics of the natural enemies not only of the associated hemiptera (Daane et al., 2007), but also of coinciding hemiptera that do not produce honeydew and are not involved in the mutualism (James et al., 1999).

Mediterranean ant communities are organized in hierarchies of dominant and subordinate species where interspecific competition and tolerance to physical factors leads to temporal (daily and seasonal) separations in foraging activity (Cros et al., 1997; Cerdá et al., 1997; Cerdá et al., 1998; Retana and Cerdá, 2000). Various studies have examined the ant community present in Mediterranean citrus ecosystem (Rosen, 1967; Panis, 1981; Tumminelli et al., 1996; Vanaclocha et al., 2005; Alvis and García-Marí, 2006; Urbaneja et al., 2006; Cerdá et al., 2009). However, the temporal and spatial interspecific interactions as well as the feeding sources of ants on citrus canopies remain unclear. What it is known from other systems is that seasonal changes in honeydew demand (Sudd and Sudd, 1985) as well as the quantity and quality of honeydew (Völkl et al., 1999, Mailleux et al., 2003) may influence ant activity and behavior.

In the citrus ecosystem, ants carry out different ecological functions that may affect the dynamics of citrus production. Although *Oecophylla* sp. workers have been employed to suppress pest populations since ancient times (Way and Khoo, 1992), it is generally thought that ants disrupt (directly or indirectly) the biological control of citrus pests because very often they have been associated with population outbreaks of their honeydew-producing mutualistic partners (Bartlett, 1961; Itioka and Inoue, 1996; James et al.,

1999), as well as other herbivores not directly associated (Moreno et al., 1987).

Thus, in this paper we examine: i) the ants' daily and seasonal foraging patterns on citrus canopies; ii) their temporal and spatial interspecific interaction; iii) the quantification of the temporal and spatial niche overlap between the ant species iv) their main feeding sources; iv) the role of citrus nectar on their seasonal activity; and v) their associations with the honeydew producing hemiptera naturally occurring in citrus orchards.

5.2. Materials and methods

Study area

We conducted our study in a 20 years old citrus orchard *Citrus cinensis* (L.) Osbeck, variety Navelina), approximately 0.6 ha, located 30 km south of Valencia, at the center of the main citrus growing area of Spain (39º 12´ 2´´ N, 0º 20´ 52´´ W). The climate is of a Mediterranean type with mild winters and dry summers. Management practices consisted in mowing in May every year and the orchard was furrow-irrigated. From May to October soil was maintained free of weeds by applying herbicides locally (Finale®, active ingredient: glufosinate ammonium). Between mid autumn (November) and spring (April) *Oxalis pescaprae* L. was the main plant species present in the understory. No chemical treatments were applied for pest control during the last six years.

A total of 64 trees belonging to four plots were sampled. Each plot contained 16 trees (four rows by four trees) and was separated from the adjacent plot by four rows of buffer trees. Ants nested in the ground near each trunk. To ensure that the tree trunk was the only access into the tree we trimmed the ground vegetation and lightly pruned all the test trees to prevent branches from touching the ground. We also trimmed the tree branches from the test plots that were interlaced with those from the buffer rows. We continued pruning and trimming of the vegetation during the study when necessary.

Seasonal and daily foraging patterns

Ant activity was quantified as the number of ants moving up and down the tree trunk during a 2min period. Ants climbing the trees were identified to the species level. To determine the ants´ seasonal foraging pattern we counted ant activity once a month, from March to December during two years (2007 and

2008). We did not sample during winter because ant activity ceases during this period in the study area (Alvis and Garcia-Marí, 2006; Urbaneja et al., 2006). Observations were made between 10:00 hours and 12:00 hours.

To determine the daily foraging pattern we monitored ant activity at 2-hour intervals over a 24 hour period in the 64 trees. Observations were conducted over a 2-day period during representative days for spring, summer and autumn 2008: on 22 and 24 of May (mean daily, minimum and maximum temperatures in the canopy were 21 ^{o}C, 13 ^{o}C and 30 ^{o}C, respectively), 14 and 24 of July (25^{o}C, 18^{o}C and 34^{o}C) and 4 and 6 of November (16 ^{o}C, 10 ^{o}C and 22 ^{o}C).

In order to assess the factors (interspecific competition or environmental variation) that shaped the observed patterns of ant activity and spatial distribution we used null model analyses (Gotelli and Graves, 1996). We compared the observed patterns with those expected from a community where species are randomly associated one with another. We measured the species overlap in activity patterns (seasonal and daily) and spatial distribution using the Czechanowski index (Albrecht and Gotelli, 2001):

$$O_{12} = O_{21} = 1 - 0.5 \left(\sum^{n}_{i=1} \left| p_{i1} - p_{i2} \right|\right)$$

where O_{12} is the overlap of species 1 on species 2; p_{i1} and p_{i2} are the respective proportions of resource utilization in the period i of the species 1 and 2 respectively. For species having identical resource use the index approaches 1.0 while for species that do not share resources the index approaches 0. We used the data from our observations to construct matrixes where rows represented ant species and columns resource states (trees, months, hours). All calculations were performed using EcoSim (Gotelli and Entsminger, 2009). We examined the following patterns of overlap:

1. Spatial overlap: the entries in the matrix consisted of the abundance of each species summed over the 24 hour observations (rows) on each of the 64 trees (columns). The data obtained for spring, summer and autumn 2008 were analyzed separately.
2. Seasonal activity overlap: entries in the matrix consisted of the abundance of each species (rows) every month (columns). Data for 2007 and 2008 were analyzed separately.
3. Daily activity overlap: entries in the matrix consisted of the abundance of each species (rows) at each hour of observation (columns). Data for spring, summer and autumn 2008 were analyzed separately.

We calculated the observed mean and variance for every data matrix. Then we compared the observed means and variances with those derived from

1000 simulated communities. For the seasonal activity overlap we considered that all resource states (trees) could be used by all species, thus we used the algorithm RA3 from Albrecht and Gotelli (2001). In this algorithm the niche breadth of the species is retained but it allows utilization of the potential resource states. For the spatial and daily activity overlap we considered that species could not occur on a tree or hour that it was not found during the 24hr observations. Thus, we used the algorithm RA4 from Albrecht and Gotelli (2001) where only the non-zero entries in each row of the matrix were reshuffled. All simulations were performed using EcoSim (Gotelli and Entsminger, 2009). We calculated the two-tailed probability that the observed means and variances were different than the simulated. Interspecific competition should cause mean niche overlap to be less than expected by chance while abiotic factors (temperature) should cause mean niche overlap to be greater than expected (Albrecht and Gotelli, 2001). Also, variance in the niche overlap superior than the expected informs about a guild structure in the community studied (Albrecht and Gotelli, 2001).

Food sources on the canopy

Nectar

We tested if citrus floral nectar represents a food source for ants. We checked for the presence of ants feeding on citrus floral nectar in 40 flowers on each one of the 64 test trees (10 flowers at various heights at each of the four quadrants; 2560 flowers in total). This sampling took place on 20 of March of 2008 (when the majority of the flowers were open) between 10:00 and 17:00 hours. Mean daily, minimum and maximum temperatures in the canopy were 13.2^{o}C, 9.5^{o}C and 17^{o}C, respectively. We also monitored ant activity every two hours over a 24-hour period before (March 13th) and during the flowering period (March 20th) to check if ant activity increased during the flowering period. For each ant species differences in activity before and during the flowering period were tested by ANOVA with sampling date (before vs. during flowering) as the main factor. Alternative nectar sources were not present during the flowering period since citrus do not posses extrafloral nectaries (Agustí, 2000) and *O. pescaprae* flourishes between December and February.

Solid food items

We collected all the ants that were descending the trees carrying solid food items between their mandibles during the 2 min observations of the daily activity (24 hour observations at two hour intervals in May, July and November 2008; for details see above). Ants and solid items were sampled

with a hand aspirator, stored in 70% ethanol and transferred to the laboratory for identification.

Hemipteran-honeydew

To determine the ant-hemiptera associations each of the 64 trees was examined for a maximum of 15 min or until four honeydew sources were found. In each case we recorded the species and number of individuals of the honeydew producing hemiptera and, if present, the species and number of the attending ants. These observations were carried out between 10:00 and 18:00 in spring, summer and autumn (within the same week that the ant's daily activity). Care was taken to ensure that trees were never observed at the same hour among the sampling days in order to avoid bias due to the ants' daily activity rhythm. Differences in occupation of the honeydew sources by ants among the sampling days were tested by a chi-square contingency test.

We considered the number of ants per honeydew source (absolute ant-attendance) as a measure of potential honeydew preference because ants respond more intensively to a more profitable source (see Mailleux et al., 2003). Also, the number of attending ants divided by the number of hemipterans per honeydew source (relative ant-attendance) was calculated because the effectiveness of ant protection varies with hemipteran density (Itioka and Inoue, 1996; Harmon and Andow, 2007). In both cases the intensity of ant-attendance was compared by one-way ANOVA, with the hemiptera species as the main factor. The significance level was adjusted by sequential Bonferroni correction.

5.3. Results

Ant species

We found four ant species foraging for food on the tree canopies. The most abundant species was the dimorphic ant *Pheidole pallidula* (Nylander) (Myrmicinae), with a total of 5964 and 3989 individuals counted, representing 67% and 54% of the total number of foraging ants in 2007 and 2008 respectively. It was foraging on trunk trails. This species was present in the major part of the study area with maximal incidence in August (presence on 82% of the trees). The second most abundant species was *Plagiolepis schmitzii* Forel (Formicinae) with 2206 (25%) and 2609 (36%) individuals counted in 2007 and 2008 respectively. Its tiny workers exhibited an individual foraging on the tree trunk. Maximal incidence for this species was registered in May and June samplings (presence on 90% of the trees). The third most important species in terms of abundance was *L. grandis* Forel (Formicinae) with 702

(8%) and 691 (9.5%) of individuals counted in 2007 and 2008 respectively. *Lasius grandis* workers were seen foraging individually on the tree trunk. This species was found in a smaller territory, (14 out of the 64 trees sampled) with maximal incidence in May-June. We also found the species *Tapinoma nigerrimum* (Nylander) (Dolichoderinae) visiting the trees but only rarely (less than 0.5% of the total ants counted in 2007 and 2008) and therefore it was excluded from the analysis.

Spatial distribution

The observed mean overlap for the spatial distribution was significantly lower than the generated by null models in spring, summer and autumn (Table 1) providing strong evidence of spatial interspecific competition. This was particularly evident for the two dominants *L. grandis* and *P. pallidula* that were found in separate territories and had a very low overlap in their spatial distribution (Czechanowski index *L. grandis*-*P. pallidula*: 0.0088, 0.0095 and 0.01 for spring, summer and autumn respectively). The subordinate *P. schmitzii* was regularly found on the same tree with one of the two dominants, mostly with *P. pallidula* (Czechanowski index *P. schmitzii* -*P. pallidula*: 0.318, 0.443 and 0.29 for spring, summer and autumn; *P. schmitzii* –*L. grandis*: 0.296, 0.232 and 0.124).

Although not conclusive, our data provide no evidence for guild structure in spatial distribution because the observed variances of the spatial overlap did not differ significantly from the expected (Table 1).

Table 1. Observed and expected mean and variance of spatial overlap of the canopy foraging ants. The expected values for mean and variances were calculated from niche overlap indices from 1000 randomized data sets using the algorithm RA3 (see Material and methods for details).

Season	Observed vs expected means of spatial niche overlap	Tail probability	Observed vs expected variance of spatial niche overlap	Tail probability
Spring	0.208 < 0.245	0.025	0.029 > 0.019	0.086
Summer	0.228 < 0.308	0.001	0.047 > 0.039	0.206
Autumn	0.141 < 0.177	0.033	0.019 < 0.022	0.415

Patterns of seasonal and daily activity

Ants were active on citrus canopies from April until November in the two years of the study (Figure 1). Activity of both *L. grandis* and *P. schmitzii* peaked in late spring-early summer. A small second increase in their activity was registered in August, for *L. grandis* and in September-October (2008) for *P.*

schmitzii. The observed mean of the overlap for the seasonal activity was significantly greater than the expected for the two years of observations (Table 2) suggesting that environmental variation rather than competition shaped the ants' activity patterns. Also, the observed variance did not differ significantly from expected (Table 2).

The three species exhibited distinct daily activity patterns that varied throughout the year (Figure 2). *Plagiolepis schmitzii* was exclusively active during daytime avoiding foraging during the night. This diurnal pattern peaked between 12:00 and 14:00 hours. *Lasius grandis* was active during the whole day displaying a clearly nocturnal peak around 22:00 hours. This pattern was repeated in spring, summer and autumn. The most abundant species, *P. pallidula*, displayed a more complex pattern. In the spring it was active during day and night and it did not exhibit a clear activity peak. During the summer and autumn we observed a shift in the daily activity rhythm towards nocturnal foraging. Peak activities were registered between 22:00 hours and 2:00 hours in summer and around 20:00 hours in autumn sampling (early November). Major workers were also seen foraging in the canopies mostly during night but they represented only a very small fraction (0.7 %) of the whole forager's population.

The observed mean of the overlap for the daily activity varied among seasons (Table 2). In spring, it was significantly higher than the expected by chance meaning that daily activity patters were influenced by abiotic factors. In summer and autumn, the observed mean of the overlap for the daily activity did not differ from the expected.

The observed variance was significantly higher than the simulated in summer and autumn revealing a guild structure of the species studied (Table 2). In spring variance did not differ from random.

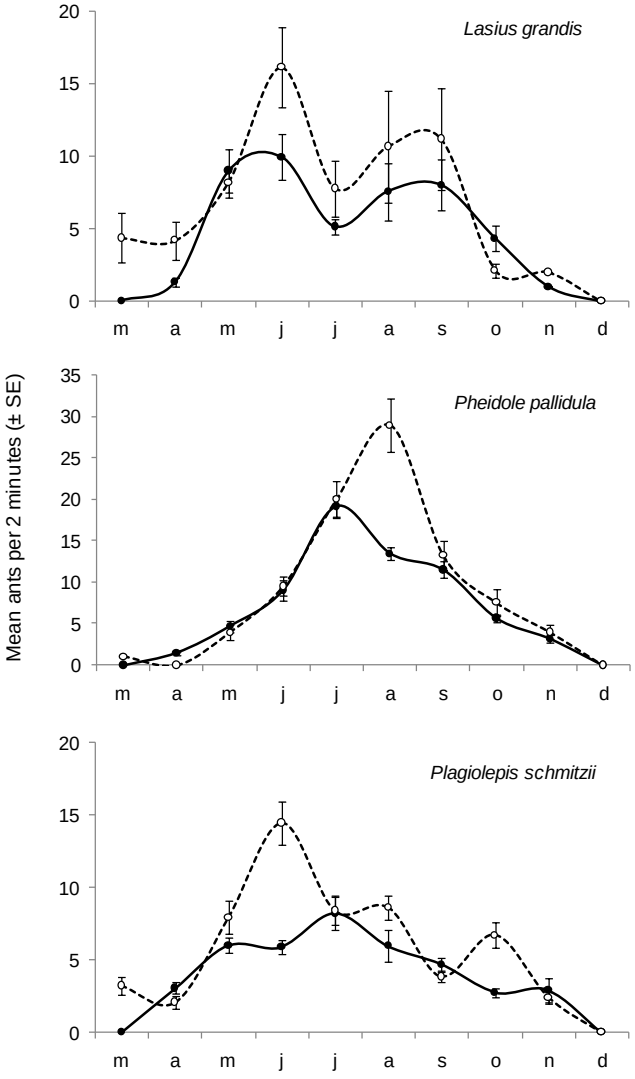

Fig. 1. Seasonal activity of *L. grandis, P. pallidula* and *P. schmitzii* foraging on citrus canopies in 2007 and 2008 based on monthly observations between 10:00 and 12:00 hours. Activity is represented as the mean (± SE) of the ants ascending and descending the tree trunk during two minutes. We only considered those trees on which there was activity during the monthly observation for each species. Closed circles and continuous line 2007 (*L. grandis*: 14, *P. pallidula* 52 and *P. schmitzii* 38 trees); open circles and dashed line 2008 (*L. grandis* 19, *P. pallidula* 53 and *P. schmitzii* 58 trees).

Table 2. Observed and expected mean and variance of A) seasonal and B) daily activity overlap. The expected values for mean and variances for daily and seasonal activity are calculated from niche overlap indices from 1000 randomized data sets using the algorithm RA3 and RA4 respectively.

	Observed vs expected mean of niche overlap	Tail probability	Observed vs expected variance of niche overlap	Tail probability
A) Seasonal activity				
YEAR				
2007	0.668 > 0.496	0.006	0.007 > 0.011	0.424
2008	0.606 > 0.461	0.02	0.03 > 0.014	0.087
B) Daily activity				
SEASON				
Spring	0.774 > 0.734	0.041	0.005 < 0.006	0.469
Summer	0.659 < 0.670	0.442	0.028 > 0.015	0.045
Autumn	0.549 > 0.508	0.194	0.079 > 0.025	0.010

Feeding sources on the canopy

Nectar

Nectar from citrus flowers did not represent an important feeding source for any of the three species. The number of ants found feeding on nectar was extremely low. We registered only three *L. grandis* workers imbibing nectar out of 2560 (0.1%) flowers checked. Moreover, we found no significant differences in ant-activity before and during flowering for none of the three species visiting the canopies (one way ANOVA, ant activity before vs. during flowering, MEAN ± SE: *L. grandis*, 12.2 ± 3.7 vs. 11.5 ± 3.95, $F_{1,19} = 0$, $P = 0.95$; *P. pallidula,* 2.33 ± 1.41 vs. 1.33 ± 0.54, $F_{1,16} = 1.14$, $P = 0.3$; *P. schmitzii*, 5.2 ± 0.85 vs. 5.52 ± 1.08, $F_{1,53} = 0.06$, $P = 0.81$).

Solid food items

Similarly, the proportion of the solid food items carried to the nest was very low. We observed only 42 cases (out of more than 26000 ants observed ascending and descending the trees) of workers descending the tree trunks with solid items between their mandibles during the whole study period. *Pheidole pallidula* was registered in 20 of the above cases (representing 0.12% of the total *P. pallidula* workers observed), *P. schmitzii* 14 (0.24%) and *L. grandis* 8 (0.22%). The type of food items collected consisted principally of fragments of other arthropods: Araneida (8 cases), Diptera (6), Hemiptrera

(5), Neuroptera (4), Psocoptera (4), Hymenoptera (3), Acarida (1), Thysanoptera (1), plant material (2), unidentified (8).

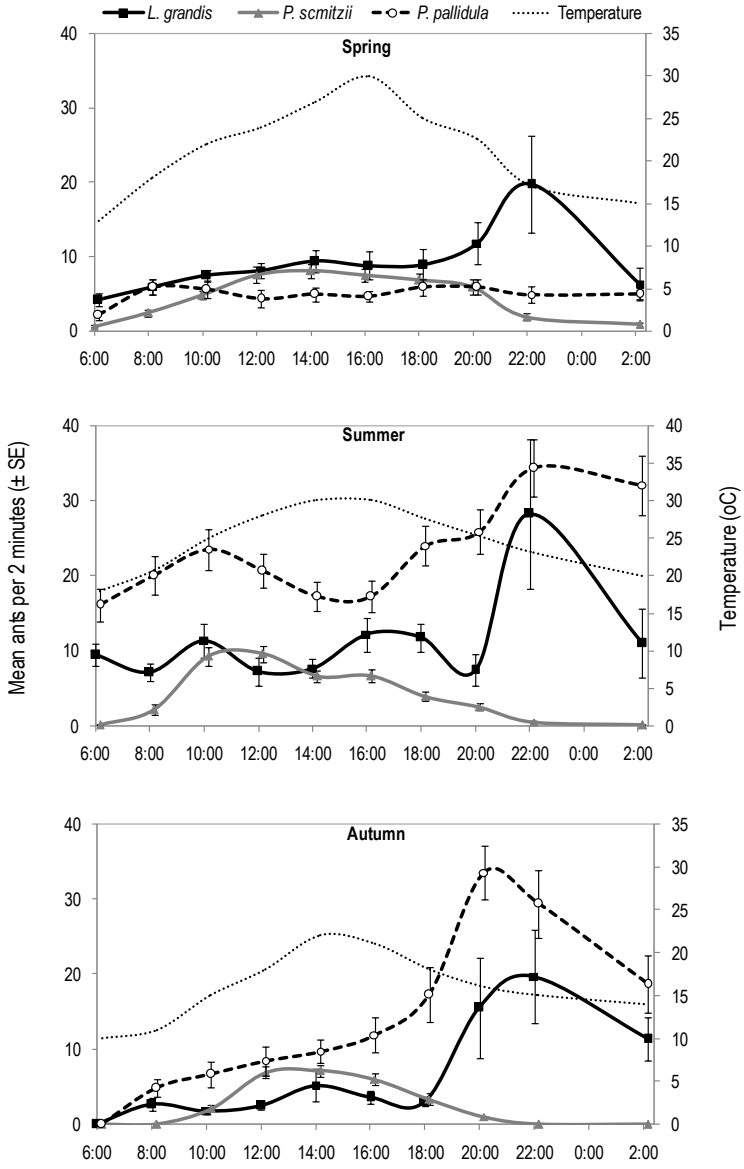

Fig. 2. Air temperature under the canopy every sampling hour and daily activity of *L. grandis, P. pallidula* and *P. schmitzii* foraging on citrus canopies in spring, summer and autumn 2008. Activity is represented as the mean (± SE) of the ants ascending and descending the tree trunk during two minutes. We only considered those trees on which there was activity along the day for each species (*L. grandis* 19, *P. pallidula* 53 and *P. schmitzii* 58 trees).

Hemipteran-honeydew

Hemipteran-honeydew was the principal food source for the ants on the canopies. The three ant species were found in trophobiotic association with honeydew-producing hemiptera in the canopies. Ants were observed collecting honeydew throughout the day, an activity easily identifiable because of their expanded gasters. Honeydew sources were always available during the observation period, although their abundance varied along the year. In summer and autumn, there was at least one honeydew source on the canopy of every individual tree examined (64 trees examined see materials and methods). In spring, this occurred on 85% of the trees.

The percentage of the honeydew sources occupied by ants varied also among the seasons. Thus, it was significantly higher in spring (62%) and summer (73%) than in autumn (23%) (chi-square contingency test: spring vs. summer, $\chi^2_1 = 0.59$, $P = 0.44$; spring vs. autumn, $\chi^2_1 = 19.19$, $P < 0.0001$; summer vs. autumn, $\chi^2_1 = 36.91$, $P < 0.0001$). Pooling the data from all the sampling dates, ants occupied half of all the available honeydew sources observed (Table 3). The most attended trophobiont was the citrus mealybug *Planoccocus citri* (Risso) (Hemiptera: Pseudococcidae) with 100% of its honeydew sources occupied by ants. By contrast only 12% of the honeydew sources produced by *Aphis spiraecola* Patch (Hemiptera: Aphididae) were ant-attended.

Table 3. Total number of hemipteran honeydew sources observed (Obs) and attended (Att) by ants in citrus canopies in spring, summer and autumn 2008 and overall percentage of attended honeydew sources (% Att).

Hemiptera species	Spring		Summer		Autumn		% Att
	Obs	Att	Obs	Att	Obs	Att	
Planococcus citri	3	3	15	15	0	0	100
Aleurothrixus floccosus	4	4	127	101	128	41	56
Icerya purchasi	21	15	28	15	6	0	55
Ceroplastes cinensis	39	25	16	5	1	0	54
Saissetia oleae	35	16	2	2	0	0	49
Aphis spiraecola	0	0	1	1	83	9	12
Total	102	63	190	139	218	50	49

Absolute ant-attendance (number of ants per honeydew source) and relative ant-attendance (number of attending ants divided by the number of hemipterans in each honeydew source) varied with hemipteran species (Figure 3). Thus, honeydew sources produced by *P. citri* were the most intensively visited by *L. grandis*, with an average of six ants per *P. citri* honeydew source (one-way ANOVA $F_{4,87} = 4.34$, $P = 0.003$). The maximum relative ant-attendance for *L. grandis* was observed for *P. citri* honeydew (2.65 ± 0.76 ants per *P. citri* individual) and the minimum for *Aleurothrixus floccosus*

(Maskell) (Hemiptera: Aleyrodidae) honeydew (0.26 ± 0.03) (Figure 3) (one-way ANOVA $F_{4,87} = 10.83$, $P < 0.0001$).

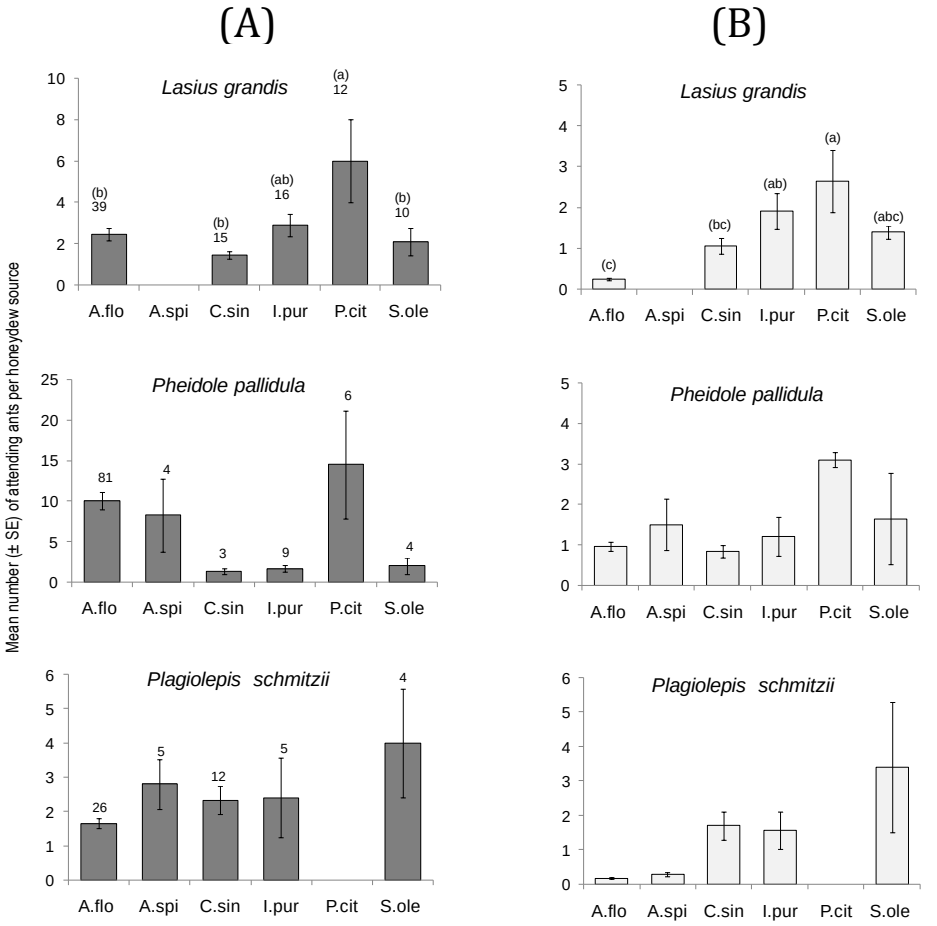

Fig. 3. (A) Absolute ant-attendance (total number of ants per honeydew source) and **(B)** relative ant-attendance (total number of attending ants divided by the number of hemipterans per honeydew source) (mean± SE) for *L. grandis*, *P. pallidula* and *P. schmitzii* when attending six hemipteran species feeding on citrus. Values above columns represent number of observations. Means sharing the shame letter do not differ significantly (one way ANOVA with the significance level adjusted by sequential Bonferroni corrections). A.flo= *Aleurothrixus floccosus*, A.spi= *Aphis spiraecola*, C.sin= *Ceroplastes sinensis*, I.pur= *Icerya purchasi*, P.cit= *Planococcus citri*, S.ole= *Saissetia oleae*.

Although the unequal sample size of the observations did not permit us to perform a statistical analysis, the relative and absolute ant-attendance for *P. pallidula* and *P. schmitzii* tend to be more intense when recollecting honeydew of *P. citri* and *Saissetia oleae* (Olivier) (Hemiptera: Coccidae) respectively.

We never observed any co-occurrence of different ant species on the same honeydew source.

5.4. Discussion

Spatial distribution

Interspecific competition has been found to be a key mechanism in structuring ant communities (Hölldobler and Wilson, 1990). Species can lessen competition pressures by segregating resources like food or territory (Hölldobler and Wilson, 1990). The results in our study provide strong evidence for territoriality since the observed mean spatial overlap was always significantly lower than the expected by random (see Albrecht and Gotelli, 2001). This outcome was influenced by the extremely low spatial overlap between the two dominants. Indeed, *L. grandis* and *P. pallidula* established in mutually exclusive territories and were very rarely found foraging on the same tree. This may be the result of a mutual aversion or exclusion by means of active fighting until the two species remained in clearly separated areas (Hölldobler and Wilson, 1990). This spatial separation permits the two species to exploit similar food sources on the canopies (hemipteran honeydew) and highly overlap in their daily activity rhythms. The subordinate *P. schmitzii* was found foraging on the same trees with one of the two dominants suggesting that the later defended spatio-temporal rather than absolute territories (*sensu* Hölldobler and Wilson, 1990). This community structure where dominants maintain mutually exclusive territories and, additionally, a set of non-dominant or submissive species coexist with each of the dominants, has been described as "ant mosaic" (Majer, 1972; Blüthgen and Stork, 2007). This mosaic distribution could also be the result of processes other than competition, like for example habitat type (Ribas and Schoereder, 2002). In our study we registered only three ant species (four if we include *T. nigerrimum*), an extremely poor diversity especially if it is compared with natural communities in nearby areas (see Cerdá et al., 2009). Thus, the observed distribution might be considered an effect of habitat homogenization typical in agricultural ecosystems rather than competition between the dominants. However, we consider that this is not the case since *P. pallidula* has been described as dominant in several studies of ant communities conducted in natural areas in the Mediterranean (see Cerdá et al., 1998; Retana and Cerdá, 2000; Santini et al., 2007). To our knowledge, there are not specific studies for the *L. grandis* dominance status in natural ecosystems; however, it is considered as aggressive as its sibling *L. niger* (Seifert, 1992), which is highly dominant (Hölldobler and Wilson, 1990). Moreover, a study conducted in a cork oak system, at ant community level, showed that soil type strongly influenced *P. pallidula* capacity to colonize a territory, especially when facing

competition with dominant (invasive) species (Way et al., 1997). In our study, the soil type was apparently uniform across the orchard.

Patterns of seasonal and daily activity

Tolerance to physical factors, interspecific competition, and colony nutritional needs have been found to determine foraging rhythms in ants (Fellers, 1989; Cerdá et al., 1998). In our study, interspecific competition does not structure the activity patterns of the three ant species studied since the seasonal activity overlap was significantly greater than the expected by chance for the two years studied. Temperature is considered the principal abiotic factor determining temporal patterns in ants (Fellers, 1989; Cros et al., 1997; Albrecht and Gotelli, 2001). In our observations temperature may also influence the seasonal patterns observed since the activity of the three species ceases during winter months (Alvis and Garcia-Marí, 2006; Urbaneja et al., 2006). Moreover, *P. schmitzii* decreased its activity during the hottest months whereas *P. pallidula* peaked in summer, probably because it is of Mediterranean origin and therefore it might be better adapted to high summer temperatures (Palacios et al., 1999). However, the activity patterns that we observed might be also associated with foraging for food in the canopies that in turn reflects the nutritional demands of the colony. In all three species maximal activity coincided with their mating period in the study area (personal observations). In citrus and other ecosystems several authors have documented a similar coincidence between maximal foraging activity on the trees and colony reproduction for other ant species (Markin, 1970b; Sanders, 1972; Abril et al., 2007). Probably, carbohydrates in the form of hemipteran honeydew may supply the workers with the additional energy required during this period. For *L. grandis* and *P. schmitzii*, the number of workers foraging on the canopies decreased during July and August. It is likely that a shift towards a more protein-based diet was produced associated with the presence of developing larvae in the nest (Edwards, 1951). The slight increase in activity observed for these two species in autumn may be due to honeydew recollection that will be stored as lipid in the fat-body of workers for the overwintering period (Sudd and Sudd, 1985). We did not notice such an increase for *P. pallidula* perhaps because of its omnivorous diet (Detrain, 1990; Retana et al., 1992; Cerdá et al., 1997); probably it obtains the required energy for surviving during winter from other sources.

The daily activity of *P. schmitzii* was strictly diurnal while *L. grandis* and *P. pallidula* were active during the entire 24 hour period. Additionally, the activity of *L. grandis* and *P. pallidula* was maximal at the beginning of the night. This daily pattern might be attributed to a foraging strategy for maximizing honeydew collection. *Lasius grandis* is well-known for its habit of obtaining a

substantial portion of its diet from hemipteran honeydew (Paris and Espadaler, 2009). Thus, as a honeydew-dependent and dominant species, *L. grandis* is expected to use the best honeydews, in terms of quantity and quality, present on the citrus canopies. Degen and Gersani (1989) reported that for weaver ants fresh matter honeydew collection is minimum during the hottest part of the day because of water evaporation from honeydew and maximum in the morning and night. Thus, considering that the number of foraging ants is directly related with the honeydew production (Mailleux et al., 2003) the maximum of fresh honeydew in combination with air temperature could be the reason why *L. grandis* is more active at the beginning of the night in citrus canopies. Although *P. pallidula* is not so strictly dependent on hemipteran honeydew because of its omnivorous diet (Detrain, 1990; Retana et al., 1992; Cerdá et al., 1997), its daily activity pattern on the canopies, where honeydew is the main food source, might also be attributed to a strategy for maximizing honeydew collection.

Finally, the seasonal activity patterns are based on monthly observations between 10:00 and 12:00 hours, consequently the daily activity pattern of each species is not taken into account. Obviously, this might lead to over (for *P. schmitzii* exhibits maximal activity between 10:00 and 12:00 hours) or underestimation (*L. grandis*, *P. pallidula*) of the influence of month on the total foraging effort.

Food sources on the canopy

Honeydew represents a spatially and temporally constant resource with a relatively high nutritional value (Yanoviak and Kaspari, 2000; Blüthgen et al., 2000, 2004) and was evidently the main food source for the canopy foraging ants in this study. The importance of arthropod exudates in ant diet has been pointed out by Tobin (1994). Moreover, we never observed ant species sharing the same honeydew source. As suggested by Blüthgen et al. (2000, 2004), the exploitation of a rich and predictable resource like honeydew apparently compensate the costs invested for resource monopolization.

Among the honeydew producers, the mealybug *P. citri* was always attended by the dominant ants *L. grandis* and *P. pallidula*. This result suggests a potential ant preference for the honeydew produced by *P. citri*. Markin (1970a) observed the same preference for workers of the Argentine ant *Linepithema humile* Mayr (Hymenoptera: Formicidae) in California citrus. Several factors may explain this potential preference. As demonstrated by Völkl et al. (1999) and Mailleux et al. (2003) honeydew quality and quantity influences ant foraging decisions. Thus, *L. grandis* and *P. pallidula* may prefer to attend *P. citri* because it might produce a higher quantity of honeydew than

other hemiptera present in citrus canopies. The other possible non-excluding factor might be the chemical composition of *P. citri* honeydew, which may attract or be phagostimulant for ants. For example, Völkl et al. (1999) showed that over two aphid species with similar honeydew production, *Lasius niger* L. ants attended more the one with higher sugar concentration and with presence of oligosaccharides in its honeydew. Finally, the subordinate *P. schmitzii* was also found tending hemiptera. However, its honeydew preference-ranking was completely different than the one observed for the dominants. This might be a result of either different nutritional requirements or competitive exclusion.

Of particular interest is the fact that natural enemies also use honeydew in the field (see Wäckers and Steppuhn, 2003; Heimpel et al., 2004; Steppuhn and Wäckers, 2004; Wäckers, 2008). Given that citrus do not possess extrafloral nectaries and citrus floral nectar is available only during the end of March and the beginning of April, honeydew represents the predominant sugar source for natural enemies during the rest of the year. In our study, honeydew exploitation by ants was continuous not only along the day but also during the season (from April to November), being high during spring and summer when 62 and 73% of the honeydew sources, respectively, were occupied by ants. Further research is needed to examine if the ant-hemiptera interaction, under circumstances of reduced sugar availability that is usual in agro-ecosystems (Wäckers, 2005; 2008), has the potential to affect carbohydrate availability for natural enemies and consequently the efficacy of biological control.

Ants supplement their diet with protein either by preying or scavenging, usually on other arthropods (Carrol and Jansen, 1973). Predation by ants on almond tree canopies has been found to be an important cause of mortality for adult parasitoids (Heimpel et al., 1997). Paris and Espadaler (2009) in a study on holm oak trees reported that almost 10% of the workers of *L. grandis* carried items (insects, part of insects) back to their nests. However, during our observations we collected an extremely low number of workers descending from the canopy carrying solid food. Furthermore we consider that ants acted more as scavengers since the majority of the collected items were fragments of other arthropods or dried insects. A possible explanation for the low contribution of solid food to the dietary spectrum of canopy foraging ants could be that both predation and scavenging in canopy are unpredictable and energetically expensive food sources (Carrol and Jansen, 1973). Also, it is possible that no direct relationship between solid food retrieval and prey consumption exists since ants may transport prey internally after consuming insects in the place they are found. Cannon and Fell (2002) reported that although evidence for insect prey consumption was almost absent (less that

1% of the workers carrying solid items) crop-borne nitrogenous food made up nearly half of all food collected by the foragers.

None of the three ant species was seen feeding on citrus nectar. Some plants employ chemical and mechanical defenses in order to repel ants from the flowers (Ghazoul, 2001). However, we consider that the non-exploitation of citrus nectar by ants is due to temperature and/or the nutritional state of the colony rather than to plant defenses. In our observations, citrus flowered in March when ant activity was null or very low, probably because the reserves stored in the fat-body of the workers were not depleted and/or temperatures were still too low. Moreover, *L. grandis*, under green-house conditions (personal observation), and the Argentine ant *L. humile* (Markin, 1970a) have been observed feeding regularly on floral nectar, indicating that citrus does not contain mechanical or chemical defenses to repel ants.

In conclusion, the dominant ants, *P. pallidula* and *L. grandis* forage in mutually exclusive territories within the same citrus field however both coexist with the subordinate *P. schmitzii* forming an "ant-mosaic". There is ant activity on the citrus canopies continuously along the day and from April to November. Hemipteran-honeydew is the main food source for the ants on the canopies. The dominants present a potential preference for *P. citri* honeydew and the presence of fresh honeydew at the beginning of the night, when temperature is lower, might influence their daily foraging patterns. Finally, more than 60% of the total honeydew sources were tended by ants during spring and summer.

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Chapter 6

Effect of Mediterranean ants (Hymenoptera: Formicidae) on California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae) populations in citrus orchards

Effect of Mediterranean ants (Hymenoptera: Formicidae) on California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae) populations in citrus orchards

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Abstract: We conducted an ant-exclusion experiment in a citrus orchard to evaluate the overall impact of three ant species native in the Mediterranean, *Pheidole pallidula* (Nylander), *Plagiolepis schmitzii* Forel and *Lasius grandis* Forel, on populations of *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) (California red scale). The ant-exclusion was carried out in four experimental plots from March 2007 to November 2008. Another subset of four plots, adjacent to the ant-excluded plots, was used as control. We measured scale densities and percent parasitism on fruits at harvest in 2007 and 2008. Additionally, we sampled the seasonal trend of the scale on twigs and fruits in both treatments during 2008. CRS densities in the ant-excluded treatment began to be significantly lower than in the ant-allowed control in May (one month after ant activity began) and this difference increased until November. Thus, the effect of the ants on CRS density appears to be accumulative. At harvest, scale densities on fruits were significantly lower in the ant-excluded treatment. However, percent parasitism on fruits was similar between treatments. Finally, scale densities on the fruits of the ant-allowed plots were positively correlated with the number of ants that climbed to the citrus canopy. These results suggest that increases of scale densities induced by Mediterranean ants depend on the intensity of the ant-activity on citrus canopies.

6.1. Introduction

It has long been known that ants are associated with the disruption of biological control of arthropod pest species in agro-ecosystems (DeBach, 1951; Bartlett, 1961; Way, 1963; Buckley, 1987). The most frequently documented mutualistic association involves ants and honeydew-producing hemipterans, where ants collect the honeydew excreted by hemipterans and in return they offer protection against predators and parasitoids (Way, 1963; Buckley, 1987). Ants, moreover, have also been found to disrupt the activity of parasitoids and predators of hemipterans that do not produce honeydew. In citrus, Flanders (1945) demonstrated that the Argentine ant *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) disturbed the activity of the endoparasitoid *Comperiella bifasciata* Howard (Hymenoptera: Encyrtidae), resulting in higher infestations of its host, the diaspidid *Aonidiella citrina* Coquillet (Hemiptera: Diaspididae). Also in citrus, Haney et al. (1987) reported that *L. humile* promoted a population increase of the citrus red mite *Panonynchus citri* (McGregor) (Acarina: Tetranychidae) by means of interference with its predator *Stethorus picipes* Casey (Coleoptera: Coccinellidae). In almond trees, Heimpel et al. (1997) reported that predation by *L. humile* was a significant source of mortality for *Aphytis aonidiae* (Mercet) and *A. vandenboschi* DeBach and Rosen (Hymenoptera: Aphelinidae), parasitoids of *Quadraspidiotus perniciosus* (Comstock) (Hemiptera: Diaspididae).

California red scale (CRS) *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) is one of the most important citrus pests worldwide. It does not produce any type of honeydew and therefore is not tended by ants. However, it has been found that ant activity, mostly the argentine ant *L. humile*, may stimulate California red scale populations and damage by reducing the efficiency of its parasitoids (DeBach, 1951; Steyn, 1954; Moreno et al., 1987; James et al., 1997). It is assumed that ants disrupt or kill CRS parasitoids as an indirect consequence of ant-attendance to a coincident honeydew producer (DeBach, 1951; Moreno et al., 1987; James et al., 1997). Nevertheless, under laboratory conditions, Martinez-Ferrer et al. (2003) demonstrated that ants reduce parasitism rates of California red scale by its parasitoids *Aphytis melinus* De Bach (Hymenoptera: Aphelinidae) and *C. bifasciata* even when a honeydew producer is not present.

The Argentine ant is a highly invasive species that has spread in all ecosystems with Mediterranean climate causing serious problems in agriculture and natural ecosystems (Suarez et al., 2001; Carpintero et al., 2005; Daane et al., 2007). This species was first cited in Spanish citrus in 1923 associated with honeydew-producing hemipterans (Font de Mora, 1923;

Garcia Mercet, 1923). Currently, *L. humile* is present in Spanish citrus groves but it is limited to areas with high anthropogenic activity (Palacios et al., 1999, Espadaler and Gómez, 2003). The two most abundant and widely distributed ant species in Western Mediterranean citrus are the natives *Lasius grandis* Forel and *Pheidole pallidula* (Nylander) (Palacios et al., 1999; Alvis, 2003; Vanaclocha et al., 2005; Urbaneja et al., 2006; Cerdá et al., 2009). Both ants are behaviorally dominant, but they coexist within the same orchard in separate territories and have been found tending honeydew-producing hemipterans on citrus trees (see chapter 5). However, their interaction with the non-honeydew producing *A. aurantii* is unknown. This is particularly interesting given that ant aggressiveness and behavior against natural enemies varies among species (Way, 1963; Martinez-Ferrer et al., 2003; Mgocheki and Addison, 2009).

Thus, the aim of this study was to examine the effect of native Mediterranean ant species on CRS populations in citrus orchards through an ant-exclusion experiment. We, firstly, tested the ant exclusion methodology. We, then, assessed the effect of such exclusion on parasitism rates and the seasonal trend of CRS along the year by comparing ant-excluded and ant-allowed treatments. Finally, we have related the number of ants that climbed to the citrus canopies with scale densities at harvest.

6.2. Material and methods

Study site

The study was conducted in a mature orange citrus orchard *Citrus sinensis* (L.) Osbeck, (variety Navelina), of 0.6 ha, located 30 km south of Valencia, at the center of the main citrus growing area of Spain. The climate is of a Mediterranean type with mild winters and dry summers. Management practices consisted of mowing in May every year and the orchard was furrow-irrigated. From May to October soil was maintained free of weeds by applying herbicides locally. Between mid autumn (November) and spring (April) *Oxalis pescaprae* L. was the main plant species present in the understory. No chemical treatments were applied for pest control during the last six years. From a previous study (see chapter 5) we knew the ant species foraging on the tree canopies as well as their spatial distribution. In order of abundance the ant species present were *P. pallidula*, *Plagiolepis schmitzii* Forel and *L. grandis*. *Pheidole pallidula* and *L. grandis* were never found on the same tree, they established in separate trees. *Lasius grandis* was present mostly in the experimental block 4 (see below) but during late spring, when its activity peaks, it was also present on some trees of block 3. *Pheidole pallidula* was present in the experimental blocks 1, 2 and 3. The subordinate *P. schmitzii* was

present in all the experimental blocks, frequently foraging on the same tree with one of the two dominant ant species (Table 1). Ant species were identified according to Seifert (1992) (*L. grandis* workers) and Bolton (1995) (*P. pallidula* and *P. schmitzii* workers).

Table 1. Relative abundance of the three ant species in the four plots of the ant-allowed treatment, based on cumulative 24-h ant activity (ant activity counts at 2-h intervals over a 24 h period in spring, summer and autumn).

		Ant species (%)		
	Total number of ants observed	***Lasius grandis***	***Pheidole pallidula***	***Plagiolepis schmitzii***
Plot 1	939	0	81	19
Plot 2	954	0	76	24
Plot 3	2569	7	81	12
Plot 4	1611	54	20	26

Ant-exclusion and ant activity

Ant-exclusion began in March 2007 and was continued until November of 2008. The experimental design was a randomized block with four replicates of two treatments: ant-allowed or ant-excluded. Each treatment contained 16 trees (four rows by four trees). For ant exclusion, the tree trunk was wrapped with gaffer tape demarcating a zone 15 cm wide at approximately 30 cm above ground. The wrapped zone was coated with Tangle-Trap® Insect Trap Coating (Tanglefoot, Biagro, Valencia-Spain). Tanglefoot was renewed once a month. Each block was separated from the adjacent block by four rows of buffer trees. To ensure that the tree trunk was the only access of ants into the tree we trimmed the ground vegetation and lightly pruned all the ant-excluded trees to prevent branches from touching the ground. The tree branches from the test plots that were interlaced with those from the buffer rows were also trimmed. Pruning and trimming of the vegetation was continued when necessary during the study.

We defined as ant activity the number of ants moving up and down an imaginary horizontal line on the tree trunk during a 2 min period. The effectiveness of the ant-exclusion method was monitored every month, from April until November for 2007 and 2008, by comparing ant activity between the ant-allowed and ant-excluded treatments. Observations were made between 1000 and 1200 hours in all the sixteen trees of each plot. Cumulative 24-h ant activity was evaluated by sampling the four central trees in each plot, in a representative day of the spring, summer and autumn 2008. On each sampling date, ant activity was monitored at 2-h intervals over a 24-h period in every tree.

Parasitism rates of CRS on fruits

In the study site, California red scale is attacked by the native *Aphytis chrysomphali* Mercet and the introduced *A. melinus* DeBach (both Hymenoptera: Aphelinidae). We sampled a minimum of five fruits infested with CRS per tree from the four central trees in each plot in July, August and September of 2007 and 2008. The fruits were transported to the laboratory and they were processed within the next 24 h using a stereomicroscope. The number of unparasitized (considering only stages susceptible to parasitism) and parasitized CRS stages was determined. Parasitism rates on fruits were estimated as number of parasitized scales) / (number of parasitized scales + number of unparasitized scales) X 100 (Murdoch et al., 1995). A minimum of 100 susceptible stages were observed per sample. The parasitoid pupae found were identified according to their coloration (Rosen and DeBach, 1979) and, when unrecognizable parasitoid stages were found (eggs, larvae and prepupae) they were transferred to crystal vials for rearing, emergence of adults and identification (Rosen and De Bach, 1979).

CRS population densities

In November 2007 and 2008, just before harvest, CRS population levels on fruit were estimated from the four central trees on each plot by counting the number of scales on 50 randomly selected fruits per tree.

In 2008, CRS populations were periodically sampled on twigs (diameter of twigs was lower than 10 mm) and fruits. The number of scales on 20 twigs, randomly selected on each of the four central trees per plot, was counted once a month from April until November. The same procedure was applied to 20 fruits from July (when fruits were available) until November.

Statistical analysis

The effectiveness of the ant-exclusion method was tested using repeated measures analyses of variance. The effect of ant exclusion on the population densities and fruit damage caused by California red scale was calculated for each sampling date separately using generalized linear models. We assumed Poisson error variance for the number of scales per fruit. The assumed error structures were assessed by a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If we detected an over- or under-dispersion, we re-evaluated the significance of the explanatory variables using an *F* test after rescaling the statistical model by a Pearson's chi-square divided by the residual degrees of freedom (Crawley, 2007). We present the means of untransformed proportion and count data (in preference

to less intuitive statistics such as the back-transformed means of logit transformed data) (see Mayhew and van Alphen, 1999; Tena et al., 2008). We analyzed the differences in parasitism rates between treatments using Yates´ chi-square tests. Cumulative 24-h ant activity among plots where ants were present was compared using ANOVAs (assuming normally distributed error variances). An exponential function was fitted to the values of scales per fruit before harvest and cumulative ant activity on the sampling trees. All statistical analyses were conducted with the R statistical package (Ihaka and Gentleman 1996, http://www.R-project.org/).

6.3. Results

Ant-exclusion

Tanglefoot sticky barriers were effective in excluding the majority of the ants from the tree canopy. Ants were almost absent from the canopies of the ant-excluded plots during the two years of the study (repeated measures ANOVA, 2007: $F = 51.49$; d.f. = 1, 6; $P = 0.0004$; 2008: $F = 26.97$; d.f. = 1, 6; $P = 0.002$) (Figure1). Conversely, ants ascended to the canopies of control trees along the two years of the experiment. Ant activity increased steadily from April until July-August, and then it descended until November when ant activity became null.

Parasitism rates on fruits

Overall, 560 parasitized scales were collected from both treatments during the period of the study. From these scales, emerged 188 parasitoids of two species, *A. melinus* and *A. chrysomphali,* accounting for 67% and 33% of the parasitoids, respectively. We also registered 372 parasitoid eggs and larvae that failed to develop to pupae or adults and therefore could not be identified.

Parasitism rates on fruits were similar on ant-excluded trees and ant-allowed trees in both years when considering data from the three sampling dates together [(2007: ant-allowed vs. ant-excluded: 24.01% vs. 27.07%; $\chi^2 = 0.39$, $P = 0.53$; 2008: 27.28% vs. 27.72%; $\chi^2 = 2.39$, $P = 0.12$) (Figure 2). Similarly, no significant differences were found between treatments when parasitism rates were compared on each sampling date along the infestation period (ants-allowed vs. ant-excluded, 2007: July: $\chi^2 = 2.12$, $P = 0.14$, August: $\chi^2 = 0.75$, P= 0.38, September: $\chi^2 = 0.06$, $P = 0.8$; 2008: July: $\chi^2 = 0.01$, $P = 0.92$, August: $\chi^2 = 0.14$, $P = 0.7$, September: $\chi^2 = 0.67$, $P = 0.41$).

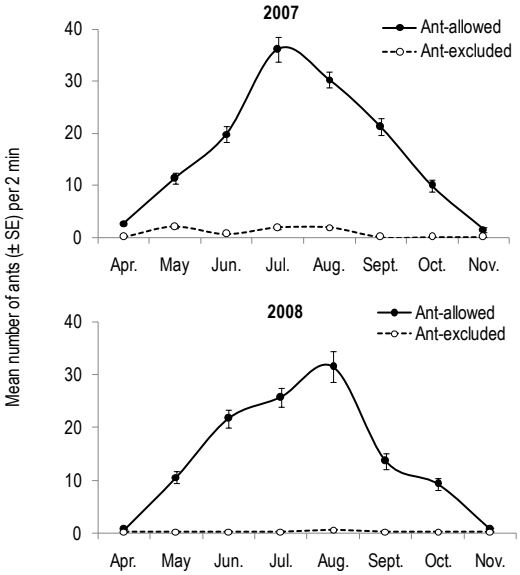

Fig. 1. Seasonal ant activity (mean ± SE) in ant-allowed and ant-excluded treatments in 2007 and 2008 (N = 32 trees per treatment).

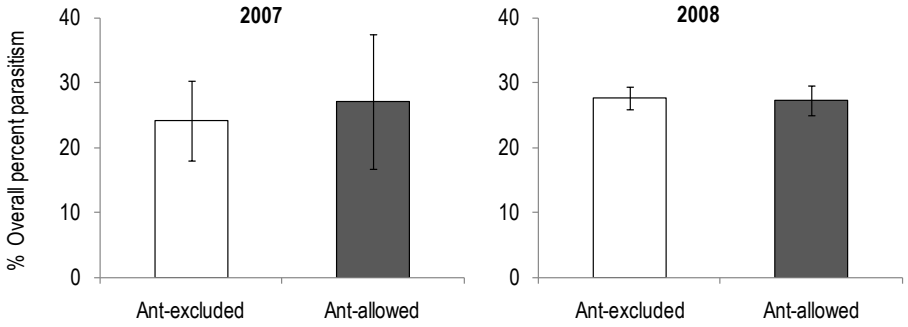

Fig. 2. Percent parasitism of *Aonidiella aurantii* (mean ± SE) in fruits of ant-allowed and ant-excluded trees in 2007 and 2008.

CRS population densities on fruits and twigs

CRS density on fruits before harvest (when the cosmetic damage caused by the pest is important) was significantly lower in the ant-excluded than in the ant-allowed treatment in both years (GLM based on semi-Poisson distribution: 2007: F = 24.49; d.f. = 1, 1599; P < 0.001; 2008: F = 4.84, d.f. = 1, 639; P = 0.028) (Figure 3). We found also a positive relationship between CRS density on fruits before harvest and cumulative ant-activity when comparing populations in individual ant-allowed trees in 2008 (GLM based on semi-Poisson distribution: F = 11.63; d.f. = 1, 15; r = 0.55; P = 0.004; *Scale number* =

Exp ((0.001* (*ant number*) + 1.62)) (Figure 4). This relationship occurred apparently above a certain threshold of ant activity. Thus, when cumulative ant-activity was lower than 500, ants did not affect CRS population levels.

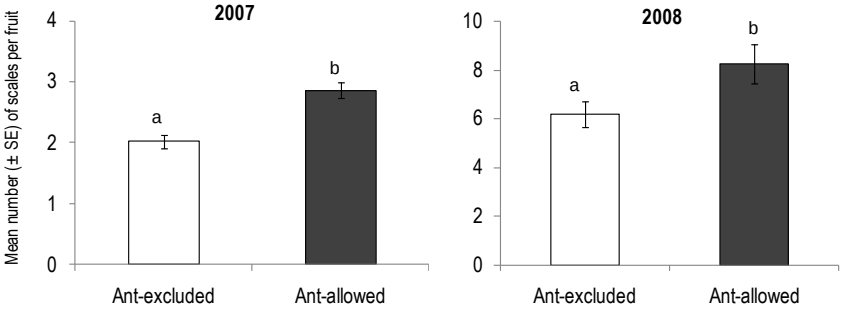

Fig. 3. Number of *Aonidiella aurantii* scales per fruit (mean ± SE) before harvest in 2007 and 2008.

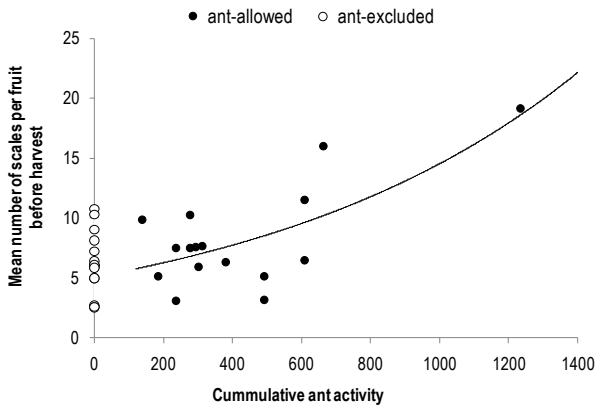

Fig. 4. Mean number of *Aonidiella aurantii* per fruit before harvest relative to cumulative 24-hr ant activity. Ant-allowed treatment: *Scale number* = Exp ((0.001* (*ant number*) + 1.62)); $r = 0.55$; $P = 0.004$.

We also determined the seasonal trend of CRS on twigs and fruits along 2008. At the beginning of the ant-exclusion period, population densities of the scale on twigs and fruits were similar in both treatments but afterwards they were significantly lower in the ant-excluded treatment (Figure 5). On twigs, we observed two peaks during June and September, which coincided with the crawler dispersal and settlement of the second and third generation. CRS densities in the ant-excluded treatment began to be significantly lower than in the ant-allowed treatment in May (one month after ant activity began) and this difference increased until November. Thus, the effect of the ants on CRS density appears to be accumulative, and consequently it was maximum at the end of the season. Pooling the data from the last three samplings, there were 62% more scales per twig in the ant-allowed than in the ant-excluded

treatment. Likewise, CRS population levels on fruits were significantly lower in the ant-excluded treatment all along the sampling period except in July, when CRS populations on fruits were still very low. Pooling the data from the last three samplings, we found 43% more scales per fruit on the ant-allowed treatment than on the ant-excluded treatment.

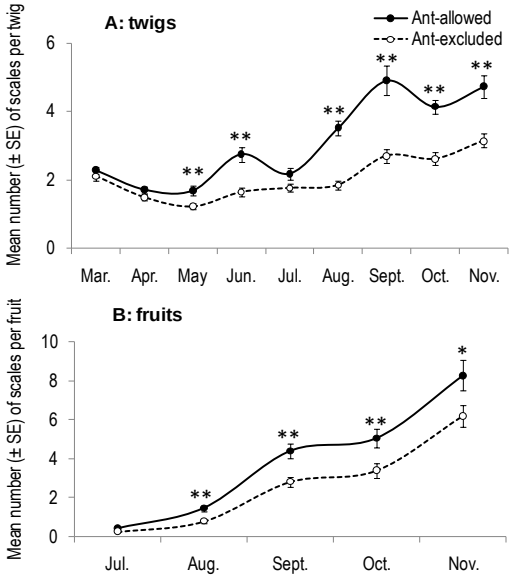

Fig. 5. Seasonal trend of *Aonidiella aurantii* (mean ± SE) on twigs (A) and fruits (B) in ant-allowed and ant-excluded treatments. For each sampling date significant differences between treatments are denoted with (*) (Generalized Linear Model based on semi-Poisson distribution); Significance levels: * $P < 0.05$; ** $P < 0.01$.

When we analyzed the seasonal trend of CRS density on twigs and fruits along the year separately on each of the four blocks in which the experiment had been replicated, we only observed a clear ant-effect on CRS density in block 3 (Figure 6). Pooling the data from the last three samplings in block 3, we registered 146% more scales on twigs and 157% more scales on fruits in the ant-allowed that in the ant-excluded treatment (Repeated measures ANOVA, twigs: $F = 11.75$; d.f. = 1, 6; $P = 0.014$; fruits: $F = 15.27$; d.f. = 1, 6; $P = 0.007$), whereas no significant differences were found in the other three blocks (Repeated measures ANOVA on twigs: Block 1: $F= 2.80$; d.f. = 1, 6; $P = 0.145$; Block 2: $F = 2.67$; d.f. = 1, 6; $P = 0.15$; Block 4: $F = 4.69$; d.f. = 1, 6; $P = 0.07$; on fruits: Block 1: $F = 3.76$; d.f. = 1, 6; $P = 0.1$; Block 2: $F = 0.03$; d.f. = 1, 6; $P = 0.861$; Block 4: $F = 0$; d.f. = 1, 6; $P = 0.98$). Coincidentally, ants were also more abundant in the canopy of the ant-allowed trees of block 3 (see table 1). Cumulative 24-h ant activity (ant activity counts at 2-h intervals over a 24 h period in spring, summer and autumn) was significantly higher in the ant-

allowed plot of the block 3 than in the other ant-allowed plots ((Ant activity: repeated measures ANOVA (ants log-tranformed: F = 4.59; d.f. = 3, 13; P = 0.004)).

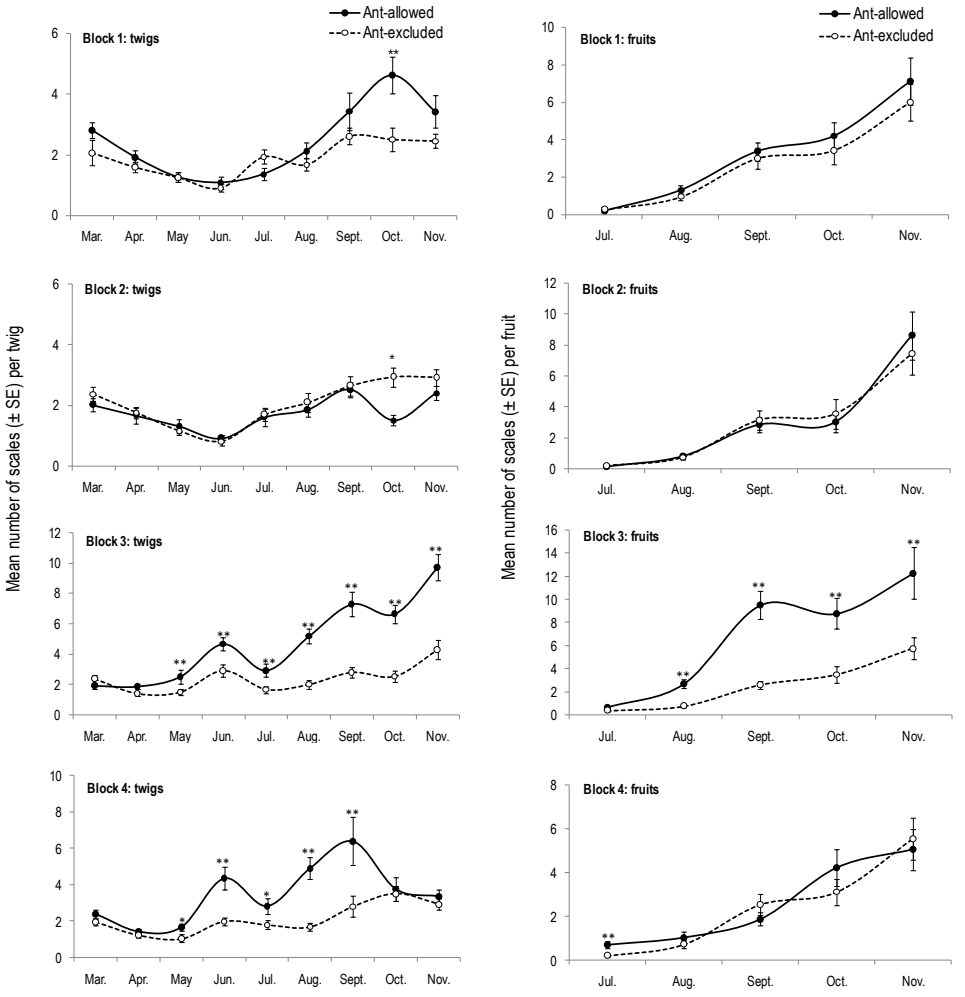

Fig. 6. Seasonal trend of *Aonidiella aurantii* (mean ± SE) on twigs and fruits in ant-allowed and ant-excluded treatments in each experimental plot. For each sampling date significant differences between treatments are denoted with (*) (Generalized Linear Model based on semi-Poisson distribution); Significance levels: * P < 0.05; ** P < 0.01.

6.4. Discussion

Since Flanders (1945) demonstrated that ants may disrupt the activity of parasitoids and predators of hosts and/or preys which do not produce honeydew, several studies have confirmed this interaction (Haney et al., 1987;

Heimpel et al., 1997). One of the most representative examples is the interference of the Argentine ant with the activity of the natural enemies of CRS. Although CRS does not produce any type of honeydew, the activity of the Argentine ant when it tends co-occurring honeydew producers induces higher scale populations (DeBach, 1951; Moreno et al., 1987; James et al., 1997). As in other citrus producing areas, CRS is the main citrus pest in Mediterranean basin (Franco et al., 2006). However, the Argentine ant is not widespread in the western Mediterranean area where *L. grandis* and *P. pallidula* are the commonest ants on citrus (Palacios et al., 1999; Alvis, 2003; Vanaclocha et al., 2005, Urbaneja et al., 2006; Cerdá et al., 2009). Thus, in this study we have examined the effect of these Mediterranean ant species on the population densities of CRS.

CRS density on fruits was positively related with the activity of *P. pallidula*, *L. grandis* and *P. schmitzii*. These ants climb up to citrus canopies to feed on honeydew secreted by hemipterans (see chapter 5). During their foraging for honeydew, they may interfere and disrupt the natural enemies of CRS. For example, *A. melinus*, the main parasitoid of CRS in our study, easily ceases oviposition when it is disrupted by ants (Martinez-Ferrer et al., 2003). Moreover, the probability of being disrupted by ants during oviposition may be high because it needs more than 6 minutes to lay an egg and between 8 and 17 minutes to feed upon scales' fluids in order to obtain the nutrients necessary for egg maturation (Casas et al., 2004; Martinez-Ferrer et al., 2003). During their foraging, ants might also create spatial refuges in the vicinity of ant-tended honeydew producing colonies where parasitoid foraging is hindered (Wyckhuys et al., 2007). In the orchard where we conducted our study, 62% and 73% of the total hemipteran-colonies were tended by ants during spring and summer respectively (see chapter 5). These small-scale spatial refuges probably serve as reservoirs for future infestation by the scale.

Our results also show that the overall impact of these Mediterranean citrus ants on CRS populations is lower when compared with that of the Argentine ant (DeBach, 1951; James et al., 1997). The Argentine ant is a highly invasive species that has spread to areas with Mediterranean-type ecosystems all around the world (Suarez et al., 2001). In the invaded regions, the Argentine ant usually displaces other native ant species mainly thanks to its numerical superiority (Holway, 1999; Holway et al., 2002). One of the factors responsible of its remarkably high abundance on citrus is the establishment of mutualistic associations with honeydew-producing Hemiptera (Holway et al., 2002). Indeed, in Californian citrus orchards, Markin (1970) estimated that more than 300,000 Argentine ant workers entered a total of four citrus trees during 24 h in summer, largely for honeydew collection. In Spain, the Argentine ant has also been found to maintain higher activity levels than the native species

in natural ecosystems (Abril et al., 2007). Similarly, Paris and Espadaler (2009) found that another invasive species, *L. negletus* Van Loon, Boomsma et Andrásfalvy, maintained higher activity and was more efficient in honeydew recollection than *L. grandis* in Catalonia, Northeastern Spain. Under our field conditions, the activity of *L. grandis*, *P. pallidula*, and *P. schmitzii* was positively correlated with the presence of honeydew-producing Hemiptera on the citrus canopies (see chapter 5), but the number of ascending ants per tree is much lower compared with the argentine ant. According to our results, Mediterranean native ants induced an increase of CRS only in those trees in which ant activity was high along the year, as shown by the differences among our experimental blocks. Thus, the impact of the ants on the scale population depends, principally, on the intensity of ant activity. This might explain why the argentine ant, which shows higher levels of activity, is so disruptive for biological control.

In previous studies, it has been demonstrated (DeBach, 1951; Martinez-Ferrer et al., 2003) or implicitly assumed (Moreno et al., 1987; James et al., 1997) that CRS parasitism rates are lower in the presence of ants. In our study, however, the possible ant-interference with the oviposition activity of the parasitoids was not translated in differences in parasitism levels on fruits. We consider that there are various explanations for this to happen. Firstly, percent parasitism based on monthly samplings might not be an adequate measure of the parasitoid potential as measures should be made on a generation time-scale (Jervis and Kidd, 1996). Secondly, the comparison of parasitism rates between treatments only in fruits probably is not consistent with the overall parasitoid performance. Parasitism rates on twigs and leaves might have also been examined. Finally, mortality due to host feeding between treatments was not included because it is difficult to estimate it in the field.

According to our results, native Mediterranean ant species are able to induce increases in the densities of CRS populations, and these increases depend on the intensity of ant activity. Under circumstances of high ant activity, ant-exclusion resulted in a significant reduction in fruit damage before harvest. The sticky barriers used in this study (together with pruning of the branches) offer a very effective and environmentally friendly method of ant-exclusion. However, this method is too laborious to be applied on large areas and further studies should be made to develop and improve ant-exclusion methodologies on commercial plantations. In conclusion, the present study underlines that ant-exclusion should be considered as a component of the strategy to reduce CRS populations and consequently fruit damage in citrus orchards.

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Chapter 7

Nutritional state and food sources used by adult *Aphytis melinus* parasitoids in the field

Nutritional state and food sources used by adult *Aphytis melinus* parasitoids in the field

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Abstract: We used high performance liquid chromatography (HPLC) to determine the nutritional state and food sources used by adults of the parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) in the field. We compared the overall sugar content and the glucose-fructose ratio between field-collected parasitoids and individuals that received reference feeding treatments in the laboratory. According to our results, nine out of eleven field-collected individuals were characterized as "fed". Moreover, they contained oligosaccharides like erlose, melezitose, melibiose and raffinose that are principally present in hemipteran honeydews. Given that laboratory reared parasitoids did not synthesize oligosaccharides after sugar feeding, these results suggest that adult *A. melinus* use hemipteran honeydew as a food source in the field.

7.1. Introduction

Parasitoids are the most important group of natural enemies for the biological control of insect pests (van Driesche et al., 2008). The majority of adult parasitoids require plant provided food, either directly, in the form of nectar or pollen, or indirectly, in the form of honeydew produced by hemipterans (Jervis et al., 1996; Wäckers, 2005). Consumption of plant-derived food provides carbohydrates (sugars) that are essential for adult maintenance and locomotion. It has been demonstrated that in absence of sugar feeding longevity, fecundity and searching capacity of many parasitoid species are negatively affected (Wäckers, 1994; Takasu and Lewis, 1995; Wäckers, 2001; Tylianiakis et al., 2004; Wäckers, 2005). Given that sugar feeding influences various parasitoid fitness parameters, it is likely to have also consequences for their efficacy as biological control agents (Tylianakis et al., 2004; Wäckers et al., 2008).

Aphytis melinus DeBach (Hymenoptera: Aphelinidae) is the most successful biological control agent of California red scale *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), one of the most important citrus pests worldwide (Rosen and DeBach, 1979). *Aphytis melinus* is a facultatively gregarious (Rosen and DeBach, 1979), synovigenic, (Opp and Luck, 1986) idiobiont, primary ectoparasitoid of diaspidid scales (Rosen and DeBach, 1979). Also, it engages in host-feeding causing considerable additional mortality of its host (DeBach et al., 1969). In the lab, and in absence of sugar feeding, longevity of adult *A. melinus* did not exceed three days and similarly, fecundity was also seriously compromised (Heimpel et al., 1997). In the same study it was found that host-feeding alone cannot enhance longevity and fecundity, it can do so only when the wasps have in addition access to a sugar source. Nevertheless, no information is available regarding the nutritional state and the food sources that adult *A. melinus* use in the field.

Overall carbohydrate levels provide an accurate measure of parasitoid nutritional state (Fadamiro and Heimpel, 2001; Giron and Casas, 2003). Using high performance liquid chromatography (HPLC), the overall carbohydrate levels, as well as an ample range of specific carbohydrates, can be quantified for individual parasitoids (Wäckers and Steppuhn, 2003; Heimpel et al., 2004; Steppuhn and Wäckers, 2004). For instance, Wäckers and Steppuhn (2003) reported that field collected parasitoids presented higher carbohydrate levels than newly eclosed individuals suggesting that the former obtained sugars in the field. Besides overall sugar content, the glucose-fructose ratio, enables the discrimination between unfed parasitoids from those that fed in the past (Steppuhn and Wäckers, 2004; Hogervorst et al., 2007). Finally, HPLC analysis enables determination of honeydew feeding by parasitoids. This is based on

the detection of specific sugars (di- and oligosaccharides), typical of hemipteran honeydews, in field collected individuals (Wäckers and Steppuhn, 2003; Hogervorst et al., 2007). However, Wäckers et al. (2006) demonstrated that *Diadegma* sp. parasitoids can also synthesize oligosaccharides after sucrose feeding. Therefore, in order to determine honeydew feeding in field collected parasitoids it is necessary to test if they can synthesize oligosaccharides after sugar feeding in the laboratory (Hogervorst et al., 2007).

Given the importance of sugar feeding on longevity and fecundity of *A. melinus* it is essential to determine the nutritional state of wild individuals and on what type of sugars this species uses in the wild. Knowledge of these food sources will help clarify the wasp´s feeding ecology and will contribute to improve the biological control of the scale. Therefore, this study has two objectives: (i) determine the nutritional state of field collected *A. melinus* and (ii) determine the food sources the adult parasitoids use in the field.

7.2. Material and methods

Wild *A. melinus* were collected from one unsprayed citrus grove, located in the township of Alzira (39º 8´ 46.28´´ N; 0º 27´ 33.96´´W) in the citrus growing area of Valencia (eastern Spain). We examined the lower side of the leaves in various trees in the grove, and when parasitoids were detected they were sampled with a hand aspirator. Immediately afterwards, they were placed in ethanol 70%. In the laboratory, parasitoids were place individually in Eppendorf tubes with 1 ml of ethnanol 70% and were kept in room temperature until HPLC analysis. Sampling was performed in May 22th between 10 and 14 hours.

Laboratory parasitoids were reared on *A. aurantii* settled on orange fruits. Orange fruits infested with third instar females of *A. aurantii* were exposed for 3 days in a cage with adult *A. melinus* collected in the field. After this period, the fruits were kept individually at room temperature for one week. Then, parasitoid pupae were isolated in glass vials (3.0 by 0.8cm) and checked daily until parasitoid emergence. Upon emergence, parasitoids were randomly assigned to a feeding treatment (see below).

To determine the nutritional status of field collected *A. melinus* we compared their overall sugar content with that of parasitoids that received reference feeding treatments in the laboratory. These treatments included newly eclosed, starved for 2 days after emergence until, and continuously fed parasitoids (access to a sugar source during 3 days). We also included a treatment in which parasitoids were given access to a sugar source during 24

hours and then they were starved during 24 hours in order to have a reference for parasitoids that had fed in the past. A 2M sucrose solution was used as food source (Wäckers, 2001). Parasitoids of all treatments were kept individually in glass vials (0.3 by 0.8 mm) at room temperature. For both field collected and laboratory reared parasitoids only females were analyzed. Field-collected *A. melinus* were characterized as "fed" if their total sugar content exceeded the maximum (mean plus two times standard deviation) of starved parasitoids and their glucose-fructose ratio was below the minimum of starved and newly eclosed parasitoids (mean minus two times standard deviation) (Steppuhn and Wäckers, 2004; Hogervorst et al., 2007).

For HPLC analysis, the parasitoids were crushed with a pestle and one millilitre of the supernatant was filtered through a micro filter. From each sample, 10 µl were injected into a Dionex DX 500 HPLC system. The system was equipped with a GP 40 gradient pump, a Carbopack PA 1 guard (4 x 250 mm^2), and an ED 40 electrochemical detector for pulsed amperimetric detection (PAD). The column was eluted with 1 M NaOH and Mill Q-water and kept at 20 ^oC during the analysis. Daily reference curves were obtained for glucose, fructose, galactose, sucrose, trehalose, raffinose, melibiose, erlose, melezitose, ramnose, stachyose, mannitol and sorbitol, by injecting calibration standards with concentrations of 2.5 p.p.m, 5 p.p.m., 7.5 p.p.m. and 10 p.p.m. of these sugars. The overall sugar content of a parasitoid was represented by the sum of the concentrations of individual sugars. To adjust the concentrations for insect size, they were expressed relative to the wasp´s hind tibia length that provides an accurate measure of adult *A. melinus* size (Opp and Luck, 1986).

7.3. Results and discussion

The total sugar content of newly eclosed parasitoids was 0.83 ± 0.22 ng (mean ± 1SE; $n = 5$) per µm of hind tibia (Figure 1). Their sugar spectrum was dominated by glucose that represented 50% of the total sugar concentration. Additionally, sucrose (29%) and low levels of fructose (5%) and the sugar alcohol mannitol (7.5%) were present. Their glucose-fructose ratio was very high (0.93; Figure 1). In starved parasitoids, the overall sugar content declined significantly to 0.28 ± 0.07 ($n = 6$) ng per µm of hind tibia. Starved parasitoids retained the high glucose-fructose ratio (0.8). Parasitoids that had access to a 2M sucrose solution presented higher total sugars levels (1.18 ± 0.19 ng per µm of hind tibia) than starved individuals. Additionally, the glucose-fructose ratio in continuously fed parasitoids was more balanced (0.63) than in newly eclosed and starved parasitoids. The overall sugars levels of fed and subsequently starved wasps were similar with those of fed and newly eclosed parasitoids. However, they differed from newly eclosed parasitoids by their

glucose-fructose ratio, which was similar to the one observed in fed parasitoids. Sugars typical of honeydews, like erlose, melezitose, melibiose and raffinose, were never detected in laboratory reared parasitoids. Thus, *A. melinus* apparently cannot synthesize oligosaccharides after sugar feeding, as was previously observed in other insects (Wäckers et al., 2006; Hogervorst et al., 2007). Consequently, presence of "honeydew sugars" in field collected parasitoids can be used as an indicator of honeydew feeding for *A. melinus*.

Fig. 1. Total sugar content and glucose-fructose ratio of *Aphytis melinus* females receiving different feeding treatments in the laboratory. Bars sharing the same letter do not differ significantly at $P > 0.05$ (Kruskal-Wallis ANOVA).

Based on the comparison of the total sugar level and the glucose-fructose ratio between starved and fed parasitoids, nine out of the eleven field collected parasitoids were classified as "fed" (overall sugar levels above 0.695 ng/hind tibia wasp and glucose-fructose ratio below 0.665) (Figure 2).

Interestingly, all the field collected parasitoids characterized as "fed" contained sugars typical of honeydews. More concretely, they contained melezitose (four individuals, with melezitose comprising on average 2.87% of their total sugar content), raffinose (three individuals, 0.76%), erlose (one individual, 0.33%) or melibiose (one individual, 0.10%). This finding suggests that they had recently consumed honeydew. They also contained sucrose, glucose and fructose suggesting that the possibility of additional nectar feeding cannot be excluded. Nevertheless, our results highlight the importance of honeydew as food source for *A. melinus* under field conditions. Honeydew is likely to be the most abundant sugar source in Mediterranean citriculture that is characterized by low plant diversity. Further research is needed to test the suitability of each honeydew type for *A. melinus.* Previous work with the sibling species *Aphytis coheni* DeBach suggests that the nutritional value of honeydew may be highly variable, even toxic in some cases (Avidov et al., 1970). Moreover, important implications for multitrophic interactions may arise given that honeydew is also a key food-source for ants on the citrus canopies (Pekas et al., 2010). Finally, *A. aurantii*, the host of *A. melinus* does not produce any type of honeydew, and therefore, a host patch does not provide food for adult wasps. As a result, *A. melinus* has to allocate time

between host-searching and food searching and this may have serious consequences for its efficacy as biological control agent.

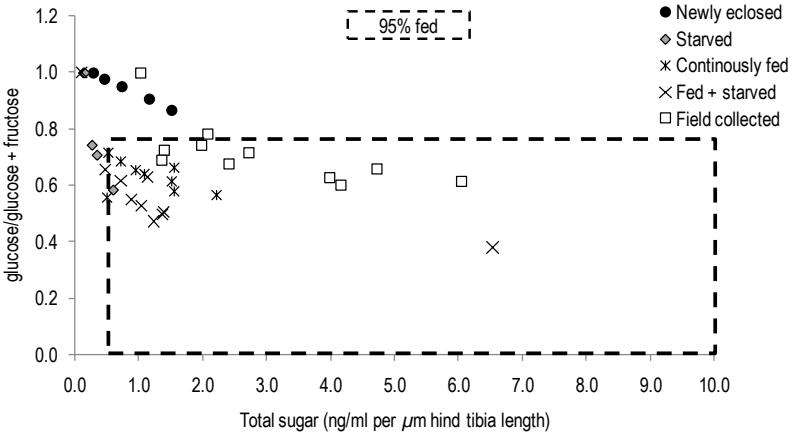

Fig. 2. Plot of the total sugar content (in ng/μm of hind tibia) versus the glucose-fructose ratio of adult female *Aphytis melinus* collected in a citrus orchard in eastern Spain. Dashed line indicates the thresholds including the 95% of fed individuals.

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Chapter 8

Conclusions

Factors affecting the size of California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae) under field conditions

i. Seasonal variation, due to temperature fluctuations along the year was the most important factor affecting *A. aurantii* size; smaller body sizes were observed when temperatures were higher, during summer and autumn.

ii. Among plant substrates, scales were larger on fruits than on leaves or branches.

iii. The size of *A. aurantii* varied significantly among orchards.

iv. A positive correlation was registered between body size of some *A. aurantii* instars and leaf content in potassium.

Influence of host size on parasitism by *Aphytis chrysomphali* and *A. melinus* (Hymenoptera: Aphelinidae) in Mediterranean populations of California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae)

i. *Aphytis chrysomphali* was recovered mostly from *A. aurantii* second instars (0.152-0.300 mm^2 in body area), but parasitized more heavily third instars (larger than 0.325 mm^2).

ii. *Aphytis melinus* developed mostly, and parasitized more heavily, third instars.

iii. Within each *A. aurantii* instar, percent parasitism was positively related with host size, peaking at ~10% for *A. chrysomphali* (scales sized between 0.80-0.85 mm^2) and ~30% for *A. melinus* (scales sized between 0.70-0.75 mm^2).

iv. Gregariousness and parasitoid size were positively influenced by host size.

v. *Aphytis melinus* laid male eggs on small hosts (less than 0.4 mm^2) and female eggs on large hosts (larger than 0.4 mm^2).

vi. The host size at which the sex ratio of *A. melinus* turned female biased remained constant (around 0.4 mm^2) whether relatively small or large hosts were available.

vii. From May until October most scales are too small to produce female *A. melinus*.

Spatio-temporal patterns and interactions with honeydew-producing hemiptera of ants in a Mediterranean citrus orchard

i. The dominant ant species *Pheidole pallidula* and *Lasius grandis* forage in mutually exclusive territories within the field, but they both share their territory with the subordinate *Plagiolepis schmitzii*, a distribution pattern known as "ant mosaic".

ii. Ants ascended to the canopies from April until November; their seasonal activity patterns were shaped by the colony nutritional requirements and temperature.

iii. The daily activity pattern of *P. schmitzii* was strictly diurnal whereas *L. grandis* and *P. pallidula* were active during the entire day.

iv. Citrus nectar and predation/scavenging did not represent important food sources for the ants on the canopies.

v. Hemipteran-honeydew was the principal food source for the ants on the canopies: more than 60% of the total honeydew sources, and 100% of the citrus mealybug *Planococcus citri* colonies, were tended by ants during spring and summer.

Effect of Mediterranean ants (Hymenoptera: Formicidae) on California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae) populations in citrus orchards

i. Ant-exclusion resulted in significantly lower *A. aurantii* densities on twigs compared with the ant-allowed control one month after ant activity began.

ii. The difference in *A. aurantii* densities between the two treatments increased until the harvest suggesting that the effect of ants is cumulative.

iii. Ant-exclusion resulted in significantly lower scale densities on fruits at harvest compared with the ant-allowed control.

iv. No differences in parasitism rates on fruits were detected between the ant-excluded and ant-allowed treatments.

v. Scale densities on fruits of the ant-allowed treatment were positively correlated with the number of ants that climbed to the canopies suggesting that the ant effect depends on the intensity of the ant-activity.

Nutritional state and food sources used by adult *Aphytis melinus* parasitoids in the field

i. The laboratory reared parasitoids did not synthesize oligosaccharides.

ii. Nine out of the eleven field collected *A. melinus* had recently consumed carbohydrates and were characterized as "fed".

iii. The field collected parasitoids characterized as "fed" contained oligosaccharides typicall of hemipteran honeydews like erlose, melezitose, melibiose and raffinose, suggesting that *A. melinus* use hemipteran-honeydew as food source in the field.

Overall, the factors considered in this study are of great relevance in order to improve the biological control of *A. aurantii* is eastern Spain citrus. Augmentative (inundative) releases of *A. melinus* parasitoids in early spring and summer are likely to compensate the scarcity of suitable hosts for the production of female progeny that this species suffers during great part of the year. Moreover, ant-manipulation decisions based on the ecology and species of ant(s) present should be an important consideration despite the fact that ant-exclusion from the canopies suffers important practical difficulties to apply in commercial orchards. Alternative approaches, easier to apply, involving chemical baits/prays or even semiochemicals should be explored. Finally, the biological control of *A. aurantii* can be improved by enhancing the food sources available for adult *Aphytis*. Direct provision of resources by means of food-sprays and/or long-term strategies based on habitat management, are likely to improve survival and consequently efficacy of *Aphytis* parasitoids. Successful biological control of *A. aurantii* can potentially provide a range of benefits to landholders and at the same time meet the increasingly growing consumer demand and legislation for safe agricultural products.