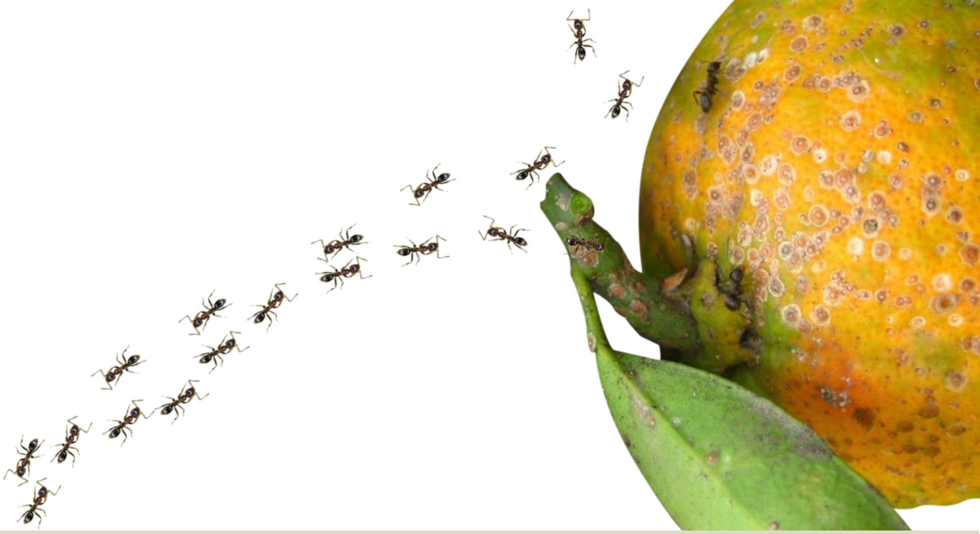

ANTS, PESTS AND NATURAL ENEMIES IN MEDITERRANEAN CITRUS

Ecological interactions and practical implications for biological control

DOCTORAL THESIS
Presented by: Altea Calabuig Gomar
Directed by: Ferran Garcia Marí and Apostolos Pekas

València, May 2015

UNIVERSITAT POLITÈCNICA DE VALÈNCIA
Escola Tècnica Superior d'Enginyeria Agronòmica i del Medi Natural

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Departament d'Ecosistemes Agroforestals

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A Dídac

“Mucha gente pequeña en lugares pequeños,

haciendo cosas pequeñas pueden cambiar el mundo”

Eduardo Galeano

Aknowledgments

Després de 4 anys de feina em vénen al cap un munt de persones sense les quals, directament o indirectament, no hagués pogut acabar aquesta tesi i a les quals m'agradaria mostrar el meu agraïment.

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INDEX

Abstract

Ants constitute an important component of the citrus agroecosystem fauna acting simultaneously as predators and as hemipteran mutualists. Thus, ants in citrus are in the center of a complex food web affecting the composition and the population dynamics of a wide arthropod community including honeydew and non-honeydew producing herbivores as well as their natural enemies. In eastern Spain the most abundant and widespread ant species are the natives *Lasius grandis* Forel and *Pheidole pallidula* (Nylander), whereas the invasive *Linepithema humile* (Mayr) is also present but not widespread. We have conducted ant-exclusion experiments in three commercial citrus orchards, each one dominated by one ant species (*Pheidole pallidula*, *Lasius grandis* or *Linepithema humile*) during two consecutive growing seasons (2011 and 2012) with the aim to disentangle the ecological interactions with honeydew and non-honeydew producing pests and with natural enemies at the community-level. We discuss the implications of the results for biological pest control.

We quantified the effect of the ant-exclusion on the infestation levels and parasitism of three of the most important citrus pests in the area, the honeydew producer *Aleurothrixus floccosus* (Maskell) (woolly whitefly) and the non-honeydew producers *Aonidiella aurantii* (Maskell) (California red scale) and *Phyllocnistis citrella* (Stainton) (citrus leafminer). California red scale densities on fruits were significantly lower in the two seasons and in the three orchards in the ant-excluded treatment. Similarly, the percentage of shoots occupied by *A. floccosus* was significantly lower in the ant-excluded plots in the orchards dominated by *P. pallidula* and *L. humile*. The percentage of leaf surface loss caused by *P. citrella* was similar on ant-allowed and ant-excluded treatments in the three orchards. Interestingly, no significant differences were found in the percent parasitism between ant-allowed and ant-excluded treatments for the honeydew and non-honeydew producing herbivores. These results suggest that factors other than parasitoid disruption might explain the increased pest populations observed in the presence of ants.

Thus, in the same orchards we compared the abundance, species richness, diversity and community structure of predators and parasitoids between the ant-allowed and ant excluded treatments. A total of 176,000 arthropods belonging to 81 taxa were captured and identified. Regarding abundance, the response of natural enemies to ant-exclusion was species specific. When examining functional groups, parasitoids showed higher abundances in the ant-allowed treatment whereas most generalist predators were less abundant. The abundance of some species from the 4th trophic level (hiperparasitoids) also differed between treatments. Similarly, the species richness (S) and the Shannon diversity index (H) of parasitoids were higher in the ant-allowed treatment, whereas the species richness (S) of predators was significantly lower. The community structure of predators and parasitoids was not significantly different between treatments. Thus, ants were not associated with a dramatic and/or general decrease in natural enemy abundance or biodiversity; on the contrary we found higher parasitoid species richness and diversity in the presence of ants. On the other hand, the negative impact of ants on generalist predators may have important implications for the regulation of pest populations.

Finally we tested a novel hypothesis examining the potential competition between ants and natural enemies for honeydew produced by Hemiptera. Through the use of high performance liquid chromatography (HPLC) we related the level of ant activity with the energy reserves and feeding history of individual specimens collected in the field during representative days of spring, summer and autumn. Out of 145 *Aphytis chrysomphali* (Mercet) parasitoids, 65% were classified as sugar-fed and 24.7% as honeydew-fed. A significant negative correlation between ant activity and the total sugar content and honeydew feeding incidence by *A. chrysomphali* was found in summer, when ant activity peaked. Out of 47 individuals of the predator *Chrysoperla carnea* s.l., 55.3% were classified as sugar-fed. Ant activity was negatively correlated with the sugar feeding incidence by *C. carnea* in spring. This is a previously undocumented indirect interaction in food webs in which ants interfere with the physiological state of the natural enemies. Given that the absence of sugar feeding is detrimental for the fitness of many species of predators and parasitoids, this interaction may have important consequences for the arthropod community composition and practical implications for biological control.

Resum

Les formigues són un element important de l'agroecosistema dels cítrics, en el que poden actuar simultàniament com a insectes depredadors i com a mutualistes d'hemípters. Com a conseqüència, les formigues es troben al centre d'una complexa xarxa tròfica en la que poden afectar a la composició i la dinàmica poblacional d'un ampli grup d'artròpodes, incloent herbívors productors i no productors de melassa així com els seus enemics naturals. Les espècies de formigues més abundants i esteses als cítrics de l'est de la Península Ibèrica són les espècies natives *Lasius grandis* Forel i *Pheidole pallidula* (Nylander) mentre que l'espècie invasora *Linepithema humile* (Mayr) es troba també present però no de forma estesa. S'han dut a terme estudis d'exclusió de formigues en tres parcel·les comercials de cítrics en les que, en cada una d'elles, predominava una de les tres espècies de formigues (*Pheidole pallidula*, *Lasius grandis* o *Linepithema humile).* El principal objectiu ha estat el d'esclarir les interaccions ecològiques existents entre les formigues i els artròpodes productors i no productors de melassa així com els enemics naturals a nivell de comunitat. Les implicacions que els resultats obtinguts poden tenir sobre el control biològic de plagues són discutides.

S'ha quantificat l'efecte de l'exclusió de formigues sobre els nivells d'infestació i el parasitisme de tres de les plagues més importants dels cítrics de la zona, la plaga productora de melassa *Aleurothrixus floccosus* (Maskell) (mosca blanca) i les plagues no productores de melassa *Aonidiella aurantii* (Maskell) (poll roig de California) i *Phyllocnistis citrella* (Stainton) (minador dels cítrics). Les densitats poblacionals del Poll roig de Califòrnia en fruits foren significativament menors en el tractament d'exclusió de formigues els dos anys i en les tres parcel·les de l'estudi. Igualment, el percentatge de brots ocupats per *A. floccosus* fou significativament menor en el tractament d'exclusió de formigues en les parcel·les en les que predominava *P. pallidula* i *L. humile*. La incidència de *P. citrella* fou similar en exclusió i presència de formigues en les tres parcel·les. Curiosament, no es trobaren diferències en el percentatge de parasitisme entre els dos tractaments per a ninguna de les plagues estudiades. Aquests resultats suggereixen que altres factors, més enllà de la interferència amb els parasitoides, podrien explicar els

increments poblacionals de plagues observats en presència de formigues.

Davant aquests resultats, es va comparar l'abundància, riquesa d'espècies, diversitat i estructura de la comunitat de depredadors i parasitoides entre els tractaments d'exclusió i presència de formigues. Es capturaren i identificaren un total de 176,000 artròpodes pertanyents a 81 taxons distints. En quant a l'abundància, la resposta dels enemics naturals a l'exclusió de formigues fou específica per a cada espècie. Agrupant els grups funcionals, els parasitoides mostraren majors abundàncies en presència de formigues mentre que la majoria de depredadors generalistes foren menys abundants. L'abundància d'algunes espècies pertanyents al 4t nivell tròfic (hiperparasioides) també fou diferent entre tractaments. De la mateixa forma, la riquesa d'espècies i l'Índex de diversitat de Shannon (H) dels parasitoides foren majors en presència de formigues mentre que la riquesa d'espècies (S) dels depredadors fou menor. L'estructura de la comunitat de depredadors i parasitoides fou similar entre tractaments. Conseqüentment, les formigues no es veuen associades a una reducció important i/o generalitzada de l'abundància o la biodiversitat de parasitoides en presència de formigues. Per altra banda, l'impacte negatiu de les formigues sobre els depredadors generalistes pot tenir importants implicacions per a la regulació de les poblacions de plagues.

Finalment s'estudià una novedosa hipòtesi en la que es va plantejar l'existència de competència per la melassa que produeixen els hemípters entre les formigues i els enemics naturals. Mitjançant l'ús de cromatografia líquida d'alta resolució (HPLC) es va relacionar l'activitat de les formigues amb les reserves energètiques i les fonts alimentàries utilitzades per espècimens d'enemics naturals recol·lectats al camp durant dies representatius de primavera, estiu i tardor. Es va trobar una correlació significativament negativa entre l'activitat de les formigues i el contingut total de sucres i l'alimentació a base de melassa del parasitoide *Aphytis chrysomphali* (Mercet) en estiu, moment en el que l'activitat de les formigues era màxima. L'activitat de les formigues es va correlacionar negativament amb l'alimentació a base de sucres del depredador *Chrysoperla carnea* s.l. en primavera. Aquesta interacció indirecta en la que les formigues interfereixen en l'estat fisiològic dels enemics naturals no ha sigut documentada prèviament. Donat que l’absència de sucres en l’alimentació de moltes espècies de depredadors i parasitoides és perjudicial per al seu estat físic, aquesta interacció pot tenir importants implicacions pràctiques per al control biològic de plagues.

Resumen

Las hormigas constituyen un elemento importante del agroecosistema de los cítricos, en el que actúan simultáneamente como insectos depredadores y como mutualistas de hemípteros. Como consecuencia, las hormigas se encuentran en el centro de una compleja red trófica en la que pueden afectar a la composición y a la dinámica poblacional de un amplio grupo de artrópodos, en los que se incluye herbívoros productores y no productores de melaza, así como a sus enemigos naturales. Las especies de hormigas más abundantes y extendidas en los cítricos del este de la Península Ibérica son las especies nativas *Lasius grandis* Forel y *Pheidole pallidula* (Nylander), mientras que la especie invasora *Linepithema humile* (Mayr) se encuentra también presente pero no de forma extendida. Se han llevado a cabo estudios de exclusión de hormigas en tres parcelas comerciales de cítricos en las que, en cada una de ellas, predominaba una de las tres especies de hormigas (*P. pallidula*, *L. grandis* o *L. humile).* El principal objetivo ha sido el de esclarecer las interacciones ecológicas existentes entre las hormigas y los artrópodos productores y no productores de melaza, así como con los enemigos naturales a nivel de comunidad. Se discuten las implicaciones que los resultados obtenidos pueden tener sobre el control biológico de plagas.

Se ha cuantificado el efecto de la exclusión de hormigas sobre los niveles de infestación y el parasitismo de tres de las plagas más importantes de los cítricos de la zona, la plaga productora de melaza *Aleurothrixus floccosus* (Maskell) (mosca blanca) y las plagas que no producen melaza *Aonidiella aurantii* (Maskell) (piojo rojo de California) y *Phyllocnistis citrella* (Stainton) (minador de los cítricos). Las densidades poblacionales del piojo rojo de California en frutos fueron significativamente menores en el tratamiento de exclusión de hormigas en los dos años y en las tres parcelas del estudio. De igual modo, el porcentaje de brotes ocupados por *A. floccosus* fue significativamente menor en el tratamiento de exclusión de hormigas en las parcelas en las que predominaba *P. pallidula* y *L. humile.* La incidencia de *P. citrella* fue similar en exclusión y presencia de hormigas en las tres parcelas.

Curiosamente, no se encontraron diferencias en el porcentaje de parasitismo entre los dos tratamientos para ninguna de las plagas estudiadas. Estos resultados sugieren que otros factores, más allá de la interferencia con los parasitoides, podrían explicar los incrementos poblacionales de plagas observados en presencia de hormigas.

Por ello, se comparó la abundancia, riqueza de especies, diversidad y estructura de la comunidad de depredadores y parasitoides entre los tratamientos de exclusión y presencia de hormigas. Se capturaron e identificaron un total de 176,000 artrópodos pertenecientes a 81 taxones distintos. En cuanto a la abundancia, la respuesta de los enemigos naturales a la exclusión de hormigas fue específica para cada especie. Analizando los grupos funcionales, los parasitoides mostraron mayores abundancias en presencia de hormigas, mientras que la mayoría de los depredadores generalistas fueron menos abundantes. La abundancia de algunas especies pertenecientes al 4º nivel trófico (hiperparasitoides) también fue diferente entre tratamientos. De igual modo, la riqueza de especies (S) y el Índice de diversidad de Shannon (H) de los parasitoides fueron mayores en presencia de hormigas, mientras que la riqueza de especies (S) de los depredadores fue menor. La estructura de la comunidad de depredadores y parasitoides no difirió entre tratamientos. Consecuentemente, las hormigas no se ven asociadas a una reducción importante y/o generalizada de la abundancia o la biodiversidad de enemigos naturales; por el contrario encontramos una mayor riqueza de especies y biodiversidad de parasitoides en presencia de hormigas. Por otro lado, el impacto negativo de las hormigas sobre los depredadores generalistas puede tener importantes implicaciones sobre la regulación de las poblaciones de plagas.

Finalmente, se estudió una novedosa hipótesis en la que se planteó la existencia de competencia por la melaza que producen los hemípteros, entre las hormigas y los enemigos naturales. Mediante el uso de cromatografía líquida de alta resolución (HPLC) se relacionó la actividad de las hormigas con las reservas energéticas y las fuentes alimenticias utilizadas por especímenes de enemigos naturales colectados en el campo durante días representativos de primavera, verano y otoño. Se encontró una correlación significativamente negativa entre la actividad de las hormigas y el contenido total de azúcares y la alimentación a base de melaza del parasitoide *Aphytis chrysomphali* (Mercet) en verano, momento en el que la actividad de las hormigas fue máxima. La actividad de las hormigas se correlacionó negativamente con la alimentación a base de azúcares del depredador *Chrysoperla carnea* s.l en primavera. Esta interacción indirecta en la que las hormigas interfieren con el estado fisiológico de los enemigos naturales no ha sido documentada previamente. Dado que la ausencia de azúcares en la alimentación de muchas especies de depredadores y parasitoides es perjudicial para su estado físico, esta interacción puede tener importantes consecuencias para la composición de la comunidad de artrópodos e implicaciones prácticas sobre el control biológico de plagas.

Chapter 1

Introduction

1. INTRODUCTION

1.1 The importance of ants

Ants (Hymenoptera: Formicidae) are among the most abundant animals in terrestrial ecosystems and they are present in practically all terrestrial biotopes. It is estimated that the number of ants in the world is around 10^{15} individuals (Wilson, 1971). They represent 15-20% of the total terrestrial animal biomass, reaching 25% in tropical areas (Schultz, 2000). Currently, 12,990 species have been identified (Agosti & Johnson, 2005) and it is estimated that a great number of species still remain to be identified. As eusocial insects, ants are organized in colonies in which two or more adult generations coexist. They are divided in reproductive and non-reproductive castes and they care for the brood cooperatively (Hölldobler & Wilson, 1990).

Ants have evolved various feeding habits and foraging strategies. They can be predators of other insects, seed harvesters, scavengers or feed directly and indirectly on plants (Carroll & Janzen, 1973; Hölldobler & Wilson, 1990; Way & Khoo, 1992). Nevertheless, most ant species are omnivorous and combine the protein obtained from predation or scavenging with carbohydrates, principally obtained from the honeydew produced by plant feeders (Way, 1963; Carroll & Janzen, 1973).

Due to their abundance, ubiquity and feeding habits, ants may alter the environment in which they inhabit, acting as ecosystem engineers and playing a great diversity of roles. Ants may act as soil tillers since they change the structure and chemical properties of the soil by constructing subterranean galleries or mounds (Folgarait, 1998). They are also involved in soil improvement and nutrient cycling (Lobry De Bruyn, 1999). The accumulation of plant and animal remains in their nests results in an increase of carbon, nitrogen and phosphorus in the nest area, resulting in decomposition processes being greater in ant mounds (Hölldobler & Wilson, 1990; Folgarait, 1998).

Ants are involved in numerous mutualistic relationships with plants. They play an important role in natural ecosystems as seed dispersers and pollinators (Beattie, 1985). Numerous plants have developed seed appendages, elaiosomes, to promote dispersal by ants in what is called Myrmecochory (Beattie, 1985; Hughes *et al.*, 1994). Ants transport the seeds to their nest and after they consume the elaiosome they discard the seed in a safe and nutrient rich area (Rissing, 1986; Giladi, 2006). As predators, ants may play an important role as plant biotic defenses. Since Janzen (1966) demonstrated that ants could protect *Acacia* trees against herbivores and parasites in exchange for food and shelter, numerous works have been published evidencing the existence of a protective ant-plant mutualism involving numerous ant and plant species (Bentley, 1977; Way & Khoo, 1992; Rosumek *et al.*, 2009). As a result of this protective mutualism, several ant species are known to reduce pest populations and plant damage in natural and managed ecosystems (Way & Khoo, 1992; Karhu, 1998; Styrsky & Eubanks, 2007). Ants are also involved in a widespread and extensively documented mutualism with other insects that produce honeydew (Way, 1963). Honeydew is a nutritive excretion, rich in carbohydrates, produced by some insects of the Hemiptera Sternorrhyncha (whiteflies, aphids, mealybugs and scales) and Auchenorrhyncha (treehoppers and leafhoppers) suborders after feeding on phloem sap (Wäckers, 2001). The honeydew produced by these insects is a valuable source of food for almost all groups of ants (Carroll & Janzen, 1973; Tobin, 1994). In exchange for honeydew, ants offer several benefits to their partners such as protection against natural enemies, shelter, transport and dispersal and sanitation. As a result, the plant feeders reach higher reproductive and developmental rates and eventually increased populations under ant attendance (Way, 1963; Buckley, 1987; Stadler & Dixon, 1999, 2005).

1.2 Ants in agricultural ecosystems

The role of ants in agricultural ecosystems may be of high relevance due to their impact on the herbivore populations, interactions with the wider arthropod community and impact on plant health (Styrsky & Eubanks, 2007).

In most terrestrial habitats ants are top predators of other insects (Hölldobler & Wilson, 1990). They can reduce populations of herbivores by consuming them as source of protein (Figure 1.1). This protective and beneficial role of ants is widespread and has been extensively documented in agricultural ecosystems for a great number of ant species (Way & Khoo, 1992; Karhu, 1998; Styrsky & Eubanks, 2007; Rosumek *et al.*, 2009; Olotu *et al.*, 2013). Species in the genera of *Oecophylla, Dolichoderus, Anoplolepis, Wasmannia, Azteca, Solenopsis, Formica, Tetramorium, Pheidole* and *Dorymyrmex* have been reported to offer a good protection against pests, especially Hemiptera and

Coleoptera, in coconut, oil palms, coffee and cacao in different regions of the world (Way & Khoo, 1992; Philpott & Armbrecht, 2006; Olotu *et al.*, 2013). For example, the predatory ant, *Formica neoclara* (Emery) can reduce *Cacopsylla pyricola* (Foerster) populations in pear orchards in Washington (Paulson & Akre, 1992). The African weaver ant *Oecophylla longinoda* Latreille effectively controls sap-sucking pests in cashew in Tanzania (Olotu *et al.*, 2013). Finally, *Oecophylla smaragdina* Fabricius controls Jarvis's fruit fly *Bactrocera jarvisi* (Diptera: Tephritidae) in mango orchards in Australia (Peng & Christian, 2006).

Fig. 1.1. Worker of the ant *Linepithema humile* preying on a *Prays citri* larva.

However, in agroecosystems, the protective mutualism between ants and honeydew producers may have a negative impact on plants, especially because many of the ant-tended hemipteran species are serious crop pests. Indeed, many of the world's major pests are hemipterans, such as aphids, mealybugs and scale insects, and factors influencing their populations have important economic implications (Buckley, 1987). One of the best known and studied ant-hemipteran mutualism is the existing between ants and certain aphid species

(Figure 1.2). Increased populations of aphids in ant-attended colonies have been shown in apple (Stewart-Jones *et al.*, 2008; Miñarro *et al.*, 2010), bean (Banks, 1962), banana (Stechmann *et al.*, 1996) and cotton (Kaplan & Eubanks, 2002). Mealybugs are one of the most important pests in vineyards (Daane *et al.*, 2008) and ants are strongly associated with them. The invasive Argentine ant, *Linepithema humile* (Mayr) was associated with higher mealybug densities and fruit infestation in California (Daane *et al.*, 2007) and South Africa vineyards (Mgocheki & Addison, 2010). Moreover, ants have been found to induce populations increases of pests that do not produce honeydew, such as *Aonidiella citrina* Craw (Hemiptera: Diaspididae) (Flanders, 1945), *Aonidiella aurantii* Murdoch (Hemiptera: Diaspididae) (Moreno *et al.*, 1987; Pekas *et al.*, 2010b; Dao *et al.*, 2014) and *Panonychus citri* (McGregor) (Acari: Tetranychidae) (Haney *et al.*, 1987).

Fig. 1.2. Ants tending a colony of aphids.

Therefore, ants may act as pest predators offering a beneficial service, while on the other hand, due to their association with the honeydew producers they are considered as indirect pests. In many cases both effects coexist. The presence of honeydew producers on a plant attracts ants that boost their populations but, at the same time, predate on other more harmful herbivores coexisting on the same host plant (Messina, 1981; Floate & Whitham, 1994; Perfecto & Vandermeer, 2006; Styrsky & Eubanks, 2010). Thus, the net effects of ant-hemipteran interactions on host plant health constitutes a balance between the benefits obtained from herbivore suppression due to ant predation and the costs caused by the increased pest populations (Kaplan & Eubanks, 2005; Styrsky & Eubanks, 2007).

1.2.1 Ants in citrus agroecosystems

Ants are a major component of the citrus agroecosystem fauna (Bodenheimer, 1951; Samways *et al.*, 1982; Alvis & Garcia-Mari, 2006). Haney (1988) recorded 295 ant species in citrus orchards from 50 countries around the world. In South Africa, Samways *et al.*, (1982) recorded 123 ant species in citrus orchards from which 44 foraged in the trees and 25 were observed tending honeydew-producers.

The weaver ant *Oecophylla* sp. has long been used in citrus to control a wide range of potential pests in what is known as the first example of biological control, dating from 304 AD (VanMele & Cuc, 2000; VanMele, 2008). In Hawaii, Wong & McInnis (1984) reported that the Argentine ant *L. humile* predates on the fruit fly *Ceratitis capitata* (Wiedemann) pupae in the soil. However, the presence of ants in citrus ecosystems has been generally related with population outbreaks of honeydew-producing pests. Furthermore, ant activity in citrus has been found to increase populations of pests that do not produce honeydew.

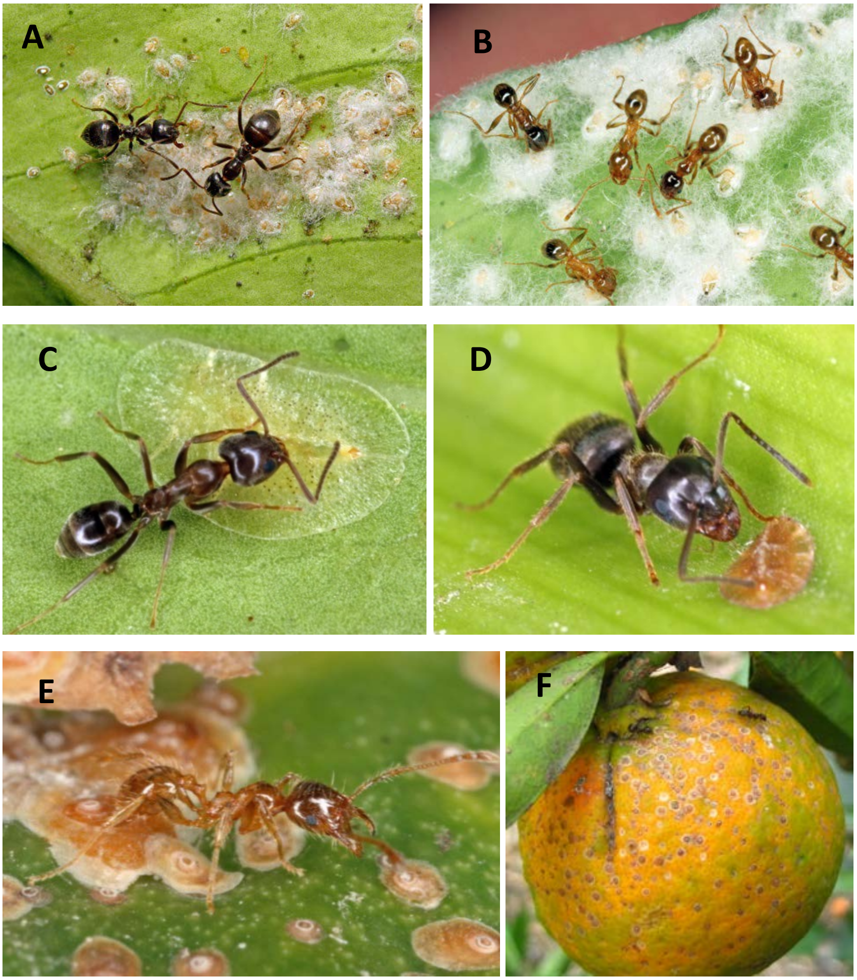

Fig. 1.3. A. Workers of the ant *Lasius grandis* tending a colony of *Aleurothrixus floccosus*. B. Workers of the ant *Pheidole pallidula* tending a colony of *A. floccosus*. C. *Linepithema humile* on *Coccus hesperidium*. D. *Lasius grandis* on *Saissetia oleae*. E. *Pheidolle pallidula* on *Aonidiella aurantii*. F. *Lasius grandis* workers foraging on an *A. aurantii* infested fruit.

Flanders (1945) reported increased populations of the non-honeydew producer *A. citrina* due to the presence of the Argentine ant *L. humile* tending the honeydew producer *Coccus hesperidum* L. on the same trees (Hemiptera: Coccidae). The abundance of the scales, *Saissetia* *oleae* Olivier (Hemiptera: Coccidae) and *C. hesperidum* (Bartlett, 1961), and *A. aurantii* declined after ant removal. Moreno *et al.* (1987) also found that the exclusion of *L. humile* resulted in lower mealybug and whitefly populations and reduced fruit infestation caused by *A. aurantii*. Yoo *et al.* (2013) confirmed that the exclusion of *L. humile* from citrus canopies resulted generally in decreased populations of *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), *Coccus hesperidium* L. (Hemiptera: Coccidae), *A. floccosus* and *A. aurantii* in an organic lemon orchard.

In South Africa, the brown house ant *Pheidole megacephala* F. was associated with higher infestations of *A. aurantii* in orange orchards (Steyn, 1954). The canopy-foraging ant *Iridiomyrmex rufoginer* (Lowne) stimulated the populations of *C. hesperidum* and *A. aurantii* (James, 1997) and black scale *S. oleae* (Dao *et al.*, 2014) in citrus orchards in Australia. In Japan, the exclusion of *Lasius niger* L. produced a 94% decrease in the populations of the mealybug *Pseudococcus citriculus* Green (Itioka & Inoue, 1996a) and of the wax scale insect *Ceroplastes rubens* Maskell (Hemiptera: Coccidae) (Itioka & Inoue, 1996b) in a Satsuma orange orchard. In the same way, *Aphis gossypii* Glover (Hemiptera: Aphididae) colonies were bigger when attended by *L. niger* in a mandarin orchard (Kaneko, 2002).

1.3 Mechanisms underlying the increases of the herbivore populations induced by ants

1.3.1. Sanitation and stimulation of the herbivores

The increased populations of honeydew producers induced by ants may be a consequence of several mechanisms. The sanitation effect, i.e. the elimination of honeydew, has been found to benefit honeydew producers by reducing mortality caused by fungal attack (Buckley, 1987). If not removed by ants, the accumulation of honeydew produced by the aphid *Chaitophorus populicola* Thomas may be one of the causes of declined aphid populations in cottonwood (Wimp & Whitham, 2001). The exclusion of *I. rufoniger* caused high mortality of the black scale *S. oleae* through asphyxiation by its own honeydew (Dao *et al.*, 2014).

Moreover, ants may have direct effects on the reproduction and fitness of the attended Hemiptera (Way, 1963). For example, the reproductive performance of aphids increased under ant attendance (El-Ziady, 1960; McPhee *et al.*, 2012). Flatt & Weisser (2000) showed that ant-tended aphids lived longer, matured earlier, and had a higher rate of reproduction and a higher expected number of offspring than aphids that are not tended by ants. Nevertheless, the aphid-ant mutualism may also result in costs for aphids that are detrimental for the colony growth (see Stadler & Dixon 1998).

1.3.2 Interference of ants with the activity of the natural enemies

The association between ants and honeydew producers is considered a protective mutualism given that the major benefit that ants offer to their partners is protection against predators and parasitoids (Buckley, 1987). Numerous studies have shown that the population increases of the honeydew and non-honeydew producers are caused by the interference of ants with their natural enemies.

Banks (1962) showed that *L. niger* protected *Aphis fabae* Scopoli against several coccinellid, chrysopid and sirfid predators on bean plants. In *Pluchea indica* (L.) plants *Pheidole megacephala* (Fanricius) reduced the parasitism and the attacks of the predatory coccinellids of *Coccus viridis* (Green) (Hemiptera: Coccidae) (Bach, 1991). The presence of *L. humile* in oleander (*Nerium oleander* L.) resulted in lower parasitism rates and increased populations of the scale *S. oleae* (Barzman & Daane, 2001). In the case of non-honeydew producers, such as *A. aurantii*, it is supposed that ants disrupt their parasitoids as an indirect effect of ant-attendance on honeydew producers present on the same tree (Flanders, 1945; Moreno *et al.*, 1987; James, 1997).

Similarly, in citrus agroecosystems pest outbreaks have been associated with the protection of ants against natural enemies (Figure 1.4). The population increase of the mealybug *Pseudococcus citriculus* Green (Hemiptera: Pseudococcidae) was caused by the aggressive behavior of *L. niger* against *Chilocorus kuwanae* (Silvestri) (Coleoptera: Coccinellidae) and a predatory chrysopid species (Neuroptera: Chrysopidae) (Itioka & Inoue, 1996a). The aggressive behavior of *L.*

niger against the coccinellid *Harmonia axyridis* Pallas resulted in increased aphid numbers in *A. gossypii* colonies (Kaneko, 2002). The parasitism of *A. aurantii* caused by *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) decreased and the *A. aurantii* population increased with the number of ants ascending to the trees in a lemon orchard (Yoo *et al.*, 2013).

Fig. 1.4. Ants attacking the predatory ladybirds *Chilocorus bipustulatus* (left) and *Coccinella septempunctata* (right).

Nevertheless, the aggressiveness of ants as well as the tolerance of parasitoids and predators to ant attack are highly variable for both ant and natural enemy species. Some parasitoid species are highly affected by ants while other species can cope effectively with ant-aggression (Flanders, 1951, 1958; Way, 1963; Völkl & Mackauer, 1993). In the same way, the response of predators to ants varies depending on the species involved. Ants impact negatively numerous coccinellid and chrysopid species (Bartlett, 1961; Itioka & Inoue, 1996a; Kaplan & Eubanks, 2002) while some other coccinellid species are not affected (Flanders, 1958; Vanek & Potter, 2010). Some natural enemy species even benefit from ants tending hemipterans through elimination of other competitors (Flanders, 1951) or via morphological or behavioral adaptations. For example, wax covers in larvae of *Scymnus* species (Völkl & Vohland, 1996), myrmecomorphy of *Pilophorus* sp. (Piñol *et al.*, 2012b; Sánchez & Ortín-Angulo, 2012) or chemical mirmicy in *Lysiphlebus cardui* (Marshall) (Hymenoptera: Braconidae) (Völkl & Mackauer, 1993) constitute camouflage strategies that allow these species to exploit food sources tended by ants.

All the aforementioned studies reporting an impact of ants on the abundance and activity of natural enemies examine mostly pairwise interactions. Nevertheless, ants may have wider community-level consequences (Styrsky & Eubanks, 2007). Ants may alter simultaneously the abundance or spatial distribution of different species, affecting therefore the biodiversity and community structure of natural enemies. Recently, the community-level consequences of the ant-hemipteran mutualism have received more attention and several authors have focused on the impact of ants on diversity, community structure or abundance of several natural enemies in different ecosystems (Table 1, Chapter 4). The results obtained are highly context-dependent and differ depending on the ant and natural enemy species as well as the ecosystem in which the study took place.

Whereas some of these studies have demonstrated a negative impact of ants on several groups of natural enemies, specially predators (James *et al.*, 1999; Eubanks, 2001; Kaplan & Eubanks, 2005; Piñol *et al.*, 2012a), some other authors do not find any effect (Offenberg *et al.*, 2005; Philpott *et al.*, 2008; Chong *et al.*, 2010) or even report positive effects of ants on the natural enemy abundance and diversity (Stewart-Jones *et al.*, 2008; Peng & Christian, 2013). It is important to mention that many of the above studies include numerous natural enemies often grouped at the order level. Little is known about the impact of ants on the community of predators and parasitoids at the species level, particularly in the case of parasitoids which are often grouped as a single group in Hymenoptera order.

1.3.3 A new hypothesis: impact of ants on the physiological state of the natural enemies?

The honeydew produced by hemipterans is a valuable source of energy not only for ants, but also for many entomophagous arthropods in natural and managed ecosystems. Most species of parasitoids and numerous predators consume carbohydrates (Jervis & Kidd, 1986; Wäckers *et al.*, 2008; Tena *et al.*, 2013c). Honeydew is the most prevalent and available sugar source in agricultural ecosystems (Wäckers, 2005) and its consumption enhances the longevity and fecundity of parasitoids (Wäckers, 2001; Faria *et al.*, 2008; Pekas *et al.*, 2010c; Tena *et al.*, 2013b). Thus, the presence and availability of honeydew in agroecosystems may be a key element for the fitness and performance of natural enemies in the field.

The presence of ants may increase honeydew availability as a result of the increased populations of the honeydew-producers. This eventually may result in improved sugar feeding opportunities for the natural enemies. Evans & England (1996) showed in field experiments that the availability of honeydew produced by aphids increased parasitism levels by the wasp *Bathyplectes curculionis* Thomson (Hymenoptera: Ichneumonidae) on the alfalfa weevil *Hypera postica* (Gyllenhal) and highlighted the importance of this indirect interactions for biological control. However, given that ants, as well as many species of parasitoids and predators, rely on honeydew to fulfil their energetic needs, they may interact competitively through the shared honeydew exploitation (Figure 1.5). In such an interaction, the access of natural enemies to honeydew might be hampered. Thus, their nutritional state and eventually their effectiveness as biological control agents may be seriously compromised. Nevertheless, this hypothesis has never been tested.

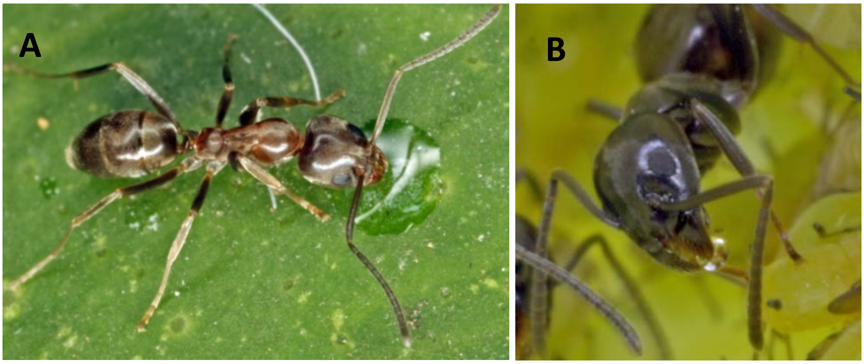

Fig. 1.5. A. *Linepithema humile* feeding on a honeydew droplet. B. *Lasius grandis* feeding on honeydew from an aphid cauda.

1.4 Ants in Mediterranean citrus agroecosystems

1.4.1 Species composition

Up to 55 ant species have been reported in Mediterranean citrus (Pekas, 2011). Nevertheless, the species richness and diversity in citrus orchards is poor when compared to natural communities (Cerdá *et al.*, 2009). Studies performed on the Mediterranean coast of the Iberian Peninsula report 26 different species of ants in citrus orchards (Table 1).

The most abundant and widespread ant species in Mediterranen citrus are *Lasius grandis* and *Pheidole pallidula* (Figure 1.6). The Argentine ant *Linepithema humile* (Figure 1.6) is present but not widely distributed in the Mediterranean citrus. Nevertheless, when present in an orchard *L. humile* is very abundant.

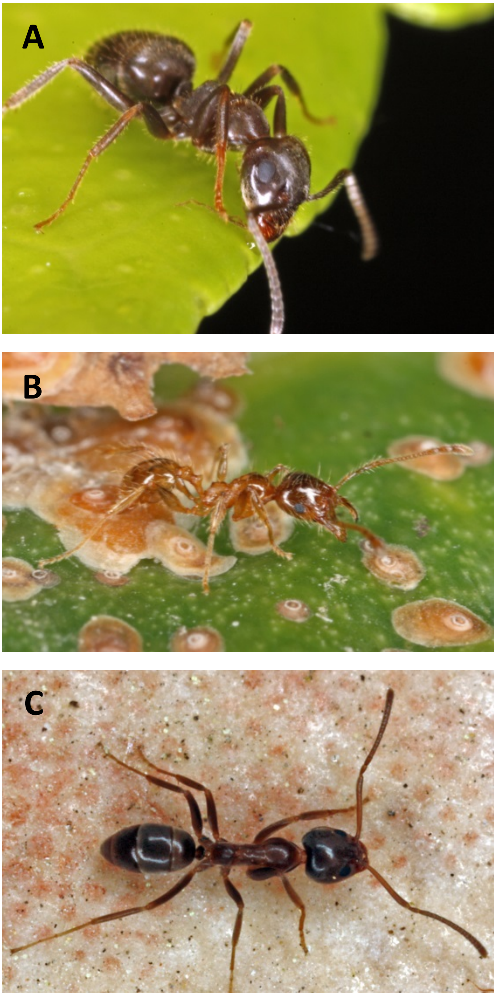

Fig. 1.6. A. A worker of *Lasius grandis*. B. A worker of *Pheidole pallidula*. C. A worker of *Linepithema humile*.

Table 1. Ant species reported in citrus orchards in the Mediterranean coast of the Iberian Peninsula.

Subfamily	Ant species	References**
Dolichoderinae	*Linepithema humile* (Mayr) *	1,2,3,4
	Tapinoma nigerrimum (Nylander) *	1,3,5
	Tapinoma erraticum (Latreille) *	2,3,4
	Tapinoma simrothi Krausse *	3
Formicinae	*Camponotus foreli* (Emery) *	1,3
	Camponotus pilicornis (Roger) *	1
	Camponotus sylvaticus (Olivier) *	2,3,4
	Formica cunicularia Latreille	1
	Formica gerardii Bondroit	3
	Formica rufibarbis Fabricius	2,4
	Lasius grandis Forel *	2,4,5
	Lasius niger L. *	1,3
	Plagiolepis pygmaea (Latreille) *	2,3,4
	Plagiolepis schmitzii Forel *	1,3,5
Myrmicinae	*Aphaenogaster senilis* Mayr	1
	Cardioncodyla batesii Forel	4
	Cardioncodyla elegans Emery	4
	Cardiocondyla mauritanica Forel	2,3
	Cataglyphis gadeai De haro & Collingwood	2,4
	Diplorhoptrum robusta Bernard	1
	Messor barbarous L.	1,2,4
	Myrmica scabrinodis Nylander	1
	Pheidole pallidula (Nylander) *	1,2,3,4,5
	Tetramorium caespitum L.	1
	Tetramorium semilaeve André	3,4
Ponerinae	*Hypoponera eduardi* (Forel)	1,2,4

* Canopy-foraging ant species

** (1) Palacios *et al.* 1999; (2) Vanaclocha *et al*. 2005; (3) Alvis 2006; (4) Urbaneja *et al.* (2006); (5) Pekas *et al.* (2011)

In Mediterranean ecosystems interespecific competition and thermal tolerance shape ant communities in hierarchies of dominant and subordinate species (Cerdá *et al.*, 1997; Retana & Cerdá, 2000). Dominant species have larger colonies and defend food sources more fiercely than subordinates (Arnan *et al.*, 2012). On the other hand, subordinate species can coexist with dominant species since they are heat-tolerant and are active in a wider range of temperatures than dominant species (Cerdá *et al.*, 1997, 1998). In citrus orchards in Valencia (Spain) Pekas *et al.* (2011) reported that ants also show interspecific competition and distribute in what is known as "ant-mosaic", in which dominant species maintain exclusive territories and subordinate species coexist with the dominant ones.

Lasius grandis

This species was identified for the first time by Forel (1909) as a "strain" of *Lasius niger* (Linnaeus). Posteriorly it was separated and identified as *Lasius grandis* Forel by Seifert (1992) who indicated that *L. grandis* is the most abundant species of this genus in the Iberian Peninsula. Thus, many studies about *L. niger* in the Iberian Peninsula apparently correspond to *L. grandis*. *Lasius grandis* has low tolerance to high temperatures and therefore it is found in sheltered and humid places avoiding sun-exposed and xerothermic places (Seifert 1992). It is a monogenic (i.e. one queen per nest), aggressive and dominant species that principally feeds on honeydew (Paris & Espadaler, 2009; Pekas *et al.*, 2011) and has been associated with outbreaks of honeydew producers (Palacios et al. 1999; Pekas et al. 2011). Interestingly, this species was associated with increased population densities of the non-honeydew producer *A. aurantii* (Pekas *et al.*, 2010b).

Pheidolle pallidula

The dimorphic species *Pheidole pallidula* is a native and dominant species in Mediterranean ecosystems. It is found in a great variety of ecosystems and nests mostly in arid or sunny areas with low vegetation (Detrain, 1990). It is an omnivorous species that combines different foraging strategies, using an efficient recruitment system when an important food source is found (Detrain, 1990; Retana & Cerdá, 1992). Its Mediterranean origin makes *P. pallidula* better adapted to high temperatures (Palacios *et al.*, 1999).

Linepithema humile

The Argentine ant *Linepithema humile* is a polygenic (multiple queens per nest) and unicolonial invasive ant species from South America. It has spread in many parts of the world with Mediterranean-type climates (Suarez *et al.*, 2001), and has colonized many ecosystems. In Spain it was recorded for the first time in 1923 associated with honeydew-producing hemipterans in citrus orchards (Font de Mora, 1923) and nowadays it is distributed over the entire coast of the Iberian Peninsula (Espadaler & Gómez, 2003). Its presence however is restricted mostly close to disturbed areas and it requires high humidity and temperate climate (Palacios *et al.*, 1999; Espadaler & Gómez, 2003). *Linepithema humile* is a dominant and very aggressive species that displaces native ants and affects other arthropods (Human & Gordon, 1997; Holway *et al.*, 2002). Although *L. humile* is an omnivorous species it shows a strong preference for liquid food, especially honeydew from hemipterans (Markin, 1970a; Abril *et al.*, 2007) and has been associated with population outbreaks of honeydew (Bartlett, 1961; Yoo *et al.*, 2013;

Dao *et al.*, 2014) and non-honeydew producers (Flanders, 1945; Moreno *et al.*, 1987) in citrus agroecosystems.

1.4.2. Impact on citrus pests

In the Mediterranean area, citrus is one of the most important crops and several authors have studied the impact of ants on pest populations. In Italy, several ant species have been associated with pest outbreaks in citrus (Tumminelli *et al.*, 1996). In Israel, (Rosen, 1967) found 13 ant species associated with honeydew producers in citrus orchards, although the populations of the hemipterans rarely acquired serious pest levels.

In the Iberian Peninsula, field observations indicated that *L. niger* disturbed parasitoids of the citrus mealybug *P. citri* reducing the parasitism levels about 35% in a study conducted in Tarragona, (Campos *et al.*, 2006). Pekas *et al.* (2010b) demonstrated that the native ants *L. grandis* (Forel), *P. pallidula* (Nylander) and *P. schmitzii* produced increases of *A. aurantii* in citrus orchards in Valencia. In Tarragona, and contrary to the expected, (Piñol *et al.*, 2012a) found lower populations of aphids in the presence of ants.

1.4.3. Impact on natural enemies

Little is known about the interactions involving ant species and natural enemy species in Mediterranean citrus orchards. Piñol & Espadaler (2010) showed that almost all arthropod orders they studied were more abundant when ants were excluded from the citrus canopies. In the same way, in an 8-year study, Piñol *et al.* (2012a) observed that ant-exclusion modified the arthropod community. More specifically, they showed that ants altered the abundance of predatory Heteroptera in four years of the study (out of 8 years) and negatively affected the spider assemblages and the abundance of several spider species in some years (Mestre *et al.*, 2013).

Besides the aforementioned studies, there is no information regarding the impact of ants in the Mediterranean citrus on predator and parasitoid species simultaneously and on the biodiversity and community structure of natural enemies.

1.5 Justification and objectives

Ants constitute an important component of citrus agroecosystems given their abundance and ubiquity. Despite the fact that they may provide a positive service as generalist predators, ants, due to their mutualism with honeydew producers, are often associated with population outbreaks of honeydew and non-honeydew producing pests. In this context, we proposed the following objectives:

i) *Evaluate the influence of ants on pest populations and their parasitism rates*

In the western Mediterranean, where we conducted our research, the most abundant species foraging in citrus canopies are the native *L. grandis* and *P. pallidula*. Despite the fact that these species have been reported to induce population increases of *A. aurantii*, little is known about their associations with other honeydew and non-honeydew producing herbivores. In the same way, information regarding the interference of ants with predators and parasitoids in Mediterranean citrus is rather scarce. Therefore, we conducted ant-excluded experiments with the objective of determine whether the ants species present in Mediterranean citrus, the native *L. grandis* and *P. pallidula* and the invasive *L. humile*, may induce population increases of honeydew and non-honeydew producing pests. The interference of ants with natural enemies has been demonstrated as one of the principal mechanisms through which ants increase pest populations. Consequently, we examined whether ants interfere with the activity of parasitoids of the selected honeydew and non-honeydew producer herbivores by comparing the parasitism levels between the ant-allowed and ant-excluded treatments.

ii) *Study the impact of ants on predator and parasitoid populations*

The ant-hemipteran mutualism may have wider community-level consequences since ants interact directly or indirectly with multiple arthropod species. Of particular interest is the impact of ants on natural enemy assemblages in citrus agroecosystems given the potential practical implications for biological pest control. Most of the studies examining the impact of ants on natural enemy communities up to date have focused on different natural enemies at the order level and little is known about the impact of ants on predator and particularly parasitoid species. In Mediterranean citrus, information regarding the impact of ants on natural enemy assemblages and concrete species is rather scarce. Therefore, another objective of our study was to determine the impact of *L. grandis*, *P. pallidula* and *L. humile* on the assemblage of the natural enemies by comparing the abundance, diversity, species richness and community structure of predator and parasitoid species between ant-allowed and ant-excluded treatments.

iii) *Determine whether ant activity affects the honeydew exploitation and energy reserves of natural enemies*

The honeydew produced by hemiptera is the most abundant sugar source in agricultural ecosystems and a valuable food for ants as well as for natural enemies. Thus, ants and natural enemies may interact competitively via the shared honeydew exploitation. In that sense, ant-tended honeydew sources may be inaccessible for natural enemies and consequently their physiological state negatively affected due to ant competition. No previous studies have tested this hypothesis. Thus, by using High Performance Liquid Chromatography (HPLC) we compared the energy reserves, sugar and honeydew feeding incidence of predators and parasitoids in the ant-allowed and ant-excluded treatments.

Chapter 2

Ants affect the infestation levels but not the parasitism of honeydew and non-honeydew producing pests in citrus

Ants affect the infestation levels but not the parasitism of honeydew and non-honeydew producing pests in citrus

Calabuig, A., Garcia-Marí, F. & Pekas, A. (2013) Ants affect the infestation levels but not the parasitism of honeydew and non-honeydew producing pests in citrus. *Bulletin of Entomological Research*, **104**, 405–417.

Doctoral thesis adapted version.

Abstract: Ants act simultaneously as predators and as hemipteran mutualists, and thereby may affect the composition and population dynamics of a wide arthropod community. We conducted ant-exclusion experiments in order to determine the impact of ants on the infestation levels and parasitism of three of the most important citrus pests of western Mediterranean citrus: the honeydew producer *Aleurothrixus floccosus* Maskell (woolly whitefly) and the non-honeydew producers *Aonidiella aurantii* Maskell (California red scale; CRS) and *Phyllocnistis citrella* (Staiton) (citrus leafminer). The study was conducted in three commercial citrus orchards, each one dominated by one ant species (*Pheidole pallidula, Lasius grandis* or *Linepithema humile*) during two consecutive growing seasons (2011 and 2012). We registered a significant reduction of the CRS densities on fruits in the ant-excluded treatment in the three orchards and in the two seasons, ranging from as high as 41% to as low as 21%. Similarly, the percentage of shoots occupied by *A. floccosus* was significantly lower in the ant-excluded plots in the orchards dominated by *P. pallidula* and *L. humile*. No significant differences were registered in the percentage of leaf surface loss caused by *P. citrella* between ant-allowed and ant-excluded treatments in any case. We found no significant differences in the percent parasitism between ant-allowed and ant-excluded treatments for honeydew and non-honeydew producing herbivores. These results suggest that i) ant management should be considered in order to reduce herbivore

populations in citrus and ii) mechanisms other than parasitism (e.g. predation) might explain the differences in herbivore infestation levels between treatments.

2.1 Introduction

Ants (Hymenoptera: Formicidae) are broadly distributed in terrestrial ecosystems and they are among the leading predators of other insects (Hölldobler & Wilson, 1990). Since Janzen (1966) reported that ants could act as biotic defences protecting plants against herbivores and parasites, several authors observed that the predatory action of ants against phytophagous insects benefited plants (Karhu, 1998; Styrsky & Eubanks, 2007; Rosumek *et al.*, 2009; Olotu *et al.*, 2013). However, most ant species are omnivorous and combine the protein obtained through predation and scavenging with plant-derived carbohydrates. Ants collect carbohydrates from floral and extrafloral nectar, food bodies, elaiosomes and especially honeydew produced by plant-feeding Hemiptera with which they have evolved mutualistic associations (Way, 1963; Carroll & Janzen, 1973; Hölldobler & Wilson, 1990; Wäckers, 2005). Thus, by acting simultaneously as predators and as hemipteran mutualists, ants are at the centre of a complex food web affecting the composition and the population dynamics of a wide arthropod community (Kaplan & Eubanks, 2005; Styrsky & Eubanks, 2007).

In the ant-Hemiptera mutualism, the net benefits for each partner are context dependent (Stadler & Dixon, 2005; Yoo & Holway, 2011). It is typically assumed that ants obtain honeydew, a food source that is copious, nutritive and spatiotemporally constant and in exchange, ants protect the honeydew producers from their natural enemies or other competing herbivores (Flanders, 1951; Bartlett, 1961; Way, 1963; Buckley, 1987; Rosumek *et al.*, 2009). Under ant protection, honeydew producers usually perform better and more quickly develop larger populations which eventually results in greater plant damage. This is particularly evident in agricultural ecosystems, where numerous studies have reported decreased populations of ant-attended honeydew producers and lower crop damage following ant-exclusion experiments (Flanders, 1951; Bach, 1991; Itioka & Inoue, 1996a; James, 1997; Daane *et al.*, 2007; Mgocheki & Addison, 2010). In citrus crops, Moreno *et al.* (1987) reported that the exclusion of the Argentine ant *Linepithema humile* (Mayr) was associated with lower densities of the citrus mealybug *Planococcus citri* Risso (Hemiptera: Pseudococcidae) and of the woolly whitefly *Aleurothrixus floccosus* Maskell (Hemiptera: Aleyrodidae). Itioka & Inoue (1996) reported that the ant *Lasius niger* L. showed an aggressive behavior towards natural enemies of the mealybug *Pseudococcus citriculus* Green (Hemiptera: Pseudococcidae) resulting in a drastic (94%) decrease in a mealybug population when ants were excluded. An ant-exclusion experiment revealed that ant-attendance caused an increase in the population growth rate of *Ceroplastes rubens* Maskell (Hemiptera: Coccidae) due to a decrease in the percentage of parasitism by *Anicetus beneficus* Ishii et Yasumatsu (Hymenoptera: Encyrtidae) (Itioka & Inoue, 1996b).

Surprisingly, ants have been reported to induce population increases, and concomitant plant damage, of non-honeydew producing insect herbivores (Bartlett, 1961). For example, Flanders (1945) demonstrated that the activity of *L. humile* resulted in higher infestations of the diaspidid *Aonidiella citrina* Coquillet (Hemiptera: Diaspididae). Similar population increases were reported for the California red scale (hereafter CRS) *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) caused by the action of *Pheidole megacephala* F. in Letaba (South Africa) (Steyn, 1954), *L. humile* in California (Moreno *et al.*, 1987), *Iridomyrmex rufoniger* gp. sp. in Australia (James, 1997) and *Lasius grandis* (Forel) and *Pheidole pallidula* (Nylander) in Valencia (Spain) (Pekas *et al.*, 2010b). Finally, Haney *et al.* (1987) reported a population increase of the citrus red mite *Panonynchus citri* (McGregor) (Acarina: Tetranychidae) in the presence of *L. humile*. In the above studies, it is assumed that the underlying mechanism is indirect interference by the ants (while searching for honeydew) with the natural enemies of the non-honeydew producers.

The outcome of the interaction among ants, Hemiptera (both honeydew and non-honeydew producers) and natural enemies is likely to depend on the particular characteristics of the species involved. For example, the degree of protection against natural enemies provided to hemipterans varies depending on the ant species (Martínez-Ferrer *et al.*, 2003; Styrsky & Eubanks, 2007; McPhee *et al.*, 2012). Several authors attribute these differences among ant species to biological traits such as foraging activity, numerical abundance, aggressiveness and territoriality (Buckley & Gullan, 1991; Kaneko, 2003a; Paris & Espadaler, 2009; McPhee *et al.*, 2012). Likewise, susceptibility of parasitoids and predators to ant activity differs greatly among species (Flanders, 1958; Bartlett, 1961; Völkl, 1992; Daane *et al.*, 2007).

The citrus agro-ecosystem, due to its perennial character, provides ideal conditions for the proliferation of insect herbivores, many of which are honeydew producers (Bodenheimer, 1951; Garcia-Marí, 2012). At the same time, ants are among the most abundant arthropods in citrus (Bodenheimer, 1951; Samways *et al.*, 1982; Samways, 1983; Alvis &

Garcia-Mari, 2006). In western Mediterranean citrus, where we conducted our study, the two most abundant and widely distributed ant species are the native *L. grandis* and *P. pallidula* (Palacios *et al.*, 1999; Alvis-Dávila, 2003; Vanaclocha *et al.*, 2005; Cerdá *et al.*, 2009; Pekas *et al.*, 2011). Interestingly, Tena *et al.* (2013c) showed that mixed populations of these species were associated with increases in the densities of CRS populations. The invasive *L. humile* has been present in Spanish citrus orchards since 1923 (Font de Mora, 1923; García-Mercet, 1923), but it appears only occasionally here (Alvis & Garcia-Mari, 2006). In other citrus-growing areas it is associated with strong increases in the abundance of both honeydew and non-honeydew producing hemipterans (Steyn, 1954; Moreno *et al.*, 1987; Daane *et al.*, 2007).

In the present study we conducted ant-exclusion experiments in the field in order to determine the impact of three species of ants on the infestation levels and parasitism of three of the most important citrus pests in western Mediterranean citrus: the honeydew producer *A. floccosus* and the non-honeydew producers *A. aurantii* and *Phyllocnistis citrella* (Staiton) (Lepidoptera: Gracillaridae). Concretely we asked the following questions: i) are ants able to induce population increases of herbivores in citrus; ii) is the impact of ants different for honeydew and non-honeydew producing herbivores; and iii) is the parasitism of the honeydew and non-honeydew producing herbivores affected by ants?

2.2 Materials and methods

Study sites

The study was conducted during two consecutive growing seasons, from April 2010 to November 2011, in three commercial citrus orchards located in an extensive citrus-growing region located 30 km south of Valencia, eastern Spain (39º 12’ N, 0º 20’ W; 39º 11’ N, 0º 20’ W and 39º 14’ N, 0º 15’ W). The climate in the study area is Mediterranean, with mild winters, and dry, hot summers. From now on we will refer to the orchards according to the acronym of the predominant ant species present, PP (*Pheidole pallidula*), LG (*Lasius grandis*) and LH (*Linepithema humile*). Two orchards (PP and LG) were of sweet orange *Citrus sinensis* L. Osbeck (cv. Navelina) and one (orchard LH) of a mixture of two species, sweet orange *C. sinensis* (cv. Navelina) and Clementine mandarin *Citrus reticulata* Blanco (Cv. Clementina Fina). In orchard PP, the most abundant ant species ascending to the citrus canopies was *P. pallidula*, which was present in all of the trees. It was frequently found foraging on the canopy of the same tree together with *Plagiolepis schmitzii* (Forel) and to a much lesser extent with *Tapinoma nigerrimum* (Nylander). In orchard LG, the most abundant and predominant ant species was *L. grandis*, coexisting in some trees with *P. schmitzii* and *T. nigerrimum,* except in one experimental where *L. grandis* and *P. pallidula* were similarly abundant. *Lasius grandis* was never found foraging on the same tree with *P. pallidula*, as the two species are dominant and mutually exclusive (Pekas *et al.*, 2011). In orchard LH, *L. humile* was the only ant species present and foraging on the tree canopies.

The three orchards were flood irrigated and weeds were controlled by local application of herbicides (Glyphosate®, Bayer CropScience, Spain). No chemical treatments for pest control were applied during the two years prior to initiation of the experiments, neither during the two seasons of the experiments. In the three orchards, the ants were nesting in the soil beneath the trees. Orchards were selected based on previous studies (Pekas *et al.*, 2010b, 2011) and previous field observations that revealed the spatial distribution of the ant species ascending to the tree canopies in each orchard.

Experimental design, ant exclusion and ant activity

For each orchard, the experimental design was fully randomized with four replicates (plots) of two treatments: ant-allowed and ant-excluded, with four adjacent repetitions per treatment. Each plot contained 16 trees (four rows by four trees). Ant-exclusion began in April 2011 in orchards PP and LG and in May 2011 in orchard LH and was maintained until November 2012 (19 months). During the first season (2011), ant exclusion was achieved by applying an insecticidal paint in a micro-encapsulated formulation (Inesfly FITO© (chlorpyrifos 3%)), Industrias Químicas Inesba S.L., Paiporta, Spain) to the trunk. In previous studies in the same citrus area Inesfly FITO© effectively excluded ants from citrus canopies (Juan-Blasco *et al.*, 2011). Inesfly FITO© was applied by painting a 25-cm wide band (starting from the ground) on the tree trunks of ant-excluded treatments. To ensure that no ants reached the tree canopies, ant-excluded trees were inspected every month and the band repainted if ants were observed crossing the band. Due to the fact that we observed ants crossing the painted bands in some of the trees during the first growing season we changed the ant exclusion method during the subsequent season. Thus, during 2012 ant exclusion was conducted by applying Tangle-trap (Tanglefoot, Biagro, Valencia, Spain) sticky barriers on the tree trunks. The Tanglefoot was applied using a spatula on a 15 cm wide adhesive plastic tape fixed around the trunk and starting 30 cm above ground and was renewed every two months. No adverse effect on tree development was observed due to trunk painting or sticky barriers. In order to ensure that ants could not reach the canopies through alternative ways during the two seasons of the experiment, all trees were pruned periodically to prevent branches from touching the ground and the ground vegetation was trimmed.

Ant activity was defined as the number of ants moving up and down crossing an imaginary horizontal line on the tree trunk during one minute. We monitored ant activity monthly from April 2011 until November 2012 by observing the trunk of the four central trees on each plot between 9:00 and 12:00 a.m., a period of the day where ants are actively foraging on the canopies (Pekas *et al.*, 2011). Thus, for each sampling date and in each orchard, we sampled ant activity on 16 ant-allowed and 16 ant-excluded trees.

Herbivore infestation levels in the ant-allowed and the ant-excluded treatments

California red scale

CRS infestation on twigs was assessed monthly by observing four twigs (a 20 cm long terminal part of a 1-2 year-old branch) per tree, taken at hand height from the four central trees on each plot. Infested twigs were ranked according to the following scale: 0 = 0 scales; 1 = 1-3 scales; 2 = 4-10 scales; 3 = 11-30 scales; 4 = 31-100 scales; 5 > 100 scales per twig. The infestation level was evaluated using the formula (Townsend & Heuberger, 1943):

$$I\ (\%) = \frac{\sum(nv)}{NV} \times 100$$

Where n – levels of infestation according to the scale

v – number of twigs or fruits in each level of infestation

V – total number of twigs or fruits screened

N – highest level of the scale infestation (5 in our case)

This sampling was performed in the three orchards from May to July in 2011 and 2012.

CRS population densities on fruits were determined monthly by applying the same scale to 20 fruits per tree, randomly selected from the four central trees on each plot. This sampling was performed in the three orchards from August to November 2011 and 2012, i.e. when fruits were available.

Citrus woolly whitefly

A. floccosus infestation was determined by estimating the percentage of shoots occupied by *A. floccosus* larvae. On each plot we observed 40 shoots, 10 per tree randomly selected at hand's height from the periphery of the four central trees of the plot, and counted the number of shoots with *A. floccosus* presence. The selected shoots were new and tender, with its leaves which had just reached its full size. This sampling was performed once a month from July to October in 2011 and 2012, whenever *A. floccosus* was observed in the orchards.

Citrus leafminer

P. citrella infestation was estimated by calculating the percentage of leaf area damaged. To do so, we randomly sampled 10 young shoots per plot from the four central trees, containing between 5 and 10 leaves each. Shoots were transferred to the laboratory, where we scored the damage on each leaf by visually estimating the percentage of reduction in surface area caused by *P. citrella* larvae, in 10% intervals from 0 to 100% (Schaffer *et al.*, 1997). The above process was performed once in August and October 2011 and in October 2012 for orchards PP and LG, as well as once in August 2011 and October 2012 for orchard LH.

Percent parasitism in the ant-allowed and the ant-excluded treatments

California red scale

CRS parasitism was assessed by sampling a minimum of 5 twigs and, when available, 5 fruits infested with CRS per tree from the four central trees of each plot. The samples were carried to the laboratory where we observed under a stereomicroscope 50 to 100 (depending on the availability) individuals of CRS stages susceptible to parasitism (second instar males, second instar females and third instar females) and determined the number of parasitized and unparasitized scales. In the cases where CRS population was very low, between 30 and 50 individuals were considered sufficient. In the study area CRS is parasitized by *Aphytis chrysomphali* (Mercet) and *Aphytis melinus*

DeBach (Hymenoptera: Aphelinidae) (Pekas *et al.*, 2010a; Pina *et al.*, 2012). Parasitism was identified by the presence of parasitoid eggs, larvae, prepupae or pupae. Percent parasitism was established as the number of parasitized scales x 100 / (number of parasitized scales + number of unparasitized scales) (Pekas *et al.*, 2010a). The above procedure was repeated once in June and July 2011, and July 2012 for assessing parasitism on twigs. On fruits, the percent parasitism was assessed once in September and November 2011 and September and October 2012 for orchards PP and LG and once in September and November 2011, and September, October and November 2012 for orchard LH.

Citrus woolly whitefly

Parasitism of *A. floccosus* was determined by sampling a maximum of 20 leaves (when available) infested by *A. floccosus* from the four central trees per plot. Samples were placed in plastic bags and transported to the laboratory where they were processed within the next 24 hours. Under a stereomicroscope, the number of parasitized and unparasitized nymphs was counted in a 1 cm^2 circular surface randomly selected inside the area covered by the whitefly colony on each leaf. In the study area *A. floccosus* is parasitized by *Cales noacki* Howard (Hymenoptera: Aphelinidae) (Soto *et al.*, 2001; Garcia-Marí, 2012). Parasitized whiteflies were identified by the presence of swollen nymphs without waxy secretion (Soto *et al.*, 2001). Percent parasitism was established as number of parasitized x 100 / (number of parasitized + number of unparasitized) whiteflies. The above procedure was repeated once in July and September 2011 and October 2012 for orchards PP and LG and once in July, August and September 2011 and July and August 2012 for orchard LH.

Citrus leafminer.

Parasitism of *P. citrella* was assessed by sampling 10 young shoots per tree from the four central trees on each plot. Samples were transferred to the laboratory and were processed within the next 24 hours. Under a stereomicroscope we observed a maximum of 50 (when available) leafminer individuals of stages susceptible to parasitism and counted the number of parasitized and unparasitized ones. In the study area *P. citrella* is mostly attacked by *Citrostichus phyllocnistoides* (Narayan) (Hymenoptera: Eulophidae) which accounts for more than the 97% of the parasitoids (Vercher *et al.*, 2000; Garcia-Marí *et al.*, 2004; Karamaouna *et al.*, 2010). *Citrostichus phyllocnistoides* attacks principally the second and third instars of *P. citrella*. Larval stages and parasitism were identified by visual observation, determining the presence of eggs, larvae or pupae of *C. phyllocnistoides*. Percent parasitism was calculated as: number of parasitized leafminers x 100 / (number of parasitized + number of unparasitized). The above procedure was repeated once in September 2011 and 2012 when young shoots (the preferred plant substrate by the leafminer) were available.

Statistical analysis

The effectiveness of the ant-exclusion methods was tested using Repeated Measures analysis of variance (ANOVA) with the data log-transformed in order to meet normality assumptions. Treatment (ant-excluded versus ant-allowed) was the fixed factor, sampled tree nested into ant-exclusion was the random factor and sampling date was the

Repeated Measures factor. The effects of the ant-exclusion on the herbivore infestation levels and percent parasitism on each sampling date were analyzed using one-way analysis of variance (ANOVA). The season-long effects of ant-exclusion on herbivore infestations (CRS on twigs, CRS on fruits, citrus woolly whitefly and citrus leafminer) and percent parasitism were analyzed using Repeated Measures Analysis of Variance (ANOVA). Treatment (ant-excluded versus ant-allowed) was the fixed factor, sampled tree nested into ant-exclusion was the random factor and sampling date was the Repeated Measures factor. Data were [arcsin$\sqrt{x}$] transformed in order to meet normality assumptions. Means were compared by using Fisher's least significant difference (LSD) test with the significance level set at α=0.05. All statistical analyses were performed using Statgraphics 5.1 software (Statgraphics, 1994).

2.3 Results

Ant Activity

When examining the ant activity registered in each orchard, the invasive *L. humile,* predominant in orchard LH, showed the highest activity levels during the two years of the study (Fig. 1). In both years its activity peak was registered in July, when 139.8 ± 29.1 (2011) and 118.3 ± 24.4 ants/min/tree (2012) ascended to or descended from the tree canopies. The native *P. pallidula* and *L. grandis,* predominant in orchards PP and LG, respectively, showed considerably lower activity levels than *L. humile* (Fig. 1). *Pheidole pallidula* showed an activity peak in August in both years, with 13.9 ± 1.6 (2011) and 19.8 ± 2.8 ants/min/tree (2012) ascending to or descending from the citrus canopies. *Lasius grandis* exhibited an activity peak in July in 2011 (9.2 ± 2.3 ants/min/tree) and in June in 2012 (17.3 ± 2.4 ants/min/tree). It is important to highlight that *L. humile* was active throughout the whole year, whereas almost no workers of *P. pallidula* or *L. grandis* were observed foraging on the tree canopies during the winter months, from December until March.

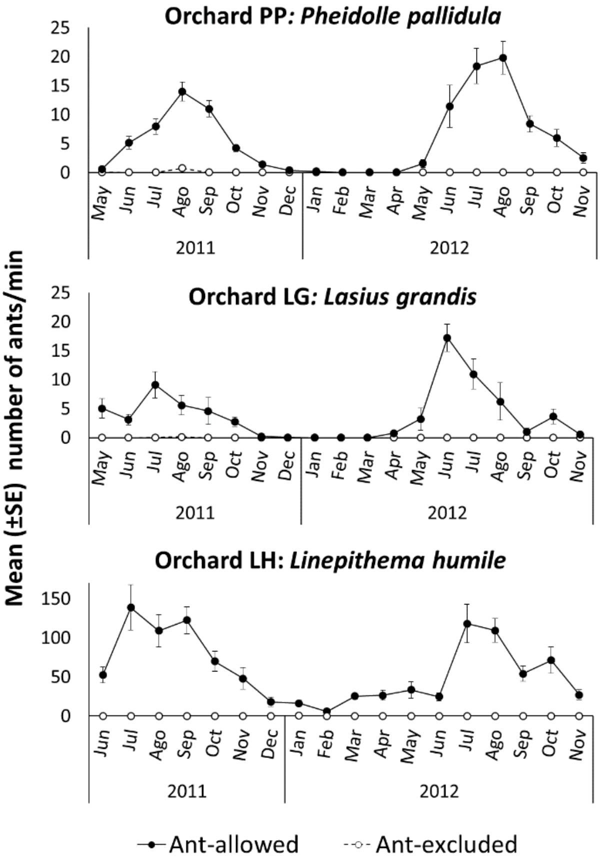

Fig. 1. Mean (±SE) ant activity (number of ants ascending or descending the tree trunk per minute) in ant-allowed and ant-excluded trees in 2011 and 2012 in three citrus orchards in eastern Spain, each with presence of *Pheidole pallidula*, *Lasius grandis* or *Linepithema humile*.

In the ant-excluded treatment, ants were effectively excluded from the tree canopies during the two years of the study. From April 2011 to March 2012, when we used Inesfly FITO® paint for ant exclusion, ants were absent from almost all the tree canopies, except in a few trees for the three orchards studied (ant-allowed versus ant-excluded: orchard PP: 4.07 ± 0.44 vs. 0.07 ± 0.05; repeated-measures ANOVA: $F_{1,\ 18}$ = 367.74; P < 0.0001; orchard LG: 2.41 ± 0.39 vs. 0.017 ± 0.01; repeated-measures ANOVA: $F_{1,\ 18}$ = 74.46; P = 0.0001; orchard LH: 60.64 ± 5.7 vs. 0.125 ± 0.05; repeated-measures ANOVA: $F_{1,18}$ = 218.71; P < 0.0001)(Fig. 1). From April 2012 to November 2012 we used Tangle-trap sticky barriers for ant exclusion and ants were totally absent from all the tree canopies, showing thus 100% effectiveness in ant-exclusion (Fig. 1).

Herbivore infestation levels

California red Scale infestation on twigs and fruits

Overall, CRS infestation on twigs was significantly lower (5% in 2011 and 18% in 2012) in the ant-excluded than in the ant-allowed trees in orchard LG, whereas no significant differences between treatments were found for orchards PP and LH (pooled data from all sampling dates; Repeated Measures ANOVA: $F_{1,\ 5}$ = 4.92; P = 0.035, $F_{1,\ 5}$ = 9.30; P = 0.34 and $F_{1,\ 5}$ = 2.94; P = 0.097, respectively) (Fig. 2a). When examining each sampling date separately, no significant differences in CRS densities were found for any sampling date for the three orchards (Fig. 2a, Table 1).

Table 1. Results of one-way analysis of variance for the effect of ant-excluded and ant-allowed treatments on A) *Aonidiella aurantii* populations on twigs B) *A. aurantii* populations on fruits, C) percentage of shoots occupied by *Aleurothrixus floccosus* and D) percentage of leaf loss caused by *Phyllocnistes citrella* in ant-allowed and ant-excluded trees in 2011 and 2012 in three citrus orchards in eastern Spain, each with presence of *Pheidole pallidula, Lasius grandis* or *Linepithema humile* (n.d. = not determined).

		Orchard PP (*Pheidole pallidula*)			**Orchard LG (*Lasius grandis*)**			**Orchard LH (*Linepithema humile)***		
Herbivore species	**Month/Year**	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
***A) Aonidiella aurantii* on twigs**										
	May 2011	1, 6	0.08	0.785	1, 6	0.51	0.503	n.d	n.d	n.d
	June 2011	1, 6	0.34	0.584	1, 6	0	0.953	1, 6	0.78	0.4125
	July 2011	1, 6	0.23	0.646	1, 6	0.79	0.408	1, 6	0.08	0.7924
	May 2012	1, 6	1.99	0.208	1, 6	0.42	0.541	1, 6	0.18	0.6868
	June 2012	1, 6	0.26	0.626	1, 6	0.89	0.382	1, 6	0.41	0.5454
	July 2012	1, 6	0.46	0.521	1, 6	5.11	0.065	1, 6	0.3	0.6038
***B) Aonidiella aurantii* on fruits**										
	August 2011	1, 6	0.61	0.463	1, 6	7.29	0.036	1, 6	13.83	0.0099
	September 2011	1, 6	1.23	0.310	1, 6	3.77	0.100	1, 6	9.26	0.0227
	October 2011	1, 6	6.61	0.042	1, 6	16.36	0.007	1, 6	9.78	0.0204
	November 2011	1, 6	13.36	0.011	1, 6	25.01	0.002	1, 6	0.89	0.3821
	September 2012	1, 6	3.77	0.100	1, 6	7.95	0.030	1, 6	1.85	0.2226
	October 2012	1, 6	0.91	0.377	1, 6	0.97	0.363	1, 6	0.67	0.4437
	November 2012	n.d	n.d	n.d	n.d	n.d	n.d	1, 6	0.39	0.5549
C) Aleurothrixus floccosus										
	July 2011	1, 6	3.68	0.104	1, 6	0.01	0.927	1, 6	7.8	0.0315
	August 2011	1, 6	1.38	0.285	1, 6	0.14	0.718	1, 6	2.74	0.149
	September 2011	1, 6	3.4	0.115	1, 6	0.01	0.925	1, 6	11.57	0.0145
	July 2012	n.d	n.d	n.d	n.d	n.d	n.d	1, 6	2.21	0.1875
	August 2012	n.d	n.d	n.d	n.d	n.d	n.d	1, 6	4.09	0.0896
	October 2012	1, 6	7.98	0.030	1, 6	3.62	0.106	n.d	n.d	n.d
D) Phyllocnistis citrella										
	August 2011	1, 6	0.02	0.894	1, 6	0.6	0.438	1, 6	0.65	0.451
	October 2011	1, 6	0.19	0.682	1, 6	0.09	0.774	n.d	n.d	n.d
	October 2012	1, 6	0.09	0.7787	1, 6	0.02	0.8928	1, 6	0.45	0.5286

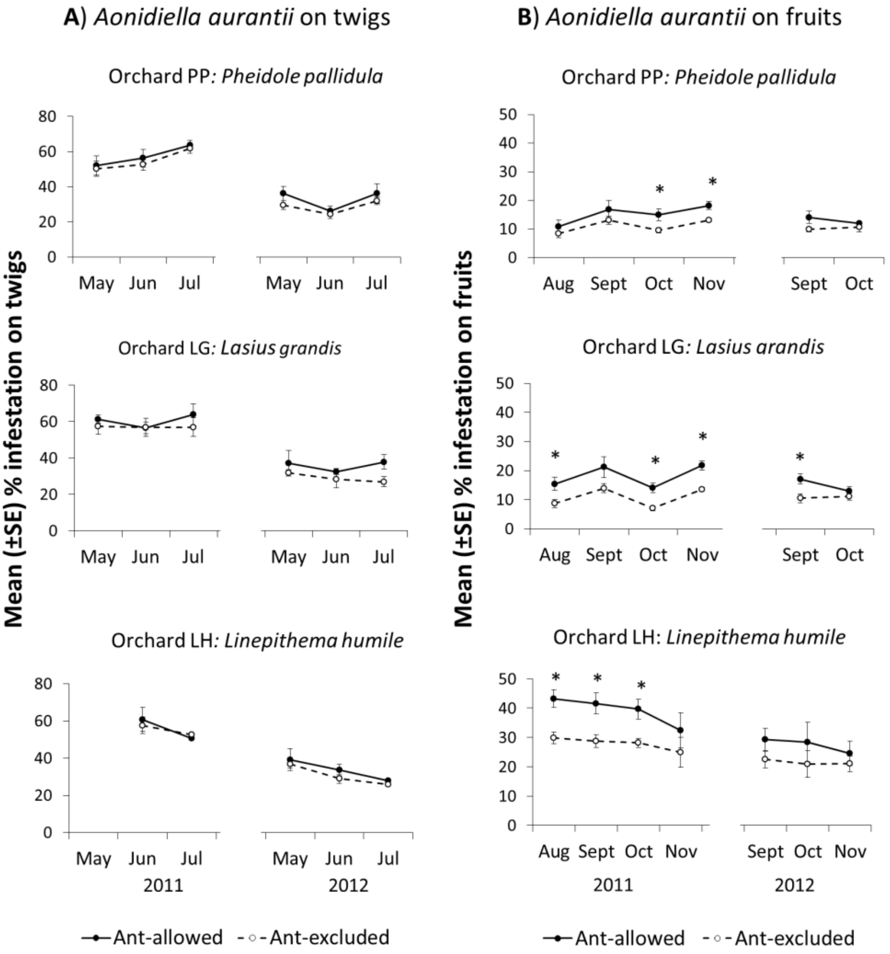

Fig. 2. Mean (± SE) California red scale infestation index on (A) twigs and (B) fruits in ant-allowed and ant-excluded treatments in 2011 and 2012 in three citrus orchards in eastern Spain, each with presence of *Pheidole pallidula*, *Lasius grandis* or *Linepithema humile*. For each sampling date, asterisk indicates significant differences between treatments (p <0.05).

CRS infestation on fruits was lower in the ant-excluded treatment for the three orchards (pooled data from all sampling dates; Repeated Measures ANOVA: orchard PP: $F_{1,5} = 11.45$; $P = 0.002$; orchard LG: $F_{1,5} = 34.91$; $P < 0.0001$; orchard LH: $F_{1,6} = 10.86$; $P = 0.003$). When examining each sampling date separately, CRS densities on fruits were significantly lower in the ant-excluded treatment in 9 out of 19 sampling dates (Fig. 2b, Table 1). Overall, we registered a significant reduction of the CRS densities on fruits in the ant-excluded treatment: 41% and 26% in 2011 and 2012, respectively, for orchard LG (where *L. grandis* was predominant), 28% and 21% for orchard PP (*P. pallidula),* and 27% and 21% in orchard LH (*L. humile)*.

Citrus woolly whitefly

The percentage of shoots occupied by *A. floccosus* was significantly lower in the ant-excluded treatment in the case of orchards PP and LH. On the other hand, no significant differences were found between treatments in the case of orchard LG (pooled data from all sampling dates; Repeated Measures ANOVA: orchard PP: $F_{1,3} = 9.43$; $P = 0.0045$; orchard LG: $F_{1,3} = 0.22$; $P = 0.646$; orchard LH: $F_{1,4} = 18.65$; $P = 0.0002$) (Fig. 3).

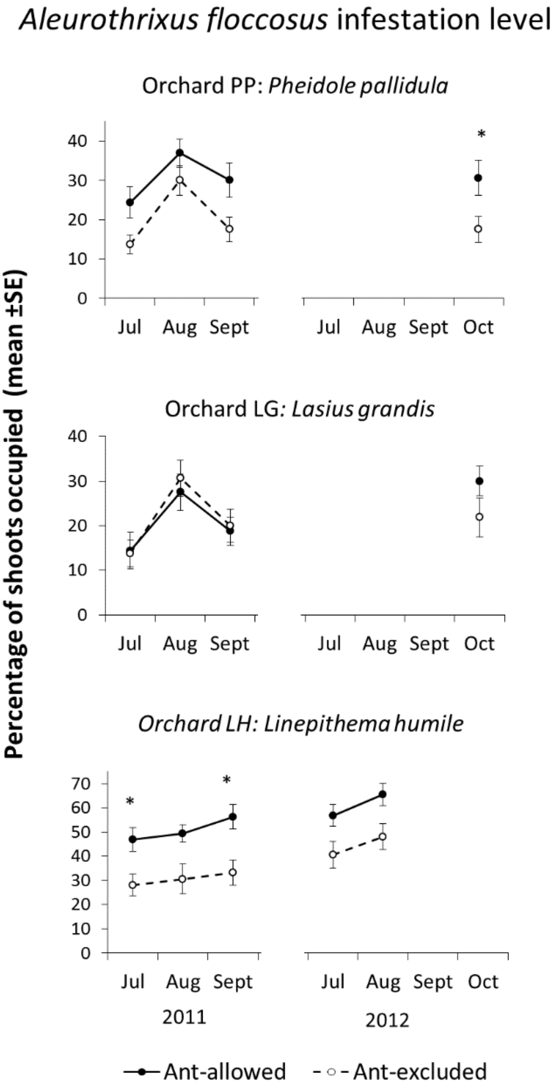

Figure 3. Mean (± SE) percentage of shoots occupied by *Aleurothrixus floccosus* in ant-allowed and ant-excluded treatments in 2011 and 2012 in three citrus orchards in eastern Spain, each with presence of *Pheidole pallidula, Lasius grandis* or *Linepithema humile*. For each sampling date, asterisk indicates significant differences between treatments (p<0.05).

When comparing each sampling date separately, the percent occupation of shoots was significantly higher in the ant-allowed treatment in one of the four dates (October 2012) for orchard PP and in 2 out of 5 sampling dates for orchard LH (Fig. 3, Table 1). Overall, the mean reduction of shoots occupied by *A. floccosus* in the ant-excluded treatment was 35% in 2011 and 43% in 2012 for orchard PP (*P. pallidula)* and 40% in 2011 and 26% in 2012 for orchard LH (*L. humile)*.

Citrus leafminer

We found no significant differences in the percent of leaf surface loss caused by larvae of *P. citrella* between ant-allowed and ant-excluded treatments for any of three orchards (pooled data from all sampling dates; Repeated Measures ANOVA: orchard PP: $F_{1,\ 2} = 1.6$; $P = 0.223$; orchard LG: $F_{1,\ 2} = 0.01$; $P = 0.9327$; orchard LH: $F_{1,\ 1} = 0.03$; $P = 0.8709$) (Fig. 4). When comparing each sampling date separately, no significant differences in the percent of leaf surface loss were found for any sampling date for the three orchards (Fig. 4, Table 1).

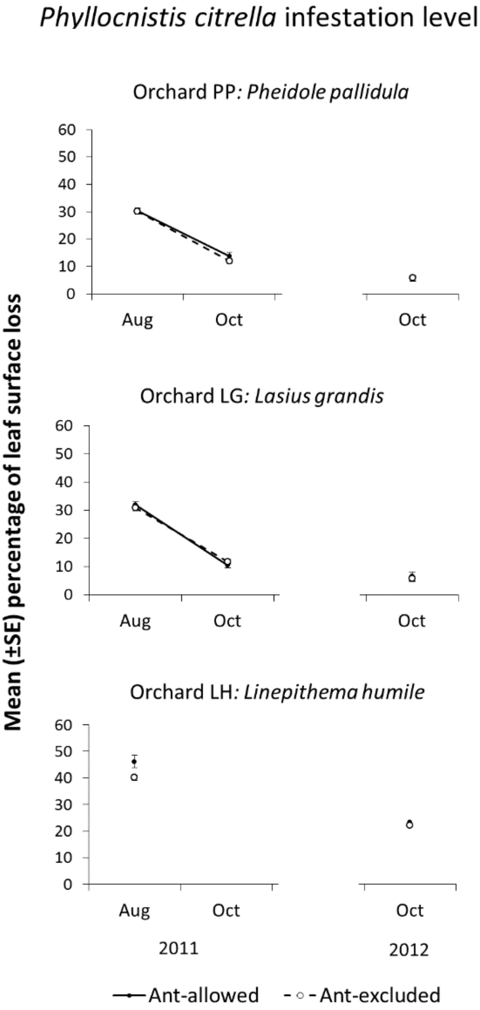

Figure 4. Mean (± SE) percentage of leaf surface loss caused by *Phyllocnistis citrella* larvae in ant-allowed and ant-excluded treatments in 2011 and 2012 in three citrus orchards in eastern Spain, each with presence of *Pheidole pallidula, Lasius grandis* or *Linepithema humile*. For each sampling date, asterisk indicates significant differences between treatments (significance level: $p<0.05$).

Percent parasitism

California red scale on twigs and fruits

The mean (±SE) percent parasitism of CRS on twigs peaked in July and reached 13.4% (± 2.07), 9.6% (± 3.3) and 11.4% (± 3.16) in orchards PP, LG and LH respectively. The mean (±SE) percent parasitism of CRS on fruits peaked in September and was considerably higher than on twigs, reaching 45.6% (± 3.6), 42.7% (± 3.33) and 38.0% (± 2.5) in orchards PP, LG and LH respectively.

On twigs we found no differences in percent parasitism of CRS between ant-allowed and ant-excluded treatments in any of the three orchards studied when pooling data from all sampling dates (Repeated Measures ANOVA; orchard PP: $F_{1,2} = 1.61$; $P = 0.2512$; orchard LG: $F_{1,2} = 2.75$; $P = 0.1481$; orchard LH: $F_{1,2} = 1.81$; $P = 0.2271$). When comparing each sampling date separately, we found significantly higher percent parasitism in the ant-excluded treatment in orchard LH in one of three dates examined (July 2011) (Table 2).

Likewise, percent parasitism of CRS on fruits was similar between the ant-allowed and the ant-excluded treatments for the three orchards (Repeated Measures ANOVA: orchard PP: $F_{1,3} = 0.26$; $P = 0.6288$; orchard LG: $F_{1,3} = 0.02$; $P = 0.8970$; orchard LH: $F_{1,4} = 4.54$; $P = 0.0772$). Furthermore, no significant differences in percent parasitism on fruits between treatments were found when comparing each sampling date separately (Table 2). In the orchard LH (*L. humile*) percent parasitism on fruits was consistently higher in the ant-excluded treatment; however, differences between treatments were marginal.

Citrus woolly whitefly

No significant differences in percent parasitism of *A. floccosus* were detected between ant-excluded and ant-allowed treatments in any of the three orchards studied (pooled data from all sampling dates; Repeated Measures ANOVA: orchard PP: $F_{1,2} = 0.71$; $P = 0.4053$; orchard LG: $F_{1,2} = 0.07$; $P = 0.7951$; orchard LH: $F_{1,4} = 0.65$; $P = 0.4428$). Similarly, no significant differences were found between treatments when comparing the data separately on each sampling date (Table 2), except on one of the five dates examined in orchard LH. On this particular date we found significantly higher percent parasitism in the ant-allowed treatment (17.73% ± 3.40) than in the ant-excluded treatment (9.46% ± 2.60) (Table 2).

Citrus leafminer

Percent parasitism of *P. citrella* was significantly higher in the ant-excluded plots in orchard LG, whereas no significant differences between treatments were found for orchards PP and LH (pooled data from all sampling dates; Repeated Measures ANOVA: $F_{1,1} = 15.11$; $P = 0.0081$; $F_{1,1} = 0.07$; $P = 0.7995$; $F_{1,1} = 0.75$; $P = 0.4197$, respectively). No significant differences between treatments were found for any of the three ant species when comparing each sampling date separately (Table 2).

Table 2: Results of one-way analysis of variance for the effect of ant-excluded and ant-allowed treatments on mean (±SE) percent parasitism of A) *Aonidiella aurantii* on twigs. B) *A. aurantii* on fruits. C) *Aleurothrixus floccosus* and D) *Phylocnistes citrella* in ant-allowed and ant-excluded trees in 2011 and 2012 in three citrus orchards in eastern Spain, each with presence of *Pheidole pallidula, Lasius grandis* or *Linepithema humile* (n.d. = not determined).

		Orchard PP (*Pheidole pallidula*)					**Orchard LG (*Lasius grandis*)**					**Orchard LH *(Linepithema humile)***				
Herbivore species	**Month/Year**	Ant-excluded	Ant-allowed	df	*F*	*P*	Ant-excluded	Ant-allowed	df	*F*	*P*	Ant-excluded	Ant-allowed	df	*F*	*P*
A) *Aonidiella aurantii* on twigs	Jun 2011	3.23±1.2	4.06±0.7	1, 6	0.6	0.470	1.61±1.6	0	1, 6	1	0.356	2.47±0.8	0.76±0.76	1, 6	2.15	0.193
	Jul 2011	17.7±3	18.92±3.6	1, 6	0.13	0.732	26.16±8.8	6.7±3.0	1, 6	5.63	0.055	16.95±3.6	3.64±2.4	1, 6	8.71	0.026
	Jul 2012	4.96±3.4	12.06±3.4	1, 6	3.35	0.12	1.14±1.1	4.3±2.5	1, 6	0.86	0.388	15.59±10.4	9.38±6	1, 6	0.28	0.614
B) *Aonidiella aurantii* on fruits	Sep 2011	75.74±8.5	55.62±3.2	1, 6	2.27	0.182	59.28±7.9	56.74±4.7	1, 6	0.09	0.769	57.9±5.7	39.48±6.3	1, 6	4.75	0.072
	Nov 2011	35.22±4.4	24.76±6	1, 6	1.68	0.24	26.7±10.1	31.57±8	1, 6	0.22	0.65	36.43±9.4	29.77±10.8	1, 6	0.32	0.595
	Sep 2012	28.42±6.8	32.44±4.4	1, 6	0.27	0.62	25.98±3.5	31.25±2	1, 6	1.47	0.271	52.72±6.6	41.66±4.7	1, 6	1.8	0.228
	Oct 2012	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	41.4±3.9	35.94±4.1	1, 6	0.9	0.379
	Nov 2012	48.33±7.9	64.03±5.5	1, 6	2.67	0.154	58.32±10.4	51.42±3.8	1, 6	0.38	0.559	25.2±2.7	17.32±2.7	1, 6	4.25	0.085
C) *Aleurothrixus floccosus*	Jul 2011	14.72±3.0	15±2.4	1, 6	0	0.947	16.46±3.5	10.4±2.4	1, 6	3.76	0,125	32.24±5.2	25.33±3.5	1, 6	0.76	0.416
	Aug 2011	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.46±2.6	17.73±3.4	1, 6	10.79	0.017
	Sep 2011	28.3±9.7	41±11.4	1, 6	0.06	0.823	24.6±8.5	25.24±5.6	1, 6	0.41	0,550	23.3±6.8	23.31±7.3	1, 6	0.01	0.941
	Jul 2012	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.64±1.6	9.81±2	1, 6	0.45	0.527
	Aug 2012	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	28.38±3.7	20.5±3	1, 6	2	0.207
	Oct 2012	31.76±4.7	33.93±4.6	1, 6	1.02	0.352	24.93±4.7	25.1±4.1	1, 6	0.14	0,723	n.d.	n.d.	n.d.	n.d.	n.d.
D) *Phyllocnistes citrella*	Sep 2011	57.75±9	63.94±13.7	1, 6	0.33	0.586	62.69±8.3	43.46±6.03	1, 6	3.58	0.1075	40.83±0.6	37.03±3.6	1, 6	0.56	0.481
	Sep 2012	62.26 ±5.9	55.35±5.3	1, 6	0.78	0.411	65.01±6.1	56.4±4.2	1, 6	1.32	0.2936	60±16.6	67.3±2.4	1, 6	0.14	0.720

2.4 Discussion

CRS is one of the worst citrus pests worldwide and its presence on fruits is highly undesirable, especially for countries whose production goes to fresh fruit market. Our results showed that fruit infestation caused by CRS was higher in the ant-allowed treatment in the three orchards of the study. These results are in agreement with previous studies which showed that ants may induce population increases of CRS on fruits (DeBach *et al.*, 1951; Steyn, 1954; Moreno *et al.*, 1987; James, 1997; Pekas *et al.*, 2010b). CRS does not produce honeydew and therefore is not tended by ants. Thus, the CRS population increase induced by ants is considered as an indirect effect; ants disrupt biological control of CRS when they accidentally encounter the CRS natural enemies while foraging on the tree canopies or while tending coincident honeydew producers (Steyn, 1954; Samways *et al.*, 1982; Murdoch *et al.*, 1995; Dao *et al.*, 2014).

In most of the aforementioned studies the ant species involved was the Argentine ant *L. humile*, which is known as an aggressive and disruptive species for biological control (Holway *et al.*, 2002). In our study, it was much more abundant than the native species and moreover it remained active throughout the whole year. This result coincides with Monzó *et al.* (2013), who also found *L. humile* active throughout all the season in the same citrus-growing area. In general, invasive ants are usually strongly attracted to hemipteran honeydew and are more aggressive than native ants (Styrsky & Eubanks, 2007). Given these attributes, *L. humile* would be expected to induce higher CRS populations on fruits compared with the native species. On the other hand, native ant species can also differ in their capacity of biological control disturbance, which is generally related to their aggressiveness and territoriality (Buckley & Gullan, 1991; Kaneko, 2003b; Mgocheki & Addison, 2009). We cannot draw definitive conclusions whether native or invasive species affect the herbivores differently; however, the population increases of herbivores in orchard LH, dominated by the invasive *L. humile*, were not higher but similar or even lower in some cases to those of orchards PP and LG, where the native species *P. pallidula* and *L. grandis* were predominant. It should be taken into account that *L. grandis* and *P. pallidula* are dominant species in their native areas (Pekas *et al.*, 2011; Arnan *et al.*, 2012) and show aggressive behaviour as well (Seifert, 1992; Retana & Cerdá, 1994; Katayama & Suzuki, 2003).

CRS infestation on twigs was similar in the ant-allowed and ant-excluded treatments. Assessments of CRS population densities on twigs were done visually without determining whether scales were alive or they were old dead scales remaining on the bark from previous generations. This fact might have masked the real effect of ant-exclusion on CRS population on twigs. In agreement with our results, Moreno *et al.* (1987) also reported no differences in CRS infestation on twigs between ant-excluded and ant-allowed citrus trees while they did find significant differences on fruits, attributing these different results to the fact that the parasitoid *A. melinus* concentrates its activity on the periphery of the trees, where most of the fruits are located.

The woolly whitefly *A. floccosus,* as for many other honeydew producing Hemiptera, is tended by ants on the citrus canopies (Moreno *et al.*, 1987, Pekas *et al.*, 2011). In fact, Moreno *et al.* (1987) reported lower whitefly densities in citrus trees when *L. humile* was excluded from the canopies. According to our results, the percentage of shoots occupied by *A. floccosus* was significantly lower in the ant-excluded treatment in orchards PP and LH dominated by *P. pallidula* and *L. humile* respectively, whereas no differences were found in the orchard LG dominated by *L. grandis.* Given that *A. floccosus* is directly tended by ants, the outcome of the interaction between the whitefly and the ant species in our study is expected to be influenced by the seasonal activity pattern of the latter. The activity of *L. grandis* ascending to the canopies peaked in spring and decreased in July, a period when the populations of *A. floccosus* start to increase (Garcia-Marí, 2012). On the other hand, *P. pallidula* and *L. humile* were active during summer and autumn, the months of higher *A. floccosus* incidence in the field. In fact, in orchard LH where *L. humile* was predominant and exhibited high activity throughout most of the year, we found higher *A. floccosus* infestations in ant-allowed trees for all the sampling dates. Interestingly, in the case of *P. pallidula*, significantly higher *A. floccosus* infestations in the ant-allowed trees were recorded only on the sampling dates following the ant´s peak activity (September and October).

Regarding the effect of ant exclusion on *P. citrella,* in the three orchards we observed no significant differences in the percent of leaf surface loss between the ant-allowed and ant-excluded treatments. Similarly, Urbaneja *et al.* (2004) conducted an ant-exclusion study to determine the impact of *Lasius niger* (Latreille) on *P. citrella* and observed no differences in the number of *P. citrella* on leaves for ant-allowed and ant-excluded treatments. *P. citrella* produces no honeydew and moreover develops on young and tender leaves (Garcia-Marí &

Granda, 2002) where other honeydew producing hemipterans are usually not found. Therefore, although the arboreal and highly aggressive weaver ants *Oecophylla* have been reported as efficient biological control agents of the citrus leafminer in Vietnam (Van Mele & Van Lenteren, 2002), the activity of the ant species in our study apparently is not affecting the citrus leafminer populations directly or indirectly.

In previous studies examining the impact of the ants on populations of honeydew producing Hemiptera, lower parasitism rates were reported on plants with ants relative to plants without ants (DeBach *et al.*, 1951; Bartlett, 1961; Itioka & Inoue, 1996b, 1999). Moreover, in the case of non-honeydew producing Hemiptera, several studies showed that ants may disrupt parasitoid activity (DeBach *et al.*, 1951; Flanders, 1958; Murdoch *et al.*, 1995; Heimpel *et al.*, 1997a; Martínez-Ferrer *et al.*, 2003). Recently, a study conducted on Australian citrus revealed that the parasitism of CRS by *Encarsia perniciosi* (Tower) and *Encarsia citrina* Craw (Hymenoptera: Aphelinidae) was severely reduced in the presence of the ant *Iridomyrmex rufoniger* (Lowne) (Dao *et al.*, 2014). In our study, however, we rarely found differences in percent parasitism between ant-allowed and ant-excluded treatments, either for the honeydew or non-honeydew producing insect herbivores. These results were consistent in the three orchards studied, each one of them with a different predominant ant species. Only in the case of CRS on fruits we did find lower parasitism levels in ant-allowed trees of orchard LH (with *L. humile*) although this reduction only approached statistical significance. In the same way, (Pekas *et al.*, 2010b) reported no differences in the parasitism of CRS on fruits between ant-excluded and ant-allowed treatments despite the fact that higher numbers of CRS were recorded on fruits in the treatment where *L. grandis* or *P. pallidula* had access to the tree canopies. Murdoch *et al.* (1995) showed that the exclusion of *L. humile* did not affect CRS parasitism in samples taken from the exterior part of trees while they did find differences in the inner part and argued that ants were rarely seen in the exterior of trees. Urbaneja *et al*. (2004) showed no differences in percentage parasitism of *P. citrella* between ant-allowed and ant-excluded treatments. Finally, regarding *A. floccosus,* to our knowledge there are no previous studies investigating the effect of ants on parasitism of this species.

Thus, apparently the parasitoid species involved in our study are not affected by the presence of ants. However, we might have failed to detect differences in percent parasitism between treatments due to the fact that the impact of parasitoids on host populations must be determined on a generational time scale (Driesche, 1983). This is because, depending on the synchronization between parasitoids and host populations, the contribution of the former to host population mortality may be overestimated or underestimated. Furthermore, other important sources of mortality induced by parasitoids such as host feeding or probing should be considered when determining percent parasitism (Kidd & Jervis, 1996). Especially in the case of *A. melinus*, the mortality caused to CRS through host-feeding is almost equal to that due to parasitism (Rosen & DeBach, 1979).

Alternatively, factors other than parasitism not assessed in our study may have contributed to the increased CRS and *A. floccosus* populations in the presence of ants. For instance, predation is an important mortality factor which nevertheless is difficult to assess accurately in the field.

(Piñol *et al.*, 2012a) during a long-term experiment of ant exclusion in citrus in Catalonia, showed that ants had a negative effect on the abundance of various groups of predators. In Australian citrus, Dao *et al*. (2014) have recently shown that the predation of CRS by coccinellid beetles was significantly increased when the ant *I. rufoniger* was excluded. Bach (1991) reported lower mortality rates of the soft scale *Coccus viridis* (Green) (Hemiptera: Coccidae) in the presence of ants not only from parasitism but also from other undetermined causes. Interestingly, several studies have reported aggressive ant behavior against predators such as coccinellids, neuropterans or dipterans (Bartlett, 1961; DeBach & Rosen, 1991; Itioka & Inoue, 1996a, 1999; Katayama & Suzuki, 2003; Piñol & Espadaler, 2010). Vanek & Potter, (2010) reported that the exclusion of the ant *Formica subsericea* Say led to a reduction of the soft scale *Eulecanium cerasorum* (Cockerell) (Hemiptera: Coccidae) densities caused principally by increased predation by *Chrysoperla rufilabris* (Burmeister) (Neuroptera: Chrysopidae), whereas parasitism of adult scales was similar between banded and control trees. In an ant-exclusion and predator-exclusion field experiment (McPhee *et al.*, 2012) demonstrated that *Myrmica rubra* (L.) induced higher aphid abundance by reducing the impact of *Chrysoperla carnea* (Stephens). Preliminary observations in the same three orchards of our study show lower abundance of potential predators of CRS and *A. flocossus,* such as green lacewings in the ant-allowed treatment (Calabuig *et al.*, unpublished data), which might explain the results obtained in the present study.

The exclusion method was very efficient in preventing the ants from ascending to the canopies in the two years of the study. The use of

Inesfly FITO© paint during the first year of the exclusion had the advantage that one application could last for several months which is highly desirable in reducing costs as well as workload. However, we observed several trees where the ants managed to sidestep the painted barrier and eventually ascend to the canopy. Therefore, in the second year we shifted to the Tanglefoot sticky barrier which, although posing important practical difficulties to employ, is known to efficiently prevent the ants from ascending to the canopies (Pekas *et al*., 2010a). A potential drawback of the use of sticky barriers for ant-exclusion involves the possibility of excluding, apart from the ants, other non-flying predators such as earwigs and the ant-mimic bug *Pilophorus* sp., (Heteroptera: Miridae), potential predators of plant feeders in the canopy (Piñol *et al.*, 2012b; Romeu-Dalmau, 2012). In our study however, we observed no earwigs on the tree trunk close to the exclusion zone and only a few *Pilophorus* sp. were obtained in tree samplings in a parallel study on the ant-allowed trees (Calabuig *et al*. unpublished data). Moreover, we are not aware of studies reporting earwigs or *Pilophorus* sp. preying upon *A. aurantii, A. floccosus* or *P. citrella*.

In conclusion, consistently higher populations of CRS were registered on fruits in the presence of the three ant species, *L. grandis, P. pallidula* and *L. humile*. Regarding the woolly whitefly *A. floccosus,* higher populations in the ant-allowed treatments were registered in the *P. pallidula* and *L. humile* orchards. We detected no effect of ants on populations of *P. citrella* for any of the three orchards studied. Overall, the increase of herbivore infestation in the orchard dominated by the invasive and much more active *L. humile*, were not higher but similar or even lower in some cases than in the orchards where the native *P.*

pallidula and *L. grandis* predominated. Thus, irrespective of the species present, ants have the potential to increase the infestation levels of honeydew and non-honeydew producing herbivores in citrus. These results suggest that ant management should be considered in order to reduce herbivore infestations in citrus orchards. The sticky barriers used in the present study proved to be efficient in excluding ants from the canopies; nevertheless, this method might suffer practical drawbacks, e.g. increased workload when needs to be applied in commercial orchards. Alternative and environmental friendly methods based on manipulating the ant-hemiptera interaction (Nagy *et al.*, 2013) or employing semiochemicals for disrupting ant foraging (Suckling *et al.*, 2010) seem promising. Regarding the underlying mechanism, parasitism alone cannot explain the differences in the herbivore population levels between treatments observed in our study. Other factors, such as the impact of ants on predators (James *et al.*, 1999; Piñol & Espadaler, 2010) or host feeding by parasitoids are important and should be further investigated.

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Chapter 3

Ants in citrus: impact on the abundance, species richness, diversity and community structure of predators and parasitoids

Ants in citrus: impact on the abundance, species richness, diversity and community structure of predators and parasitoids

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Abstract: Ants can act as plant biotic defenses, however, in agricultural ecosystems they are often associated with outbreaks of honeydew-producing pests mainly due to the protection they offer to the plant feeders in exchange for honeydew. In this interaction ants may alter the abundance, diversity and community structure of predators and parasitoids. In the present study, we conducted ant-exclusion experiments in three commercial citrus orchards, each one dominated by one ant species (*Pheidole pallidula, Lasius grandis* or *Linepithema humile*) during two consecutive years. We then compared the abundance, species richness, diversity and community structure of predators and parasitoids between the ant-allowed and ant-excluded treatments. A total of 176,000 natural enemies belonging to 81 taxa were captured and identified. The abundance of the natural enemies showed a species specific response between treatments. When examining functional groups of natural enemies the abundance of generalist predators decreased while that of parasitoids increased in the ant-allowed treatment. The species richness was significantly lower for predators and higher for parasitoids in the ant-allowed treatment. The Shannon diversity index was not different between treatments for predators, whereas parasitoid diversity was significantly higher in the ant-allowed treatment. Finally, the community structure of predators and parasitoids was not significantly different between treatments. These results suggest that ants in citrus are not associated with a

dramatic decrease in natural enemy abundance or biodiversity; on the contrary ants were associated with increased parasitoid species richness and diversity. On the other hand, ants negatively affected the abundance of specific natural enemy species, mainly generalist predators. The impact on these predators might explain the highest pest densities associated with ants in citrus.

3.1 Introduction

Ants are keystone species affecting directly and indirectly the ecosystem structure and functioning. Ants may act as soil tillers (Folgarait, 1998; Lobry De Bruyn, 1999), seed dispersers (Rico-Gray & Oliveira, 2007), pollinators (Beattie, 1985), predators (Way & Khoo, 1992) and are involved in various mutualisms (Way, 1963; Hölldobler & Wilson, 1990; Rico-Gray & Oliveira, 2007). Mutualism has been found to have broad effects on the arthropod community affecting eventually plant health (Kaplan & Eubanks, 2005; Rosumek *et al.*, 2009; Eubanks & Finke, 2014). One of the best studied mutualisms involving ants is the relationship with honeydew producing Hemiptera, in which ants use the honeydew excreted as an important carbohydrate source and, in turn, protect Hemiptera from their natural enemies (Bartlett, 1961; Way, 1963; Carroll & Janzen, 1973; Hölldobler & Wilson, 1990). As a result, ant-tending may have wider community-level consequences by altering the abundance and distribution of the third (insect predators & primary parasitoids) (James *et al.*, 1999; Styrsky & Eubanks, 2007) and fourth trophic levels (primary hyperparasitoids) (Völkl, 1992; Kaneko, 2002). These interactions play an important role in agricultural ecosystems since biological control provided by the third trophic level may be negatively affected by ant activity (Flanders, 1945; DeBach *et al.*, 1951; Heimpel *et al.*, 1997a; Martínez-Ferrer *et al.*, 2003).

The impact of ants on natural enemy abundance, diversity or community structure varies considerably depending on the natural enemy species as well as on the species of ants involved or the ecosystem where the study took place (Table 1 and references therein).

Whereas several studies have demonstrated a negative impact of ants on the abundance of natural enemies (James *et al.*, 1999; Eubanks, 2001; Kaplan & Eubanks, 2005; Piñol *et al.*, 2012a) others find no effect (Gibb, 2003; Offenberg *et al.*, 2005; Chong *et al.*, 2010) or even find positive effects of ants on the community of natural enemies (Stewart-Jones *et al.*, 2008; Peng & Christian, 2013). The same or even greater variability is reported at the species level; natural enemies, even species belonging to closely related taxa, may be affected differently by ants. For example, several studies have shown that ants have a negative impact on certain coccinellid species (Bartlett, 1961; Itioka & Inoue, 1996a; Kaplan & Eubanks, 2002), while other coccinellids are not affected (Flanders, 1958; Vanek & Potter, 2010) or even increase their densities under ant presence (Völkl & Vohland, 1996; Daane *et al.*, 2007). Likewise, the activity of several parasitoid species is disrupted by ants (Bartlett, 1961; Martínez-Ferrer *et al.*, 2003; Mgocheki & Addison, 2009) while others are able to parasitize hemipterans tended by ants (Flanders, 1958; Völkl, 1994; Barzman & Daane, 2001).

Table 1. Studies examining the impact of ants on the diversity, community structure or abundance of natural enemies in different ecosystems.

Reference	**Ecosystem**	**IMPACT OF ANTS**		
		Diversity index	**Community structure**	**Natural enemy abundance**
Chong et al., 2010	1 Vineyard	Not determined	No impact	No impact
Eubanks, 2001	3 Cotton fields	Not determined	Not determined	Negative impact on numerous predator species and Hymenoptera (grouped as wasps).
James et al., 1999	1 Citrus orchard	Not determined	Not determined	Negative impact (in some seasons) on predatory beetles, lacewings, parasitic wasps and spiders.
Human and Gordon, 1997	Variable vegetation types	No statistical analysis	Not determined	Slightly negative impact on Araneae, Hemiptera and Cynipidae
Kaplan and Eubanks, 2002	4 Cotton fields	Not determined	Not determined	Negative impact on Chrysopids and Coccinellids
Kaplan and Eubanks, 2005	2 Cotton fields	Not determined	Not determined	Negative impact on predators
Mody and Linsenmair, 2004	*Pseudocedrela kotschyi* trees	Not determined	Not determined	Negative impact on Araneae, Coleoptera, and Hymenoptera
Offenberg et al., 2005	Mangrove trees	Not determined	No impact (only predators)	Negative impact on predators
Peng and Christian, 2013	1 Cashew and 1 Mango orchard	Ants increased diversity of natural enemies in Cashew	Not determined	No effect of ants in Mango and higher abundances in ant presence in Cashew (all natural enemies pooled)
Piñol et al., 2012	1 Citrus orchard	Not determined	Impacted (includes herbivores)	Negative impact on several orders (analyzed at order level)
Philpott et al., 2008	3 Coffee farms	Not determined	Impacted (includes herbivores)	No impact (order level)
Philpott et al., 2004	1 Coffee farm	Not determined	Impacted depending on the ant species (includes herbivores)	Negative impact on Araneae and Coleoptera, but not other orders depending on the ant species
Stewart-Jones et al., 2007	2 Apple orchards	Not determined	Not determined	Positive impact (all natural enemies pooled)
Vanek and Potter, 2010	Maple trees and Magnolias	Not determined	Not determined	Negative impact on Araneae, *Crysopa rufilabris* Burmeister and parasitic wasps (several species grouped) depending on the sampling date
Wimp and Whitham, 2001	Poplar trees	Ants reduced diversity (all arthropods)	Not determined	Negative impact on generalist predators; positive impact on aphid natural enemies

Ants are among the most abundant arthropods in citrus and it has been demonstrated that they may induce population increases of honeydew and non-honeydew producing pests as a result of their interference with natural enemies (Pekas *et al.*, 2010b, 2011; Calabuig *et al.*, 2013; Yoo *et al.*, 2013; Dao *et al.*, 2014). Several studies have examined the multitrophic interactions involving ants and natural enemies in citrus in different parts of the world (James *et al.*, 1999; Piñol & Espadaler, 2010; Piñol *et al.*, 2012a; Yoo *et al.*, 2013; Dao *et al.*, 2014). Most of the studies focus on predators from different taxa (James *et al.*, 1999; Piñol *et al.*, 2012a) but little is known about the impact of ants on the diversity and abundance of parasitoids, which are often studied as a single group in the order Hymenoptera. Further, there are no studies examining the impact of ants simultaneously on the whole community of predator and parasitoid species present in citrus orchards. Knowledge about the impact of ants on the abundance, diversity and community structure of all the arthropod natural enemies in the citrus agroecosystem will provide useful insights and can help us to clarify the role of ants in biological control.

Thus, we performed an ant-exclusion experiment during two consecutive years in three commercial citrus orchards, each one with a different dominant ant species with the following objectives: (1) to know, describe and quantify the community of arthropod natural enemies, including predators and parasitoids, in the three citrus orchards (2) to test whether ants impact the abundance of the different species of natural enemies and (3) to test whether ants impact the species richness, diversity and community structure of predators and parasitoids.

3.2 Material and methods

Study sites

The study was conducted during two consecutive growing seasons, from April 2011 to November 2012, in three commercial citrus orchards located in an extensive citrus-growing area 30 km south of Valencia, eastern Spain (39º 12’ N, 0º 20’ W; 39º 11’ N, 0º 20’ W and 39º 14’ N, 0º 15’ W). The climate is Mediterranean, with a rainy spring and autumn and a dry winter and summer. The orchards were flood irrigated and weeds were controlled by local application of herbicides (Glyphosate®, Bayer CropScience, Spain). Two orchards were of sweet orange *Citrus sinensis* (L.) Osbeck (cv. Navelina) and one of a mixture of two species, sweet orange *C. sinensis* (cv. Navelina) and Clementine mandarin *Citrus reticulata* Blanco (Cv. Clementina Fina). Trees were more than 10 years old in all orchards. No insecticides were sprayed in the previous five years or during the two-year experimental period. In each orchard it was present a behaviorally dominant ant species, i.e. ant species that attacks and exclude other ant species from food sources (Cerdá *et al.*, 1997). From now on we will refer to the orchards according to the acronym of the predominant ant species present. Thus, in the orchard PP the predominant ant species was *Pheidole pallidula* (Nylander), in the orchard LG it was *Lasius grandis* Forel and in the orchard LH *Linepithema humile* (Mayr) (mixed orchard) was the only ant species present and foraging on the tree canopies (for details see Calabuig et al., 2013).

Experimental design

At each orchard, the experimental design was composed by four replicate blocks, to which a single treatment was applied with two levels (plots): ants allowed and ants excluded. This was equivalent to 8 plots per orchard each one containing 16 trees (four rows by four trees per row). Ants were excluded in the 16 trees of the ant-excluded plots and left unaffected in the 16 trees of the ant-allowed plots. Only the four central trees of each plot were used for the samplings. With that method we ensure that arthropods captured came from the trees of the same plot and corresponding treatment. Ant-exclusion began in April 2011 and was maintained until November 2012 (19 months). During the first season (2011), ant exclusion was achieved by painting a 25-cm wide band of insecticidal paint in a micro-encapsulated formulation (Inesfly FITO© (chlorpyrifos 3%)), Industrias Químicas Inesba S.L., Paiporta, Spain) on the trunk (Juan-Blasco *et al.*, 2011). To ensure that no ants reached the tree canopies, ant-excluded trees were inspected every month and the band was repainted if ants were observed crossing the band. Due to the fact that we observed ants crossing the painted bands in some of the trees during the first growing season we changed the ant exclusion method during the subsequent season. Thus, during 2012, ant exclusion was conducted by applying Tangle-trap® (Tanglefoot, Biagro, Valencia, Spain) sticky barrier on the tree trunks. Sticky barriers were inspected every month and, if necessary, the Tanglefoot was renewed; in any case, Tanglefoot was renewed routinely every two months. Trees were pruned periodically and ground vegetation was trimmed to prevent alternative ways for ants to reach the canopies. With both methods ant exclusion was successful in the three experimental orchards (see Calabuig et al., 2013).

Arthropod sampling and classification

Arthropods on the tree canopies were sampled with yellow sticky traps and by using a suction vacuum device. In each plot, one yellow sticky trap (Bug-scan, Biobest®), 100 mm x 250 mm, was placed at 1.60 m high in the middle of the plot by hanging it on a twig. Suction samples were taken using a modified vacuum sampler (Komatsu Zenoah Co. HBZ2601) consisting on a reversed leaf-blower with a mesh bag to retain the sample (Tena *et al.*, 2008). The vacuum sampler was applied on the canopies during one minute on each one of the four central trees of the plot. The sticky traps were replaced monthly, from April 2011 to November 2012 whereas suction samples were taken monthly from April to August and bimonthly from September to December in 2011 and 2012. Samples were transferred to the laboratory and maintained in a freezer until their examination.

All natural enemies captured were counted and identified. Most of them were identified to species or morphospecies level, while a few natural enemies were identified to genera or families. The use of morphospecies is a useful tool for studies that require taxonomic identifications of a great number of invertebrates without compromising scientific accuracy (Oliver & Beattie, 1996).

Predator and parasitoid diversity

The natural enemy diversity was measured in each sampling date by calculating the species richness S (number of species or morphospecies) and the Shannon diversity index H' (Shannon & Weaver, 1949):

$$H' = -\sum_{i=1}^{i=S} p_i \cdot log_e\, p_i$$

where p_i is the proportion of individuals of each species (up to a total of S species) in each sample. In the calculation of species richness and diversity we included the natural enemies identified to species or morphospecies level.

In the calculation of species richness and diversity we included the natural enemies identified to species or morphospecies level pooling data from traps and suction. Species belonging to the fourth trophic level were not included in these analyses.

Statistical analysis

All analysis were performed pooling data from traps and suction samples for each month (i.e. May, June, July, August, October and December for 2011 and April, May, June, July, August, September and November in 2012).

To compare the abundance of specific natural enemies in ant-allowed and ant-excluded treatments we included only those species accounting for more than 0.02% of the total captured during the two seasons of the study in the three orchards. For the species richness (S), the Shannon diversity index (H’) and community structure analysis we included all natural enemies identified to species or morphospecies.

We applied repeated measures ANOVA on the abundance of every species, the species richness (S) and the Shannon diversity index (H’) either at each orchard or globally, i.e. considering the three orchards together. Treatment (ant-excluded versus ant-allowed) was the fixed factor, orchard (in the global analysis) and block (nested into orchard) were random factors and sampling date was the repeated measures factor. Data were log-transformed in order to meet normality assumptions. All ANOVAS were conducted using Statgraphics 5.1 software (Statgraphics, 1994).

To compare the community structure of natural enemies in ant-allowed and ant-excluded treatments, permutational multivariate analyses of variance (PERMANOVA) were applied to predator and parasitoid abundances (including all the species and morphospecies captured) using the *adonis* function in the vegan package (Anderson, 2001; Oksanen *et al.*, 2009) in R (R Development Core Team, 2014). A separate PERMANOVA was conducted in each orchard for each year. Distance matrices for use in PERMANOVA were constructed using the Bray-Curtis index, and P-values were generated using F-tests based on sequential sums of squares from 99999 permutations of the raw data.

3.3 Results

A total of 176,000 natural enemies belonging to 81 taxa were captured and identified in all samplings in the three orchards, including sticky traps and suction of the canopies (Appendix A.1 of Supplementary data). Of them, 53 taxa contained more than 40 individuals (0.02% of the total captured): 18 taxa of predators, 31 of parasitoids and 4 belonging to the fourth trophic level. These were the taxa included in the comparative analyses of abundance (Table 2) (Appendix A.2 of Supplementary data). Among predators, the most abundant order was Neuroptera and the most abundant species were *Semidalis aleyrodiformis* Stephens and *Conwentzia psociformis* (Curtis) (both Neuroptera: Coniopterygidae). In the case of parasitoids, all of them belonging to the order Hymenoptera, the most abundant group was the superfamily Chalcidoidea, being *Aphytis chrysomphali* (Mercet) and *Cales noacki* Howard (Aphelinidae) the most abundant species (Table 2).

Table 2. Total arthropods captured, arthropods captured in ant-allowed and ant-excluded trees (mean ± SE) and effect of ants, globally and for the three orchards separately, during two years, 2011 and 2012, in three citrus orchards.

Arthropods	**Total arthropods**	**Principal prey**	**Global**			***Ant effect / Orchard**		
			Arthropods / sample		***Ant effect**			
			Ant-allowed	**Ant-excluded**		**LG**	**LH**	**PP**
PREDATORS								
Coleoptera								
Cybocephalus sp.	131		0.67 ± 0.12	0.25 ± 0.05	+	0	+	0
Ragonycha sp.	235	generalist	0.59 ± 0.23	1.03 ± 0.32	- -	0	n.p.	0
Coccinellidae								
Clitosthetus arcuatus Rossi	258	whiteflies	1.57 ± 0.13	2.18 ± 0.18	0	0	0	0
Delphastus catalinae Horn	626	whiteflies	2.22 ± 0.38	2.15 ± 0.49	++	0	0	0
Rhizobius lophantae Blaisdell	89	*Aonidiella aurantii*	0.36 ± 0.10	0.26 ± 0.10	0	n.p.	0	n.p.
Rodolia cardinalis (Mulsant)	803	*Icerya purchasi*	2.54 ± 0.41	3.05 ± 0.50	0	0	0	- -
Scymnus subvillosus (Goeze)	1242	aphids	4.85 ± 0.41	3.83 ± 0.32	0	0	++	0
Stethorus punctillum Weise	82	spider mites	0.26 ± 0.07	0.31 ± 0.05	- -	0	0	0
Diptera								
Platypalpus sp.	1338		3.07 ± 0.83	6.20 ± 1.88	0	0	0	0
Heteroptera								
Campyloneura virgula Herrich-Schäffer	745	generalist	2.04 ± 0.62	3.13 ± 0.71	- -	0	- -	-
Cardiasthetus sp.	368	generalist	1.03 ± 0.15	1.53 ± 0.17	- -	- -	0	0
Pilophorus sp.	43	generalist	0.21 ± 0.04	0.09 ± 0.02	++	+	0	0
Ploearia sp.	78	spider mites	0.27 ± 0.07	0.28 ± 0.06	0	0	0	0
Neuroptera								
Chrysopidae								
Chrysopa septempuctata Wesmael	40	generalist	0.07 ± 0.02	0.21 ± 0.04	- -	n.p.	-	- -
Chrysoperla carnea (Stephens)	591	generalist	1.39 ± 0.19	2.70 ± 0.33	- -	- -	- -	- -
Coniopterigidae								
Coniopteryx sp.	65	generalist	0.20 ± 0.04	0.25 ± 0.06	0	0	n.p.	0
Conwentzia psociformis (Curtis)	4395	generalist	14.77 ± 3.65	15.84 ± 3.39	0	0	-	0
Semidalis aleyrodiformis Stephens	29987	generalist	107.39 ± 11.37	101.68 ± 10.46	0	0	0	0

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			Global			*Ant effect / Orchard		
			Arthropods / sample					
Arthropods	**Total arthropods**	**Principal prey**	**Ant-allowed**	**Ant-excluded**	***Ant effect**	**LG**	**LH**	**PP**
PARASITOIDS								
Hymenoptera								
Ceraphronoidea								
Ceraphronidae	1083		4.26 ± 0.54	3.30 ± 0.34	++	++	0	n.p.
Megaspilidae	349		1.18 ± 0.18	1.25 ± 0.22	0	0	0	0
Chalcidoidea								
Ablerus sp.**	645	diaspidid parasitoids	3.65 ± 0.96	0.90 ± 0.23	++	n.p.	++	n.p.
Anagyrus sp.	281	pseudococcids	1.43 ± 0.36	0.54 ± 0.11	++	++	++	++
Aphelinus sp.	127	aphids	0.21 ± 0.04	0.09 ± 0.02	0	0	0	0
Aphytis chrysomphali (Mercet)	50638	*Aonidiella aurantii*	167.94 ± 20.29	184.64 ± 21.83	0	-	++	0
Aphytis hispanicus (Mercet)	7534	*Parlatoria pergandii*	35.34 ± 6.08	17.47 ± 1.92	++	++	++	++
Aphytis melinus DeBach	11694	*Aonidiella aurantii*	39.96 ± 5.53	41.50 ± 7.12	0	0	0	0
Cales noacki	18448	whiteflies	67.66 ± 8.16	61.01 ± 6.71	0	0	+	+
Citrostichus phyllocnistoides (Naranayan)	659	*Phyllocnistis citrella*	2.74 ± 0.86	1.86 ± 0.29	+	0	0	++
Encarsia inquirenda (Silvestri)	3662	*Parlatoria pergandii*	15.21 ± 2.16	10.39 ± 1.22	++	- -	++	++
Encarsia sp. 1	1029		4.50 ± 0.63	2.71 ± 0.32	++	++	++	0
Encarsia sp. 2	178		0.69 ± 0.34	0.55 ± 0.19	0	n.p.	0	n.p.
Encarsia sp. 3	59		0.26 ± 0.05	0.15 ± 0.03	++	0	++	0
Encyrtus sp.	62	coccids	0.33 ± 0.08	0.10 ± 0.04	++	n.p.	++	n.p.
Eretmocerus sp.	102	whiteflies	0.35 ± 0.09	0.37 ± 0.09	0	n.p.	0	0
Marietta sp. **	313	coccid parasitoids	1.36 ± 0.32	0.83 ± 0.21	++	0	n.p.	++
Metaphycus flavus (Howard)	8005	coccids	29.43 ± 4.32	26.40 ± 4.03	0	0	++	0
Metaphycus helvolus (Compere)	4355	coccids	18.83 ± 3.58	11.64 ± 1.56	++	0	++	++
Metaphycus lounsburyi (Howard)	75	coccids	0.35 ± 0.12	0.17 ± 0.05	0	n.p.	0	n.p.
Microterys nietneri (Motschulsky)	184	coccids	0.54 ± 0.11	0.73 ± 0.13	0	0	+	0
Mymaridae	4932	cicadellidae	19.65 ± 2.09	14.80 ± 1.81	++	+	0	+
Pachyneuron sp.**	65	aphid parasitoids	0.22 ± 0.08	0.23 ± 0.07	0	n.p.	0	n.p.
Trichogramma sp.	75	lepidoptera	0.29 ± 0.05	0.23 ± 0.05	0	+	0	0

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Arthropods	Total arthropods	Principal prey	Global			*Ant effect / Orchard		
			Arthropods / sample		*Ant effect			
			Ant-allowed	Ant-excluded		LG	LH	PP
Chrysidoidea								
Chrysis sp.	62	hymenoptera	0.21 ± 0.09	0.22 ± 0.07	0	n.p.	0	n.p.
Cynipoidea	286		1.03 ± 0.17	0.97 ± 0.18	0	0	0	0
Ichneumonoidea								
Alysinae: Alysinii	172	leaf miners	0.59 ± 0.08	0.69 ± 0.08	0	0	0	0
Aphidius sp.	45	aphids	0.15 ± 0.04	0.17 ± 0.05	0	-	0	- -
Binodoxys sp.	3451	aphids	11.94 ± 3.33	12.11± 3.09	0	0	0	0
Ichneumonidae	1038		3.70 ± 0.44	3.54 ± 0.43	0	++	0	0
Lysiphlebus sp.	93	aphids	0.42 ± 0.13	0.24 ± 0.08	0	0	0	++
Microgastrinae	587	lepidoptera	1.82 ± 0.23	2.26 ± 0.30	0	0	0	- -
Other Braconidae	270		1.01 ± 0.17	0.87 ± 0.14	0	0	0	0
Platygastroidea								
Scelionidae	10897		39.73 ± 4.00	36.27 ± 3.11	0	0	0	0
Proctotrupoidea								
Helorus sp.**	309	crisopids	0.80 ± 0.14	1.34 ± 0.18	- -	0	- -	0

* Repeated measures analysis of variance (ANOVA) was used, with treatment as fixed factor, block as random factors and time as repeated measures factor. In the global analysis, orchard was set as random factor as well. The + indicates a slightly positive effect of ants on the abundance of the natural enemy ($P<0.1$); ++ indicates a significant positive effect of ants ($P<0.05$); - indicates a slightly negative effect of ants ($P<0.1$); - - indicates a significant negative effect of ants ($P<0.05$); n.p. indicates no presence of the natural enemy. LG: *Lasius grandis* orchard; LH: *Linepithema humile* orchard; PP: *Pheidole pallidula* orchard.

** Species belonging to the 4th trophic level.

Abundance of parasitoids and predators

When comparing the abundance of specific species or arthropod taxa between treatments we observed different responses depending on the functional group and species of natural enemy examined. From the 53 comparisons of particular taxa of natural enemies between ant-allowed and ant-excluded trees, 21 (40%) showed a significant difference between treatments. When separated according to functional groups, 44% of the predator and 37% of the parasitoid taxa were affected by ants (Table 2). Further, in the comparisons obtained considering orchards individually, the percentage of taxa of natural enemies significantly affected by ants was 38% in orchard LH, 31% in orchard PP and 27% in orchard LG (Table 2).

In those cases where we detected significant differences between treatments we observed that in the ant-allowed treatment predator (concretely generalist predator) abundance was usually lower (seven species decreased and four increased in at least one orchard), whereas parasitoid abundance was usually higher (four species decreased and 18 increased in at least one orchard) (Table 2).

Differences in abundance between ant-allowed and ant-excluded treatments occurred in some of the most abundant species of natural enemies all along the sampling period and in the three orchards, as can be seen by examining their seasonal population trend (Fig. 1). For example, the abundance of the generalist predator *Chrysoperla carnea* sensu lato (Stephens) (Neuroptera: Chrysopidae) was consistently lower in the ant-allowed trees. On the contrary, the abundance of parasitoids such as *Aphytis hispanicus* (Mercet), parasitoid of *Parlatoria* *pergandii* Comstock (Hemiptera: Diaspididae), and *Anagyrus* sp. (Girault) (Hymenoptera: Encyrtidae), parasitoid of pseudococcids, remained usually higher in the ant-allowed trees during the sampling period (Fig. 1). Conversely, the response of other species was not so consistent and several taxa showed similar abundance in ant-allowed and ant-excluded treatments (Table 2).

Among true bugs (Heteroptera) the abundance of the myrmecomorphic *Pilophorus* sp. (Heteroptera: Miridae) was significantly higher in the ant-allowed treatment, especially in the orchard dominated by *L. grandis* (LG), whereas the abundance of other true bug species such as *Cardiasthetus* sp. (Heteroptera: Anthocoridae) or *Campyloneura virgula* (Herrich-Schäffer) (Heteroptera: Miridae) (Fig. 1) was significantly lower in the ant-allowed treatment (Table 2).

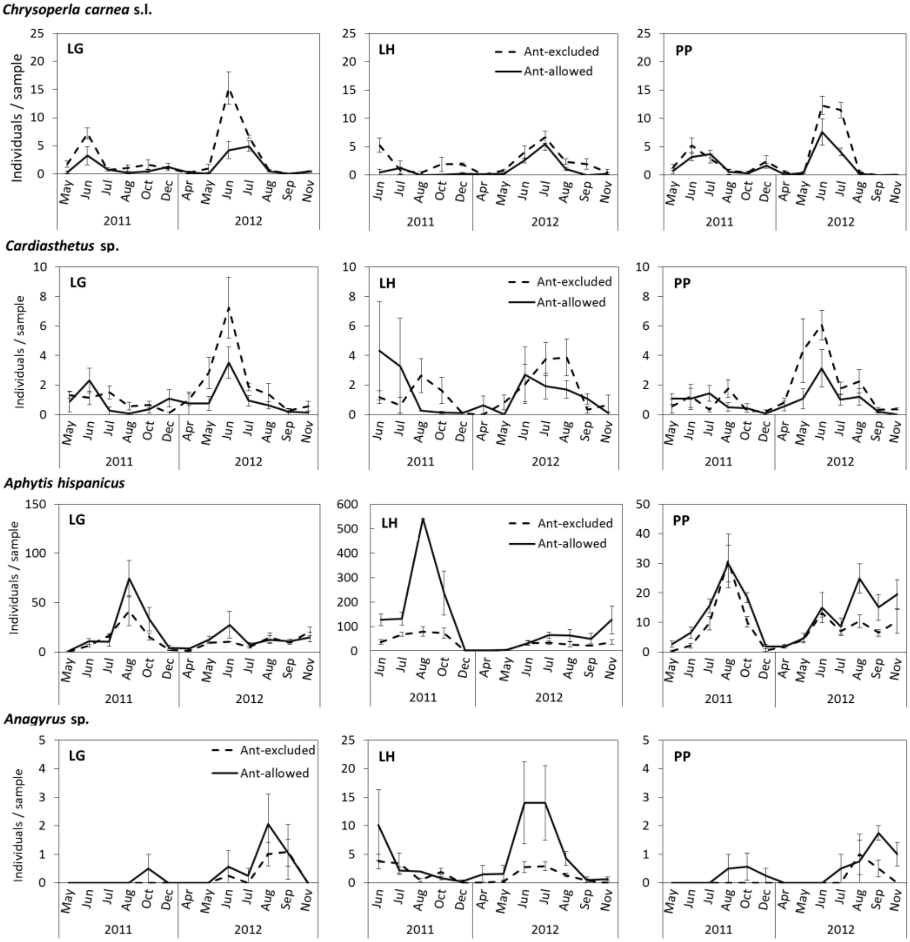

Figure 1. Abundance of *C. carnea* s.l., *Cardiasthetus* sp., *A. hispanicus* and *Anagyrus* sp*.* in ant-allowed and ant-excluded trees during 2011 and 2012. Abundance is measured as the mean number of individuals captured in one sticky trap and four aspirations (one/tree) in each experimental plot (each plot consisted in 16 trees and only the four central trees were sampled; each orchard contained 8 plots, 4 ant-allowed and 4 ant-excluded).

Species belonging to the 4th trophic level (most of them identified as morphospecies) showed mixed responses to ant presence. *Marietta* sp. (Hymenoptera: Aphelinidae), hyperparasitoid of coccid parasitoids, and

Ablerus sp. (Hymenoptera: Aphelinidae), hyperparasitoid of diaspidid parasitoids, were significantly more abundant in the ant-allowed treatment. The abundance of *Pachyneuron* sp. (Hymenoptera: Pteromalidae), hyperparasitoid of aphid parasitoids, was not significantly different between treatments. *Helorus* sp. (Hymenoptera: Heloridae), a parasitoid of chrysopid eggs, was less abundant in the ant-allowed treatment.

Species richness, diversity and community structure of predators and parasitoids

Overall, in the ant-allowed treatment the species richness (S) was significantly lower for predators and higher for parasitoids when compared with the ant-excluded trees. The impact of ants was, nevertheless, significant only in one orchard for predators and two orchards for parasitoids when examining the three orchards separately (Table 3).

The Shannon diversity index (H) was not different between the ant-allowed and ant-excluded treatments in the case of predators, whereas in the case of parasitoids diversity was significantly higher in the ant-allowed treatment both in the global analysis and in the three orchards analyzed individually (Table 3).

Table 3. Impact of ants on Species richness (S) (mean ± SE) and Shannon diversity index (H) (mean ± SE) of predators and parasitoids, globally and for the three orchards separately.

Species richness (S)

	Predators					Parasitoids				
Orchard	Ant-excluded	Ant-allowed	d.f.	*F*	*P*	Ant-excluded	Ant-allowed	d.f.	*F*	*P*
LG	7.61 ± 0.37	6.96 ± 0.35	1,71	4.34	0.059*	11.02 ± 0.30	11.76 ± 0.33	1,71	5.50	0.037**
LH	8.42 ± 0.43	8.55 ± 0.42	1,56	0.05	0.828	13.54 ± 0.56	15.50 ± 0.60	1,56	14.08	0.003**
PP	7.35 ± 0.34	7.10 ± 0.38	1,75	0.76	0.401	11.58 ± 0.30	11.27 ± 0.31	1,75	0.78	0.396
Global	7.77 ± 0.22	7.44 ± 0.23	1,250	4.93	0.045**	12.03 ± 0.24	12.59 ± 0.28	1,250	14.02	0.003**

Shannon diversity (H)

	Predators					Parasitoids				
Orchard	Ant-excluded	Ant-allowed	d.f.	*F*	*P*	Ant-excluded	Ant-allowed	d.f.	*F*	*P*
LG	0.66 ± 0.06	0.62 ± 0.07	1,71	0.16	0.699	1.41 ± 0.04	1.51 ± 0.03	1,71	5.98	0.031**
LH	1.32 ± 0.08	1.45 ± 0.06	1,56	1.99	0.183	1.40 ± 0.05	1.54 ± 0.04	1,56	6.67	0.022**
PP	0.69 ± 0.06	0.60 ± 0.06	1,75	1.50	0.245	1.37 ± 0.05	1.45 ± 0.05	1,75	5.28	0.040**
Global	0.88 ± 0.04	0.84 ± 0.05	1,250	0.09	0.773	1.39 ± 0.03	1.49 ± 0.02	1,250	33.58	<0.001**

Repeated measures analysis of variance (ANOVA) was used, with treatment as fixed factor and time and block as random factors. In global analysis, orchard was set as random factor as well. ** indicates a significant effect of ants (P<0.05) and * indicates a marginally significant effect (P<0.1). LG: *Lasius grandis* orchard; LH: *Linepithema humile* orchard; PP: *Pheidole pallidula* orchard.

The multivariate test showed that the community of parasitoids changed significantly in the ant-allowed treatment only in 2012 in the orchard LH (Table 4). In the other orchards and/or years the community structure was not different between treatments.

Table 4. Summary of the PERMANOVA results of the effect of ants on the predators and parasitoids communities in 2011 and 2012 in orchards LG, LH and PP.

		Predators		Parasitoids	
Year	Orchard	R^2	P	R^2	P
2011	LG	0.077	0.742	0.105	0.630
	LH	0.301	0.059	0.210	0.288
	PP	0.372	0.057	0.199	0.200
2012	LG	0.050	0.828	0.107	0.544
	LH	0.166	0.371	0.332	0.029**
	PP	0.395	0.085	0.043	0.943

3.4 Discussion

Our results show that the overall community structure of predators and parasitoids was not significantly different between the ant-allowed and the ant-excluded treatments. When analyzing the effect of ants on the abundance of particular species of natural enemies, there are many cases of significant differences across taxa or species. In general terms, we observed lower numbers of generalist predators and higher numbers of parasitoids in the ant-allowed treatment compared to the ant-excluded treatment. Most crucially, the species richness and diversity of parasitoids was higher in the ant-allowed treatment whereas the diversity of predators was not different between treatments.

Abundance of predators and parasitoids

The abundance of most parasitoid species in our study was either not affected by ants or higher in the ant-allowed treatment. This is of particular interest especially if we consider the widely held assumption that ant-attendance offers hemipterans a protective service against parasitoids (Flanders, 1951; Steyn, 1954; Buckley, 1987). This effect seems to be related, at least in some cases, with the impact of ants on the parasitoid host populations, the relaxation of intraguild predation and/or with the ability of the particular species to cope with ant aggression (Barzman & Daane, 2001). Often, honeydew producing pests are more abundant under ant protection and eventually this might explain the higher abundance of their parasitoids in the ant-allowed treatment. In our study, parasitoids of honeydew producing pests, such as the soft scale parasitoids *Metaphycus helvolus* Compere, *Metaphycus flavus* Howard and *Encyrtus* sp. (Hymenoptera: Encyrtidae) or the mealybug parasitoid *Anagyrus* sp. were in general more abundant in the ant-allowed treatment, especially in the orchard LH. Additionally, the lower abundance of predators in ant-allowed trees may result in lower intraguild predation upon parasitized hosts, resulting in increased populations of some parasitoid species.

It was surprising to see that *Encarsia inquirenda* Silvestri and *A. hispanicus* (Hymenoptera: Aphelinidae), parasitoids of *P. pergandii*, an armored scale that does not produce honeydew and therefore is not tended by ants, were more abundant in the ant-allowed treatment both globally and on each orchard analyzed individually (except in the orchard LG for *E. inquirenda*). Apparently, ant presence is associated with increased abundances of *P. pergandii,* as already found for other armored scales (DeBach *et al.*, 1951; Pekas *et al.*, 2010b; Calabuig *et al.*, 2013; Yoo *et al.*, 2013). Other parasitoids of non-honeydew producers did not follow this trend. In the case of *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) and *A. chrysomphali*, parasitoids of *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae), we observed no differences in their abundance between treatments except in the orchard LH where the populations of *A. chrysomphali* were significantly higher in the ant-allowed treatment.

One of the most important findings in our study is the fact that the abundance of the generalist predators was lower in the ant-allowed treatment. It is important to highlight the case of the chrysopids (green lacewings) *C. carnea* sensu lato and *C. septempunctata*. These species are considered relevant biological control agents in many agroecosystems (Senior & McEwen, 2001) and are among the most abundant predators in Mediterranean citrus orchards, preying upon a wide range of pests (Garcia-Marí, 2012). Our results are in agreement with other studies which have also found lower densities of chrysopids in ant-allowed treatments (James *et al.*, 1999; Kaplan & Eubanks, 2002; Vanek & Potter, 2010; McPhee *et al.*, 2012). Several authors reported aggressive behavior of different ant species against chrysopids (Bartlett, 1961; Vanek & Potter, 2010) or ant predation on chrysopid eggs (Dreistadt *et al.*, 1986; Morris *et al.*, 1998) which may result in lower chrysopid populations. For the two most abundant species of predatory Heteroptera (true bugs) *Cardiasthetus* sp. and *Campyloneura virgula* we also registered lower populations in the ant-allowed treatment. In an 8-year study, Piñol et al. (2012b) also found lower abundance of predatory Heteroptera, especially *Cardiasthetus fasciiventris,* in the ant-allowed trees. It is interesting to mention the higher populations of *Pilophorus* sp. in the ant-allowed treatment. This species exhibits mirmecomorphy that allows it to benefit from ant presence as was already reported in other studies between the ant *L. grandis* and *Pilophorus* sp. (Piñol *et al.*, 2012b; Sánchez & Ortín-Angulo, 2012).

Regarding the impact of ants on coccinellids (ladybirds), which are mostly specialists, our results show great variability depending on the species examined. The response of coccinellids to ant attacks differs between species (Jiggins *et al.*, 1993) as some species can cope with ant aggression through morphological, behavioural or chemical adaptations. For example, Völkl and Vohland (1996) found higher populations of *Scymnus* sp. in ant attended resources due to the protective wax cover of the *Scymnus* larvae which allow them to predate upon honeydew producers tended by ants. Wimp and Whitham (2001) found that the aphid-ant mutualism had a negative impact on generalist predators and a positive effect on specialist enemies of aphids. Apparently, specialist predators have evolved the mechanisms necessary in order to deal with ant aggressiveness (Way, 1963; Völkl, 1995) which is not the case for the generalists.

The use of ant-exclusion barriers on the trunk might potentially exclude other non-flying predators from climbing on the citrus canopies. In the study area the only predators that could have been excluded by the barriers are earwigs; concretely the species *Forficula auricularia* L. (Dermaptera: Forficulidae). However, this species is of very low abundance in citrus in the study area (Alvis & Garcia-Mari, 2006; Bru & Garcia-Marí, 2008). In addition we observed no earwigs or other predators on the trunks of the ant-allowed treatment. Thus, our ant-exclusion method did not affect the abundance of the natural enemies on the canopies.

The abundance of some species from the 4th trophic level was also found to be different between the ant-allowed and ant-excluded treatments. This was apparently related with the abundance of their primary hosts. *Ablerus* sp., hyperparasitoid of *A. chrysomphali*, as well as *Marietta* sp., hyperparasitoid of encyrtids, was more abundant in the ant-allowed treatment. On the other hand, *Helorus* sp., parasitoid of chrysopid eggs, was less abundant in the ant-allowed trees. Several studies have demonstrated that some parasitoids benefit from ant attendance because ants may reduce hyperparasitism by disturbing hyperparasitoids (Völkl, 1992; Sanders & Frank Van Veen, 2010). Additionally, intraguild predation caused by higher abundance of predators in the ant-excluded trees, may result in a decrease of the hyperparasitoid populations (Novak, 1994; Kaneko, 2002, 2006).

Species richness, diversity and community structure of predators and parasitoids

To our knowledge, the present study is the first to demonstrate a significant increase on species richness as well as on the Shannon diversity index for parasitoids in the ant-allowed treatment. Previous studies in several ecosystems show usually a decrease of arthropod diversity as a result of ant activity (Human & Gordon, 1997; Wimp & Whitham, 2001). Nevertheless, it is difficult to compare these results with ours given that the previous studies focused on overall arthropod communities including different guilds such as herbivores. On the other hand, Peng and Christian (2013) found that weaver ants either had no impact or increased the diversity of natural enemies in cashew and mango trees.

Our results show that the community structure of predators and parasitoids on the citrus canopies was similar between treatments. Previous studies also found that ants did not affect the overall arthropod communities in vineyards (Chong *et al.*, 2010), peaches (Mathews *et al.*, 2009) or coffee (Philpott *et al.*, 2008). Conversely, Piñol et al. (2012a), in an 8-year exclusion experiment, reported that ants changed the arthropod community in a citrus orchard in some years of their study. Differences in the results obtained in the different studies might be attributed to the species of ants present and/or the characteristics of the experimental orchards.

The present study was not designed to compare the effects of specific ant species on the natural enemy community. Perhaps more replicates, i.e. orchards dominated from each ant species, should take place in order to make any inferences about the effect of the ant species. This however would be logistically very complex to undertake in a field study. Nevertheless, and despite the fact we cannot draw any definitive conclusions when it comes to comparison of species it is interesting to highlight that the impact of the three ant species on the community structure of natural enemies was quite similar. Despite *L. humile* having been described as an aggressive and very disruptive ant species for biological control (Markin, 1970b), in our study we found no significant effects of this invasive species on the community structure of natural enemies. The same can be said of the native species, *L. grandis* and *P. pallidula.* In a previous study it was found that the three ant species induced similar population increases of the herbivore *A. aurantii* and *A. floccosus* (Calabuig *et al.*, 2013).

Conclusion

In conclusion, our ant exclusion study revealed that ants in citrus were not associated with a dramatic and overall decrease in natural enemy abundance or biodiversity at the community level. The impact of ants on the natural enemies depended mostly on the species of natural enemy; even closely related species showed different or opposite responses to ant activity. In spite of the species specific response of natural enemies, we detected a general tendency related to functional groups: generalist predator abundance decreased whereas parasitoid abundance and diversity increased in the ant-allowed treatment. These results may have practical implications for biological pest control. Despite the fact that ants had no negative impact on the abundance and diversity of predators and parasitoids at the community level their impact on specific natural enemy species may explain the highest pest densities associated with ant presence in citrus (Pekas *et al.*, 2010b; Calabuig *et al.*, 2013; Yoo *et al.*, 2013; Dao *et al.*, 2014).

3.5 Aknowledgments

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3.6 Supplementary material

Appendix A.1. Total arthropods captured and arthropods captured in ant-allowed and ant-excluded trees (pooling data from sticky traps and suction samples) during two years, 2011 and 2012, in three citrus orchards.

	Arthropods captured		
Arthropods	**TOTAL**	**Ant-allowed**	**Ant-excluded**
PREDATORS			
Araneae	673	381	292
Coleoptera			
Cybocephalus sp.	131	95	36
Ragonycha sp.	235	84	151
Coccinellidae			
Clitosthetus arcuatus Rossi	258	124	134
Coccinella septempunctata Linnaeus	7	0	7
Cryptolaemus montrouzieri Mulsant	1	1	0
Delphastus catalinae Horn	626	312	314
Nephus bipunctatus (Kugelann)	2	1	1
Propylaea quatuordecimpunctata (Linnaeus)	19	8	11
Rhizobius lophantae Blaisdell	89	51	38
Rhyzobius litura (Fabricius)	7	4	3
Rodolia cardinalis (Mulsant)	803	358	445
Scymnus interruptus Goeze	32	16	16
Scymnus rufipes (Fabricius)	2	1	1
Scymnus subvillosus (Goeze)	1242	684	558
Stethorus punctillum Weise	82	37	45
Diptera			
Platypalpus sp.	1338	433	905
Heteroptera			
Campyloneura virgula Herrich-Schäffer	745	288	457
Cardiasthetus sp.	368	145	223
Orius spp.	24	8	16
Pilophorus sp.	43	29	14
Ploearia sp.	78	38	40
Neuroptera			
Chrysopidae			
Chrysopa septempuctata Wesmael	40	10	30
Chrysoperla carnea (Stephens)	591	196	395
Coniopterigidae			
Coniopteryx sp.	65	28	37
Conwentzia psociformis (Curtis)	4395	2082	2313
Semidalis aleyrodiformis Stephens	29987	15142	14845

PARASITOIDS			
Hymenoptera			
Ceraphronoidea			
Ceraphronidae	1083	601	482
Megaspilidae	349	167	182
Chalcidoidea			
Ablerus sp.	645	514	131
Anagyrus sp.	281	202	79
Aphelinus sp.	127	62	65
Aphytis chrysomphali (Mercet)	50638	23680	26958
Aphytis hispanicus (Mercet)	7534	4983,53	2550,08
Aphytis melinus DeBach	11694	5635	6059
Cales noacki Howard	18448	9540	8908
Cheiloneurus sp.	4	3	1
Citrostichus phyllocnistoides (Naranayan)	659	387	272
Elasmus sp.	6	2	4
Encarsia inquirenda (Silvestri)	3662	2145	1517
Encarsia sp. 1	1029	634	395
Encarsia sp. 2	178	98	80
Encarsia sp. 3	59	37	22
Encarsia sp. 4	9	5	4
Encyrtus sp.	62	47	15
Eretmocerus sp.	102	49	53
Homalotylus flaminius Dalman	33	19	14
Isodromus flaviscutum Hoffer & Trjapitzin	20	14	6
Leptomastidea sp.	40	21	19
Leptomastix sp.	1	1	0
Marietta sp.	313	192	121
Metaphycus flavus (Howard)	8005	4150	3855
Metaphycus helvolus (Compere)	4355	2655	1700
Metaphycus lounsburyi (Howard)	75	50	25
Microterys nietneri (Motschulsky)	184	77	107
Mymaridae	4932	2771	2161
Pachyneuron sp.	65	31	34

	Arthropods captured		
Arthropods	**TOTAL**	**Ant-allowed**	**Ant-excluded**
Scutellista caerulea (Foscolombe)	31	16	15,
Tetranecmoidea sp.	13	1	12
Trichogramma sp.	75	41	34
Other Encyrtidae	1475	879	596
Other Eulophidae	343	195	147
Other Pteromalidae	294	186	108
Chrysidoidea			
Chrysis sp.	62	30	32
Cynipoidea	286	145	141
Ichneumonoidea			
Alysinae: Alysinii	172	83	89

Aphidius sp.	45	21	24
Binodoxys sp.	3451	1683	1768
Diaeretiella sp.	4	2	2
Ephedrus sp.	5,00	1	4
Ichneumonidae	1038	521	517
Lipolexis sp.	37	18	19
Lysiphlebus sp.	93	59	34
Microgastrinae	587	257	330
Praon sp.	4	1	3
Other Braconidae	270	143	127
Platygastroidea			
Scelionidae	10897	5602	5295
Proctotrupoidea			
Helorus sp.	309	113	196

Appendix A.2. Results of repeated measures ANOVA for the effect of ants on the abundance of natural enemies, globally and for the three orchards separately, during two years, 2011 and 2012, in three citrus orchards.

	Global			**Orchard LG**			**Orchard LH**			**Orchard PP**		
Arthropods	df	F	P	df	F	P	df	F	P	df	F	P
PREDATORS												
Coleoptera												
Cybocephalus sp.	1, 9	4.71	0.0581	1, 6	2.49	0.1652	1, 7	5.29	0.0533	1, 6	1.92	0.2148
Ragonycha sp.	1,3	1.17	0.3573	1, 3	0.52	0.5211	n.p.	n.p.	n.p.	1, 3	1.21	0.3522
Coccinellidae												
Clitosthetus arcuatus Rossi	1, 12	2.10	0.1721	1, 6	0.97	0.3622	1, 10	0.46	0.5096	1, 6	0.35	0.5746
Delphastus catalinae Horn	1,11	6.08	0.0309	1, 6	0.42	0.5415	1, 11	0.79	0.3393	1, 6	0.42	0.5429
Rhizobius lophantae Blaisdell	1, 9	1.93	0.1962	n.p.	n.p.	n.p.	1, 9	1.93	0.1962	n.p.	n.p.	n.p.
Rodolia cardinalis (Mulsant)	1, 10	1.22	0.2937	1, 7	0.49	0.5076	1, 7	0.56	0.4720	1,6	6.82	0.0400
Scymnus subvillosus (Goeze)	1, 12	1.89	0.1934	1, 9	0.23	0.6407	1, 11	4.81	0.0473	1, 9	1.21	0.3008
Stethorus punctillum Weise	1, 8	5.48	0.0472	1, 3	1.83	0.2688	1, 5	0.07	0.8069	1, 5	0.01	0.9189
Diptera												
Platypalpus sp.	1, 7	2.02	0.1964	1, 4	0.00	0.9532	1, 5	2.73	0.1558	1, 5	3.07	0.1403
Heteroptera												
Campyloneura virgula Herrich-Schäffer	1, 5	16.36	0.0072	1, 3	2.98	0.1826	1, 2	26.14	0.0586	1, 4	6.47	0.0637
Cardiasthetus sp.	1,12	5.75	0.0334	1, 11	6.03	0.0317	1, 9	2.08	0.1803	1, 10	2.39	0.1530
Pilophorus sp.	1, 5	11.86	0.0171	1, 5	4.57	0.0855	1, 2	1.66	0.2246	1, 6	0.14	0.7464
Ploearia sp.	1, 6	0.26	0.6302	1, 4	0.00	0.9794	1, 1	1.37	0.4498	1, 2	0.36	0.6101
Neuroptera												
Chrysopidae												
Chrysopa septempuctata Wesmael	1, 11	5.67	0.0363	n.p.	n.p.	n.p.	1, 4	6.51	0.0548	1, 3	118.54	0.0017
Chrysoperla carnea (Stephens)	1, 12	19.26	0.0009	1, 11	9.06	0.0118	1,9	16.79	0.0025	1, 9	8.50	0.0172
Coniopterigidae												
Coniopteryx sp.	1, 10	0.37	0.5576	1, 5	0.22	0.6621	n.p.	n.p.	n.p.	1, 4	0.50	0.5167
Conwentzia psociformis (Curtis)	1, 12	2.19	0.1634	1, 8	1.35	0.2769	1, 5	4.95	0.0536	1, 10	0.33	0.5774
Semidalis aleyrodiformis Stephens	1, 11	1.06	0.3254	1, 11	2.62	0.2859	1, 9	0.16	0.6978	1, 11	0.00	0.9678

	Global			Orchard LG			Orchard LH			Orchard PP		
Arthropods	df	F	P	df	F	P	df	F	P	df	F	P
PARASITOIDS												
Hymenoptera												
Ceraphronoidea												
Ceraphronidae	1, 12	5.97	0.0301	1, 11	11.88	0.054	1, 10	1.94	0.1917	1, 12	0.31	0.5878
Megaspilidae	1, 10	0.01	0.9088	1, 8	0.35	0.5718	1, 3	0.95	0.3995	1, 9	0.68	0.4317
Chalcidoidea												
Ablerus sp.	1, 8	24.98	0.0004	n.p.	n.p.	n.p.	1, 5	24.98	0.0004	n.p.	n.p.	n.p.
Anagyrus sp.	1, 10	18.96	0.0014	1, 10	6.27	0.0309	1, 10	7.25	0.0204	1, 10	4.79	0.0536
Aphelinus sp.	1, 11	0.12	0.7403	1, 1	0.20	0.7324	1, 5	1.55	0.2636	1, 2	0.00	1,0000
Aphytis chrysomphali (Mercet)	1, 12	0.10	0.7602	1, 12	3.54	0.0840	1, 11	5.62	0.341	1, 12	0.01	0.9187
Aphytis hispanicus (Mercet)	1, 12	24.64	0.0000	1, 12	4.63	0.0522	1, 11	25.94	0.0002	1, 12	13.36	0.0033
Aphytis melinus DeBach	1, 12	2.47	0.1414	1, 12	1.83	0.2007	1, 11	1.40	0.2553	1, 12	1.22	0.2917
Cales noacki	1, 12	0.75	0.4019	1, 11	0.81	0.3870	1, 11	4.45	0.0520	1, 10	3.66	0.0847
Citrostichus phyllocnistoides (Naranayan)	1, 10	3.57	0.0864	1, 8	0.39	0.5502	1, 9	0.71	0.4143	1, 7	9.56	0.0175
Encarsia inquirenda (Silvestri)	1, 12	5.17	0.0415	1, 11	6.90	0.0234	1, 10	30.15	0.0001	1, 11	6.60	0.0261
Encarsia sp. 1	1, 12	7.22	0.0195	1, 10	5.71	0.0375	1, 11	20.24	0.0007	1, 10	0.74	0.4089
Encarsia sp. 2	1, 5	1.55	0.2636	n.p.	n.p.	n.p.	1, 5	1.55	0.2636	n.p.	n.p.	n.p.
Encarsia sp. 3	1, 10	9.93	0.0097	1, 2	3.95	0.1854	1, 5	8.20	0.0328	1, 3	0.00	0.9847
Encyrtus sp.	1, 9	15.21	0.0035	1, 9	1.76	0.2169	1, 9	10.57	0.0083	n.p.	n.p.	n.p.
Eretmocerus sp.	1, 8	0.01	0.9070	n.p.	n.p.	n.p.	1, 7	0.24	0.6328	1, 2	0.25	0.6667
Marietta sp.	1, 10	15.71	0.0250	1, 6	1.44	0.2808	n.p.	n.p.	n.p.	1, 10	13.55	0.0042
Metaphycus flavus (Howard)	1, 12	1.01	0.3350	1, 12	0.01	0.9248	1, 11	5.58	0.0347	1, 12	0.01	0.9440
Metaphycus helvolus (Compere)	1, 11	21.76	0.0005	1, 11	2.72	0.1272	1, 10	9.48	0.0097	1, 11	21.14	0.0008
Metaphycus lounsburyi (Howard)	1, 5	3.93	0.1034	1, 5	1.89	0.2273	1, 5	2.20	0.1937	1, 5	0.24	0.6466
Microterys nietneri (Motschulsky)	1, 11	2.92	0.1147	1, 11	3.49	0.0885	1, 10	0.15	0.7076	1, 11	0.92	0.3570
Mymaridae	1, 12	12.47	0.0039	1, 12	4.73	0.0501	1, 11	3.57	0.0820	1, 12	3.90	0.0717
Pachyneuron sp.	1, 3	0.76	0.4474	1, 3	1.95	0.2574	1, 3	0.72	0.4582	1, 3	8.80	0.0592
Trichogramma sp.	1, 9	1.13	0.3159	1, 9	3.24	0.1054	1, 9	0.25	0.6303	1, 9	0.00	1.0000
Chrysidoidea												
Chrysis sp.	1, 6	0.02	0.8998	n.p.	n.p.	n.p.	1, 5	0.02	0.9063	n.p.	n.p.	n.p.

	Global			Orchard LG			Orchard LH			Orchard PP		
Arthropods	df	F	P	df	F	P	df	F	P	df	F	P
Cynipoidea	1, 12	1.32	0.2723	1, 5	1.15	0.3307	1, 8	0.84	0.3839	1, 5	0.75	0.4260
Ichneumonoidea												
Alysinae: Alysinii	1, 12	0.01	0.9437	1, 9	0.01	0.9275	1, 4	4.12	0.1057	1, 10	0.18	0.6793
Aphidius sp.	1, 2	0.46	0.5606	1, 3	8.95	0.058	1, 3	0.75	0.4496	1, 3	3.81	0.2707
Binodoxys sp.	1, 6	0.00	0.9491	1, 3	1.77	0.2747	1, 5	2.02	0.2136	1, 5	3.00	0.1433
Ichneumonidae	1, 12	1.84	0.1990	1, 11	5.27	0.0422	1, 11	0.10	0.7595	1, 11	0.42	0.5196
Lysiphlebus sp.	1, 2	2.44	0.2584	1, 3	2.66	0.2011	1, 1	1.18	0.4739	1, 3	16.89	0.0261
Microgastrinae	1, 12	2.60	0.1321	1, 8	0.13	0.7307	1, 10	0.10	0.7599	1, 8	5.48	0.0473
Other Braconidae	1, 11	0.46	0.5115	1, 9	0.01	0.9230	1, 9	1.62	0.2309	1, 9	0.86	0.3785
Platygastroidea												
Scelionidae	1, 12	0.22	0.6441	1, 12	0.05	0.8264	1, 11	0.46	0.5102	1, 12	0.00	0.9829
Proctotrupoidea												
Helorus sp.	1, 10	13.66	0.0039	1, 8	0.05	0.8242	1, 9	7.73	0.0197	1, 8	2.41	0.1591

Chapter 4

Ants impact the energy reserves of natural enemies through the shared honeydew exploitation

Ants impact the energy reserves of natural enemies through the shared honeydew exploitation

Calabuig, A., Tena, A., Wäckers, F. L., Fernández-Arrojo, L., Plou, F.J., Garcia-Marí, F., Pekas, A. (2015) Ants impact the energy reserves of natural enemies through the shared honeydew exploitation. Submitted to *Ecological Entomology.* Decision: Minor revision

Abstract: Ants as well as many species of parasitoids and predators rely on sugar-rich foods such as honeydew to fulfil their energetic needs. Thus, ants and natural enemies may interact through the shared honeydew exploitation. Here we performed ant-exclusion experiments in a citrus orchard to test the hypothesis that ants may impact the energy reserves of predators and parasitoids through the competition for honeydew sources. Through the use of high performance liquid chromatography (HPLC) we related the level of ant activity with the energy reserves and feeding history of individual specimens collected in the field during representative days of spring, summer and autumn. Out of 145 *Aphytis chrysomphali* parasitoids captured in the field, 65% were classified as sugar-fed and 24.7% as honeydew-fed. In summer, when ant activity peaked, there was a significant negative correlation between the level of ant activity and the total sugar content and honeydew feeding incidence by *A. chrysomphali*. Out of 47 individuals of the predator *Chrysoperla carnea* s.l., captured in the field, 55.3% were classified as sugar-fed. We found a significant negative effect of the level of ant activity on the sugar feeding incidence by *C. carnea* in spring.This study provides evidence that ants can interfere with the energy reserves of natural enemies. This interaction may be widespread in various ecosystems with important consequences for the arthropod community composition and with practical implications for biological control given that absence of sugar feeding is detrimental for the fitness of many species of predators and parasitoids.

4.1 Introduction

Ecological communities are complex systems that consist of species interacting directly and indirectly (Miller, 1994; Bascompte *et al.*, 2006; Ohgushi, 2008; Eubanks & Finke, 2014). It has been long known that sugar rich food sources, such as floral and extrafloral nectar or honeydew excreted by plant feeders, mediate species interactions over several trophic levels. For example, pollinators interact with birds (Laverty & Plowright, 1985) or ants (LeVan *et al.*, 2014) when they share a common nectar source. Extrafloral nectar sources may affect the plant, the herbivore and the herbivore´s natural enemies abundance at the community level (Rudgers & Gardener, 2004). Lately, the importance of honeydew in shaping multitrophic interactions has gained increased attention (Kaplan & Eubanks, 2005; Styrsky & Eubanks, 2007; Yoo *et al.*, 2013). Honeydew can be involved in a protective mutualism; ants protect the plant feeders from their natural enemies in exchange for honeydew (Way, 1963; Carroll & Janzen, 1973; Hölldobler & Wilson, 1990). Honeydew is a valuable energy source for numerous organisms including the third trophic level, i.e. natural enemies such as predators and parasitoids, in natural (Zoebelein, 1956) and agricultural ecosystems (Hogervorst *et al.*, 2007; Wäckers *et al.*, 2008; Tena *et al.*, 2013b, 2013c). In this context, ants and natural enemies may interact through the shared energy sources in the form of honeydew. Understanding this interaction will provide useful insights from an ecological but also applied perspective given that it may impact the fitness of the natural enemies and eventually the efficacy of biological control. However, no studies have examined the potential impact of ants on the energy reserves of predators and parasitoids.

Honeydew is a sugar-rich fluid excreted by plant feeders (mostly hemipteran species) after feeding on phloem sap. Honeydew is especially relevant in agricultural ecosystems where it is the principal carbohydrate source since the presence of other sugar sources, such as nectar, is limited and variable in space and time, being available almost exclusively during the flowering season (Wäckers *et al.*, 2008). Honeydew contains a mixture of phloem sugars, such as sucrose, fructose and glucose, and oligosaccharides synthesized by the plant feeders, such as erlose and melezitose (Völkl *et al.*, 1999; Wäckers, 2000). Its composition makes honeydew an important carbohydrate source for a wide range of insects in the field, among which ants hold a predominant position (Hölldobler & Wilson, 1990; Wäckers, 2005). Most ant species are omnivorous and obtain protein from animal matter and carbohydrates from plant products such as floral and extrafloral nectar, food bodies, plant sap and above all honeydew (Way, 1963; Carroll & Janzen, 1973; Tobin, 1994). Honeydew is crucial for the ant colony growth and, usually, honeydew producers thrive when ant-tended (Hölldobler & Wilson, 1990). In fact, honeydew exploitation is an indication of behavioural dominance in ants: dominant ant species exclude subordinate species and monopolize the honeydew sources (Blüthgen *et al.*, 2004; Pekas *et al.*, 2011).

Honeydew is not only crucial for ants. A broad range of entomophagous arthropods, including parasitoids and predators, uses honeydew as an energy source (Jervis & Kidd, 1986; Jervis *et al.*, 1993; Wäckers, 2001; Steppuhn & Wäckers, 2004; Tena *et al.*, 2013c). Honeydew consumption enhances the longevity (Wäckers, 2001; Wäckers *et al.*, 2008) and fecundity of parasitoids (Faria *et al.*, 2008;

Tena *et al.*, 2013b), eventually resulting in increased efficacy of biological control (Faria *et al.*, 2008; Wäckers *et al.*, 2008). Therefore, it is likely that honeydew, due to its availability, nutritional quality and impact on fitness, may mediate direct and indirect competitive interactions between ants and parasitoids or predators. For example, ants may negatively affect the natural enemies by excluding them from the honeydew sources in the same way ants have been found to exclude floral visitors from nectar sources (Lach, 2007). On the other hand, predators and parasitoids may benefit if the probabilities for sugar feeding increase due to the higher abundance of the honeydew producers under ant-attendance.

The citrus agro-ecosystem provides a suitable environment for several honeydew producers (Garcia-Marí, 2012) that are usually ant-attended (Pekas *et al.*, 2011; Tena *et al.*, 2013a) and also harbours a complex of naturally occurring parasitoids and predators (Garcia-Marí, 2012). In the present study, we test the hypothesis that ants might impact the energy reserves of predators and parasitoids through the exploitation of honeydew: positively, by increasing the opportunities for honeydew feeding due to the positive feedback between the ants and the abundance of the honeydew producers or negatively, due to the direct competition ants exert by monopolizing the honeydew sources. To test our hypothesis we carried out an ant-exclusion experiment in a citrus agroecosystem where we related the level of ant activity with the energy reserves and feeding history (sugar and/or honeydew feeding incidence) of individual natural enemies. We included in the study two of the most important entomophagous arthropods in terms of abundance and biocontrol potential on the citrus canopy belonging to two different guilds, one parasitoid and one predator species.

Study system:

We conducted our study in the main Mediterranean citrus-growing area (Valencia, Spain), where the two most abundant and widely distributed ant species are the native *Lasius grandis* (Forel) and *Pheidole pallidula* (Nylander) (Cerdá *et al.*, 2009; Pekas *et al.*, 2011). Both species are behaviourally dominant and are in close association with honeydew producers; they are active from March until November, with *L. grandis* peaking its activity in June and *P. pallidula* in July-August (Pekas *et al.*, 2011). The most abundant honeydew producers present in Mediterranean citrus orchards are the citrus aphid *Aphis spiraecola* Patch (Hemiptera: Aphididae) in early spring, soft scales such as *Coccus hesperidium* L. and *Saissetia oleae* Olivier (Hemiptera: Coccidae), and the citrus mealybug *Planoccocus citri* (Risso) (Hemiptera: Pseudococcidae) during summer, whereas the most abundant honeydew producer in autumn is the woolly whitefly *Aleurothrixus floccosus* (Maskell) (Hemiptera: Aleyrodidae) (Pekas *et al.*, 2011; Tena *et al.*, 2013c).

Parasitoids of genus *Aphytis* (Hymenoptera: Aphelinidae) are the most important natural enemies of the California red scale (CRS) *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae), a major pest in citrus worldwide (Rosen & DeBach, 1979). *Aphyti*s are synovigenic ectoparasitoids that engage in host-feeding for egg maturation (Heimpel & Collier, 1996; Heimpel *et al.*, 1997b); however, host feeding cannot substitute sugar feeding because CRS contains very low amounts of sugar (Tena *et al.*, 2013c). In the lab, in the absence of sugar feeding, *Aphytis* fecundity and longevity are seriously compromised and survival does not exceed three days (Avidov *et al.*, 1970; Heimpel *et al.*, 1997b; Tena *et al.*, 2013b). Furthermore, Tena *et al.* (2013c) demonstrated that honeydew is the main sugar source for *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) in the field, despite the fact that its host does not produce honeydew. Honeydew feeding enhances the longevity and realized-fecundity of *A. melinus* (Tena *et al.*, 2013b). The species of our study, *Aphytis chrysomphali* Mercet (Hymenoptera: Aphelinidae), is native to the Mediterranean and is one of the most important parasitoids of CRS (Pekas *et al.*, 2010a). Most probably it also feeds on honeydew in the field, though this has never been demonstrated.

Neuroptera belonging to the family Chrysopidae are among the most abundant generalist predators present in Mediterranean citrus (Garcia-Marí, 2012). *Chrysoperla carnea* sensu lato (Stephens) (Neuroptera: Chrysopidae) is the most important species in agricultural ecosystems (Stelzl & Devetak, 1999). *Chrysoperla carnea* larvae prey upon aphids (Hemiptera: Aphididae), tetranychid mites (Acari: Tetranychidae), whiteflies (Hemiptera: Aleyrodidae) and the citrus leaf miner (Lepidoptera: Gracillariidae) in citrus (Garcia-Marí, 2012), whereas adults are non-predaceous and consume nectar, pollen and honeydew (Principi & Canard, 1984; Hogervorst *et al.*, 2007). Sheldon and MacLeod (1971) reported that honeydew is a major food source for adult *C. carnea* in the field. Honeydew enhances fecundity (Finney, 1948; Neumark, 1952) and acts as an arrestment stimulus for *C. carnea* (McEwen *et al.*, 1993).

4.3. Materials and Methods

Study site and experimental design

The study was conducted in a commercial citrus orchard of sweet orange *Citrus sinensis* L. Osbeck (cv. Navelina) of approximately 0.7 ha, located in an extensive citrus-growing area 30 km south of Valencia, eastern Spain (39º 12'2" N, 0º 20'52" W). The climate is Mediterranean, with a rainy spring and autumn and a dry winter and summer. The orchard was flood irrigated and weeds were controlled by local application of herbicides (Glyphosate®, Bayer CropScience, Spain). No weeds or other plant species potential hosts for honeydew producers were present in the understory or in the periphery of the orchard. No insecticides were sprayed in the previous nine years, or during the experiment. According to previous studies, the ant species foraging on the tree canopies were *P. pallidula, Plagiolepis schmitzii* and *L. grandis* (Pekas *et al.*, 2010b, 2011). Details about the seasonal activity and spatial distribution of the ant species can be found in the aforementioned studies as well as in Calabuig *et al.* (2013).

The experimental design was a randomized block with eight replicates (blocks) of two adjacent treatments (plots): ant-allowed and ant-excluded trees. Each treatment contained 16 trees (four rows by four trees). Ants were excluded in the 16 trees of each plot in the ant-allowed treatment. In both treatments only the four central trees were sampled. Ant-exclusion began in January 2013 and was maintained until November 2013 by placing sticky barriers based on Tangle-trap® (Tanglefoot, Biagro, Valencia, Spain) on the tree trunks at 30 cm above ground. Tanglefoot was applied using a spatula on a 15 cm wide adhesive plastic tape fixed around the trunk. Sticky barriers were inspected every month and if necessary the Tanglefoot was renewed; in any case, Tanglefoot was renewed routinely every two months. Trees were pruned periodically and ground vegetation was trimmed to prevent alternative ways for ants to reach the canopies.

Ant activity

Ant activity was defined as the number of ants (all species) moving up and down crossing an imaginary horizontal line on the tree trunk during one minute. We monitored ant activity by observing the trunk of the four central trees on each plot between 10:00 and 14:00 h, a period of the day when ants are actively foraging on the canopies (Pekas *et al.*, 2011). Ant activity was monitored on the same day that the natural enemies were collected (see below) in spring (3 June), summer (10 August), and autumn (5 November).

Honeydew producers

To determine the honeydew sources present in the orchard, a 0.52 m-diameter ring was randomly thrown on the four compass directions (N, S, E and W) of the canopy of the sampled. The number of honeydew producers in the ring was counted from the outer part of the canopy to the centre of the trunk (Tena *et al.*, 2013c). The sampling unit consisted of the four rings thrown per tree. Honeydew producers were sampled within the same week that ant activity was monitored.

Sampling parasitoids and predators

Adult parasitoids and predators were collected between 11.00 and 14.00 hours by sampling the whole canopy of the four central trees of each plot until at least four individuals were captured per plot. To collect adult *Aphytis* spp., the branches were hand-beaten in order to make arthropods fall onto the white surface. *Aphytis* were then captured with a brush soaked in ethanol and transferred to an Eppendorf tube with 70% ethanol to preserve them individually. To collect the predators, we actively searched on the canopies. When adult *C. carnea* were detected, we captured them within a small plastic pot and transferred them individually into an Eppendorf tube with 70% ethanol. No *C. carnea* individuals were captured in the autumn sampling.

In the laboratory, the hind tibia length of each individual used in the HPLC analyses, was measured under a stereomicroscope in order to adjust the results to arthropod size (Tena *et al.*, 2013c). Each arthropod was maintained individually in Eppendorf tubes with 70% ethanol and stored at 5ºC until HPLC analysis.

HPLC sugar analysis

Prior to the analysis, the samples were homogenized in the ethanol solution using a pestle. Then, the ethanol was evaporated in a vacuum centrifuge for 60-120 min. After that, the samples were rehydrated by adding 20µl of 70% ethanol and 80µl of Milli-Q water for *A. chrysomphali* and 200µl of 70% ethanol and 800µl of Milli-Q water for *C. carnea* The samples were mixed by vibration and filtered (0.2 µm PVDF membrane). Analysis was carried out by high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). 25µl of each sample were injected into a ICS3000 Dionex system (Dionex Corp., Sunnyvale, CA) consisting of a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode. All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 x 250 mm Carbo-Pack PA-1 column (Dionex) connected to a CarboPac PA-1 guard column was used at 30 °C. For eluent preparation, MilliQ water and 50% (w/v) NaOH (Sigma-Aldrich) were used. Daily reference curves were obtained for sorbitol, mannitol, trehalose, galactose, glucose, sucrose, mannose, fructose, melezitose, raffinose, erlose and maltose by injecting calibration standards with concentrations of 2.5, 5, 7.5 and 10 ppm of each of these sugars. The peaks were analysed using Chromeleon software. Identification of the different carbohydrates was done based on standards commercially available.

A total of 46 (spring), 44 (summer) and 55 (autumn) *A. chrysomphali* were analysed. The total number of *C. carnea* analysed was 25 (spring) and 22 (summer).

Interpretation of HPLC data

The “total sugar content” for each insect was obtained from the sum of the concentrations of all the sugars detected and adjusted to the parasitoid size by the hind tibia length, expressed as µg/mm hind tibia length (Tena *et al.*, 2013c).

In order to determine whether an insect had fed on sugar, we used the total sugar content and the glucose-fructose ratio as classification parameters (Steppuhn & Wäckers, 2004). The total sugar content indicates the current nutritional state of an individual whereas the glucose-fructose ratio becomes fructose dominated after sugar feeding. The combination of both parameters allows to classify an individual that has recently fed as “sugar-fed”(Hogervorst *et al.*, 2007). The glucose-fructose ratio was calculated as the glucose fraction of the sum of both monosaccharides. An insect was classified as “sugar-fed” when the total sugar content was above an established threshold and the glucose-fructose ratio was below an established threshold. The thresholds used to classify an insect as “sugar-fed” were obtained from previous studies with laboratory insects. For *A. chrysomphali,* we used the thresholds obtained for the sibling species *A. melinus* established at 1.5 µg/mm hind tibia length for total sugar content and 0.63 for glucose-fructose ratio (Tena *et al.*, 2013c). For *C. carnea* s.l., we used the thresholds obtained by Hogervorst *et al*. (2007) for the total sugar content and glucose-fructose ratio, established at 59.2 µg/lacewing and 0.85 respectively. Individuals with total sugar content below or glucose-fructose ratio above the established thresholds were considered as “unfed or starved”.

Erlose and melezitos are considered honeydew “signature sugars”. Nevertheless, the species in our study can synthesize erlose and melezitose after feeding on sucrose and therefore the mere presence of these sugars cannot be used to identify honeydew-feeding. Instead, the erlose-melezitose ratio allow us to determine recent honeydew consumption for some species (Hogervorst *et al.*, 2007; Tena *et al.*, 2013c). We used the erlose-melezitose ratio to determine “honeydew consumption” by *A. chrysomphali* using the threshold obtained in laboratory by Tena *et al*. (2013c) for *A. melinus*, which was established at 0.32. In the case of *C. carnea* it is not possible to apply this method since this species synthesizes erlose and malezitose in a ratio similar to that found in honeydew (Hogervorst et al. 2007).

Statistical analysis

We used a one-way ANOVA to check for differences in ant activity and abundance of honeydew producers among seasons. Normality assumption was assessed using Shapiro test, and homoscedasticity assumption was assessed with Levene test. When necessary, data were log transformed in order to fulfil normality and homoscedasticity assumptions.

In the ant-excluded treatment the tanglefoot barriers were very effective in completely excluding the ants from climbing to the canopy, however, ant activity was null or very low in some of the ant-allowed trees. Therefore, for the analysis average ant activity per plot was used as explanatory variable in place of the categorical treatment variables ant-exclusion and ant-allowed (see Yoo *et al*. 2013).

We applied generalized linear mixed modeling techniques assuming Gamma error variance to construct a model with the abundance of honeydew producers as depended variable, ant activity as the explanatory variable and block as random factor. Abundance of honeydew producers from the four sampled trees was averaged to obtain a mean for each plot.

We also applied generalized linear mixed modeling techniques assuming Gamma error variance for the total sugar content and binomial error structure for sugar-feeding or honeydew-feeding occurrence to construct models with ant activity as the explanatory variable and block as random factor using the *glmer* function (Bates, 2010). In all models, ant activity from the four sampled trees in the ant–allowed treatment was averaged to obtain a mean for each plot. Different models were constructed for each sampling date given that ant activity as well as the composition of species of the honeydew producers was significantly different among seasons. All statistical analyses were conducted with R (R Development Core Team, 2014).

4.3. Results

Ant activity

In the ant-excluded treatment, the sticky barriers excluded the ants from climbing to the canopy; ant activity was null in all the trees and samplings dates. In the ant-allowed trees, three ant species were identified foraging on the canopies: *P. pallidula* (accounting for the 56% of the total ants counted), *P. schmitzii* (31%) and *L. grandis* (13%). Ant activity was different among seasons ($F_{2, 21} = 44.42$; $P < 0.0001$), being significantly higher in summer (11.7 ± 1.8 ants/min) than in spring (6.4 ± 1.1 ants/min) or autumn (1.5 ± 0.3 ants/min) (Fig. 1a).

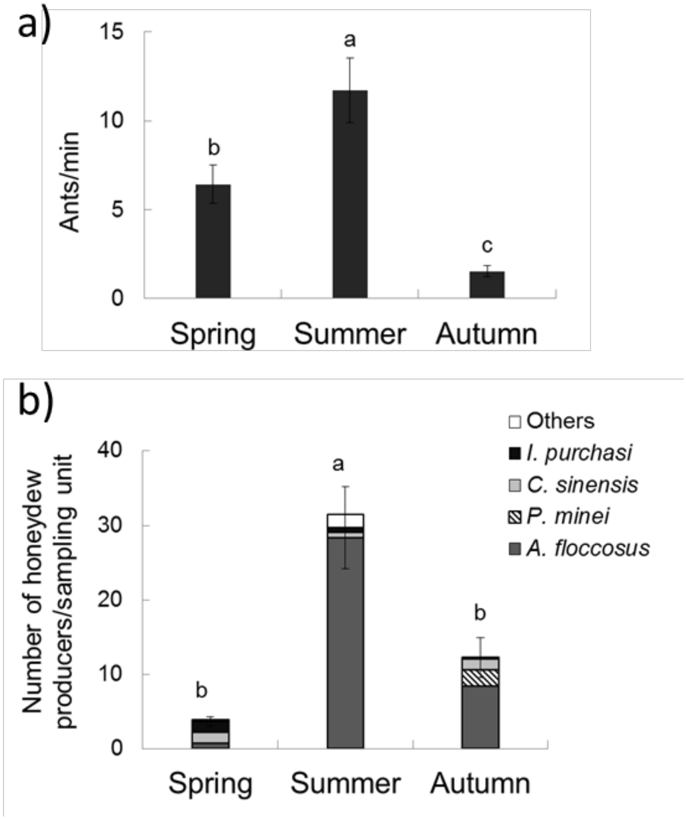

Fig. 1. (a) Mean (±SE) ant activity (number of ants per minute) in representative days of spring, summer and autumn. Means with different letter differ significantly at $P < 0.05$. (b) Mean (± SE) number of hemipteran honeydew producers per sampling unit in representative days of spring, summer and autumn. Means with different letter differ significantly at $P < 0.05$.

Abundance of honeydew producers

Honeydew producers were present in the three seasons in the ant-allowed and ant-excluded treatments. The abundance of the honeydew producers was different among seasons, being significantly higher in summer than spring and autumn ($F_{2,\,45} = 19.1$; $P < 0.0001$) (Fig. 1b).

Honeydew producer abundance increased with ant activity in summer ($\chi^2 = 7.93$, $P = 0.005$), whereas no relationship was found in spring ($\chi^2 = 0.26$, $P = 0.607$) or autumn ($\chi^2 = 0.001$, $P = 0.965$).

The species composition of the honeydew producers differed among seasons. In spring, the principal honeydew producers were *Ceroplastes sinensis* Del Guercio (Hemiptera: Coccidae) (accounting for the 40% of the total honeydew producers) and *Icerya purchasi* Maskell (Hemiptera: Monophlebidae) (36%), whereas the whitefly *Aleurothrixus floccosus* (Maskell) (Hemiptera: Aleyrodidae) was the most abundant species in summer (90%) and autumn (70%) (Fig. 1b).

Sugar spectrum of natural enemies

The sugar spectrum revealed that glucose and fructose were the predominant sugars detected in *A. chrysomphali* and *C. carnea* (Table 1). In addition, both species contained the honeydew specific sugars erlose and melezitose.

Table 1. Total amount of sugars (µg/mm tibia length) of *Aphytis chrysomphali* and *Chrysoperla carnea* captured in the field (number of individuals is shown in parentheses).

	Total sugar (µg/mm tibia length)	
Sugar	*Aphytis chrysomphali* (145)	*Crysoperla carnea* (47)
Erlose	0.170 ± 0.041	1.351 ± 0.471
Fructose	1.100 ± 0.099	19.728 ± 3.797
Galactose	0.005 ± 0.002	0.050 ± 0.040
Glucose	1.105 ± 0.073	22.146 ± 1.847
Maltose	0.183 ± 0.017	3.477 ± 0.891
Mannitol	0.068 ± 0.008	1.250 ± 0.204
Mannose	0	0.157 ± 0.034
Melezitose	0.511 ± 0.099	7.381 ± 1.824
Melibiose	0.021 ± 0.003	2.856 ± 0.503
Raffinose	0.002 ± 0.001	0.576 ± 0.133
Sorbitol	0.011 ± 0.004	1.307 ± 0.223
Sucrose	0.716 ± 0.107	4.885 ± 1.190
Trehalosae	0.045 ± 0.018	0.381 ± 0.130
Total	3.936 ± 0.290	66.687 ± 9.389

Effect of ants on total sugar content

Pooling all the captured individuals, the total sugar content of *A. chrysomphali* in the field ranged from 0.15 to 17.12 µg/hind tibia length, with a mean value of 3.94 ± 0.29 µg/hind tibia length. A significant negative relationship between ant activity and the total sugar content of *A. chrysomphali* was found in summer ($\chi^2 = 5.88$, $P = 0.015$) (Fig. 2). Ant activity had a marginally significant effect on the total sugar content of *A. chrysomphali* in spring ($\chi^2 = 3.55$, $P = 0.059$) whereas no significant relationship between ant activity and the total sugar content was found in autumn ($\chi^2 = 0.168$, $P = 0.682$).

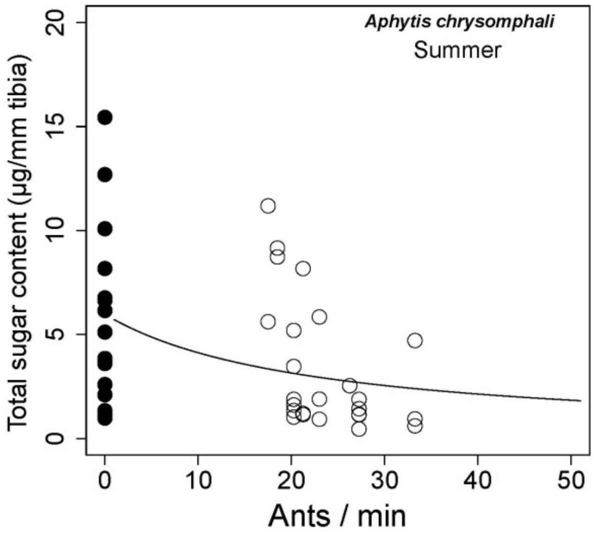

Fig. 2. Relationship between the level of ant activity (ants/minute) and the total sugar content (µg/mm tibia length) of *Aphytis chrysomphali* in representative days of summer. (Open circles, ant-allowed trees; solid circles, ant-excluded trees).

The total sugar content of *C. carnea* ranged from 1.6 to 337.8 µg/hind tibia length, with a mean value of 66.7 ± 9.4 µg/hind tibia length (pooling all the captured individuals). The relation between ant activity and total sugar content of *C. carnea* was non-significant in spring (χ^2 = 2.58, *P* = 0.108) or summer (χ^2 = 0.05, *P* = 0.82). It is also important to highlight the (4 times) higher total sugar content in *C. carnea* individuals captured in summer in comparison with the ones captured in spring.

Effect of ants on sugar-feeding occurrence

Overall, out of 146 *A. chrysomphali* captured in the field in the three seasons, 65% were classified as sugar-fed. Ant activity had a marginally negative significant effect on the occurrence of "sugar-fed" individuals of *A. chrysomphali* in summer ($\chi^2 = 3.64$, $P = 0.056$) whereas no significant relationship between ant activity and sugar feeding occurrence was found in spring ($\chi^2 = 0.23$, $P = 0.630$) or autumn ($\chi^2 = 0.26$, $P = 0.607$).

Overall, out of 47 *C. carnea* captured in the field in the three seasons, 55.3% were classified as sugar-fed. The effect of ant activity on the sugar feeding occurrence for *C. carnea* was negative in spring ($\chi^2 = 4.82$, $P = 0.028$) (Fig. 3) whereas it was non-significant in summer ($\chi^2 = 1.12$, $P = 0.290$).

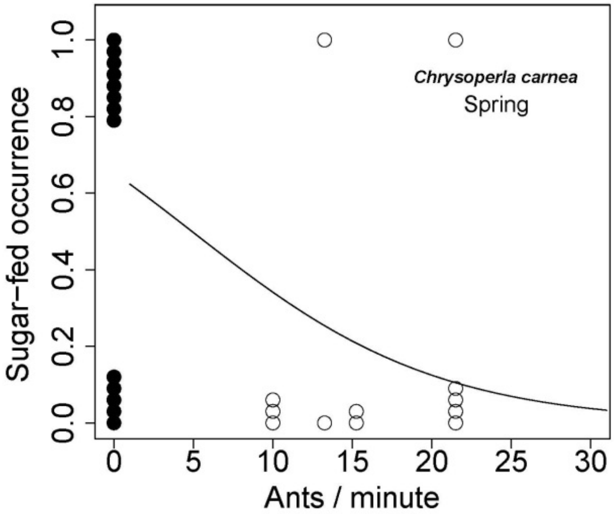

Fig. 3. Relationship between ant activity (ants/minute) and sugar-feeding occurrence by *Chrysoperla carnea* in representative days of spring. Data are slightly displaced from their originally binary positions in order to better represent sample size. (Open circles, ant-allowed trees; solid circles, ant-excluded trees).

Effect of ants on honeydew-feeding occurrence

Overall, out of 146 *A. chrysomphali* captured in the field in the three seasons, 24.7% was classified as honeydew-fed. Honeydew-feeding in *A. chrysomphali* was negatively correlated with ant activity in summer ($\chi^2 = 4.99$, $P = 0.026$) (Fig. 4), whereas no relationship was found in spring ($\chi^2 = 0.06$, $P = 0.808$) or autumn ($\chi^2 = 2.13$, $P = 0.144$).

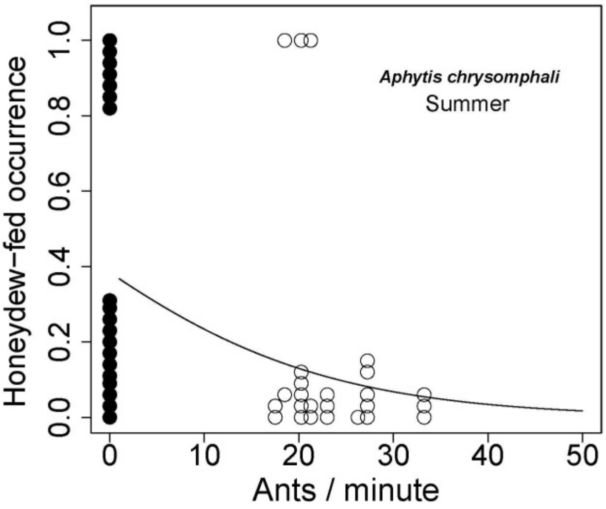

Fig. 4. Relationship between ant activity (ants/minute) and honeydew-feeding occurrence by *Aphytis chrysomphali* in representative days of summer. Data are slightly displaced from their originally binary positions in order to better represent sample size. (Open circles, ant-allowed trees; solid circles, ant-excluded trees).

Despite the fact that the mere presence of erlose and melezitose cannot be used for determination of honeydew feeding, we detected a considerable percentage of *C. carnea* (63.8% of individuals captured in spring and summer) with presence of honeydew signature sugars.

4.5 Discussion

Our study delves into the understanding of indirect, multi-trophic interactions mediated by honeydew excreted by plant feeders. Concretely, we tested the hypothesis that the mutualistic relationship between ants and honeydew producers may impact positively or negatively the energy reserves and access to honeydew of natural enemies. Herein, for the first time we provide evidence that ants can have a negative impact on the energy reserves and access to honeydew of the parasitoid *A. chrysomphali*, and the predator *C. carnea*. The impact of ant activity on the energy reserves of natural enemies is likely to be widespread in natural and managed ecosystems with potential effects for the arthropod community composition and biological control.

When examining the interference of ants with the total sugar content of *A. chrysomphali,* we found a negative relationship in summer, the period when both ant activity and abundance of honeydew producers peaked. Similarly, the sugar and honeydew-feeding incidence by *A. chrysomphali* were also negatively affected by ant activity in summer. Apparently, *Aphytis* spp. are highly susceptible to ant aggression. In fact, there are studies demonstrating that ants prey upon them in the field (Heimpel *et al.*, 1997a) or disturb them during host-feeding and oviposition (Martínez-Ferrer *et al.*, 2003). *Aphytis melinus* females spend more than 300 seconds per honeydew feeding bout (Tena *et al.*, 2013b). Assuming that *A. chrysomphali* behaves similarly feeding on honeydew, it is likely that ants may interfere with the sugar acquisition by *A. chrysomphali* during that feeding time. The impact of ant activity on the feeding history and energy reserves may be more significant for small parasitoids such as *Aphytis* species because of their limited capacity to engage in long flights (Campbell, 1976; Zappalà *et al.*, 2012; Tena *et al.*, 2015) to exploit other non-tended honeydew sources. Honeydew sources in citrus orchards are commonly ant tended. Therefore, *Aphytis* spp. should avoid ant-tended honeydew sources to prevent attacks by ants. Finally, our results show that the effect of ants on the energy reserves of parasitoids depended on the level of ant activity. Therefore, we expect that this interference may be more pronounced when honeydew producers are tended by aggressive ant species and with high levels of activity, as is the case of many invasive ant species (Holway *et al.*, 2002). All this being said, we propose that ant interference might be more detrimental for those parasitoid species that are not tolerant to ant aggression, have limited dispersion capacity and/or whose host does not excrete honeydew.

Overall, the energy reserves of the predator *C. carnea* were not affected by ant activity either in spring or in summer. On the other hand, sugar-feeding occurrence was negatively correlated with ant activity in spring. We suggest that this result may be interpreted in terms of honeydew availability in combination with the behavior of *C. carnea.* As the populations of the honeydew producers were very low in spring, sugar availability was scarce. Under this scenario, adult *C. carnea* may find occasional honeydew droplets in absence of ants which would slightly increase their sugars levels but would be enough to distinguish them as sugar-fed. Nevertheless, under conditions of increased ant activity, it is likely that most honeydew sources would be occupied by ants, leaving very little chance to *C. carnea* adults to feed on sugars. In summer, the higher availability of honeydew allows lacewings to feed and reach high energy reserves, despite the presence of ants. The flight behavior, in particular the migration flights of *C. carnea* might give an additional explanation for the results obtained in our study. In the first three nights after emergence, the lacewings fly downwind in adaptive dispersal flights, irrespective of the availability of food (Duelli, 1980a, 1980b). This behavior implies that young adults captured after these flights will show very low levels of energy reserves regardless of the availability of honeydew or the disturbance of ants. Later, the sexually mature adults respond to kairomones signaling honeydew and perform a stepwise flight against the wind to approach the source of attractant (Duelli, 1980b). Although larvae and adults of green lacewings are heavily attacked by ants and the adults show a strong tendency to avoid ants (Bartlett, 1961) the capacity of adults for dispersion apparently plays an important role in overcoming the disturbance by ants in summer since they can search for non-ant tended honeydew sources. For example, adult *C. carnea* may move up to 1 km away from the release point after one day (Duelli, 1980b). Consequently, the energy reserves of *C. carnea* are less susceptible to be influenced by the level of local ant activity compared to *Aphytis* parasitoids.

It is also important to highlight that the energy reserves and incidence of sugar or honeydew feeding were never enhanced in the presence of ants neither for *A. chrysomphali* nor for *C. carnea* This is even more surprising if we consider the fact that the present as well as other studies conducted in citrus (Moreno *et al.*, 1987; Yoo *et al.*, 2013) have demonstrated that under increased ant activity levels there are greater numbers of honeydew producers on the citrus canopies. This means that predators and parasitoids should have higher probabilities of accessing honeydew sources in the presence of ants. However, this was not the case in our study. Possibly the direct ant competition might explain this result given that in citrus orchards colonies of honeydew producers are usually ant tended (Pekas *et al.*, 2011). Moreover, competition with other honeydew feeders such as pollinators and other unintended consumers might also have an influence (Wäckers & Fadamiro, 2005).

All in all, we report a novel interaction between ants and natural enemies mediated by the shared honeydew exploitation. As already known, honeydew collecting ants may modify natural enemy abundance (Bartlett, 1961; James *et al.*, 1999), enhance hemipteran populations (Bartlett, 1961; Samways, 1990) and, eventually, impact plant health (Rosumek *et al.*, 2009). In a previous study it was shown that red imported fire ants indirectly affected the fecundity of native ants by excluding them from aphid-provided carbohydrates (Wilder *et al.*, 2013). Herein, for the first time we quantify the effects that ants can have on the energy reserves, sugar and honeydew feeding of entomophagous arthropods. This type of interaction may be widespread in various ecosystems with important consequences at the community-level and with practical implications for the biocontrol services that natural enemies provide (Eubanks & Finke, 2014). Absence of sugar feeding has been found to be detrimental for the fitness of many species of predators and parasitoids and is likely to affect also their abundance, the abundance of the herbivores and ultimately plant quality resulting in top-down trophic cascades (Pace *et al.*, 1999). These trophic cascades may be particularly important in agroecosystems, where the availability of sugar sources is usually limited in space and time (Wäckers *et al.*,

2008) resulting in a deficient pest control. Ideally, this lack of sugar sources may be compensated by means of artificial sugar sprays (Wade *et al.*, 2008; Tena *et al.*, 2015) and/or habitat management to enhance the functional biodiversity that will support the beneficial organisms to maximize the ecosystem services provided (Olson & Wäckers, 2006).

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Chapter 5

General discussion

General discussion

Our ant-exclusion experiments revealed that the three most abundant ant species in Mediterranean citrus orchards are able to induce higher populations of California red scale and wooly whitefly, i.e. populations of honeydew and non-honeydew producing pests. In order to disentangle the underlying mechanisms explaining the increased pest populations we examined various hypotheses: the interference of ants with the activity of parasitoids, the impact of ants on natural enemy abundance and community structure, and the competition between ants and natural enemies for honeydew.

Interestingly, the increased pest populations observed were not apparently the result of ant interference with the activity of the parasitoids given that parasitism was similar in ant-allowed and ant-excluded treatments in the three orchards studied. It is important to highlight that previous studies in the same area reported higher populations of *A. aurantii* on citrus fruits in the ant-allowed treatment while finding no differences in the percent parasitism between the ant-allowed and ant-excluded treatments (Pekas *et al.*, 2010b). Thus, factors other than parasitoid disruption might explain the increased pest populations induced by ants.

Crucially, in chapter 2 we report that, overall, ants did no impact the community structure of natural enemies in citrus. However, ants altered the abundance of many natural enemy species revealing a previously undocumented pattern: the abundance of most parasitoid species increased while the abundance of most generalist predators decreased in the ant-allowed treatment. Especially for parasitoids we also registered higher species biodiversity in the ant-allowed treatment, apparently as a result of the higher populations of their hosts under ant-attendance. These results suggest that the role of generalist predators may be highly relevant for pest control in citrus and thus the decrease in predator abundance as a consequence of ant interference might explain the higher pest populations in the ant-allowed treatment. According to Symondson (2002) native generalist predators are efficient biological control agents which eventually could be more important than parasitoids The capacity of generalist predators to regulate pest populations in Mediterranean citrus has received little attention. Their non-specificity poses a serious technical difficulty when designing an experiment and might explain the lack of studies quantifying their biocontrol potential in the field. This fact highlights the need for future, detailed research on the role of generalist predators in citrus agroecosystems as biological control agents and the potential of ants to disturb their activity.

Finally, we showed that ants interact indirectly with predators and parasitoids through the common honeydew exploitation. High level of ant activity was negatively associated with the total sugar content and the sugar and honeydew feeding occurrence of the parasitoid *A. chrysomphali* and the predator *C. carnea* s.l. at certain times of the year. Absence of sugar feeding is detrimental for the fitness of predators and parasitoids. Therefore, the reported interaction is expected to have consequences for the wider arthropod community, affecting the abundance and biocontrol potential of the natural enemies. To our knowledge, this study is the first to report these indirect effects in food webs, in particular the impact on the physiological state of the impacted species. Application of artificial sugar sprays may alleviate the sugar-deprived parasitoids and increase their populations as well as the parasitism rates as recently demonstrated for *Aphytis melinus* in citrus (Tena *et al.*, 2015).

Ants are generalist predators and can act as biocontrol agents in various ecosystems (Hölldobler & Wilson, 1990). In our study, the native ant species *L. grandis* and *P. pallidula* may act as predators to some extent (Cerdá & Retana, 1988; Paris & Espadaler, 2009), however, no data exist demonstrating their biocontrol potential which seems to be rather low. Similarly, there are no studies quantifying their potential contribution to biological pest control in Mediterranean citrus. Thus, in view of the increased pest populations and the negative impact on the abundance of the generalist predators observed in the ant-allowed treatment, the exclusion of ants may be recommended in citrus orchards. Several methods can be used to exclude ant populations from citrus canopies. In this study we have used sticky barriers and micro-encapsulated insecticidal paint applied to the trunk. Both methods have been found to be adequate to exclude ants from tree canopies. Unfortunately the use of sticky barriers is a very laborious and expensive method, whereas the insecticidal paint shows some constrains that are limiting its use (Juan-Blasco *et al.*, 2011). A sustainable alternative might be the provisioning of artificial sugar sources to distract the ants from tending the honeydew producing herbivores. Thus the effectiveness of the natural enemies is expected to be enhanced, increasing additionally ant predation on herbivores, when ants are satiated with sugars. The supply of artificial sugar sources has been found to be effective in altering ant attendance and eventually reducing aphid populations in apple (Nagy *et al.*, 2013) and citrus orchards (Sánchez-Alberola *et al.*, 2013).

The mutualistic relationship between ants and honeydew-producing hemiptera has broad effects and community-level consequences in the citrus agroecosystem. Ants impact direct or indirectly the second (herbivores), third (natural enemies) and fourth trophic levels (hiperparasitoids). Although not directly quantified in the present study, ants apparently also impact the first trophic level (primary producers) since the increased herbivore populations in the ant-allowed treatment are expected to affect negatively the plant health and productivity.

Chapter 6

Conclusions

Ants affect the infestation levels but not the parasitism of honeydew and non-honeydew producing pests in citrus

i) The infestation level of *Aonidiella aurantii* on twigs was lower in the ant-excluded treatment in the orchard dominated by *L. grandis* whereas in the orchards dominated by *P. pallidula* and *L. humile* it was similar in ant-allowed and ant-excluded treatments.

ii) Ant-exclusion resulted in decreased *A. aurantii* densities on fruits in the three orchards of the study, ranging from as high as 41% to as low as 21%.

iii) The percentage of shoots occupied by *A. floccosus* in the orchards dominated by *P. pallidula* and *L. humile* was lower in the ant-excluded treatment.

iv) The incidence of *P. citrella* was similar on ant-allowed and ant-excluded treatments in the three orchards of the study.

v) Parasitism levels were similar in the ant-allowed and the ant-excluded treatments for both the non-honeydew producing *A. aurantii* and the honeydew producing *A. floccosus*. These results suggest that mechanisms other than parasitism (e.g.

predation) might explain the differences in herbivore infestation levels between treatments.

Ants in citrus: impact on the abundance, species richness, diversity and community structure of predators and parasitoids

i) The impact of ants on the abundance of natural enemies showed a species specific response.

ii) We observed a general pattern related to functional groups: there were lower numbers of generalist predators and higher numbers of parasitoids in the ant-allowed treatment compared to the ant-excluded treatment.

iii) In the ant-allowed treatment the species richness (S) of predators was significantly lower whereas the species richness (S) and the Shannon diversity index (H) of parasitoids were higher.

iv) The community structure of predators and parasitoids was not significantly different between treatments.

v) The negative impact of ants on specific generalist predators may explain the increases of hemipteran populations observed.

Ants impact the energy reserves of natural enemies through the shared honeydew exploitation

i) We detected a significant negative correlation between the ant activity and the total sugar content and honeydew feeding incidence by *A. chrysomphali* in summer.

ii) The sugar feeding incidence by *C. carnea* was negatively correlated with the level of ant activity in spring.

iii) Our study provides the first evidence that ants can interfere with the energy reserves of natural enemies through the common honeydew exploitation.

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