Running head: Impact of ants on population abundance and parasitism of citrus herbivores

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

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Abstract:

Ants can act simultaneously as predators and as hemipteran mutualists, and thus may affect the composition and the 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 in western Mediterranean citrus: the honeydew-producer *Aleurothrixus floccosus* (woolly whitefly) and the non-honeydew-producers *Aonidiella aurantii* (California red scale; CRS) and *Phyllocnistis citrella* (citrus leafminer). The study was conducted in three commercial citrus orchards (A, B, C) during two consecutive growing seasons (2011 and 2012). *Pheidole pallidula, Lasius grandis,* and *Linepithema humile* were the most abundant ant species in orchards A, B and C respectively. In the three orchards we registered a significant reduction of the CRS densities on fruits in the ant-excluded treatment, ranging from 41% for orchard B in 2011 to 21% for orchards A and B in 2012. Similarly, the percentage of shoots occupied by *A. floccosus* was significantly lower in the ant-excluded plots in orchards A (*P. pallidula*) or C (*L. humile*). No significant differences were registered in the percentage of leaf surface loss caused by larvae of *P. citrella* between ant-allowed and ant-excluded treatments in any case. However, we found no significant differences in the percent parasitism between ant-allowed and ant-excluded treatments for honeydew and non-honeydew producing insect herbivores, suggesting that parasitism cannot explain the differences in the herbivore population levels between treatments.

Keywords: *Lasius grandis*, *Pheidole pallidula*, *Linepithema humile*, *Aonidiella aurantii, Aleurothrixus floccosus*, *Phyllocnistis citrella*,

Introduction

Ants (Hymenoptera: Formicidae) are broadly distributed on terrestrial ecosystems and they are among the leading predators of other insects (Hölldobler and 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 and Eubanks, 2007; Olotu *et al*., 2012; Rosumek *et al*., 2009). However, most ant species are omnivorous and combine the protein obtained through predation and scavenging with plant-derived carbohydrates such as 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 and Jansen, 1973; Hölldobler and Wilson, 1990; Wäckers, 2005). Thus, by acting simultaneously as predators and as hemipteran mutualists, ants are in the centre of a complex food web affecting the composition and the population dynamics of a wide arthropod community (Kaplan & Eubanks, 2005; Styrsky and Eubanks, 2007).

In the ant-hemiptera mutualism, the net benefits for each partner are context dependent (Stadler & Dixon, 1999; Yo and Holway, 2012). It is typically considered that ants obtain honeydew, a copious, nutritive and constant in time and space food source, whereas in exchange they 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 develop faster higher populations which eventually result 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 and Inoue, 1996a;

James *et al*., 1997; Daane *et al*., 2007; Mgocheki and 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 and Inoue (1996a) 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 decrease by 94% in a mealybug population when ants were excluded. An ant-exclusion experiment revealed that ant-attendance caused an increase in the 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 and 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. (Steyn 1954) in Letaba (South Africa), *L. humile* in California (Moreno *et al*., 1987), *Iridiomyrmex rufoniger* in Australia (James *et al*., 1997) and *Lasius grandis* (Forel) and *Pheidole pallidula* (Nylander) in Valencia (Spain) (Pekas *et al*., 2010a). 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 the indirect interference (while searching for honeydew) of the ants 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 (Martinez-Ferrer *et al*., 2003; Styrsky and 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 and Gullan, 1991; Kaneko, 2003; Paris and 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, 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-Mari, 2012). At the same time, ants are among the most abundant arthropods in citrus (Bodenheimer 1951; Samways *et al*., 1982; Samways 1983; Alvis and García-Marí, 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, Pekas *et al*. (2010a) showed that mixed populations of these species were associated with increases of the densities of CRS populations. The invasive *L. humile* is present in Spanish citrus groves since 1923 (Font de Mora, 1923; García Mercet 1923) but it appears only occasionally in citrus orchards (Alvis and García-Marí, 2006). In other citrus growing areas it is associated with strong increases of 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, the native *L.grandis* and *P. pallidula* and the invasive *L. humile*, on the infestation levels and parasitism of three of the most important citrus pests in western Mediterranean citrus: the honeydew-producer *Aleurothrixus floccosus* and the non-honeydew-producers *A. aurantii* and *Phyllocnistis citrella* (Staiton) (Lepidoptera: Gracillaridae).

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. The climate in the study areas is Mediterranean, with mild winters and dry summers. Two orchards (A and B) were of sweet orange *Citrus sinensis* L. Osbeck (cv. Navelina) and one (orchard C) of a mixture of two species, sweet orange *C. sinensis* (cv. Navelina) and Clementine mandarin *Citrus reticulata* Blanco (Cv. Clementina Fina). In orchard A, the most abundant ant species ascending on 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 extend with *Tapinoma nigerrimum* (Nylander). In orchard B, the most abundant and predominant ant species was *L. grandis*, coexisting in some trees with *P. schmitzii* and *T. nigerrimum,* except in the experimental block 6 (see below) where *L. grandis* and *P. pallidula* where 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 C, *L. humile* was the only ant species present and foraging on the tree canopies.

The three orchards were furrow-irrigated and weeds were controlled by local application of herbicides (Glyphosate®). No chemical treatments for pest control were applied during the previous two years before the onset 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*., 2010a, 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

At each orchard the experimental design was a randomized complete block 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 A and B and in May 2011 in orchard C and was maintained until November 2012 (19 months). During the first season (2011), ant exclusion was achieved by applying to the trunk an insecticidal paint in a micro-encapsulated formulation (Inesfly FITO© (chlorpyrifos 3%)), Industrias Químicas Inesba S.L., Paiporta, Spain). In previous studies in the same citrus area, Inesfly FITO© effectively excluded ants from citrus canopies (Juan-Blasco *et al*., 2010). 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. In order to ensure that ants could not reach the canopies trough alternative ways along 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. 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 per tree from the four central trees on each plot of the ant-allowed and the ant-excluded treatments. 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 and Heuberger, 1943):

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

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 randomly selected per tree from the four central trees on each plot of the ant-allowed and the ant-excluded treatments. This sampling was performed in the three orchards from August to November 2011 and 2012, i.e. when fruits were available.

Citrus woolly whitefly

Aleurothrixus floccosus infestation was determined by estimating the percentage of shoots occupied. Once a month we observed 10 shoots randomly selected per tree from the four central trees on each plot of the ant-allowed and the ant-excluded treatments and counted the number of shoots with *A. floccosus* present. This sampling was performed in the three orchards from July to October in 2011 and 2012, whenever *A. floccosus* was observed in the orchards.

Citrus leafminer

Phyllocnistis citrella infestation was estimated by calculating the percentage of damaged leaf area. In order to do so, we randomly sampled once a month 10 young shoots, containing between 5 and 10 leaves each, from the four central trees per plot of the ant-allowed and the ant-excluded treatments. Shoots were transferred to the laboratory, where we scored the damage in 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 in August and October 2011 and in October 2012 for the orchards A and B, as well as in August 2011 and October 2012 for the orchard C.

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

California red scale

CRS parasitism was assessed once a month 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 of the ant-allowed and the ant-excluded treatments. The samples were carried to the laboratory where we observed under a stereomicroscope 50 to 100 (depending on the availability) CRS stages susceptible to parasitism and determined the number of parasitized and unparasitized scales. In some cases where CRS population was very low, between 30 and 50 stages were considered sufficient. In the study area CRS is parasitized by *Aphytis chrysomphali* (Mercet) and *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) (Pekas *et al*., 2010b; 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 in June and July 2011, and July 2012 for assessing parasitism on twigs. In fruits, the percent parasitism was assessed in September and November 2011 and September and October 2012 for orchards A and B and in September and November 2011, and September, October and November 2012 for the orchard C.

Citrus woolly whitefly

Parasitism of *A. floccosus* was determined once a month by sampling a maximum of 20 leaves (when available) infested by *A. floccosus* from the four central trees per plot of the ant-allowed and the ant-excluded treatments. 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 larvae 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 whitefly pupae were identified by the presence of swollen larvae 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 in July and September 2011 an October 2012 for orchards A and B and in July, August and September 2011 and July and August 2012 for orchard C.

Citrus leafminer.

Parasitism of *P. citrella* was assessed once a month by sampling 10 young shoots per tree from the four central trees on each plot of the ant-allowed and the ant-excluded treatments. 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) immature leafminer 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* 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 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 infestation 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).

Results

Ant Activity

When examining the ant activity registered in each orchard, the invasive *L. humile,* predominant in orchard C, 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 A and B, respectively, showed considerably lower activity levels than *L. humile*. *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.

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 A: 4.07 ± 0.44 vs. 0.07 ± 0.05; repeated-measures ANOVA: $F = 367.74$; df = 1, 6; $P < 0.0001$; orchard B: 2.41 ± 0.39 vs. 0.017 ± 0.01; repeated-measures ANOVA: $F = 74.46$; df = 1, 6; $P = 0.0001$; orchard C: 60.64 ± 5.7 vs. 0.125 ± 0.05; repeated-measures ANOVA: $F = 218.71$; df = 1, 6; $P < 0.0001$) (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 B*,* whereas no significant differences between treatments were found for orchards A and C (pooled data from all sampling dates; Repeated measures ANOVA: $F = 4.92$; df = 1, 30; $P = 0.035$, $F = 9.30$; df = 1, 30; $P = 0.34$ and $F = 2.94$; df = 1, 30; $P = 0.097$, respectively) (Fig. 2a). When examining each of the three sampling dates separately, CRS density on twigs was significantly lower in the ant-excluded treatment in July 2011 and 2012 for orchard B and in May 2012 for orchard A (Fig. 2a) (Table 1).

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 A: $F = 11.45$; df = 1, 30; $P = 0.002$; orchard B: $F = 34.91$; df = 1, 30; $P < 0.0001$; orchard C: $F = 10.86$; df = 1, 30; $P = 0.003$). When examining each sampling date separately, CRS densities on fruits were significantly lower in the ant-excluded treatment in 14 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 B (where *L. grandis* was predominant), 28% and 21% for orchard A (*P. pallidula),* and 27% and 21% in orchard C (*L. humile)*. Thus, all three ant species were able to induce higher populations of CRS on fruits.

Citrus woolly whitefly

The percentage of shoots occupied by *A. floccosus* was significantly lower in the ant-excluded treatment in the case of orchards A (*P. pallidula*) and C (*L. humile*). On the other hand, no significant differences were found between treatments in the case of orchard B (*L. grandis*) (pooled data from all sampling dates; Repeated Measures Anova: orchard A: $F = 9.43$; df = 1, 30; $P = 0.0045$; orchard B: $F = 0.22$; df = 1, 30; $P = 0.646$; orchard C: $F = 18.65$; df = 1, 30; $P = 0.0002$) (Fig. 3). When comparing each sampling date separately, the percent occupation of shoots was significantly higher in ant-allowed treatment in two of the four dates (September 2011 and October 2012) for orchard A and in all five sampling dates for orchard C (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 A (*P. pallidula)* and 40% in 2011 and 26% in 2012 for orchard C (*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 A: F = 1.6; df = 1, 6; P = 0.223; orchard B: F = 0.01; df = 1, 6; P = 0.9327; orchard C: F = 0.03; df = 1, 6; P = 0.8709) (Fig. 4). When comparing each sampling date separately, the percent of leaf surface loss was significantly lower in the ant-excluded treatment in one of three dates (October 2011) in the case of orchard A, in one of three (October 2012) for orchard B and in one of two (August 2011) in orchard C (Table 1).

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 A, B and C respectively. The mean (±SE) percent parasitism of CRS on fruits peaked in September and was considerable higher than on twigs, reaching 45.6% (± 3.6), 42.7% (± 3.33) and 38.0% (± 2.5) in orchards A, B and C 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 A: F = 1.61; df = 1, 6; P = 0.2512; orchard B: F = 2.75; df = 1, 6; P = 0.1481; orchard C: F = 1.81; df = 1, 6; P = 0.2271). When comparing each sampling date separately, we found significantly higher percent parasitism in the ant-excluded treatment in orchard C in one of three dates examined (July 2011). In this particular date we found a percent parasitism of 16.9% (± 3.63) in ant-excluded treatment versus 3.64% (± 2.40) in the ant-allowed treatment (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 A: $F = 0.26$; df = 1, 6; $P = 0.6288$; orchard B: $F = 0.02$; df = 1, 6; $P = 0.8970$; orchard C: $F = 4.54$; df = 1, 6; $P = 0.0772$) (Fig. 5b). Furthermore, no significant differences in percent parasitism on fruits between treatments were found when comparing each sampling date separately (Table 2). In the orchard C (*L. humile*) percent parasitism on fruits was consistently higher in the ant-excluded treatment; however, differences between treatments only approached statistical significance.

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 A: $F = 0.71$; df = 1, 6; $P = 0.4053$; orchard B: $F = 0.07$; df = 1, 6; $P = 0.7951$; orchard C: $F = 0.65$; df = 1, 6; $P = 0.4428$) (Fig. 6). Similarly, no significant differences were found between treatments when comparing the data separately on each sampling date (Table 2).

Citrus leafminer

Percent parasitism of *P. citrella* was significantly higher in the in the ant-excluded plots in orchard B (*L. grandis*), whereas no significant differences between treatments were found for orchards A and C (pooled data from all sampling dates; Repeated Measures ANOVA: $F = 15.11$; df = 1, 6; $P = 0.0081$; $F = 0.07$; df = 1, 6; $P = 0.7995$; $F = 0.75$; df = 1, 6; $P = 0.4197$, respectively) (Fig. 7). No significant differences between treatments were found for any of the three ant species when comparing each sampling date separately (Table 2).

Discussion

In the present study we examined the impact of ants on the population densities and parasitism rates of three citrus insect herbivores: the honeydew producer *A. floccosus* and the non-honeydew producers CRS and *P. citrella*. The infestation levels of the honeydew producer *A. floccosus* and of the non-honeydew producer CRS were higher in the presence of ants. Regarding the underlying mechanism responsible for these increases, we found no evidence relating the presence of the ants with reduced parasitism levels of the herbivores studied.

The exclusion method was very efficient in preventing the ants from ascending to the canopies the two years of the study. The use of IGR 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, the second year we shifted to the Tanglefoot sticky barrier which, although poses important practical difficulties to employ, is of known efficiency for preventing 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 on the canopy (Piñol *et al*., 2012a; Romeu-Dalmau *et al*., 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*.

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, 1951; Steyn 1954; Moreno *et al*., 1987; James *et al*., 1997; Pekas *et al*., 2010a). CRS is not producing 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*., 2013). In most of the afore mentioned 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. Invasive ants are usually strongly attracted to hemipteran honeydew and are more aggressive than native ants (Styrsky and Eubanks, 2007). 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 and Gullan, 1991; Kaneko, 2003; Mgocheki and Addison, 2009). Therefore, and given that *L. humile* is aggressive and maintains high levels of activity all year round, it would be expected to induce higher CRS populations on fruits compared with the native species. We cannot draw definitive conclusions whether native or invasive species affect differently the herbivores; however, the population increases of herbivores in Orchard C, dominated by the invasive *L. humile*, were not higher but similar or even lower in some cases to that of orchards A and B, 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 and Cerdá, 1994; Katayama and Suzuki, 2003).

It is important to highlight that, unlike *L. grandis* and *L. humile,* which are considered hemipteran honeydew specialists foraging on the tree canopies (Markin, 1970; Paris and Espadaler, 2009), *P. pallidula* is an omnivorous species which obtains great part of its diet by foraging on the ground (Retana *et al*., 1992; Piñol *et al*., 2008). However, in our study *P. pallidula* ascended to the citrus canopies and tended honeydew-producing hemipterans as well. This might be due to the fact that there was no permanent ground cover in our experimental orchard that could offer alternative food sources in the ground surface.

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 *Aleurothrixus floccosus,* as 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 A and C dominated by *P. pallidula* and *L. humile* respectively, whereas no differences were found in the orchard B 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 C where *L. humile* was predominant and exhibited high activity during 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 in the sampling dates following the ant´s peak activity (September and October).

Regarding the effect of ant exclusion on *Phyllocnistis 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. *Phyllocnistis citrella* produces no honeydew and moreover develops on young and tender leaves (García-Marí, 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 and Van Lenteren, 2002), the activity of the ant species in our study apparently are not affecting directly or indirectly the citrus leafminer populations.

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, 1951; Bartlett, 1961; Itioka and Inoue, 1996b and 1999). Moreover, in the case of non-honeydew-producing hemiptera, several studies showed that ants may disrupt parasitoid activity (DeBach, 1951; Flanders 1958; Murdoch *et al*., 1995; Heimpel *et al*., 1997; Martínez-Ferrer *et al*., 2003). Recently, a study conducted in Australian citrus revealed that the parasitism of CRS by *Encarsia perniciosi* (Tower) and *Encarsia citrina* Craw was severely reduced in the presence of the ant *Iridomyrmex rufoniger* (Lowne) (Dao *et al*. 2013). 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 found lower parasitism levels in ant-allowed trees of orchard C (with *L. humile*) although this reduction only approached statistical significance. In the same way, Pekas *et al*. (2010a) 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 ant presence. 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 generation time scale (Van 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 (Jervis and Kidd, 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 and DeBach, 1979).

Alternatively, factors other than parasitism, not assessed in our study may have contributed to the increased CRS and *A. floccosus* populations in ant presence. For instance, predation and host-feeding are two important mortality factors which nevertheless are difficult to assess accurately in the field. Piñol *et al*. (2012b), 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*. (2013) 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* 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 and Rosen, 1991; Itioka and Inoue, 1996a; Itioka and Inoue, 1999; Katayama and Suzuki, 2003; Piñol *et al*., 2010). Vanek and Potter (2010) reported that the exclusion of the ant

Formica subsericea Say led to a reduction of the soft scale *Eulecanium cerasorum* (Cockerell) densities caused principally by increased predation by *Chrysoperla rufilabris* (Burmeister), 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.

In conclusion, ants were associated with increased populations of honeydew and non-honeydew producing insect herbivores in the three citrus orchards studied. Consistently higher populations of *A. aurantii* were registered on fruits in the presence of the three ant species, *L. grandis, P. pallidula* and *L. humile*. Regarding the woolly whithefly *A. floccosus,* higher populations in the ant-allowed treatments were registered in orchards A (*P. pallidula*) and C (*L. humile*). We detected no effect of ants on populations of *P. citrella* for any of the three orchards studied. Overall, the population increases of herbivores in Orchard C, dominated by the invasive and much more active *L. humile*, were not higher but similar or even lower in some cases to that of orchards A and B, where native ants predominated. However, percent parasitism was generally similar between ant-allowed and ant-excluded treatments suggesting that parasitism cannot explain the differences in the herbivore population levels between treatments observed in our study. Apparently, the effects of ants on predators, host feeding by parasitoids or other unknown causes are important and should be further investigated.

Aknowledgments:

This work was supported by the project (RTA2010-00012-C02-02) assigned to F.G.M from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) of Spain.

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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 fruits B) A. aurantii populations on twigs, C) percentage of shoots occupied by *Aleurothrixus floccosus* and D) percentage of leaf loss caused by *Phyllocnistes citrella* in three citrus orchards in eastern Spain in 2011 and 2012 each one of them with presence of *Lasius grandis*, *Pheidole pallidula* or *Linepithema humile* (n.d. = not determined).

		Orchard A (*Pheidolle pallidula*)			**Orchard B (*Lasius grandis*)**			**Orchard C (*Linepithema humile)***		
Herbivore species	**Month/Year**	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
A) Aonidiella aurantii on twigs										
	May 2011	1,127	0.59	0.4451	1,127	2.37	0.1261	n.d	n.d	n.d
	June 2011	1,127	0.69	0.4078	1,127	0.01	0.9388	1,127	0.75	0.3872
	July 2011	1,127	0.42	0.5199	1,127	4.51	0.0357	1,127	0.37	0.5456
	May 2012	1,127	5.52	0.0204	1,127	2.03	0.1567	1,127	0.43	0.5121
	June 2012	1,127	0.8	0.3737	1,127	2.11	0.1491	1,127	2.56	0.1119
	July 2012	1,127	1.3	0.2557	1,127	8.4	0.0044	1,127	0.56	0.4576
B) Aonidiella aurantii on fruits										
	August 2011	1,626	3.05	0.0806	1,459	24.7	<0.0001	1,578	54.02	<0.0001
	September 2011	1,639	8.17	0.0043	1,639	28.12	<0.0001	1,639	52.88	<0.0001
	October 2011	1,639	20.33	<0.0001	1,628	43.33	<0.0001	1,631	39.13	<0.0001
	November 2011	1,634	11.83	0.0006	1,613	41.4	<0.0001	1,639	15.54	0.0001
	September 2012	1,639	11.76	0.0006	1,638	23.15	<0.0001	1,607	10.07	0.0015
	October 2012	1,639	0.92	0.3378	1,639	1.08	0.298	1,599	15.91	0.0001
	November 2012	n.d	n.d	n.d	n.d	n.d	n.d	1,639	3.28	0.0701
C) Aleurothrixus floccosus										
	July 2011	1,31	3.29	0.0796	1,31	0.12	0.733	1,31	8.01	0.0082
	August 2011	1,31	1.17	0.2877	1,31	0.22	0.6436	1,31	7.61	0.0098
	September 2011	1,31	5.86	0.0218	1,31	0.03	0.8667	1,31	9.6	0.0042
	July 2012	n.d	n.d	n.d	n.d	n.d	n.d	1,31	5.03	0.0324
	August 2012	n.d	n.d	n.d	n.d	n.d	n.d	1,31	6.02	0.0202
	October 2012	1,31	4.58	0.0405	1,31	3.4	0.0752	n.d	n.d	n.d
D) Phyllocnistis citrella										
	August 2011	1,873	0.04	0.839	1,834	0.6	0.4375	1,691	20.86	<0.0001
	October 2011	1,730	10.34	0.0013	1,752	1.45	0.2279	n.d	n.d	n.d
	October 2012	1,503	0.07	0.7975	1,472	4.52	0.034	1,513	0.2	0.6514

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 three citrus orchards in eastern Spain in 2011 and 2012 each one of them dominated by *Lasius grandis*. *Pheidole pallidula* or *Linepithema humile* (n.d. = not determined).

		Orchard A (*Pheidolle pallidula*)					**Orchard B (*Lasius grandis*)**					**Orchard C (*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	June 2011	3.23 ± 1.2	4.06 ±0.7	1,7	0.6	0.4697	1.61 ± 1.6	0	1,7	1	0.3559	2.47 ± 0.8	0.76 ± 0.76	1,7	2.15	0.1929
	July 2011	17.7 ± 3	18.92 ± 3.6	1,7	0.13	0.7322	26.16 ± 8.8	6.7 ± 3.0	1,7	5.63	0.0554	16.95 ± 3.6	3.64 ± 2.4	1,7	8.71	0.0256
	July 2012	4.96 ± 3.4	12.06 ± 3.4	1,7	3.35	0.117	1.14 ± 1.1	4.3 ± 2.5	1,7	0.86	0.3884	15.59 ± 10.4	9.38 ± 6	1,7	0.28	0.6144
B) *Aonidiella aurantii* on fruits	September 2011	75.74 ± 8.5	55.62 ± 3.2	1,7	2.27	0.1822	59.28 ± 7.9	56.74 ± 4.7	1,7	0.09	0.7691	57.9 ± 5.7	39.48 ± 6.3	1,7	4.75	0.0722
	November 2011	35.22 ± 4.4	24.76 ± 6	1,7	1.68	0.242	26.7 ± 10.1	31.57 ± 8	1,7	0.22	0.654	36.43 ± 9.4	29.77 ± 10.8	1,7	0.32	0.5946
	September 2012	28.42 ± 6.8	32.44 ± 4.4	1,7	0.27	0.623	25.98 ± 3.5	31.25 ± 2	1,7	1.47	0.2711	52.72 ± 6.6	41.66 ± 4.7	1,7	1.8	0.2283
	October 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	1,7	0.9	0.3788
	November 2012	48.33 ± 7.9	64.03 ± 5.5	1,7	2.67	0.1537	58.32 ± 10.4	51.42 ± 3.8	1,7	0.38	0.5585	25.2 ± 2.7	17.32 ± 2.7	1,7	4.25	0.0848
C) *Aleurothrixus floccosus*	July 2011	14.72 ± 3.0	15 ± 2.4	1,111	0.09	0.7613	16.46 ± 3.5	10.4 ± 2.4	1,93	2.62	0.1088	32.24 ± 5.2	25.33 ± 3.5	1,106	0.87	0.3532
	August 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	1,96	2.29	0.1335
	September 2011	28.3 ± 9.7	41 ± 11.4	1,22	0.66	0.426	24.6 ± 8.5	25.24 ± 5.6	1,39	0.02	0.8817	23.3 ± 6.8	23.31 ± 7.3	1,38	0.04	0.8389
	July 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	1,152	0.02	0.885
	August 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	1,124	2.71	0.1023
	October 2012	31.76 ± 4.7	33.93 ± 4.6	1,98	0.08	0.7795	24.93 ± 4.7	25.1 ± 4.1	1,107	0.01	0.9238	n.d	n.d	n.d	n.d	n.d
D) *Phyllocnistes citrella*	September 2011	57.75 ± 9	63.94 ± 13.7	1,7	0.33	0.5859	62.69 ± 8.3	43.46 ± 6.03	1,7	3.58	0.1075	40.83 ± 0.6	37.03 ± 3.6	1,7	0.56	0.4814
	September 2012	62.26 ± 5.9	55.35 ± 5.3	1,7	0.78	0.4113	65.01 ± 6.1	56.4 ± 4.2	1,7	1.32	0.2936	60 ± 16.6	67.3 ± 2.4	1,7	0.14	0.7198

FIGURES:

Figure 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 one of them with presence of *Lasius grandis*, *Pheidole pallidula* or *Linepithema humile.*

Figure 2. Mean (± SE) California red scale infestation index on (A) fruits and (B) twigs in ant-allowed and ant-excluded treatments in 2011 and 2012 in three citrus orchards in eastern Spain, each one of them with presence of *Lasius grandis*, *Pheidole pallidula* or *Linepithema humile*. For each sampling date, asterisk indicates significant differences between treatments (p <0.05). For the entire period, CRS infestation on twigs was significantly higher in the ant-allowed than in the ant-excluded trees in the case of orchard the orchard dominated by *L. grandis,* whereas CRS infestation on fruits was higher in the ant-allowed treatment for the three orchards for the three ant species studied (in both cases Repeated measures ANOVA, LSD test; see text for details).

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

and *L. humile* whereas no significant differences were found between treatments for *L. grandis* (repeated measures ANOVA, LSD test; see text for details).

Figure 4. Mean (± SE) percentage of leaf surface loss caused by *Phyllocnistis citrella* larvae in ant-allowed and ant-excluded treatments in three citrus orchards in eastern Spain in 2011 and 2012 each one of them with presence of *Lasius grandis*, *Pheidole pallidula* or *Linepithema humile.* For each sampling date, asterisk indicates significant differences between treatments (significance level: $p<0.05$). for the entire period we found no significant differences in the percent of leaf surface loss between ant-allowed and ant-excluded treatments for none of three ant species (repeated measures ANOVA, LSD test; see text for details).