Title: Effect of the California red scale *Aonidiella aurantii* sex pheromone on the natural parasitism by *Aphytis* spp. in Mediterranean citrus

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Abstract

Mating disruption has proved successful against California red scale (CRS), *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) in Mediterranean citrus. Although mating disruption does not affect negatively the parasitism by *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), a CRS parasitoid introduced to the Mediterranean, there is no information regarding its potential effect on the native *Aphytis* species. In the present study, the effect of CRS mating disruption on the field parasitism inflicted by *Aphytis* spp. has been assessed and compared to a mineral oil and a control treatment. In order to confirm the effectiveness of the mating disruption we also evaluated its effect on the captures of the CRS males and on fruit infestation. Moreover, the potential role of the CRS sex pheromone as kairomone for the *Aphytis* species was also evaluated by comparing captures of parasitoids on sticky traps with or without pheromone. Significantly lower CRS male captures and fruit damage were registered in the mating disruption respect to the control or oil treatments indicating that mating disruption was effective. In September, when compared to the control, parasitism by *Aphytis* spp. was significantly lower in the mating disruption and mineral oil treatments and crucially no *A. chrysomphali* were registered in the mating disruption treatment. Finally, while the captures of both *A. melinus* and *A. lepidosaphes* (Mercet) were not significantly different between traps with or without pheromone, *A. chrysomphali* Marcet captures were significantly higher in traps baited with CRS pheromone. These results suggest a possible kairomonal effect of the CRS pheromone on *A. chrysomphali*.

Keywords: *Aphytis melinus*, *Aphytis lepidosaphes*, *Aphytis chrysomphali*, kairomone, mating disruption, host recognition
1. **Introduction**

California red scale (CRS), *Aonidiella aurantii* Maskell) (Hemiptera: Diaspididae), is a major pest of citrus worldwide. Although only heavy infestations are able to kill the trees, the sole presence of scales on fruits considerably reduces their market value causing huge economic losses (Jacó et al. 2010). Currently, integrated pest management including applications of pesticides, mineral oil sprays, biological control and methods based on semiochemicals is employed to control CRS infestations in citrus orchards. The CRS sex pheromone was used exclusively for monitoring purposes, however, recently mating disruption (MD) against this pest was employed successfully in Mediterranean citrus proven at least as effective as conventional mineral oil sprays (Vacas et al., 2009, 2010). In fact, CRS presents the first case of successful mating disruption for a diaspidid scale insect.

The use of mating disruption has been found not only to be effective against CRS but was also innocuous for the parasitism caused by *Aphytis melinus* DeBach a CRS parasitoid introduced to the Mediterranean (Vacas et al., 2011, Vanaclocha et al., 2012).

Nevertheless, there is no information regarding the potential impact of mating disruption on the CRS parasitism inflicted by the *Aphytis* species native to the Mediterranean.

Alternative pest management methods have to ensure sustainability from both the socioeconomic and the environmental perspectives, which involves the conservation of beneficial insects. In general, parasitoids exploit a range of stimuli for host location which can derive from the microhabitat or the plant, from the presence of the host (i.e. frass, honeydew) or from the host itself (Godfray, 1994). In the latter case, sex or aggregation pheromones, which are deliberately emitted by the host for its own purposes, can be exploited by the parasitoids. For example, sex pheromones have been described to serve as chemical cues for host location for egg parasitoids such as *Trichogramma* spp., *Telenomus* spp. (Powell 1998) and aphid parasitoids (Powell, 1998; Birkett and Pickett, 2003; Powell...
In the concrete case of entomophagous arthropods of scale insects, the predator *Elatophilus hebraicus* Pericart (Hemiptera: Anthocoridae) is reported to be attracted to the racemic mixture of the female sex pheromone of *Matsucoccus josephi* Bodenheimer et Harpaz (Mendel et al., 1995). Similarly, the sex pheromone of *Planococcus ficus* (Signoret) acts as a kairomone for the parasitoid *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae) (Franco et al., 2008). There is also evidence that aphelinid parasitoids are attracted to the sex pheromone of their scale hosts. *Encarsia perniciosi* Tower (Hymenoptera: Aphelinidae) significantly responds to synthetic pheromone and virgin females of the San Jose scale, *Diaspidiotus perniciosus* (Comstock) (Rice and Jones, 1982, McClain et al., 1990, Bayoumy et al., 2011).

The principal natural enemies of CRS in the Mediterranean basin are the ectoparasitoids *Aphytis chrysomphali* Mercet (Hymenoptera: Aphelinidae) and *A. melinus* (Rodrigo et al., 1996, Pina, 2007, Pekas et al., 2010) and to a lesser extent some endoparasitoids and generalist predators (Vanaclocha et al., 2009, Pina, 2007). Sternlicht (1973) reported attraction of *A. melinus* and *A. coheni* DeBach to CRS female sex pheromone. This was confirmed by other studies concluding that *A. melinus* females are attracted to airborne cues from hosts, i.e. CRS virgin females (Bernal and Luck, 2007; Zappalà et al., 2012). Nevertheless, various experiments proved that the recognition and acceptance of *A. aurantii* as host by *A. melinus* is mainly based on a contact, non-volatile kairomone (Hare et al., 1993; Morgan and Hare, 1998). In laboratory experiments, Vacas et al. (2011) demonstrated that *A. melinus* mating behavior and parasitism were not affected when parasitoids were exposed inside cages to CRS pheromone concentrations even higher that in orchards where mating disruption was applied. Most crucially, Vacas et al. (2011) demonstrated the compatibility of mating disruption with augmentative releases.
of *A. melinus* in extensive field trials, where CRS mortality caused by the released parasitoids was not affected in the orchards with mating disruption treatments. Although the effect of the mating disruption treatment on the parasitism inflicted by the introduced *A. melinus* appears to be not significant, the effect on other *Aphytis* species, especially the native to the Mediterranean *A. chrysomphali*, remains unknown. Likewise, there is no information regarding the impact of the commercially available pheromone employed routinely on sticky traps for CRS monitoring purposes on *Aphytis spp.* parasitoids. Thus, in the present study we asked the following questions: i) is CRS mating disruption having an effect on the field parasitism inflicted by the *Aphytis* species native to the Mediterranean? and ii) can the CRS sex pheromone act as a kairomone for the *Aphytis* species? The first question was addressed by assessing the parasitism rate of the *Aphytis* species in citrus orchards in plots treated with mating disruption dispensers, plots receiving mineral oil sprays and untreated (control) plots. In order to test the effectiveness of the mating disruption treatment and corroborate its potential impact on the parasitoids we also evaluated its effect on the flight of the CRS males and on the fruit infestation. To answer the second question, we compared the captures of *Aphytis* species on sticky traps baited with the female CRS sex pheromone.

2. Material and methods

2.1. Mating disruption field trials

The trials were conducted in a 5-ha sweet orange (*Citrus sinensis* Osbeck, var. Lane late) orchard located in Denia (Alicante, Spain; UTM: X243500 Y4303900). The California red scale sex pheromone was released in the field by installing the mesoporous pheromone dispensers described by Vacas et al. (2009, 2010). The dispensers were developed by Universitat Politècnica de València and Ecología y Protección Agrícola (Valencia, Spain).
and are now registered and commercialized in Spain under the name Dardo® (Syngenta Agro SA, Madrid, Spain). Each dispenser consisted in a cylindrical tablet, containing 70 mg of the diastereomeric mixture (3S,6R and 3S,6S) of the 3-methyl-6-isopropenyl-9-decen-1-yl acetate.

Mating disruption dispensers were deployed, one per tree, in three 0.5 ha plot on 25 March 2009, before the first CRS males’ flight, and in three 0.5 ha plots on 11 May 2009, before the second CRS males’ flight. The trees were spaced 6×4 m apart (~420 dispensers/ha) and dispensers were placed on the internal tree branches at a height of 1.5–2.0 m. Three 0.4 ha plots received conventional mineral oil applications which were timed for the presence of crawlers. Finally, three 0.25 ha plots were left without treatment as an untreated reference (control).

2.2. Mating disruption efficacy

The efficacy of the pheromone treatment was evaluated according to the CRS male flight disruption and the fruit infestation assessment. One commercial white sticky pheromone trap (Pherocon® V Trap; Trécé Inc., Adair, OK) was placed in each plot to compare male captures between the different control strategies every 7 or 15 days, from March to November 2009. The inhibition of male captures that occurs in pheromone-treated plots is the first indicator for male disorientation. Flight Inhibition Index (FII) was calculated according to the formula $FII = \left(1 - \frac{x}{y}\right) \times 100$, where $x$ is the number of males captured in MD plot, and $y$ is the number of captures in the untreated plot. Finally, fruit infestation was evaluated on 10 November 2009, by counting the number of scales present on 40 fruits per tree (10 fruits per orientation) of the 4 central trees in each plot. The percentage of fruit with more than 5 scales was recorded as it is a common damage threshold employed for marketable fruit.
The pheromone release profile of the mating disruption dispensers was studied during the trial to determine the mean release rate and their life-span. Additional dispensers were aged under field conditions in a nearby area, in order to extract and quantify by gas chromatography (GC-FID) their residual pheromone content at different days of ageing.

2.3. Influence of mating disruption on CRS parasitism

Parasitism rate was evaluated on 9 September and 10 November 2009. On each sampling date, we collected 40 branches (less than 10 mm in diameter and bearing at least ten leaves), and 40 fruits (10 per orientation), infested by CRS, from at least ten different trees per treatment. Samples were transferred to the laboratory and were processed using a stereomicroscope. Parasitized CRS scales were identified by the presence of parasitoid eggs, larvae, prepupae or pupae. For every parasitized scale, parasitoid species was identified based on the pupae coloration (Rosen and DeBach, 1979). Eggs, larvae and prepupae were transferred to glass vials (3.0 by 0.8 cm) and maintained at 22–25 °C, 60–70% RH and 16:8 L:D photoperiod for development to pupa and identification.

2.4. Attraction of parasitoids and CRS males to pheromone baited traps

The trial was conducted in a nearby 3-ha mandarin (Citrus reticulata Blanco; var. Ortanique) orchard without mating disruption treatment. The possible kairomonal response of Aphytis sp. to the sex pheromone of CRS was tested by evaluating the attraction to traps baited with Pherocon® rubber monitoring lures (Trécé Inc., Adair, OK), loaded with 250 μg CRS female sex pheromone. The effect of trap color on captures was also tested by including commercial white sticky Pherocon® V traps and transparent traps, made from transparent PVC sheets with Tangle-Trap™ sticky coating (Biagro SL, Valencia, Spain). Thus, traps included in the trial were: (1) white with monitoring pheromone lure, (2)
transparent with monitoring pheromone lure, (3) white without pheromone, (4) transparent without pheromone. A fifth (5) white trap was included, baited with a mating disruption dispenser, to check for the effect of higher pheromone loads on parasitoid attraction. Three blocks with these five traps were installed on 12 August 2009. Traps were attached to tree branches at 1.5-2.0 m from the ground. Distance between traps was 20 m and blocks were located at least 50 m apart. The number of *Aphytis* sp individuals and *A. aurantii* males captured on the traps were recorded on 9 September, 8 October, 23 October and 5 November 2009. On each sampling date, the position of traps was rotated within each block and sticky boards were replaced by new ones. The collected boards were transferred to the laboratory and were processed using a stereomicroscope. The *Aphytis* captured on the traps were extracted, mounted, and identified under a microscope according to Rosen and DeBach (1979).

2.5. Statistical analysis

Simple regression was used to study the evolution of the GC-FID quantified residual pheromone load (mg) versus time (days) and calculate mean emission rate for the mating disruption dispensers employed. Regarding mating disruption efficacy assessment, the number of males captured per trap and day (MTD) was transformed by log(n+1) in order to homogenize variance and normalize the distributions before analysis of variance (ANOVA). Tukey HSD test (P < 0.05) was performed to assess the effect of treatment on the CRS male flight activity. In the same trial, a one-way ANOVA model was employed with arcsin (asin(sqrt(n))) transformed data of percentage of infested fruits to compare the level of infestation among treatments (Tukey HSD test at P < 0.05). The Statgraphics Centurion XVI (v. 16.1.11) package was used for these statistical analyses (Statpoint Technologies Inc., 2010).
Using generalized linear model techniques, two different models (one for each assessment date), assuming binomial error variance, were constructed to compare the rate of parasitized individuals of CRS in the different treatments. Likewise, we used generalized lineal model techniques assuming Poisson error variance to compare the number of *Aphytis* spp. parasitoids or CRS males captured per trap. Given the highly male-biased sex ratio of the captured parasitoids (see below) only the female parasitoids were considered for the analyses. For each species, we constructed different models with the number of individuals captured per trap as the dependent variable and trap type, sampling date and block and their interaction as the explanatory variables.

In all the models the significance of the explanatory variables was assessed by backward elimination of the non-significant terms from the model and subsequent comparison of the two models using the F test statistic. When significant effects were found the `glht` function in the `multcomp` package (Hothorn et al., 2008) was used to perform TukeyHSD tests for post-hoc pairwise comparisons. These statistical analyses were conducted with R (R Development Core Team, 2012).

### 3. Results

#### 3.1. Mating disruption efficacy

*Aonidiella aurantii* MD dispensers had a useful life of approximately 110 days, providing a mean release rate of approximately 402 µg/day during 15 weeks, which was consistent with the emission rates required to obtain enough pheromone concentration in the orchard to disrupt CRS male flights (Vacas et al., 2009, 2010). Indeed, the mean number of males per trap and day (MTD) captured in the monitoring traps was significantly influenced by the treatment applied in each plot ($F = 82.17; \text{df} = 3,168; P < 0.0001$). Neither block ($F = 2.44; \text{df} = 2,168; P = 0.09$) nor the interaction block x treatment ($F = 1.86; \text{df} = 6,168; P = 0.09$).
0.09) were significant. Both mating disruption treatments, employed either in March or May, obtained significantly lower CRS male captures respect to control and oil plots (Table 1). MD treatments inhibited male captures by > 90%, indicating that the mating disruption environment managed to disorientate the CRS males. It is important to mention that Aphytis individuals were observed in the monitoring traps in all the plots. Fruit infestation was also significantly affected by the different control measures applied $(F = 12.23; \text{df} = 3, 61; P < 0.0001)$. Both mating disruption treatments reduced the percentage of fruit with more than 5 scales compared to the control but MD-May achieved significantly lower infestations compared to oil treatment (Table 1).

3.2. Influence of mating disruption on the CRS parasitism

In September we registered 188 scales parasitized by *A. melinus* (80% of the total) and *A. chrysomphali* (20%) in all treatments. Treatment had a significant effect on the CRS parasitism $(F = 6.26, \text{df} = 3, 584, P = 0.0003)$ (Fig. 1). Compared to the control treatment, the CRS parasitism rate was significantly lower in the mineral oil $(P < 0.001)$ and in the mating disruption-March treatments $(P = 0.01)$ (Tukey test; adjusted P values with single step method). It is important to highlight that in September, no *A. chrysomphali* was registered in the mating disruption treatments whereas we did find it in the mineral oil and control treatments. Given that oil treatments were performed only in June, no significant differences were expected between the parasitism rate in control and oil treated plots. Thus in November, the oil treatment was not sampled. We registered 108 scales parasitized by *A. melinus* (77%) and *A. chrysomphali* (23%). The CRS parasitism rate was similar between treatments $(F = 0.0025; \text{df} = 1, 412; P = 0.96)$ and *A. chrysomphali* was found in all treatments including the mating disruption (55% of the total *A. chrysomphali* registered).
3.3. Attraction of parasitoids and CRS males to pheromone baited traps

The number of CRS males captured per trap was significantly influenced by trap type ($F = 26.86; \text{df} = 4, 75; P < 0.0001$) (Fig. 2). The effect of trap type was independent of sampling date (interaction trap x sampling date: $F = 1.09; \text{df} = 12, 55; P = 0.38$) or block (trap type x block: $F = 1.93; \text{df} = 4, 67; P = 0.12$). Overall, the number of CRS males captured was significantly higher on the traps with pheromone either white (166.06 ± 34.80) or transparent (120.5 ± 30.49) (Tukey test; adjusted P values with single step method).

The most abundant parasitoid species captured on the traps was *A. melinus* (1165 individuals; 55 females, 1110 males), followed by *A. lepidosaphes* (1145 individuals; 369 females, 776 males) and *A. chrysomphali* (84 individuals; 81 females, 3 males). The highly male-biased sex ratio of the captured parasitoids indicates a possible “calling effect” of the females captured on the traps, therefore, only the female parasitoids were considered for the analyses. Moreover, only females can inflict mortality to the host through host feeding and parasitism and in that sense they are more relevant for assessing any effects of our treatments on the biocontrol services provided by the parasitoids.

Specifically, the trap type significantly affected the number of *A. chrysomphali* captured ($F = 13.86; \text{df} = 4, 75; P < 0.0001$) (Fig. 3). The effect of the trap did not vary among sampling dates (interaction trap type x sampling date: $F = 1.12; \text{df} = 12, 55; P = 0.37$) or block (trap type x block: $F = 2.23; \text{df} = 4, 67; P = 0.08$). Overall, the number of *A. chrysomphali* captured was significantly higher in the white (2.35 ± 0.91 parasitoids per trap) and transparent traps (1.37 ± 0.49) both loaded with the CRS pheromone.

The effect of trap type on the number of *A. lepidosaphes* captured was marginally non-significant ($F = 2.46; \text{df} = 4, 75; P = 0.06$) (Fig. 4). The trap effect was independent of sampling date (interaction trap x sampling date: $F = 0.39; \text{df} = 12, 55; P = 0.95$) or block.
(trap x block: $F = 1.14; \text{df} = 4, 67; P = 0.35$). Overall, the highest number of $A. \text{lepidosaphes}$ was captured on the white traps with or without pheromone suggesting a possible role of the trap color in the attraction of this species. Finally, the number of $A. \text{melinus}$ captured was not affected by trap type ($F = 1.86; \text{df} = 4, 75; P = 0.13$) (Fig. 5).

4. Discussion

Mating disruption again has proven to be efficient in reducing CRS infestations in citrus, by inhibiting the male flight and reducing fruit infestation. When the pheromone dispensers were employed in March, before the first CRS male flight, fruit infestation was significantly reduced compared to the control, at a level similar to that in the oil spray treatment. However, mating disruption employed in May gave significantly better results, allowing for a more rational pheromone use. In this way, using the same pheromone dose dispensers’ life span will last long enough to cover the most important CRS male flights, as reported by Vacas et al. 2015. Moreover, we found that the mating disruption method, especially the one employed in May, has the additional benefit of not affecting the parasitism inflicted by $Aphytis$ spp. in the orchards where these treatments were applied. Therefore, the deployment of the dispensers in May is the optimal option in terms of reducing fruit infestation, selectivity towards natural $Aphytis$ parasitism and also from an economic point of view.

Our results show diverse responses of the $Aphytis$ spp. present in the study area to various trap types tested. Specifically, $A. \text{melinus}$ captures were not affected by the white trap color. This is in agreement with the previously reported results by Moreno et al. (1984) according to which $A. \text{melinus}$ did not distinguish opaque from transparent rectangles and, moreover, it responded less to white compared to green or yellow trap.
color. In general, *Aphytis* spp. are attracted to the yellow-green frequencies of the electromagnetic spectrum (Rosen and DeBach, 1979). Likewise, *A. melinus* captures were not affected by the presence of CRS pheromone in the traps. These results are in agreement with previous laboratory trials reporting that the CRS sex pheromone does not act as a kairomone for *A. melinus* (Morgan and Hare, 1998). Similarly, the fact that the parasitism inflicted by *A. melinus* was unaffected by the MD environment (Vacas et al., 2011, Vanaclocha et al., 2012) provides strong evidence that the CRS sex pheromone, independently of concentration or formulation, has no effect on the host location or the parasitism behavior of this parasitoid. This is of special relevance for biological control given that *A. melinus* is the most abundant parasitoid attacking CRS in the Mediterranean (Pekas et al., 2010). Finally, our results show that loading sticky traps with CRS sex pheromone for studies monitoring *A. melinus* abundance in the field is not necessary.

Regarding *A. lepidosaphes*, captures seemed to be more affected by the color than the presence of CRS pheromone on the traps although the captures between white and transparent traps were not statistically significant. *A lepidosaphes* parasitizes the armored scale *Lepidosaphes beckii* (Newman) and it has not been reported attacking the CRS. Therefore, it seems quite consistent not be attracted by the CRS sex pheromone.

On the other hand, *A. chrysomphali* captures were significantly higher in traps baited with the CRS sex pheromone. In contrast to *A. melinus*, no previous studies have examined the effect of CRS sex pheromone on *A. chrysomphali*. Our results suggest that *A. chrysomphali* may be employing the CRS sex pheromone as a kairomone for host location. Additional indirect evidence supporting this hypothesis may be provided by our MD trials where *A. chrysomphali* was not found in the MD treated plots in September. In this period, pheromone emission was still high enough to disrupt CRS flight and probably *A. chrysomphali* behavior. Conversely, in November the dispenser life span is near depletion.
(Vacas et al. 2010) and consequently, airborne pheromone concentration in the field was lower, resulting in *A. chrysomphali* individuals captured also in the mating disruption treatment. We consider that these results are not due to the variation of the *Aphytis* spp. abundance along the year because both *A. melinus* and *A. chrysomphali* peak their abundances in the study area in the period between September and November (Sorribas et al., 2008). Moreover, and given that *A. melinus* is apparently unaffected by the CRS sex pheromone the reduction of the parasitism in the mating disruption treatments in September may be due to the reduced activity of *A. chrysomphali*. *A. chrysomphali* is the second most important parasitoid of CRS in the Mediterranean citrus and any possible effects of the CRS pheromone on its behavior and parasitism may have important implications for the biological control of the scale. However, more detailed laboratory studies are needed in order to draw definitive conclusions about this issue.

It was already reported that several *Aphytis* spp. employ a kairomone from the scale cover and body in making oviposition decisions. Luck and Uygun (1986) demonstrated that *A. melinus*, *A. lignanensis* and *A. coheni* responded to water and ethanol extracts of CRS covers. Later, Millar and Hare (1993) isolated and identified this kairomonal compound as O-caffeoyltyrosine. Response of *A. melinus* to this kairomone is considered as an innate cue which may arise from its co-evolutionary background. The evolutionary host of *A. melinus* is *Aonidiella orientalis* (Newstead), which is a congener of *A. aurantii* (Morgan and Hare, 1998). Likewise, innate responses to sex pheromones are likely to happen in the case of coevolution or when the cue is shared with the evolutionary host. In Mediterranean citrus *A. chrysomphali* has been found parasitizing *Chrysocephalus dictyospermi* (Morgan), which is a closely related species of *A. aurantii* (Garcia Marí, 2012). However, no information on a sex pheromone produced by *C. dictyospermi* is available.
We conclude that mating disruption with mesoporous dispensers was confirmed once again as a solid alternative for the management of the CRS in citrus. The optimal period to place the dispensers in terms of reducing fruit infestation as well as in terms of selectivity towards the natural *Aphytis* sp. parasitism is May. The CRS sex pheromone used for monitoring and also the high concentration employed for the MD do not have an effect on *A. melinus*, however, we provide evidence for a possible effect on the sibling species *A. chrysomphali*.

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Fig. 1. Mean (±SE) parasitism rate among treatments inflicted by *Aphytis* spp. on the California red scale *Aonidiella aurantii* in September in a citrus orchard in Valencia Spain. Columns bearing different letters are significantly different at $P < 0.05$. (MD= mating disruption employed either in March or May).

Fig. 2. Mean (±SE) number of California red scale (CRS) *Aonidiella aurantii* males caught on different trap types. Columns bearing different letters are significantly different at $P < 0.05$.

Fig. 3. Mean (±SE) number of *Aphytis chrysomphali* parasitoids caught on different trap types (CRS= California red scale, *Aonidiella aurantii*). Columns bearing different letters are significantly different at $P < 0.05$.

Fig. 4. Mean (±SE) number of *Aphytis lepidosaphes* parasitoids caught on different trap types (CRS= California red scale, *Aonidiella aurantii*).

Fig. 5. Mean (±SE) number of *Aphytis melinus* parasitoids caught on different trap types (CRS= California red scale, *Aonidiella aurantii*).