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Life history parameters and scale-cover surface area of Aonidiella aurantii are altered in a mating disruption environment: implications for biological control

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Abstract

BACKGROUND: In recent years, environmentally safe measures to control *Aonidiella aurantii* (Maskell) (CRS) such as mating disruption (MD) or biological control are being successfully implemented. The goal of this study was to examine the effect of high concentrations of the CRS sex pheromone on the life history parameters and the scale cover surface area under controlled laboratory conditions.

RESULTS: The developmental time of both males and females of CRS increased with exposure to airborne pheromone. MD had an effect on both the total population progeny and on the crawler production period for females. Accordingly, the demographic parameters such as net fecundity (R_0) and intrinsic rate of increase (r_m) were significantly lower in the pheromone-treated populations. The largest scale cover surface areas were observed on the CRS reared under the pheromone environment.

CONCLUSION: A clear influence of airborne pheromone on the biology of CRS has been demonstrated. In addition to the classical benefits of this technique because of mating disruption, additional benefits, such as the increase in the duration of exposure to natural enemies and the increase in size that benefits some species of parasitoids, have been confirmed.

Key words: California Red Scale, reproductive parameters, scale cover area, natural enemies, pheromone.

1 INTRODUCTION

The California Red Scale (CRS), *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), is key pest in almost all citrus areas around the world.[1-3]In the absence of control methods, CRS may cause severe economic losses due to its pest management costs and the reduced marketability of infested fruit. Armored scales may feed on various parts of their host plants, such as twigs, leaves or fruit,[4] affecting them by removing sap and injecting toxic saliva during the feeding process.[5] The sites on which CRS feeds upon are associated with depressions, discolorations, and other distortions of host tissues such as leaf crinkling, splitting of bark, defoliation, dieback of twig terminals, and in heavy infestations, the eventual death of the host.[4]

The postembryonic development of CRS has been extensively described by several authors.[4,6-10] The crawlers emerge from beneath the scale body of the female and wander for a short time. This brief period is the only active stage during immature development. The crawler inserts its stylet into the tissue of the plant where settles and starts to feed; then, it develops as a consequence of the feeding activity. This site will be its feeding site until it becomes an adult. During the immature development, the body and the scale cover surface area increase in size. There is sexual dimorphism for *A. aurantii* development which becomes manifest in the second nymphal stage. Females go through three nymphal instars and undergo two molts. Male scales are usually smaller and distinctly different in shape, elongation, and color. In addition, the males are distinguishable by the appearance of their eyes, which are obscure and sometimes visible through the scale cover. When the female is receptive to mating, she extends the pygidium to the very edge of the scale cover and emits a sex pheromone, indicating that she is sexually mature.[9,11-13] Adult male emergence is coincident with the onset of female pheromone emission.[7] Winged adult males walk around and fly following the pheromone emitted by the females. When an attractive female is found, copulation occurs. Insemination is followed by irreversible retraction of the mature female pygidium.[8] After that,

70 the mated female secretes a waxy sheath beneath her epidermis, the epidermis sclerotizes,
71 and after several weeks, she begins to produce crawlers .[7,8,14]

72 The sex pheromone of CRS was identified as a mixture of 3-methyl-6-isopropenyl-9-decen-1-yl
73 acetate (I) and (*Z*)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate (II).[15,16]Since the
74 description of these pheromones, synthetic sex pheromone traps have been widely employed
75 as a tool for detecting and monitoring CRS populations.[17-21] Furthermore, in the late 1980s,
76 some researchers attempted to employ the MD technique for the management of CRS using
77 rubber pheromone dispensers, but the effectiveness of the technique was not clearly
78 demonstrated.[22,23] However, a new mesoporous dispenser for the MD of CRS has proven to be
79 the first effective mating disruption treatment against a diaspidid pest.[24-26] Recent field studies
80 suggested that MD delays the development of *A. aurantii* and supplied evidence for increased
81 sizes of the body and cover of CRS under this pheromone treatment.[26] The delay in the CRS's
82 life cycle could be beneficial to natural enemies because the scale's window of vulnerability is
83 increased.[27,28] Additionally, a size increase of *A. aurantii* under the influence of MD would
84 benefit its most important parasitoid in the Mediterranean basin, *Aphytis melinus* DeBach
85 (Hymenoptera: Aphelinidae), which prefers to parasitize larger second and third instars and
86 CRS prepupae.[29-33] (26-29)

87 To our knowledge, there is no previous information about whether exposure to a synthetic sex
88 pheromone can influence the life history parameters of *A. aurantii*, i.e., the developmental
89 time and the scale size. Therefore, this work focuses on the effect of the CRS mating disruption
90 pheromone on the life history parameters and the size of CRS.

91 **2 MATERIALS AND METHODS**

92 The present study was conducted under environmentally controlled conditions in the
93 laboratory (25±1ºC, 16:8 h (L:D) and 65±5% RH).

2.1 Plant and insects

Green lemon fruit (*Citrus limon* (L.) Burm f.), var. Verna, were collected in a pesticide-free lemon orchard located at the Instituto Valenciano de Investigaciones Agrarias, IVIA (Valencia, Spain). After collection, fruit were brushed under water to ensure that any pest present was removed and then were dried with absorbent paper. Approximately 2/3 of the surface of each lemon was covered with red paraffin around the mid-section to slow the drying out of the lemon. The red paraffin was prepared with a mixture of 1 kg of paraffin pearls (Parafina USP perlas, Guinama S.L., Alboraya, Spain) and 1 g of red pigment (Sudan III, Panreac Química S.A., Castellar del Vallés, Spain). Once the lemons were prepared, they were checked under a binocular stereoscope to ensure that no pests were present.

2.2 Experimental unit

To obtain a uniform cohort, lemons were placed and left undisturbed for 24 h on the CRS colony maintained at the IVIA (technique described by Tashiro in 1966[34] and modified by the University of California, Riverside[31]). Then, these lemons were removed from the colony and from the total settled crawlers, only 20 randomly selected (those with the stylet inserted into the fruit and already forming the waxy cover) were left on the lemon while the rest was removed. Lemons and nymphs were marked to track them throughout the experiment. Two different treatments were tested, one of them with the CRS pheromone environment (PhE) and the other one in absence of this pheromone (control). Each treatment consisted of five replicates of one lemon with settled *A. aurantii* crawlers. The five lemons of each treatment were kept on a tray in two identical climatic cabinets (SANYO MLR-350, Sanyo, Japan) where the experiment was conducted under the same climate conditions (25±1ºC, 65±5% RH and a photoperiod of 16:8 h (L:D)). A mesoporous MD pheromone dispenser was placed in one of the climatic cabinets to apply the pheromone treatment with the CRS sex pheromone (PhE).

2.3 MD pheromone treatment

The pheromone dispenser employed for the MD treatment was based on a mesoporous material[35,36] and consisted of a cylindrical tablet 9 mm in diameter and 10 mm in length. The formulation contained 70 mg (a.i.) of the diastereomeric mixture (3S,6R and 3S,6S) of the 3-methyl-6-isopropenyl-9-decen-1-yl acetate. The remaining pheromone load at the end of the trial was quantified by gas chromatography (GC-FID) to ascertain the total pheromone emitted.

The mesoporous dispenser was introduced inside of a 50x90 mm polypropylene (PP) basket, with a 6x5 mm mesh. This basket had a hook at the top by which it could be secured to the cabinet. The dispenser and the basket were supplied by Ecología y Protección Agrícola S.L. (Valencia, Spain).

2.4 Developmental time and survivorship of CRS

The developmental time and survivorship of CRS were calculated in the two different treatments. The CRS individuals for both treatments were checked daily under a binocular stereoscope. The developmental time and the survival rate from one stage to the next were recorded in each scale from the crawlers' settling time until adulthood or death. Scales that had not developed beyond a certain stage were considered dead. The sex was also determined in adults. In the case of the males, observations were done until adult emergence. Emerged CRS males were allowed to matewith the corresponding females. The non-mated females were observed for 69 days from the beginning of the third-nymphal instar.

2.5 Reproductive parameters

Before the females started to produce crawlers, they were isolated with a double-sided sticky plastic ring (3M Scotch®, Cergy Pontoise Cedex, France) to trap the crawlers. The observations took place daily until the end of crawler production, which coincided with the death of the mature female. Each sticky plastic ring was replaced every day, and the crawlers were

143 removed daily to record the total number of progeny. The duration of female pre-reproductive
144 and reproductive periods, the lifetime fecundity and the average daily reproduction were
145 calculated for each female.

146 **2.6 Size of CRS**

147 To estimate whether the exposure to the high pheromone concentration influences the size of
148 early and late instars, pictures of the different CRS instars were daily taken with a Leica EC3
149 digital color camera with 3.1 megapixels (Leica Microsystems GmbH, Spain) throughout the
150 duration of the experiments. Images were processed with the Imaging software for Windows
151 Operating Systems for "EZ" documentation and annotation Leica LAS EZ (Leica Microsystems
152 GmbH, Spain). In the case of the females, pictures were taken for the second and third instars.
153 In the case of the males, pictures were taken at the beginning of second instar and just before
154 male emergence. For each particular instar, images were taken at the beginning and the end of
155 the instar.

156 In both treatments, the scale cover surface area(mm^2) of females and males was measured.
157 Measurements from all the pictures were made with ImageJ. This software is a public domain
158 Java image processing program.[37]

159 **2.7 Data analysis**

160 Developmental time, survivorship, reproductive parameters and scale cover surface areas were
161 compared using Student's t-test ($P<0.05$). When the assumptions of normality and homogeneity
162 of variance could not be fulfilled and data could not be transformed to meet those assumptions,
163 the non-parametric Mann–Whitney test was applied. The Fisher exact probability test was used
164 to check for differences in the mortality between the two treatments. The life history
165 parameters values of *A. aurantii* were obtained with the age-specific survivorship, beginning

with 1-day-old crawlers and the age-specific progeny. The intrinsic rate of increase (r_m) was computed using the Euler equation,

$$\sum e^{-rm} l_x m_x \qquad [1]$$

where l_x is survivorship of the original cohort over the age interval from day $x - 1$ to day x and m_x is the mean number of female offspring produced per surviving female during the age interval x.[38] Values of m_x for the population were calculated from the mean number of crawlers produced per female per day. Other parameters, including reproductive rate (R_0) and generation time (T), were calculated as described by Birch[38] using a statistical jackknife method.[39] The significance of differences between mean values of life table parameters was determined using Student's *t* test (P<0.05).[39]

3 RESULTS

3.1 MD pheromone treatment

A. Aurantii MD dispensers provided a mean release rate of ~389 µg of pheromone per day, which was consistent with the emission rates required to obtain enough airborne pheromone for CRS disruption to take place.[24,25]

3.2 Developmental time and mortality

Except for the first-instar nymphs, where no significant differences were found between treatments, the rest of the *A. aurantii* instars took more time to complete their development under the pheromone environment (Table 1). The duration of the nymphal life cycle for both females (from first to third instar nymph) and males (from first until adult emergence) was significantly longer, by approximately 3 days, when exposed to PhE environment.

From the 100 initial individuals monitored in each treatment, 18 and 15 died before the end of the experiment in the PhE and control treatments, respectively. No significant effect on mortality was observed due to the high airborne pheromone (F= 0.325; P=0.704).

3.3 Reproductive parameters

Only 7 of the 35 third instar nymphs mated and consequently became gravid females in the pheromone treatment, whereas all third instar nymphs (n=31) mated in the control treatment (Table 1).

The pre-crawler production period was significantly longer for mated females in the pheromone environment (Table 2). In the control treatment, a total of 5.5 crawlers per day were produced from 27 females during approximately 40 days of crawler production; in contrast, 0.7 crawlers per day were produced from 33 females in the PhE treatment during approximately 56 days of crawler production. These numbers resulted in significant differences when the gross daily rates of crawler progeny were represented (Figure 1). In the control treatment, two pick of crawlers were observed at day 8 (10 crawlers/day) and at day 27 (7 crawlers/day); however, this was not observed in the pheromone treatment, where a decreasing plateau of 1 crawler per day was obtained.

3.4 Demographic parameters

The demographic parameters were significantly different between the PhE treatment and control (Table 3). Net fecundity, R_0, and intrinsic rate of increase, r_m, were significantly lower for the PhE group, whereas generation time, T, was higher (Table 3).

3.5 Size of CRS

The scale cover surface areas of females (N_2 and N_3) and prepupae males of CRS subjected to airbone pheromone were significantly larger than the scale cover surface areas of females and males subjected to the control treatment (Table 4).

4 DISCUSSION

212 The effect of MD on the reproductive behaviour of other insect species has been previously
213 studied.[40-42] As general statement, MD treatments restrict the availability of males, which
214 prevents mating in most cases or delays it in others. In some species such as the European pine
215 sawfly *Neodiprion sertifer* (Geoffr.) (Hymenoptera: Diprionidae), the codling moth *Cydia*
216 *pomonella* (L.) (Lepidoptera: Tortricidae), the European corn borer, *Ostrinia nubilalis* (Hübner)
217 (Lepidoptera: Crambidae), it was demonstrated that delayed mating has detrimental effects on
218 female fecundity, fertility and oviposition patterns.[40-42] Our study confirms the difficulties of
219 CRS males to find and mate with the CRS females exposed to an environment with high
220 concentration of CRS sex pheromone. The CRS females subjected to the pheromone treatment
221 showed low intrinsic rate of increase, r_m, as a result of a slower developmental time and a
222 lower population fecundity than the control CRS females.

223 The life history parameters and the cover surface area of CRS have been widely studied
224 previously on different plant hosts and under different climatic conditions.[7,8,10,18,30-32,43,44]
225 Nevertheless, our study is the first in which these parameters have been investigated when
226 CRS is exposed to a sex pheromone environment. For our knowledge, the delay in the CRS
227 nymphal development and the increase of the CRS cover surface area observed in this study,
228 when CRS is exposed to a sex pheromone environment, have not been previously reported on
229 other insects. The explanation of why a sex pheromone environment can lengthen the
230 developmental time while increasing the area of the scale cover surface is not entirely clear
231 and needs further investigation.

232 Our results showed that the longer the developmental time of CRS, the larger the scale cover
233 surface area. This can greatly influence and would also benefit the CRS biological control, as
234 Vacas *et al*.[26] observed under field conditions. The delay in CRS development under MD
235 environment may extend the exposure of the CRS immature developmental stages to its
236 natural enemies; therefore, lengthening the time during which they are vulnerable.[26] Although,

CRS predators reported in the Mediterranean basin are able to prey upon all nymphal stages [33] and CRS parasitoids can parasitize and feed only on some selected stages (second and third nymphal instars and male prepupae),[29-33] both of them prefer to prey and parasitize the third-instar nymphal stage.[33] Therefore, in an area subjected to MD there would be another additional advantage for the natural enemies, since the majority of the third-instar nymphal stage (young female) will remain as unmated females as an available source of preys or hosts for predators and parasitoids.

Vacas et al 2011[26] confirmed the compatibility of MD and biological control techniques. Currently augmentative biological control of CRS, by means of releases of the parasitoid *A. melinus* are being implemented. [3] The *Aphytis* genus choose to lay eggs on the larger scales in order to provide a more abundant source of food for the development of their progeny.[29] In addition, the scale size influences host selection and sex allocation by the female parasitoids,[32,51,52] and it has a strong impact on the efficiency of the parasitoids as biological control agents.[30,31] In our results, an increase in size of second and third-instar developmental stages was observed in the pheromone treatment compared to the control (0.05 mm^2 and 0.36 mm^2, respectively in the scale cover surface area), hence our results would explain the higher percentage of parasitism observed by Vacas et al (2011)[26] in the MD area in comparison to the control area.

In conclusion, the influence of high airborne pheromone amounts on the biology of CRS has been demonstrated. In addition to the classical benefits of this technique because of mating disruption, other benefits, such as the increase in the duration of exposure to natural enemies and the increase in size that benefits the parasitoids, have been confirmed. These conclusions should encourage future research on the effect of this pheromone over the interaction of CRS and natural enemies.

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Table 1. Developmental time in days (mean ± SE) for *Aonidiella aurantii* when reared on lemons subjected to the pheromone environment (PhE) and without the pheromone (control) treatment. n= number of individuals for a particular instar.

	Instar	PhE	Control	Statistical values
Female	N_1	6.04 ± 0.02 a (n=100)	6.06 ± 0.03 a (n=100)	[1]*U*= 4956; *P*=0.7385
	1^{st}molt	4.11 ± 0.11 a (n=99)	2.91 ± 0.10 b (n=99)	t_{196}= 7.69; *P*<0.0001
	N_2	6.60 ± 0.16 a (n=91)	6.16 ± 0.09 b (n=93)	[1]*U*= 3357; *P*=0.0105
	2^{nd}molt	6.65 ± 0.10 a (n=41)	5.08 ± 0.16 b (n=34)	[1]*U*= 101.0; *P*<0.0001
	N_3	7.57 ± 0.30 a (n=7)	6.61 ± 0.24 a (n=31)	t_{36}= 1.78; *P*=0.0822
	N_1-N_3	29.29 ± 0.36 a (n=7)	26.16 ± 0.20b (n=31)	t_{36}= 6.94; *P*<0.0001
Male	Prepupae and pupae	9.42 ± 0.13 a (n=49)	8.22 ± 0.15 b (n=58)	t_{105}= 5.76; *P*<0.0001
	N_1- males	26.45 ± 0.20 a (n=49)	23.55 ± 0.20 b (n=58)	t_{105}= 9.99; *P*<0.0001

Means followed by the same letter within the same row were not significantly different (t-Student, *P*<0.05).

[1]Non-parametric Mann–Whitney test was applied

*Unmated N_3instars females were not able to complete this developmental stage and they were discarded.

*Unmated N_3 instars females were discarded because remain in this instar.

443 **Table 2.** Reproductive parameters (mean ± SE) of *Aonidiella aurantii* when reared on lemons
444 subjected to the pheromone environment (PhE) and without the pheromone
445 (control)treatment.

	PhE	Control	Statistical values
Period before crawler production(days)	16.14 ± 0.26 a (n=7)	12.70 ± 0.15 b (n=30)	t_{35}= 10.08; *P*<0.0001
Period of crawler production (days)	55.80 ± 8.75 a (n=5)	39.70 ± 3.32 a (n=27)	t_{30}= 1.88; *P*=0.069
Progeny per female	42.09 ± 20.41 a (n=33)	208.22 ± 18.24 b (n=27)	t_{58}= 5.94; *P*<0.0001
Crawlers/day	0.71 ± 0.32 a (n=33)	5.55 ± 0.26 a (n=27)	t_{58}= 11.35; *P*<0.0001

446 Means followed by the same letter within the same row were not significantly different (t-Student,
447 *P*<0.05).
448

449 **Table 3**. Selected life history parameters (mean ± SE) of *Aonidiella aurantii*, generation time T
450 (days), net fecundity R_0 (female crawlers per female), intrinsic rate of increase r_m (female
451 crawlers per female per day) when reared on lemons subjected to the pheromone
452 environment (PhE) and without the pheromone (control) treatment.

	PhE	Control	Statistical values
T	70.85 ± 0.13 a (33)	55.31 ± 0.06 b (27)	t_{45}= 104.80; *P*<0.0001
R_0	13.89 ± 0.21 a (33)	56.22 ± 0.19 b (27)	t_{58}= 146.50; *P*<0.0001
r_m	0.04 ± 0.00 a (33)	0.07 ± 0.00 b (27)	t_{33}= 141.90; *P*<0.0001

453 Means followed by the same letter within the same row were not significantly different (Student's t test,
454 using a statistical jackknife technique; *P*<0.05).
455

456 **Table 4.** Scale cover surface area (mm^2) (mean ± SE) of second and third female instars and
457 male of *Aonidiellaaurantii* when reared on lemons subjected to the pheromone environment
458 (PhE) and without the pheromone (control) treatment.

459

		PhE	Control	Statistical values
Female	**N_2**	0.72 ± 0.01 a (35)	0.67 ± 0.01 b (30)	t_{63}= 2.95; *P*=0.0044
	N_3	3.02± 0.04 a (35)	2.66 ± 0.04 b (30)	t_{63}= 6.67; *P*<0.0001
Male	**Prepupae**	0.70 ± 0.01 a (49)	0.67 ± 0.01 b (58)	t_{105}= 2.01; *P*=0.05

460 Means followed by the same letter within the same row were not significantly different (t-Student,
461 *P*<0.05).
462

463

Figure 1. Gross rates of crawler production (mean ± SE) for female *Aonidiella aurantii* when reared on lemons subjected to the pheromone environment (PhE) and without the pheromone (control) treatments.