Assessment of life history parameters of *Aspidiotus nerii* (Hemiptera: Diaspididae) to improve the mass rearing of *Aphytis melinus* (Hymenoptera: Aphelinidae)

Running title: Life history of *Aspidiotus nerii*

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Assessment of life history parameters of *Aspidiotus nerii* (Hemiptera: Diaspididae) to improve the mass rearing of *Aphytis melinus* (Hymenoptera: Aphelinidae)

Abstract

The biological control of *Aonidiella aurantii* (Maskell) on citrus can be achieved with periodic releases of the parasitoid *Aphytis melinus* DeBach. *Aphytis melinus* is normally reared on parthenogenetic strains of the scale *Aspidiotus nerii* Bouché. The developmental rate, crawler production, and survival of *A. nerii* were studied at two temperatures (25±1 and 30±1 °C) and two levels of relative humidity (55±5 and 85±5%) on squash. Temperature had an important effect on developmental rate, but relative humidity did not. At 30 °C, no growth was observed and no crawlers were produced. At 25 °C, development of the L1, L2, and young females stages required 24.4±0.7, 11.1±0.8, and 13.2±0.3 days, respectively. At 25 °C, times until the appearance of mature females, the start of crawler production, and the peak of crawler production were 48.7±0.1, 50.1±0.7, and 63.3±1.0 days, respectively. Crawler production lasted 42.6±1.9 days, and the 50% production level was reached on day 13. Initially, crawler survival was higher at 30 °C (65.9±2.8 %) than at 25 °C (51.7±4.8%), but by the end of development survival was much higher at 25 than 30 °C (88.0±2.1% vs 22.9±2.7%). Relative humidity had no effect on either initial or final survival. Average progeny production was of 28.0±2.8 crawlers per female at 25 °C. The relationship between the weight of groups of crawlers and their number was: Number = 1,031 + 835,500*weight of group (g) (R² = 0.826), which can assist in colony management. The importance of selecting the correct strain of *A. nerii* is emphasised.

Keywords: *Aspidiotus nerii*; parthenogenetic strain; development; crawler survival; biology.
1. Introduction

Increasing restrictions on the use and registration of pesticides (as happen with the Council Directive 91/414/EEC, European Union 2011) has increased interest in the development of biological control options for many crops and pests worldwide. For example, the use of biological control in greenhouse crops, such as those in southeast Spain, has increased substantially in recent years, expanding from 1.3% of the surface area under protection in 2006/2007 to 44.1% in 2008/2009 (Beltrán, Parra, Roldán, Soler and Vila 2010). In certain crops, such as sweet pepper or melon, 70 to 90% of the greenhouse crop area is grown using integrated pest management criteria, relying mainly in augmentative biological control of pests (Beltrán et al. 2010; Blom, Robledo, Torres and Sánchez 2010).

In citrus production, biological control has been used against several important pests from the end of the 19th century (Kennet, McMurtry and Beardsley 1999), first in the United States and later in many other countries. Perhaps one of the most important pests in the citrus industry is *Aonidiella aurantii* (Maskell) (Hemiptera; Diaspididae), the California red scale (Jacas, Karamaouna, Vercher and Zappalà 2010). Many parasitoids and predators have been considered for use against this pest, but most useful are hymenopteran parasitoids such as *Aphytis africanus* Quednau, *Aphytis coheni* DeBach, *Aphytis lingnanensis* DeBach, *Aphytis melinus* DeBach, *Comperiella bifasciata* Howard, and *Encarsia pergiciosi* (Tower) (Kennet et al. 1999), with the importance of particular species varying by region. In orchards using integrated pest management or under organic management, biological control forms the basis of the control of California red scale. Control programs may also involve the use of several insecticides against this pest or other pests (Grafton-Cardwell 2006; Jacas et al. 2010), although mating disruption is now a promising and compatible strategy for control this pest (Vacas, Alfaro, Navarro-Llopis and Primo 2010; Vacas et al. 2012). Conservation of existing natural enemies is the most logical approach (Jacas and Urbaneja 2010), but if this is not adequate, natural enemy releases can be used to help control California red scale, and that approach is
commonly used when parasitoids are scarce at the beginning of the first scale generation due to overwintering mortality or other factors (Moreno and Luck 1992; Sorribas and García-Mari 2010; University of California 2011). This strategy, based on the mass rearing of A. melinus, is widely used in many citrus regions (Mazih, 2008; Zappalà et al. 2008; Zappalà 2010; Olivas, Lucas, Calvo and Belda 2011; University of California, 2011).

Biological control based on augmentative releases requires reliable, inexpensive production of the needed natural enemy by insectaries (Hoy 2000; van Lenteren 2003; Warner and Getz 2008). As demand for such products increases, commercial insectaries must increase production to meet these requirements. Aphytis melinus has been produced in insectaries since the 1950s with a well-known technique (DeBach and White 1960; Rose 1990) based on the use of a parthenogenetic strain of the oleander scale Aspidiotus nerii Bouché (Hemiptera: Diaspididae) as the host and various species of squash as the feeding substrate for the host.

The parthenogenetic strain of A. nerii was first described in 1901 and subsequently discussed by several authors in several regions of the world (for reviews see Gerson and Hazan 1979; Provencher, Morse, Weeks and Normark 2005). Parthenogenesis is caused by a bacterium in the genus Cardinium (Provencher et al. 2005; Gruwell, Wu and Normark 2009). The parthenogenetic strain is widely used in laboratories and insectaries for the mass rearing of different parasitoids and predators (Uygun and Elekçıoglu 1998; Raciti, Saraceno and Siscaro 2003; Silva, Guerreiro, Michelotto and Busoli 2003). However, although the demand for natural enemies reared on this host is increasing (as in the case of A. melinus), few recent studies have investigated the influence of factors on the scale’s biology and rate of progeny production (DeBach and Fisher 1956; Gerson and Hazan 1979; Rocha, Silva, Michelotto and Busoli 2006). Such information may be useful for improving the efficiency of the production of parasitoids and predators based on rearing of this scale.

For these reasons, the biology of a parthenogenetic strain of A. nerii was examined under different temperatures and levels of relative humidity to determine the effects of these factors on
crawler survival and the rate of scale development on squash. We also measured the pattern of crawler production to help in scheduling scale production. Finally, we established the relationship between weight and crawler number, which is useful in studies of mass production based on this scale.

2. Material and methods

2.1 Insect origin and mass rearing

The uniparental (parthenogenetic) strain of *A. nerii* we studied was supplied by the Institute National de la Recherche Agronomique (INRA) laboratory in Vallbone, France, in January 2009. The founding insects were received on potatoes, but crawlers were transferred to butternut squash (*Cucurbita moschata* Duch ex Lam.) for further rearing. Thereafter, our colony of this scale was reared continuously on squash at 25 ± 1 °C and 55-65% RH, following DeBach and White (1960) and Rose (1990), with minor adjustments to our local conditions, as is done in other insectaries (Raciti et al. 2003).

Squash were washed with a sponge soaked in soapy water, rinsed with tap water, dried with a paper towel, and then placed at 25 °C to reach room temperature before being infested with crawlers. In the standard procedure used for rearing this scale, scale-infested squash are maintained on steel shelves, which are positioned above uninfested squash ready to be colonised. Attracted by light, the mobile first-stage nymphs (crawlers) walk towards the tip of the mother squash to search for a place to attach and feed. They fall down onto the new squash positioned below, effecting their colonization. In our laboratory, we used a variation of this approach (as described in Raciti et al. 2003). In our method, instead of placing new squash on the lower shelves, a sheet of paper was placed there and the crawlers that accumulated on the paper were collected daily. Crawlers were then transferred onto new squash using a dispenser resembling a salt shaker. This technique produced a more regular distribution of scales on the surface of the newly infested squash.
2.2 Survival and development studies

Squash (ca 15-20 cm long by 8-12 cm in dia) were used for the development and fertility studies. Crawlers collected on that day were distributed over half of the squash surface. The second or third day after inoculation, the infested surface was divided into 15-20 zones with an indelible ink pen. Each zone contained at least 50 newly settled scales.

The squash were then held at either 25 ± 1°C or 30 ± 1°C each at one of two levels of relative humidity (55 ± 5% or 85 ± 5%) until these newly settled crawlers developed into mature females and started producing crawlers themselves. Observations were continued until F₁ crawler production ended. Two to four squash were used for each combination of temperature and relative humidity, and the entire experiment was repeated twice.

Early rates of scale survival were estimated with a stereomicroscope at days 3 and 7 after squash were inoculated. The numbers of dead crawlers and recently attached live first-instar nymphs were recorded on two or three randomly selected zones of each squash. Because the values for both dates were similar, the average value obtained on the two dates for each squash was used for further analysis. After the seventh day, one randomly selected zone on each squash was observed every 3 or 4 days and the developmental stages of all individuals in the zone were recorded. Scale covers were removed to determine whether the insect was dead or alive. This procedure continued until the first crawlers of the new generation were observed on the squash. At that point, one of the left zones on each squash was randomly selected and surrounded with a non-drying glue, the number of females in the zone recorded, and then all crawlers were counted and removed every two days. Late rates of survival were estimated by averaging survival rates for the last three recording dates before crawler production began.

2.3 Relation weight-number of crawlers
From the mass rearing colony, groups comprised of all the crawlers produced in a 24 h period were collected twice a week and weighed with an analytical balance (model HR-120, A&D Company, San Jose CA). For each sample, a subsample was taken, weighted with the same analytical balance and all crawlers counted using a grid on a Petri dish and a stereomicroscope. Seventeen pairs of such subsamples with crawler weight and number were obtained and analysed.

2.4 Statistical analysis
Developmental times for the first and second instars, the young female period, and the time until the appearance of mature females were calculated using Pontius’ method (Pontius, Boyer and Deaton 1989a, b; Manly 1990), a method that is appropriate if little or no mortality occurs. Population size is then constant for all sample times, and the proportions of the population in different stages at different times can be used to estimate the distributions of the stage durations. This method is appropriate for single-cohort analysis, and represents a simple non-parametric approach to estimate the mean times required to reach a given developmental stage, if samples are taken from the beginning of development of a cohort to the time at which all individuals are in the last stage of development (Manly 1990). The calculations were made with the program P1f (Manly 1994), which calculates both the mean time for each stage and its standard error.

Two-way ANOVA was used with the factors temperature and relative humidity to compare early and late survival, and one-way ANOVA was used to compare developmental times and progeny production with different relative humidities. Simple regression was used to study the relationship between weight and number of crawlers. The Statgraphics Centurion XVI package (StatPoint Technologies 2010) was used to analyse the results. If factors studied in the analysis of variance were significant at $P<0.05$, then the differences between the means were determined using HSD Tukey test at a 95% confidence level. The data were transformed using the arcsine of the square root for variables recorded as percentages. Crawler production from each squash was
transformed to relative values and adjusted to a sigmoidal curve with TableCurve 2D for Windows software (Jandel Scientific 1994). Because no differences between the two different levels of relative humidity within each temperature were found, both data sets within a given temperature were pooled. Survival curves at the different combinations of the two temperatures and the two relative humidities were compared using the Kaplan-Meier procedure, and the overall log-rank test was applied to know possible differences between the survival curves. The SPSS v15.0 for Windows (SPSS 2006) was used to perform this last analysis.

3. Results

3.1 Survival and development studies

Early survival (average of days 3 and 7 post inoculation) was influenced by temperature ($F_{1,16} = 6.80$, $P = 0.02$), with higher survival at 30 °C than at 25 °C (average values of 65.9±2.8% and 51.7±4.8% respectively) (Table 1), but relative humidity had no effect on survival ($F_{1,16} = 0.94$, $P = 0.35$). The interaction between temperature and relative humidity was not significant ($F_{1,16} = 2.01$, $P = 0.18$).

Late survival (the final three sample dates before the start of $F_1$ crawler production) was also influenced by temperature ($F_{1,20} = 218.9$, $P <0.001$), with much higher survival at 25 than at 30 °C (average values of 88.0±2.1% and 22.9±2.7% respectively) (Table 1). The effect of relative humidity was not significant ($F_{1,20} = 0.46$, $P = 0.51$), and no interaction occurred between temperature and relative humidity ($F_{1,20} = 0.07$, $P = 0.79$).

Changes in survival over time after crawler settling were very similar between the four possible combinations of temperature (25 and 30 °C) and relative humidity (55 and 85 %) values, until approximately day 50 (Figure 1), with an average value of approximately 80-90 % of survival. At 25 °C (in both relative humidities), crawlers started appearing after this point, but at 30 °C (in both relative humidities) survival decreased rapidly at after day 50 and no mature females were
observed. The overall log-rank test applied to the four survival curves resulted in $\chi^2 = 5.23$, d.f.=3, and $P=0.16$.

Developmental times were influenced by temperature. At 30 °C, most individuals died in or before the L2 stage, and further development was not observed. No crawlers were produced at this temperature. The developmental times of A. nerii at 25 °C differed greatly between instars (Table 2), with L1s (which includes crawler stage) requiring an average of 24.4±0.7 days, which was considerably greater than that of L2s (which required 11.1±0.8 days) (Table 2). The only developmental parameter that was statistically different between both relative humidities was the total time until crawlers were first observed ($F_{1,10} = 11.2$, $P=0.007$). The other parameters included in Table 2 showed no statistical differences, with $P \geq 0.08$. The average values (in days) for young females, time to mature female, time to maximum crawler, and duration of crawler production were 13.2±0.3, 48.7±0.1, 63.3±1.0, and 42.6±1.9 respectively.

The total number of F1 progeny produced at 25 °C was similar at the two relative humidity levels ($F_{1,10} = 1.27; P = 0.29$), with 30.1± 4.7 and 25.9 ± 3.1 crawlers per female at high (85 %) and normal (55 %) relative humidity respectively. On average, females produced 28.0 ± 2.8 crawlers at 25 °C. The pattern of crawler production did not differ between the two levels of relative humidity at 25 °C and all data were therefore pooled. A sigmoidal curve was fitted to the data (Figure 2), and the fit was statistically significant ($P <0.0001$, $R^2$ [adjusted for d.f.] = 0.926). The standard error of the estimate was 0.092. The production of crawlers reached its peak value on day 11, 50% of total crawler production was reached on day 13, and 95 % of the crawler production was reached on day 32.

3.2. Relation weight-number of crawlers

The relationship between the number of crawlers and their weight is given by this regression:
Number of crawlers = 1,031.37 + 835,500.0*weight of crawlers (g), with R² = 0.826 (n = 17) and the standard error of the estimate = 739.4.

4. Discussion

Information about the biology of *A. nerii* is important for improving the mass production of certain parasitoids used in biological control, particularly *A. melinus*, a species that is reared on *A. nerii* for control of the scales *A. aurantii* and *A. nerii* (Olivas, Lucas, Calvo and Belda 2011). The developmental times and, especially, reproductive parameters we observed indicate that the parthenogenetic strain of *A. nerii* used in this study differs from the strains used in some studies, but strongly resembles some other strains.

Several studies have compared uniparental and biparental strains of *A. nerii* (Schmutterer 1952 [cited by Gerson and Hazan 1979]; DeBach and Fisher 1956; Gerson and Hazan 1979) and have found some differences in biological parameters between the two. DeBach and Fisher (1956) found that their uniparental strain had more descendants, tended to live longer and showed higher crawler survival than the biparental strain. However, Gerson and Hazan (1979) found that the performance of the biparental strain was superior for all the parameters measured compared with the uniparental strain, especially because the biparental strain had shorter developmental time and higher fecundity.

The uniparental strain used in the current study had biological parameters similar to those of the uniparental strain used by Gerson and Hazan (1979). The similarity was especially pronounced for the development of the first and second instar. However, the young female time and the total time from egg to egg were shorter in the current study, compared with the values of approximately 30 and 62 days, respectively, reported by Gerson and Hazan (1979). The value of progeny production reported by these authors was 41.6 ± 17.7 crawlers per female at 24 °C, relatively similar to the value of 28.0 ± 2.8 crawlers per female at 25 °C observed in the current study. The developmental times of
our strain were also very similar to those found by DeBach and Fisher (1956) in their uniparental strain. However, the progeny production of 94 crawlers per female at 23.9 ºC reported by DeBach and Fisher (1956) in their uniparental strain differs substantially from the progeny production observed in our study.

Rocha et al. (2006) examined a uniparental strain of A. nerii that differed in developmental time and crawler production from our strain, with 175.5 ± 10.29 crawlers being produced per female at 25º. Overall, our uniparental strain and the uniparental strains investigated in other studies differ primarily in the degree of production of crawlers, which is probably the most important feature in order to select the appropriate strain for a commercial production of A. melinus. Provencher et al. (2005) found a greater difference between different parthenogenetic (uniparental) strains of A. nerii than that observed within biparental lineages.

The differences observed by various authors between the uniparental and biparental strains led some authors to treat the strains as sibling species, each assigned its own scientific name (as in Schmutterer 1952 [cited by Gerson and Hazan 1979] and also in Gerson and Hazan 1979). Currently, however, most authorities treat the two strains as a single species, A. nerii (Watson 2005).

High survival of crawlers is very important for population increase on squash (and thus parasitoid production) and is influenced by the environment (e.g., temperature, relative humidity). Our results indicate that within 3-7 days after squash inoculation, survival and attachment to the squash surface are influenced to a certain extent by temperature but not by relative humidity. Initial mortality of crawlers (due to failure to settle) can be high, with only 55-65 % of individuals settling on the squash surface. After the scales have settled, survival was high and did not differ between the two levels of relative humidity tested. These findings show that the scale cover provides some protection against certain environmental factors.

Temperature, instead, played an important role in development, with no development to mature females at 30ºC in our study. Temperatures only a few degrees above optimum (24 ºC)
produced consistent reductions in fertility, survival or developmental rate in the parthenogenetic strain of *A. nerii*, as shown both here and in other studies (DeBach and Fisher 1956; Gerson and Hazan 1979).

Relative humidity did not influence survival over the period of development examined in this study. Similar values of survival (85-90 % survival of the settled scale insects) were obtained at high and normal humidity at both temperatures until approximately day 50, at which time crawler production began at 25 ºC, and a rapid decrease of population occurred at 30 ºC. None of the population reared at 30 ºC developed beyond the second-instar nymph stage.

Relative humidity did not clearly influence developmental time at 25 ºC, and statistical differences were only found in the time to first crawler production. For the high humidity level, crawler production began immediately after mature females appeared, but at normal humidity, there was a delay of approximately 3.5 days. Time span of crawlers production showed no statistical differences between the two humidity levels, but was shorter at higher humidity. Relative humidity neither influenced total crawler production at 25 ºC.

Information on the timing of crawler production allows the production of *A. nerii* on squash to be more closely scheduled. The period during which squash inoculated at one time produced crawlers was relatively long (an average of 42.6±1.9 days in this study), with peak production on day 11 (very similar in both aspects to the findings of DeBach and Fisher 1956). Most crawlers were produced before day 32 (similar to the results of Raciti et al. 2003). However, our results differ substantially from the values obtained by Rocha et al. (2006) with their uniparental strain, which was also reared on squash. Finally, knowledge of the relationship between crawler number and weight is of interest for improving control of production, since it allows more convenient dosing of squash with the desired numbers of crawlers in commercial production systems. Different experiments carried out in our laboratory have indicated that a good density of *A. nerii* on the squash surface is 20 to 40 individuals per cm², which allows to maximize the production of *A. melinus* in our conditions.
Acknowledgements

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References


Jandel Scientific. AISN Software. (1994), TableCurve 2D for Windows, ver. 2.02.


Table 1. Percentage survival (mean ± SE) of *Aspidiotus nerii* at early (3 and 7 days after crawler settled) and late (last three samplings before reaching adults) developmental stages under two temperatures and two levels of relative humidity. Number of squash used in each combination of temperature and relative humidity appear between brackets.

<table>
<thead>
<tr>
<th>Relative Humidity</th>
<th>Early Survival</th>
<th>Late Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 °C</td>
<td>30°C</td>
</tr>
<tr>
<td>85 %</td>
<td>50.4±4.3</td>
<td>72.2±3.4</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>55 %</td>
<td>53.0±9.1</td>
<td>59.6±2.0</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(5)</td>
</tr>
</tbody>
</table>
Table 2. Developmental time (mean± SE, days) of *Aspidiotus nerii* at 25°C and two levels of relative humidity. Number of squash used in each combination of temperature and relative humidity appear between brackets.

<table>
<thead>
<tr>
<th>Relative humidity</th>
<th>L1 (days)</th>
<th>L2 (days)</th>
<th>Young female (days)</th>
<th>Time to mature female (days)</th>
<th>Time to first crawler production (days)</th>
<th>Time to maximum crawler production (days)</th>
<th>Duration of crawler production (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>85 %</td>
<td>23.3±0.3</td>
<td>12.2±0.3</td>
<td>13.4±0.2</td>
<td>48.9±0.2</td>
<td>48.4±0.8 a</td>
<td>61.7±0.9</td>
<td>41.1±2.9</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>55 %</td>
<td>25.4±1.0</td>
<td>9.9±1.3</td>
<td>13.1±0.6</td>
<td>48.4±0.1</td>
<td>51.9±0.7 b</td>
<td>64.9±1.6</td>
<td>44.0±2.6</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
</tr>
</tbody>
</table>

Different letters within the same column represent statistical differences between means, using Tukey’s HSD with *P* = 0.05.

*The period began with the inoculation with crawlers at the beginning of the experiment.*
Figure Legends

Figure 1. Survival over time of Aspidiotus nerii reared on squash at two different temperatures and relative humidity levels.

Figure 2. Cumulative crawler production of Aspidiotus nerii reared on squash at 25 ºC. Data are averages of the high- and normal-humidity conditions. Parameter values of the equation: a=-0.230, b=1.206, c=10.592, d=5.614
Figure 1
Figure 2

Fitted equation:

\[ y = a + \frac{b}{1 + \exp\left(-\frac{(x-c)}{d}\right)} \]