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 ºC) 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 ºC, no growth was observed and no crawlers were produced. At 25 ºC, 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 ºC, 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 ºC (65.9±2.8 %) than at 25 ºC (51.7±4.8%), but by the end of development survival was much higher at 25 than 30 ºC (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 ºC. The relationship between the weight of groups of crawlers and their number was: Number = 1,031 + 835,500*weight of group (g) (R^2 = 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 perniciosi* (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

48 commonly used when parasitoids are scarce at the beginning of the first scale generation due to
49 overwintering mortality or other factors (Moreno and Luck 1992; Sorribas and García-Marí 2010;
50 University of California 2011). This strategy, based on the mass rearing of *A. melinus,* is widely used
51 in many citrus regions (Mazih, 2008; Zappalà et al. 2008; Zappalà 2010; Olivas, Lucas, Calvo and
52 Belda 2011; University of California, 2011).

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

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

71 For these reasons, the biology of a parthenogenetic strain of *A. nerii* was examined under
72 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 ºC 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 ºC 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.

98

99 ***2.2. Survival and development studies***

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

104 The squash were then held at either 25 ± 1ºC or 30 ± 1ºC each at one of two levels of relative
105 humidity (55 ± 5% or 85 ± 5%) until these newly settled crawlers developed into mature females and
106 started producing crawlers themselves. Observations were continued until F_1 crawler production
107 ended. Two to four squash were used for each combination of temperature and relative humidity, and
108 the entire experiment was repeated twice.

109 Early rates of scale survival were estimated with a stereomicroscope at days 3 and 7 after
110 squash were inoculated. The numbers of dead crawlers and recently attached live first-instar nymphs
111 were recorded on two or three randomly selected zones of each squash. Because the values for both
112 dates were similar, the average value obtained on the two dates for each squash was used for further
113 analysis. After the seventh day, one randomly selected zone on each squash was observed every 3 or
114 4 days and the developmental stages of all individuals in the zone were recorded. Scale covers were
115 removed to determine whether the insect was dead or alive. This procedure continued until the first
116 crawlers of the new generation were observed on the squash. At that point, one of the left zones on
117 each squash was randomly selected and surrounded with a non-drying glue, the number of females in
118 the zone recorded, and then all crawlers were counted and removed every two days. Late rates of
119 survival were estimated by averaging survival rates for the last three recording dates before crawler
120 production began.

121

122 ***2.3 Relation weight-number of crawlers***

123 From the mass rearing colony, groups comprised of all the crawlers produced in a 24 h period were
124 collected twice a week and weighed with an analytical balance (model HR-120, A&D Company, San
125 Jose CA). For each sample, a subsample was taken, weighted with the same analytical balance and
126 all crawlers counted using a grid on a Petri dish and a stereomicroscope. Seventeen pairs of such
127 subsamples with crawler weight and number were obtained and analysed.
128
129 ***2.4 Statistical analysis***
130 Developmental times for the first and second instars, the young female period, and the time until the
131 appearance of mature females were calculated using Pontius' method (Pontius, Boyer and Deaton
132 1989a, b; Manly 1990), a method that is appropriate if little or no mortality occurs. Population size is
133 then constant for all sample times, and the proportions of the population in different stages at
134 different times can be used to estimate the distributions of the stage durations. This method is
135 appropriate for single-cohort analysis, and represents a simple non-parametric approach to estimate
136 the mean times required to reach a given developmental stage, if samples are taken from the
137 beginning of development of a cohort to the time at which all individuals are in the last stage of
138 development (Manly 1990). The calculations were made with the program P1f (Manly 1994), which
139 calculates both the mean time for each stage and its standard error.
140 Two-way ANOVA was used with the factors temperature and relative humidity to compare
141 early and late survival, and one-way ANOVA was used to compare developmental times and
142 progeny production with different relative humidities. Simple regression was used to study the
143 relationship between weight and number of crawlers. The Statgraphics Centurion XVI package
144 (StatPoint Technologies 2010) was used to analyse the results. If factors studied in the analysis of
145 variance were significant at $P<0.05$, then the differences between the means were determined using
146 HSD Tukey test at a 95% confidence level. The data were transformed using the arcsine of the
147 square root for variables recorded as percentages. Crawler production from each squash was

148 transformed to relative values and adjusted to a sigmoidal curve with TableCurve 2D for Windows
149 software (Jandel Scientific 1994). Because no differences between the two different levels of relative
150 humidity within each temperature were found, both data sets within a given temperature were
151 pooled. Survival curves at the different combinations of the two temperatures and the two relative
152 humidities were compared using the Kaplan-Meier procedure, and the overall log-rank test was
153 applied to know possible differences between the survival curves. The SPSS v15.0 for Windows
154 (SPSS 2006) was used to perform this last analysis.
155
156 **3. Results**
157 ***3.1 Survival and development studies***
158 Early survival (average of days 3 and 7 post inoculation) was influenced by temperature ($F_{1,16} = 6.80$,
159 $P = 0.02$), with higher survival at 30 ºC than at 25 ºC (average values of 65.9±2.8% and 51.7±4.8%
160 respectively) (Table 1), but relative humidity had no effect on survival ($F_{1,16} = 0.94$, $P = 0.35$). The
161 interaction between temperature and relative humidity was not significant ($F_{1,16} = 2.01$, $P = 0.18$).
162 Late survival (the final three sample dates before the start of F_1 crawler production) was also
163 influenced by temperature ($F_{1,20} = 218.9$, $P < 0.001$), with much higher survival at 25 than at 30 ºC
164 (average values of 88.0±2.1% and 22.9±2.7% respectively) (Table 1). The effect of relative humidity
165 was not significant ($F_{1,20} = 0.46$, $P = 0.51$), and no interaction occurred between temperature and
166 relative humidity ($F_{1,20} = 0.07$, $P = 0.79$).
167 Changes in survival over time after crawler settling were very similar between the four
168 possible combinations of temperature (25 and 30 ºC) and relative humidity (55 and 85 %) values,
169 until approximately day 50 (Figure 1), with an average value of approximately 80-90 % of survival.
170 At 25 ºC (in both relative humidities), crawlers started appearing after this point, but at 30 ºC (in
171 both relative humidities) survival decreased rapidly at after day 50 and no mature females were

172 observed. The overall log-rank test applied to the four survival curves resulted in χ^2=5.23, d.f.=3,
173 and *P*=0.16.

174 Developmental times were influenced by temperature. At 30 ºC, most individuals died in or
175 before the L2 stage, and further development was not observed. No crawlers were produced at this
176 temperature. The developmental times of *A. nerii* at 25 ºC differed greatly between instars (Table 2),
177 with L1s (which includes crawler stage) requiring an average of 24.4±0.7 days, which was
178 considerably greater than that of L2s (which required 11.1±0.8 days) (Table 2). The only
179 developmental parameter that was statistically different between both relative humidities was the
180 total time until crawlers were first observed ($F_{1,10}$=11.2, *P*=0.007). The other parameters included in
181 Table 2 showed no statistical differences, with *P*≥0.08. The average values (in days) for young
182 females, time to mature female, time to maximum crawler, and duration of crawler production were
183 13.2±0.3, 48.7±0.1, 63.3±1.0, and 42.6±1.9 respectively.

184 The total number of F_1 progeny produced at 25 ºC was similar at the two relative humidity
185 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
186 normal (55 %) relative humidity respectively. On average, females produced 28.0 ± 2.8 crawlers at
187 25 ºC. The pattern of crawler production did not differ between the two levels of relative humidity at
188 25 ºC and all data were therefore pooled. A sigmoidal curve was fitted to the data (Figure 2), and the
189 fit was statistically significant (*P* <0.0001, R^2 [adjusted for d.f.] = 0.926). The standard error of the
190 estimate was 0.092. The production of crawlers reached its peak value on day 11, 50% of total
191 crawler production was reached on day 13, and 95 % of the crawler production was reached on day
192 32.

193

194 ***3.2. Relation weight-number of crawlers***

195 The relationship between the number of crawlers and their weight is given by this regression:

196 *Number of crawlers* = 1,031.37 + 835,500.0**weight of crawlers (g)*, with $R^2 = 0.826$ (n = 17)
197 and the standard error of the estimate = 739.4.
198
199 **4. Discussion**
200 Information about the biology of *A. nerii* is important for improving the mass production of certain
201 parasitoids used in biological control, particularly *A. melinus*, a species that is reared on *A. nerii* for
202 control of the scales *A. aurantii* and *A. nerii* (Olivas, Lucas, Calvo and Belda 2011). The
203 developmental times and, especially, reproductive parameters we observed indicate that the
204 parthenogenetic strain of *A. nerii* used in this study differs from the strains used in some studies, but
205 strongly resembles some other strains.
206 Several studies have compared uniparental and biparental strains of *A. nerii* (Schmutterer
207 1952 [cited by Gerson and Hazan 1979]; DeBach and Fisher 1956; Gerson and Hazan 1979) and
208 have found some differences in biological parameters between the two. DeBach and Fisher (1956)
209 found that their uniparental strain had more descendants, tended to live longer and showed higher
210 crawler survival than the biparental strain. However, Gerson and Hazan (1979) found that the
211 performance of the biparental strain was superior for all the parameters measured compared with the
212 uniparental strain, especially because the biparental strain had shorter developmental time and higher
213 fecundity.
214 The uniparental strain used in the current study had biological parameters similar to those of
215 the uniparental strain used by Gerson and Hazan (1979). The similarity was especially pronounced
216 for the development of the first and second instar. However, the young female time and the total time
217 from egg to egg were shorter in the current study, compared with the values of approximately 30 and
218 62 days, respectively, reported by Gerson and Hazan (1979). The value of progeny production
219 reported by these authors was 41.6 ± 17.7 crawlers per female at 24 ºC, relatively similar to the value
220 of 28.0 ± 2.8 crawlers per female at 25 ºC 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 appropiate 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 cm^2, which allows to maximize the production of *A. melinus* in our conditions.

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385

386 Table 1. Percentage survival (mean ± SE) of *Aspidiotus nerii* at early (3 and 7 days after crawler
387 settled) and late (last three samplings before reaching adults) developmental stages under two
388 temperatures and two levels of relative humidity. Number of squash used in each combination of
389 temperature and relative humidity appear between brackets.
390

Relative Humidity	Early Survival		Late Survival	
	25 ºC	30ºC	25 ºC	30ºC
85 %	50.4±4.3 (5)	72.2±3.4 (5)	88.7±2.9 (7)	24.9±4.3 (5)
55 %	53.0±9.1 (5)	59.6±2.0 (5)	87.3±3.2 (7)	20.8±3.3 (5)

391
392
393
394

395 Table 2. Developmental time (mean± SE, days) of *Aspidiotus nerii* at 25ºC and two levels of relative
396 humidity. Number of squash used in each combination of temperature and relative humidity appear
397 between brackets.

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

398 Different letters within the same column represent statistical differences between means, using Tukey's HSD
399 with $P = 0.05$

400 [a] The period began with the inoculation with crawlers at the beginning of the experiment.

401

402

403 **Figure Legends**

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

406

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

410

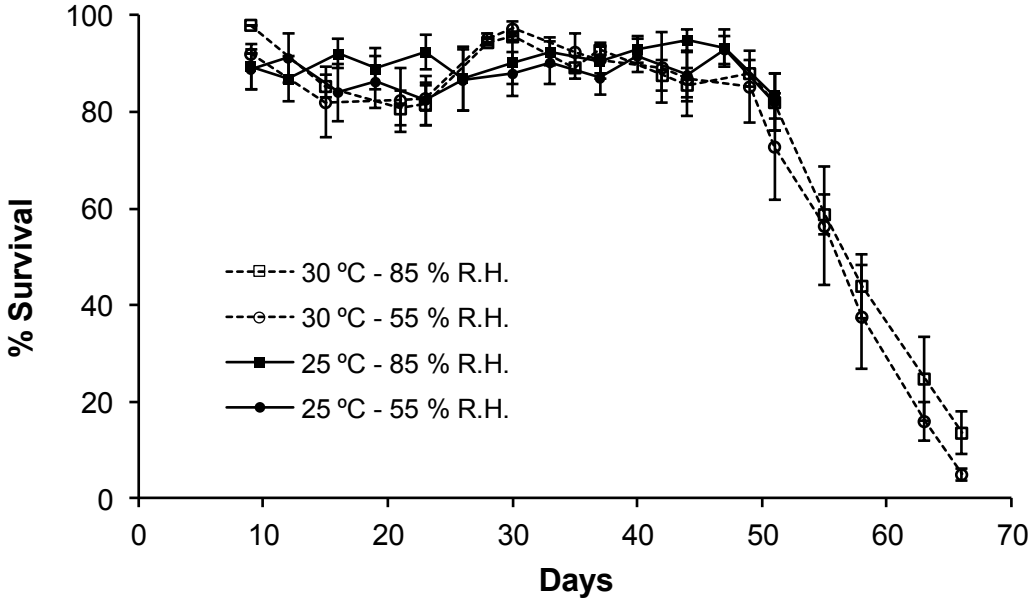

Figure 1

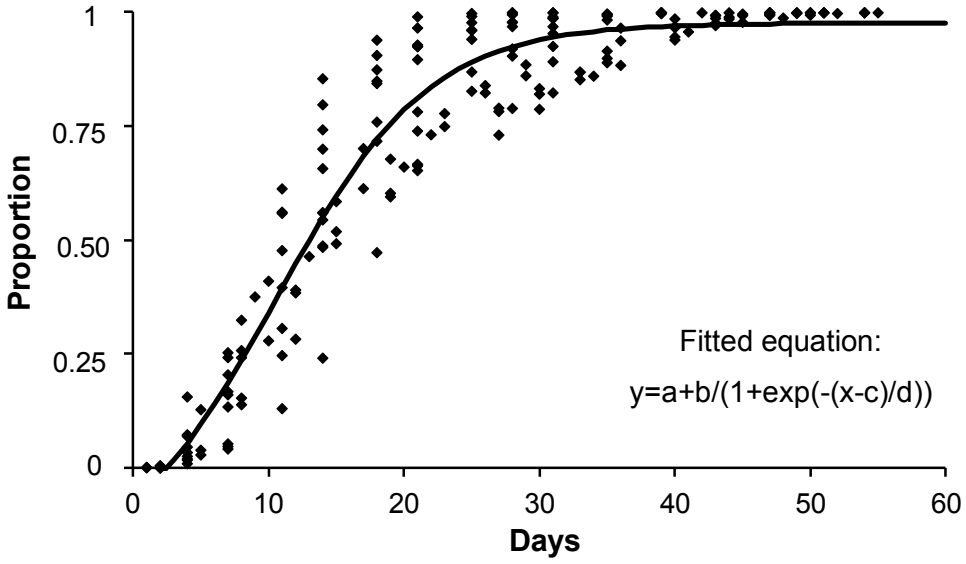

Figure 2