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[Cebolla, R., Bru, P., Urbaneja, A., Tena, A. (2017). Effect of host instar on host discrimination of heterospecific-parasitised hosts by sympatric parasitoids. *Ecological Entomology*, 43:137-145.]

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The final publication is available at

[\[http://dx.doi.org/10.1111/een.12479\]](http://dx.doi.org/10.1111/een.12479)

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Effect of host instar on host discrimination of heterospecific parasitized hosts by sympatric parasitoids

Journal:	<i>Ecological Entomology</i>
Manuscript ID	17-0148-EEN.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Cebolla, Ruth; Instituto Valenciano de Investigaciones Agrarias Bru, Pablo; Instituto Valenciano de Investigaciones Agrarias Urbaneja García, Alberto; Instituto Valenciano de Investigaciones Agrarias, Protección Vegetal y Biotecnología Tena, Alejandro; Instituto Valenciano de Investigaciones Agrarias,
Keywords:	Aphytis, Aonidiella aurantii, behavioural ecology, interspecific competition, intrinsic competition, multiparasitism

Graphical Abstract**Effect of host instar on host discrimination of heterospecific
parasitized hosts by sympatric parasitoids****Ruth Cebolla, Pablo Bru, Alberto Urbaneja, Alejandro Tena***

- The effect of host instar on the ability of parasitoids to discriminate between unparasitized and heterospecific parasitized hosts is tested using two parasitoid of genus *Aphytis*.
- Both parasitoid species were able to discriminate when they found third-instar hosts (larger size) but not when they found second-instar hosts (smaller size).
- The behavioural strategies adopted to multiparasitize third-instar hosts varied between species. *Aphytis chrysomphali* reduced its clutch size in heterospecific parasitized hosts and *A. melinus* tended to probe them for longer.

137x68mm (200 x 200 DPI)

1 *To be submitted to Ecological Entomology*

2 Running title: Effect of host instar on heterospecific discrimination

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4 **Effect of host instar on host discrimination of heterospecific**
5 **parasitized hosts by sympatric parasitoids**

6 **Ruth Cebolla, Pablo Bru, Alberto Urbaneja, Alejandro Tena**

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21 **ABSTRACT**

- 22 1. Interspecific competition among hymenopteran parasitoids may shape their
23 behavioural strategies for host resource exploitation. In order to reduce or
24 prevent competition many parasitoid species have evolved the ability to
25 discriminate between unparasitized hosts and hosts parasitized by another
26 parasitoid species (i.e. heterospecific host discrimination). However,
27 discriminatory ability might be affected by host instar.
- 28 2. Here, we report the first results on whether host instar can influence the use of
29 heterospecific parasitized hosts by sympatric parasitoids of the genus *Aphytis*
30 (Hymenoptera: Aphelinidae).
- 31 3. *Aphytis melinus* and *A. chrysomphali* discriminated between unparasitized and
32 heterospecific parasitized hosts when they found a third instar host (high
33 quality), with a tendency to multiparasitize. However, this discrimination was
34 not observed in the second instar (lower size).
- 35 4. The behavioural strategies adopted to multiparasitize third-instar hosts varied
36 between both species. *Aphytis chrysomphali* reduced its clutch size in
37 heterospecific parasitized hosts, whereas *A. melinus* tended to probe them for
38 longer than healthy hosts.
- 39 5. Overall, our results highlight the importance of host instar to study intrinsic
40 competition between parasitoids.

41

42 **Keywords:** *Aphytis*, *Aonidiella aurantii*, behavioural ecology, interspecific competition,
43 intrinsic competition, multiparasitism,

1. INTRODUCTION

Interspecific competition among hymenopteran parasitoids has shaped their behavioural strategies of host resource exploitation (Connell, 1980; Hawkins, 2000). Contrary to prey eaten by predators, immature parasitoids do not immediately consume host resources. Parasitized hosts remain *in situ* and are vulnerable to be attacked by other foraging females (Van Alphen & Visser, 1990; Godfray, 1994; Wajnberg *et al.*, 2008). When encountering parasitized hosts, female parasitoids can either reject them and look for more suitable hosts for their progeny, or accept them and lay a second egg or clutch of eggs on/in these parasitized hosts (i.e., multiparasitize them) (Goubault *et al.*, 2004; Boivin & Brodeur, 2006; Hopper *et al.*, 2013). The decision implies that the female is able to distinguish between unparasitized hosts and hosts parasitized by another parasitoid species (i.e. heterospecific host discrimination) (Turlings *et al.*, 1985; Pijls *et al.*, 1995; Collier *et al.*, 2007; Yang *et al.*, 2012). Discriminatory behaviour is facilitated through the external and internal cues left by the first female (Vinson, 1976). Pheromones left during oviposition and/or physical marks on the host body such as wounds caused to the host during the oviposition serve as external cues (Vinson, 1976; Mackauer, 1990; Hoffmeister & Roitberg, 2002). Both substances injected during oviposition by the mother and host quality changes associated with parasitism serve as internal cues for female parasitoids to detect previous parasitism (Mackauer, 1990).

Once the female parasitoids has detected that the located host is already parasitized, the final decision will be based on a combination of physiological (e.g., egg load, age, and other characteristics of the female parasitoids) and ecological (e.g., patch quality and size, structure and host abundance) parameters, as well as on the fitness consequences for the offspring (Van Alphen & Visser, 1990; Harvey *et al.*, 2013). Generally, parasitoid species which larvae are superior competitors are more likely to multiparasitize than those which larvae are inferior competitors (Van Alebeek *et al.*, 1993). Under these circumstances, the latter is expected to benefit from host

71 discrimination, as it prevents the wastage of their eggs in hosts parasitized by the
72 superior parasitoid (Pedata *et al.*, 2002; Wang & Messing, 2004). Thus, the female
73 decision to multiparasitize will depend on the probability of a second egg or clutch
74 winning the competition against the first (Netting & Hunter, 2000). In contrast to
75 unparasitized hosts, larvae in multiparasitized hosts, develop under conditions that are
76 unfavorable in both quantity and quality (Harvey *et al.*, 2009, 2013). The limited host
77 resources have to be shared with the competitor (Cusumano, 2015, 2016). Furthermore,
78 the quality of the available resources can be altered by the injection of regulatory factors
79 by the female parasitoid during oviposition (Pennacchio & Strand, 2006; Beckage &
80 Drezen, 2012). Therefore, intrinsic competition can negatively affect fitness related life
81 history traits such as immature mortality, sex ratio, developmental time or the size of
82 the offspring (Collier *et al.*, 2007; Cingolani *et al.*, 2013; Cusumano *et al.*, 2013, 2015).

83 The response to heterospecific parasitized hosts might also be affected by host
84 size/instar. Generally, within a host species, large hosts (i.e. older instars) are
85 considered higher quality for parasitoid development, as they provide more food for the
86 developing progeny than small hosts. A larger host may benefit parasitoid egg load,
87 longevity and sex ratio (Luck *et al.*, 1982; Opp & Luck, 1986; Godfray, 1994; Lampson
88 *et al.*, 1996; Bernal *et al.*, 1999; King, 2000; Harvey, 2005; Kapranas *et al.*, 2009; Silva-
89 Torres *et al.*, 2009; Pekas *et al.*, 2010). An interaction between host instar and
90 heterospecific parasitized hosts might therefore exist and affect host acceptance rates.
91 Previous experimental assays designed to test this hypothesis have not been reported to
92 our knowledge. Therefore, the aim of this study was to determine whether a female is
93 more willing to accept heterospecific parasitized third-instar hosts (large size) than
94 second-instar hosts (small size).

95 *Parasitoid-host system*

96 Parasitoids of genus *Aphytis* Howard (Hymenoptera: Aphelinidae) are
97 considered the most successful and widespread biological control agents of *Aonidiella*
98 *aurantii* (Maskell) (Hemiptera: Diaspididae) in citrus (DeBach & Rosen, 1991; Forster
99 & Luck, 1996; Pekas et al., 2010; 2016). These specialist parasitoids can reduce their
100 shared host to levels nearly 200 times lower than the average density observed in their
101 absence (DeBach et al., 1971), which suggests strong resource competition between
102 parasitoid species (Borer et al., 2004). Host size/instar has a major influence on *Aphytis*
103 fitness as it determines the maximum amount of food available for the developing
104 parasitoid (Luck & Podoler, 1985; Opp & Luck, 1986; Reeve, 1987; Walde *et al.*, 1989;
105 Hare & Luck, 1991). Furthermore, sex ratio and adult size of the offspring are positively
106 correlated with host size (Luck & Podoler, 1985; Opp & Luck, 1986; Yu *et al.*, 1990;
107 Pekas *et al.*, 2010). Indeed, larger hosts (third-instar) are more frequently used for
108 oviposition than their smaller counterparts (second-instar hosts) (Flanders, 1951;
109 Abdelrahman, 1974; Rosenheim & Rosen, 1991). *Aphytis melinus* DeBach and A.
110 *chrysomphali* (Mercet) coexist in sympatry in eastern parts of Spain (Pekas *et al.*, 2010,
111 2016). Therefore, the *Aphytis*-*A. aurantii* system is ideal to test whether host instar can
112 influence the use of heterospecific parasitized hosts by sympatric parasitoids.

113

114 2. MATERIAL AND METHODS

115 2.1 Insect rearing.

116 The phytophagous host, *Aonidiella aurantii*, was reared on lemons and supplied
117 from a colony founded in 1999 from field collected scales in Alzira (Valencia, Spain).
118 The colony has been maintained at Instituto Valenciano de Investigaciones Agrarias,
119 IVIA (Moncada, Valencia, Spain), and renewed every 2-3 years with field collected
120 scales (Tena *et al.*, 2013). Approximately 2/3 of the surface of each lemon was covered
121 with red paraffin around the mid-section to prevent desiccation. The red paraffin was

122 prepared with a mixture of 1 kg of paraffin pearls (Parafina USP Perlas; Guinama S.L.,
123 Alboraya, Spain) to 1 g of red pigment (Sudan III; Panreac Química S.A., Castellar del
124 Vallés, Spain). The remaining surface (approximately 24-cm² area) of the lemon was
125 available for colonisation by *A. aurantii*. Colonisation was achieved by exposing the
126 lemon to gravid female scales from the colony for 48 h. Once infested, lemons were
127 maintained at 26 ± 1 °C, $70 \pm 5\%$ RH in the dark until female scales reached the second
128 or third nymphal instar which were used in these assays, approximately 9-11 days or 19-
129 22 days respectively.

130 *Aphytis melinus* and *A. chrysomphali* are facultative gregarious synovigenic (i.e.
131 females mature eggs throughout their adult life), idiobionts (i.e. the host is paralyzed
132 and arrests development once parasitized) and ectoparasitoids species (Rosen &
133 DeBach, 1979). They exploit the same hosts and both feed and lay eggs on them (Pekas,
134 2010). Individuals of both species were obtained by exposing third-instar *A. aurantii*
135 reared on lemons to parasitism by the foraging adult females. The parasitoids were
136 maintained in the laboratory at 26 ± 1 °C, $60 \pm 5\%$ RH and 16:8 h (L:D) photoperiod.
137 Cultures of *A. melinus* and *A. chrysomphali* were initiated in 2008 and 2013,
138 respectively, from parasitized *A. aurantii* scales collected from citrus fields, Valencia,
139 Spain. Both cultures are renewed yearly with field collected parasitoids.

140 Between five and ten late-stage pupae of both parasitoid species were removed
141 from parasitized scales and separated into 8 mm in diameter and 35 mm long crystal
142 vials and stoppered with a cotton plug. At emergence, *A. melinus* were sexed, and males
143 and females were held together for a 24-hour period in order to obtain mated females
144 [*A. chrysomphali* reproduces parthenogenetically and hence for this species this step
145 was not performed (Gottlieb *et al.*, 1998)]. Twenty-four hours after emergence, females
146 were again isolated in crystal vials. One *A. aurantii* female body was introduced daily to
147 allow host feeding before their use 2-3 days later in behavioural assays (Heimpel *et al.*,
148 1997). A drop of honey was added on the inside wall of each vial as adults. *Aphytis*

would die within three days in the absence of a carbohydrate source (Heimpel *et al.*, 1997), and sugars are not gained from host feeding (Tena *et al.*, 2013). Vials were stored in a climatic chamber (SANYO MLR- 350; Sanyo, Japan) at $25 \pm 1^\circ\text{C}$, 50-70% RH and 14:10 h (L:D) photoperiod.

2.2 Experimental arena.

All behavioral observations were conducted on colonised lemon fruit previously described. Second- ($0.5\text{-}0.7\text{ mm}^2$) or third-instar scale ($0.8\text{-}1\text{ mm}^2$) were measured and selected (Luck & Podoler, 1985; Opp & Luck, 1986; Pekas *et al.*, 2010) under a dissecting microscope with a Leica EC 3 3.1 megapixel digital color camera (Leica Microsystems GmbH, Spain). Images were processed with Leica LAS EX imaging software for Windows (Leica Microsystems GmbH, Spain) and the area of the scales (mm^2) were measured with ImageJ, a public-domain Java Image-processing program (Rasband, 2015). The selected scale was mapped, and the remaining scales were removed using an entomological needle and a paper moistened with water.

2.3 Adult female behaviour.

Female parasitoids were introduced individually into a glass petri dish (4 cm in diameter, height 1.5 cm) and the petri dish placed over the individual selected scale. The behavior of *A. melinus* and *A. chrysomphali* was observed and recorded under two different conditions for each of the two *A. aurantii* nymphal instars (second or third instar). In the control treatment, a single female parasitoid was introduced into the experimental arena and the behaviour observed. In the competition treatment, *A. melinus* and *A. chrysomphali* were introduced sequentially in both possible orders; the female of the first species introduced to the arena was allowed to forage freely until oviposition occurred and then removed, the female of the second species was then introduced 2 h after parasitism and the behaviour recorded. Both treatments for each of the host life history stages used were replicated 30 times.

175 All behavioural observations were continuously recorded under microscopy at
176 10x to 50x magnification and a cool fibre light to illuminate the arena. Behavioural
177 recording began when the female recognized the hosts, this was determined by antennal
178 and forefoot drumming of the scale and by positioning herself on the scale cover,
179 moving from the centre to the edge and tapping the cover with the antennae and
180 sometimes mouthparts (van Lenteren, 1994). Observations terminated when the female
181 left the scale or resting behaviour continued for more than 2 min. Three separate
182 behavioural components were identified, timed and recorded: (1) rejection, (2)
183 oviposition and (3) host feeding. After drumming the scale with the antennae, the
184 female might investigate the host further by probing. The female drills the scale cover
185 with the ovipositor to explore the cavity between the scale body and cover and then
186 pierces the body to explore the body (Casas *et al.*, 2004). The parasitoid may leave the
187 host at any time during this process (hereinafter referred to as rejection), accept the host
188 (oviposition) or consume the scale's body fluids (host-feeding) (Casas *et al.*, 2004). The
189 time spent probing the host was also recorded, but probes that ended in oviposition were
190 not included. Oviposition was identified when female abdominal vibrations were
191 observed during probing behaviour and viscous droplets were seen exuded from the tip
192 of the ovipositor. The ovipositor is then withdrawn and the parasitoid may leave the
193 scale or stay to lay another egg (Pina, 2007).

194 Once the behavioural assays were complete, the parasitoid was removed from
195 the experimental arena and the lemon moved to a plastic container (14 x 14 x 8 cm)
196 covered with muslin to determine the outcome of the encounters. The containers were
197 kept in climatic chamber under the same environmental conditions ($25 \pm 1^{\circ}\text{C}$, 50-70%
198 RH and 14:10 h (L:D) photoperiod) until developing parasitoids reached the pupal
199 stage.

200 Finally, the cost of competition was determined by testing whether
201 multiparasitism imposed a time cost for either species. To achieve this, host handling
202 time in unparasitized versus heterospecific parasitized hosts was compared.

203 **Effect of host instar (size) on host discrimination**

204 The discriminatory ability of either parasitoid species between unparasitized
205 and parasitized hosts of second and third instar was determined by comparing female
206 behaviour. The following behaviours were quantified for this purpose; i) acceptance
207 rates, ii) clutch size (=number of eggs laid per host), iii) handling time and iv) duration
208 of probing. Host handling time was defined as the sum of probing duration and
209 oviposition.

210

211 **Effect of host instar (size) on immature mortality, brood size and sex ratio**
212 **in multiparasitized hosts.**

213 To evaluate whether a cost was incurred to the surviving parasitoid larvae due
214 to juvenile competition, parasitized and multiparasitized scales of both instars were
215 reared to the pupal stage (10 to 12 days). The outcome of competition was measured by
216 i) immature mortality, ii) brood size (=number of emerging parasitoid per host) and iii)
217 sex ratio. The cover of the scales was removed carefully with an entomological needle
218 under microscopy. The species was identified and number and sex of parasitoid pupae
219 were recorded. *Aphytis chrysomphali* pupae are identified by the presence of a
220 longitudinal black line on the mesosternum which is not present in *A. melinus* (Rosen &
221 DeBach, 1979).

222 **2.4 Statistical analyses.**

223 Treatments (host instar and parasitoid species) were compared using two-way
224 ANOVAs. Normality was assumed for handling time and probing duration, assumption
225 was assessed using Shapiro's test, and homoscedasticity assumption was assessed with

the Levene test. Initially binomial error of variance for proportional data (host acceptance, immature mortality and sex ratio) and a Poisson error variance for count data (clutch and brood size) was assumed. The assumed error structures were assessed by a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If over- or under-dispersion was detected, the statistical model was rescaled by a Pearson's chi-square divided by the residual degrees of freedom and the significance of the explanatory variables re-assessed using an F test (Crawley, 2007). The means of untransformed proportion and count data is presented (in preference to less intuitive statistics such as the back-transformed means of logit transformed data). All statistical analyses were performed with R studio (Version 0.98.501 – © 2009-2013 RStudio, Inc) (Ihaka & Gentleman, 1996; <https://www.rstudio.com>).

3. RESULTS

3.1 Effect of host instar (size) on host discrimination

3.1.1 Acceptance

Aphytis melinus females accepted significantly more third instar hosts (0.77 ± 0.06) than second instar hosts (acceptance ratio: 0.55 ± 0.07) ($F_{1,117} = 6.11$; $P = 0.02$) (Table 1). However, there were not significant differences between the acceptance of hosts parasitized by the competitor (*A. chrysomphali*) (0.6 ± 0.06) and unparasitized hosts (0.72 ± 0.06) ($F_{1,117} = 1.86$; $P = 0.18$) (Table 1). The interaction between host instar and competition was not significant ($F_{1,116} = 1.36$; $P = 0.25$).

Aphytis chrysomphali, on the other hand, did not display significant differences between the acceptance of third instar hosts (0.47 ± 0.07) and second instar hosts (0.53 ± 0.07) ($F_{1,117} = 0.52$; $P = 0.47$) and, between hosts parasitized by the competitor (*A. melinus*) (0.42 ± 0.06) and unparasitized hosts (0.58 ± 0.06) ($F_{1,117} = 3.28$; $P = 0.72$)

(Table 2). The interaction between host instar and competition was not significant ($F_{1,116} = 1.13$; $P = 0.72$).

3.1.2 Clutch size

Aphytis melinus females laid significantly larger clutches in third instar hosts (1.37 ± 0.09 eggs) than in second instar hosts (1.06 ± 0.04) ($F_{1,73} = 8.04$; $P < 0.01$) (Fig. 1A). However, there were not significant differences between the clutches laid in hosts parasitized by the competitor (*A. chrysomphali*) (1.28 ± 0.57) and unparasitized hosts (1.2 ± 0.07) ($F_{1,73} = 0.1$; $P = 0.75$). The interaction between host instar and competition was not significant ($F_{1,72} = 0.001$; $P = 0.98$).

Aphytis chrysomphali females laid significant larger clutches in third instar hosts (1.19 ± 0.07 eggs) than in second instar host (1.04 ± 0.04) ($F_{1,57} = 6.86$; $P = 0.011$) (Fig. 1B). On the other hand, the clutches laid in hosts parasitized by the competitor (*A. melinus*) (always laid one egg) were smaller than in unparasitized hosts (1.2 ± 0.07) ($F_{1,57} = 4.15$; $P = 0.046$) The interaction between host instar and competition was not significant ($F_{1,56} = 2.47$; $P = 0.12$).

3.1.3 Handling time

Aphytis melinus females spent significantly more time parasitizing third instar hosts (267.05 ± 15.97 sec) than second instar hosts (203.58 ± 19.07) ($F_{1,73} = 5.94$; $P = 0.017$), but they spent the same time in hosts parasitized by the competitor (*A. chrysomphali*) (253.44 ± 20.65) and unparasitized hosts (226.94 ± 15.47) ($F_{1,73} = 1.16$; $P = 0.28$) (Fig. 2A). The interaction between host instar and competition was not significant ($F_{1,72} = 4.03$; $P = 0.05$).

Aphytis chrysomphali females spent more time parasitizing third instar hosts (332 ± 18.33 sec) than second instar hosts (190.28 ± 11.74) ($F_{1,57} = 39.02$; $P < 0.0001$) but they spent the same time in hosts parasitized by the competitor (*A. melinus*) (262.8 ± 25.4) and unparasitized hosts (268.06 ± 17.15) ($F_{1,57} = 0.05$; $P = 0.82$) (Fig. 2B). The

interaction between host instar and competition was not significant ($F_{1,56} = 2.17$; $P = 0.15$).

3.1.4. Probing time

Aphytis melinus females spent more time probing in third instar hosts (141.93 ± 21.42 sec) than in second instar hosts (32.79 ± 10.24) ($F_{1,76} = 15.44$; $P < 0.001$) and also, in hosts parasitized by the competitor (*A. chrysomphali*) (137.81 ± 27.07) than in unparasitized hosts (61.62 ± 11.82) ($F_{1,76} = 8.84$; $P = 0.004$) (Fig. 2A). The interaction between host instar and competition was not significant ($F_{1,75} = 2.3$; $P = 0.13$).

Aphytis chrysomphali females spent more time probing in third instar hosts (162.41 ± 14.02 sec) than in second instar hosts (53.52 ± 6.36) ($F_{1,54} = 41.15$; $P < 0.0001$) (Fig. 2B). However, they spent the same time probing in hosts parasitized by the competitor (*A. melinus*) (116.56 ± 18.4) and unparasitized hosts (113.16 ± 13.08) ($F_{1,54} = 0.04$; $P = 0.84$). The interaction between host instar and competition was not significant ($F_{1,53} = 0.063$; $P = 0.80$).

3.2 Effect of host instar (size) on immature mortality, brood size and sex ratio in multiparasitized hosts

3.2.1 Immature mortality

Immature mortality was independent of host instar (*A. melinus*: $F_{1,76} = 3.89$; $P = 0.052$; *A. chrysomphali*: $F_{1,58} = 0.60$; $P = 0.44$) but was dependent of competition as immature mortality of *A. melinus* and *A. chrysomphali* increased significantly with multiparasitism (*A. melinus*: $F_{1,76} = 9.96$; $P < 0.001$; *A. chrysomphali*: $F_{1,58} = 12.96$; $P < 0.001$) (Table 3 and 4). The interaction between host instar and competition was not significant for any species (*A. melinus*: $F_{1,75} = 0.79$; $P = 0.78$; *A. chrysomphali*: $F_{1,57} = 0.06$; $P = 0.80$).

301 3.2.2 Brood size

302 In the case where at least one parasitoid emerged, the brood size of *A. melinus*
303 and *A. chrysomphali* was independent of host instar (*A. melinus*: $F_{1,57} = 2.63$; $P = 0.11$;
304 *A. chrysomphali*: $F_{1,33} = 2.12$; $P = 0.15$) and competition (*A. melinus*: $F_{1,57} = 0.29$; $P =$
305 0.59 ; *A. chrysomphali*: $F_{1,33} = 0.85$; $P = 0.36$) (Table 3 and 4). The interaction between
306 host instar and competition was not significant for either parasitoid species (*A. melinus*:
307 $F_{1,56} = 0.25$; $P = 0.62$; *A. chrysomphali*: $F_{1,32} = 0.49$; $P = 0.49$). In the second instar,
308 both parasitoid species behaved as solitary.

309 3.2.3 Sex ratio

310 Secondary sex ratio of *A. melinus* was dependent of host instar ($F_{1,54} = 12.60$; P
311 < 0.001) but it was not affected by competence ($F_{1,54} = 0.50$; $P = 0.48$) (Table 3). The
312 sex ratio of *A. melinus* in the second instar was male-biased, whereas it was female-
313 biased in the third instar. The interaction between host instar and competition was not
314 significant ($F_{1,53} = 0.011$; $P = 0.92$). *Aphytis chrysomphali* reproduced
315 parthenogenetically as expected, and only females were recovered.

316

317 **4. DISCUSSION**

318 **Effect of host instar (size) on host discrimination**

319 Host discrimination by *A. melinus* and *A. chrysomphali* was found to be host
320 instar (size) dependent. *Aphytis melinus* and *A. chrysomphali* were able to discriminate
321 between unparasitized and heterospecific parasitized hosts when they encountered a
322 third instar host (larger size) but this discriminatory ability was not observed in the
323 second instar (smaller size) hosts. Rejection of the lower quality second instar hosts
324 might occur under a greater range of physiological and ecological constraints (e.g.,
325 nutritional status, egg load and experience) than those under which third instar rejection

occurs (Heimpel *et al.*, 1996; Fourrier *et al.*, 2004; Goubault *et al.*, 2005; Boivin & Brodeur, 2006; Hopper, 2013). As far as we are aware, intraspecific instar dependent host discrimination has not previously been reported. Goubault *et al.* (2004), however, tested the impact of host size on female decisions with two host species which differ greatly in size. Contrary to our results, the parasitoid *Pachycrepoideus vindemmiae* Rondani (Hymenoptera: Pteromalidae) was found to discriminate between parasitized and unparasitized hosts in the smaller species *Drosophila melanogaster* (Diptera: Drosophilidae) but not in the larger host species *Delia radicum* L. (Diptera: Anthomyiidae). This was attributed to the relative difficulty to find cues on bigger hosts (Goubault *et al.*, 2004).

Previous studies have documented that some *Aphytis* species are able to discriminate conspecific parasitized hosts (Abdelrahman, 1974; Rosen & DeBach, 1979; van Lenteren & DeBach, 1981). However, heterospecific host discrimination in *Aphytis* parasitoids had not previously been investigated. Here, second instar (smaller) hosts are rejected by both *A. melinus* and *A. chrysomphali* after investigation by antennal drumming and without inserting their ovipositor. This result reinforces Morgan and Hare (1997) observations, which considered that the primary physical cue derived from the scale cover is probably cover diameter and a kairomone, *O*-caffeoyltyrosine, in the cover for initial assessment of host quality (Morgan & Hare, 1997). Hosts rejection of heterospecific parasitized third instar hosts however, occurred significantly more frequently after females had inserted the ovipositor into the host. This suggests that the recognition of heterospecific parasitized hosts occurs in response to internal cues or physiological changes in the host after oviposition. A recent study of van Lenteren (2015) show that the neurons present in the sensillum of the ovipositor tip of *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) are used for host discrimination between parasitized and unparasitized larvae of *Drosophila* spp.

(Diptera: Drosophilidae), and also to discriminate between hosts with different number of parasitoid eggs.

The behavioural strategy adopted during parasitism of a heterospecific-parasitized host was found to vary between species. *Aphytis chrysomphali* reduced its clutch size in heterospecific parasitized hosts, whereas *A. melinus* laid the same number of eggs in healthy and heterospecific parasitized hosts. When we compared the time spent parasitizing, *A. melinus* tended to probe heterospecific-parasitized hosts for longer than healthy ones, whereas there were not differences for *A. chrysomphali*. A reduction of clutch size has been widely cited in cases of superparasitism (Ikawa & Suzuki, 1982; van Dijken & Waage, 1987; Tena *et al.*, 2008), but has been documented only one in the case of heterospecific parasitism (Magdaraog *et al.*, 2013). Regarding the increased time spent probing in heterospecific-parasitized hosts, it is likely that *A. melinus* females were searching for the first clutch of eggs to commit ovicide and eliminate competitors for their offspring, as suggested in the companion manuscript (Cebolla *et al.*, 2017).

Finally, time costs may affect female propensity to multiparasitize, at least in species that are time-limited (Strand & Godfray, 1989; Van Alphen & Visser, 1990). *Aphytis* parasitoids, however, are egg-limited (Heimpel *et al.*, 1998; Casas & Nisbet, 2000; Casas & Mccauley, 2012; Tena *et al.*, 2015) and the same time is needed to multiparasitize as to parasitize a healthy host, independently of host instar. Therefore, time costs are negligible for both parasitoids when they multiparasitize.

Effect of host instar (size) on immature mortality, brood size and sex ratio in multiparasitized hosts

A high cost in terms of immature mortality was incurred under multiparasitism for both parasitoids, independent of host instar. When multiparasitism occurs, the quality of the host may be disrupted by multiple regulatory factors as the injection of polydnaviruses and virus-like particles by the female parasitoid during oviposition

(Pennacchio & Strand, 2006; Beckage & Drezen, 2012) with consequences for the developing larvae. In the most severe cases, hosts die as consequence of the attacks and with them the larvae of both parasitoid species (Godwin & Odell, 1984; Lashomb *et al.*, 1987; Desneux *et al.*, 2009; Asgari & Rivers, 2011; Abram *et al.*, 2016). Larval mortality under multiparasitism occurred in $37.5 \pm 10.1\%$ and $42.9 \pm 8.5\%$ of the second and third instar hosts that were multiparasitized, respectively. Ovicide and competition between immatures may have also affected offspring mortality (Cebolla *et al.*, 2017).

Interspecific larval competition can also impact the development of immature parasitoids and the fitness of the emerging offspring (Harvey, Poelman & Tanaka, 2013; Cusumano *et al.*, 2013). Here, multiparasitism did not affect the secondary brood size and sex ratio in either parasitoid species, independent of host instar. Although the sex ratio of *A. melinus* was significantly higher (male-biased) in the second instar than in the third, it was not altered by multiparasitism. Similarly to results presented here, the secondary sex ratio was not affected by multiparasitism in a study of two egg parasitoids of *Nezara viridula* (L.) (Hemiptera: Pentatomidae), *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) and *Trissolcus basalis* (Wollaston) (Hymenoptera: Platygasteridae) (Cusumano *et al.*, 2013). Finally, the results obtained herein, together with those of Cebolla *et al.* (2017) provide new insights into the importance of host size in the competition between the sympatric parasitoids *A. melinus* and *A. chrysomphali*. That will help us to understand better the niche differentiation and the intrinsic competition that might contribute to their coexistence and/or the displacement of *A. chrysomphali* by *A. melinus*.

Acknowledgments

The authors would like to thank A. Mockford and two anonymous reviewers for her detailed review of the manuscript, Dr. A. Pekas for his comments in a previous version of the manuscript and P. García Meri (IVIA) for technical assistance with experiments.

406 R. Cebolla received a predoctoral fellowship from the Spanish Ministry of Science and
407 Innovation (BES-2012-058937). A. Tena received postdoctoral fellowships from INIA
408 and IVIA. This work was partially funded through the Spanish Ministry of Science and
409 Innovation (AGL2011-30538-C03-02) and the Conselleria d'Agricultura, Pesca i
410 Alimentació de la Generalitat Valenciana.

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- 632

Table 1. Ratio of accepted (parasitized), rejected (before or after probing) and host-fed host (mean $\pm$ SE) when an unparasitized (control) and heterospecific parasitized second- (N2) and third-instar (N3) *Aonidiella aurantii* were exposed to *Aphytis melinus*. Different upper-case and lower-case letters denote significant differences at $P < 0.05$ between host instars and host status (unparasitized vs heterospecific parasitized) respectively.

HOST		Acceptance (Parasitized)	Rejected before probing	Rejected after probing	Host-fed
Instar	State				
N2	Parasitized by <i>A. chrysomphali</i>	0.43 $\pm$ 0.09 ^{Ba}	0.07 $\pm$ 0.05	0.1 $\pm$ 0.06	0.4 $\pm$ 0.09
	Unparasitized	0.67 $\pm$ 0.09 ^{Ba}	0.1 $\pm$ 0.06	0.1 $\pm$ 0.06	0.13 $\pm$ 0.06
N3	Parasitized by <i>A. chrysomphali</i>	0.77 $\pm$ 0.0 ^{Aa}	0.00	0.17 $\pm$ 0.07	0.07 $\pm$ 0.05
	Unparasitized	0.77 $\pm$ 0.08 ^{Aa}	0.07 $\pm$ 0.05	0.13 $\pm$ 0.06	0.03 $\pm$ 0.03

637

638 **Table 2.** Ratio of accepted (parasitized), rejected (before or after probing) and host-fed host (mean $\pm$ SE) when an unparasitized (control) and heterospecific
 639 parasitized second- (N2) and third-instar (N3) *Aonidiella aurantii* were exposed to *A. chrysomphali*. Different upper-case and lower-case letters denote
 640 significant differences at $P < 0.05$ between instars and host status (unparasitized vs heterospecific parasitized) respectively.

HOST		Acceptance	Rejected	Rejected after	Host-fed
Instar	State	(Parasitized)	before probing	probing	
N2	Parasitized by <i>A. melinus</i>	0.4 ± 0.09^{Aa}	0.27 ± 0.09	0.17 ± 0.06	0.17 ± 0.03
	Unparasitized	0.53 ± 0.09^{Aa}	0.33 ± 0.09	0.1 ± 0.07	0.03 ± 0.07
N3	Parasitized by <i>A. melinus</i>	0.43 ± 0.09^{Aa}	0.2 ± 0.03	0.33 ± 0.08	0.03 ± 0.03
	Unparasitized	0.67 ± 0.09^{Aa}	0.03 ± 0.07	0.27 ± 0.09	0.03 ± 0.03

641

642

Table 3. Effect of host instar on immature mortality, secondary brood size (emerged larvae) and sex ratio (% males) (mean $\pm$ SE) of *Aphytis melinus* when females encountered an unparasitized (control) and heterospecific parasitized second- (N2) and third-instar (N3) *Aonidiella aurantii*. Different upper-case and lower-case letters denote significant differences at $P < 0.05$ between instars and host status (unparasitized vs heterospecific parasitized) respectively.

Factor	Treatment	N2	N3
Immature mortality	Control	0.10 $\pm$ 0.07 (20) ^{Ab}	0.15 $\pm$ 0.07 (23) ^{Ab}
	Multiparasitism	0.27 $\pm$ 0.12 (13) ^{Aa}	0.57 $\pm$ 0.10 (13) ^{Aa}
2nd Brood size	Control	1 (18) ^{Aa}	1.10 $\pm$ 0.07(21) ^{Aa}
	Multiparasitism	1 (10) ^{Aa}	1.18 $\pm$ 0.18 (11) ^{Aa}
Sex Ratio	Control	0.59 $\pm$ 0.12 (18) ^{Aa}	0.13 $\pm$ 0.07 (20) ^{Ba}
	Multiparasitism	0.70 $\pm$ 0.02 (10) ^{Aa}	0.20 $\pm$ 0.09 (10) ^{Ba}

Table 4. Effect of host instar on immature mortality and secondary brood size (emerged larvae) (mean $\pm$ SE) of *Aphytis chrysomphali* when females encountered an unparasitized (control) and heterospecific parasitized second- (N2) and third-instar (N3) *Aonidiella aurantii*. Different upper-case and lower-case letters denote significant differences at $P < 0.05$ between instars and host status (unparasitized vs heterospecific parasitized) respectively.

Factor	Treatment	N2	N3
Immature mortality	Control	0.31 $\pm$ 0.12 (16) ^{Ab}	0.13 $\pm$ 0.06 (19) ^{Ab}
	Multiparasitism	0.75 $\pm$ 0.13 (12) ^{Aa}	0.69 $\pm$ 0.13 (13) ^{Aa}
2nd Brood size	Control	1 (11) ^{Aa}	1.16 $\pm$ 0.08 (19) ^{Aa}
	Multiparasitism	1 (3) ^{Aa}	1 (4) ^{Aa}

656

657
658 **Figure legends.**

659 **Fig. 1.** Clutch size (mean $\pm$ SE) of *Aphytis melinus* (Fig. 1A) and *A. chrysomphali* (Fig.
660 1B) when they accepted an unparasitized (control) and heterospecific parasitized
661 second- (N2) and third-instar (N3) of *Aonidiella aurantii*. Different upper-case and
662 lower-case letters beside bars denote significant differences at $P < 0.05$ between instars
663 and host status (unparasitized vs heterospecific parasitized) respectively.

664 **Fig. 2.** Host handling time (mean $\pm$ SE) (time spent probing + time spent parasitizing)
665 of *Aphytis melinus* (Fig. 2A) and *A. chrysomphali* (Fig. 2B) when they accepted an
666 unparasitized (control) and heterospecific parasitized second- (N2) and third-instar (N3)
667 *Aonidiella aurantii*. Different letters inside and besides the bars denote significant
668 differences at $P < 0.05$ in the time spent probing and time spent parasitizing
669 respectively. Differences between host instars are represented in different upper-case
670 letters and between host status (unparasitized vs heterospecific parasitized) in lower-
671 case letters.

672

Fig. 1A.

680

681 **Fig. 1B.**

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Fig. 2A

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690 **Fig. 2B**

1 *To be submitted to Ecological Entomology*

2 Running title: Effect of host instar on heterospecific discrimination

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4 **Effect of host instar on host discrimination of heterospecific**
5 **parasitized hosts by sympatric parasitoids**

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7

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ABSTRACT

1. Interspecific competition among hymenopteran parasitoids may shape their behavioural strategies for host resource exploitation. In order to reduce or prevent competition many parasitoid species have evolved the ability to discriminate between unparasitized hosts and hosts parasitized by another parasitoid species (i.e. heterospecific host discrimination). However, discriminatory ability might be affected by host instar.
2. Here, we report the first results on whether host instar can influence the use of heterospecific parasitized hosts by sympatric parasitoids of the genus *Aphytis* (Hymenoptera: Aphelinidae).
3. *Aphytis melinus* and *A. chrysomphali* discriminated between unparasitized and heterospecific parasitized hosts when they found a third instar host (high quality), with a tendency to multiparasitize. However, this discrimination was not observed in the second instar (lower size).
4. The behavioural strategies adopted to multiparasitize third-instar hosts varied between both species. *Aphytis chrysomphali* reduced its clutch size in heterospecific parasitized hosts, whereas *A. melinus* tended to probe them for longer than healthy hosts.
5. Overall, our results highlight the importance of host instar to study intrinsic competition between parasitoids.

Keywords: *Aphytis*, *Aonidiella aurantii*, behavioural ecology, interspecific competition, intrinsic competition, multiparasitism,

1. INTRODUCTION

Interspecific competition among hymenopteran parasitoids ~~have~~has shaped their behavioural strategies of host resource exploitation (Connell, 1980; Hawkins, 2000). Contrary to prey eaten by predators, immature parasitoids do not immediately consume ~~immediately the~~ host resources, ~~and, thus these p~~Parasitized hosts remain *in situ* ~~and are~~ vulnerable to be ~~encountered-attacked~~ by other foraging females (Van Alphen & Visser, 1990; Godfray, 1994; Wajnberg *et al.*, 2008). When encountering ~~such parasitized~~ hosts, female parasitoids can ~~either choose to~~ reject them and look for more suitable hosts for their progeny, or accept them and lay a second egg or clutch of eggs ~~on/in~~ these ~~already~~ parasitized (i.e., multiparasitize them) (Goubault *et al.*, 2004; Boivin & Brodeur, 2006; Hopper *et al.*, 2013). These decision ~~imply~~implies that the female is able to distinguish between unparasitized hosts and ~~hosts~~those parasitized by another parasitoid species (i.e. heterospecific host discrimination) (Turlings *et al.*, 1985; Pijls *et al.*, 1995; Collier *et al.*, 2007; Yang *et al.*, 2012). ~~This~~ Discriminatory behaviour is facilitated through the external and internal cues left by the first female (Vinson, 1976). ~~External cues are based on P~~pheromones left during oviposition ~~and/or~~ physical marks on the host body such as wounds caused to the host during the oviposition serve as external cues (Vinson, 1976; Mackauer, 1990; Hoffmeister & Roitberg, 2002). ~~Internal cues are originated either from Both~~ substances injected during oviposition by the mother ~~or and from~~ host quality changes associated with parasitism serve as internal cues for female parasitoids to detect previous parasitism (Mackauer, 1990).

Once the female parasitoids has ~~perceived-detected~~ that the located host is already parasitized, ~~her the~~ final decision will be based on a combination of physiological (e.g., egg load, age, and other characteristics of the female parasitoids) and ecological (e.g., patch quality and size, structure and host abundance) parameters, as well as on the fitness consequences for ~~her the~~ offspring (Van Alphen & Visser,

1990; Harvey *et al.*, 2013). ~~Regarding fitness consequences, Generally,~~ parasitoid species ~~whose~~ which larvae are superior competitors are more likely to multiparasitize than those which larvae are inferior competitors (Van Alebeek *et al.*, 1993). Under these circumstances, ~~it is expected that~~ the latter is expected to benefit from host discrimination, as it prevents the wastage of their eggs in hosts parasitized by the superior parasitoid (Pedata *et al.*, 2002; Wang & Messing, 2004). Thus, the female decision to multiparasitize will depend on the probability of a second egg or clutch winning the competition against the first (Netting & Hunter, 2000). ~~Compared-In~~ contrast to unparasitized hosts, larvae in multiparasitized hosts, develop under conditions that are unfavorable in both quantity and quality (Harvey *et al.*, 2009, 2013). The limited host resources have to be shared with the competitor (Cusumano, 2015, 2016). Furthermore, the quality of the available resources ~~available could can~~ be altered by the injection of regulatory factors by the female parasitoid during oviposition (Pennacchio & Strand, 2006; Beckage & Drezen, 2012). Therefore, intrinsic competition can negatively affect fitness related life history traits such as ~~the increase of~~ immature mortality, sex ratio, developmental time or ~~by decreasing~~ the size of the offspring (Collier *et al.*, 2007; Cingolani *et al.*, 2013; Cusumano *et al.*, 2013, 2015).

The response to heterospecific parasitized hosts might ~~therefore~~ also be affected by host size/instar. Generally, within a host species, large hosts (i.e. older instars) are considered ~~of~~ higher quality for parasitoid development, as they provide more food for the developing progeny than small hosts. A larger host ~~size may, for instance,~~ benefits parasitoid egg load, longevity and sex ratio (Luck *et al.*, 1982; Opp & Luck, 1986; Godfray, 1994; Lampson *et al.*, 1996; Bernal *et al.*, 1999; King, 2000; Harvey, 2005; Kapranas *et al.*, 2009; Silva-Torres *et al.*, 2009; Pekas *et al.*, 2010). An interaction between host instar and ~~the use of~~ heterospecific parasitized hosts might therefore exist and affect host acceptance rates. Previous experimental assays designed to test this hypothesis have not been reported to our knowledge. Therefore, the aim of this study ~~is~~

98 | ~~was~~ to determine whether a female ~~will be~~ more willing to accept heterospecific
 99 | parasitized third-instar hosts (large size) than second-instar hosts (small size).

100 | Parasitoid-host system

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101 | Parasitoids of genus *Aphytis* Howard (Hymenoptera: Aphelinidae) are
 102 | considered the most successful and widespread biological control agents of *Aonidiella*
 103 | *aurantii* (Maskell) (Hemiptera: Diaspididae) in citrus (DeBach & Rosen, 1991; Forster
 104 | & Luck, 1996; Pekas et al., 2010; 2016). These specialist parasitoids can reduce their
 105 | shared host to levels nearly 200 times lower than the average density observed in their
 106 | absence (DeBach et al., 1971), which suggests strong resource competition between
 107 | parasitoid species (Borer et al., 2004). Host size/instar has a major influence on *Aphytis*
 108 | fitness as it determines the maximum amount of food available for the developing
 109 | parasitoid (Luck & Podoler, 1985; Opp & Luck, 1986; Reeve, 1987; Walde *et al.*, 1989;
 110 | Hare & Luck, 1991). Furthermore, sex ratio and adult size of the offspring are positively
 111 | correlated with host size (Luck & Podoler, 1985; Opp & Luck, 1986; Yu *et al.*, 1990;
 112 | Pekas *et al.*, 2010). Indeed, larger hosts (third-instar) are more frequently used for
 113 | oviposition than their smaller counterparts (second-instar hosts) (Flanders, 1951;
 114 | Abdelrahman, 1974; Rosenheim & Rosen, 1991). *Aphytis melinus* DeBach and A.
 115 | *chrysomphali* (Mercet) coexist in sympatry in eastern parts of Spain (Pekas *et al.*, 2010,
 116 | 2016). Therefore, the *Aphytis*-*A. aurantii* system is ideal to test whether host
 117 | ~~quality/size/instar~~ can influence the use of heterospecific parasitized hosts by ~~these~~
 118 | ~~sympatric parasitoids. Host acceptance (multiparasitism), handling time and clutch size~~
 119 | ~~of both parasitoids were compared in detail. The discriminatory ability of both~~
 120 | ~~parasitoid species between unparasitized and heterospecific parasitized hosts in both the~~
 121 | ~~smaller sized second instar (0.5–0.7 mm³) and larger sized third instar (0.8–1 mm³) was~~
 122 | ~~tested. The impacts of host size and multiparasitism on immature mortality, brood size~~
 123 | ~~and sex ratio, were also determined.~~

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125 | **2. MATERIAL AND METHODS**

126 | **2.1 Insect rearing.**

127 The phytophagous host, *Aonidiella aurantii*, was reared on lemons and supplied
128 from a colony founded in 1999 from field collected scales in Alzira (Valencia, Spain).
129 The colony has been maintained at Instituto Valenciano de Investigaciones Agrarias,
130 IVIA (Moncada, Valencia, Spain), and renewed every 2-3 years with field collected
131 scales (Tena *et al.*, 2013). Approximately 2/3 of the surface of each lemon was covered
132 with red paraffin around the mid-section to prevent desiccation. The red paraffin was
133 prepared with a mixture of 1 kg of paraffin pearls (Parafina USP Perlas; Guinama S.L.,
134 Alboraya, Spain) to 1 g of red pigment (Sudan III; Panreac Química S.A., Castellar del
135 Vallés, Spain). The remaining surface (approximately 24-cm² area) of the lemon was
136 available for colonisation by *A. aurantii*. Colonisation was achieved by exposing the
137 lemon to gravid female scales from the colony for 48 h. Once infested, lemons were
138 maintained at 26 ± 1 °C, 70 ± 5% RH in the dark until female scales reached the second
139 or third nymphal instar which were used in these assays, approximately 9-11 days or 19-
140 22 days respectively.

141 *Aphytis melinus* and *A. chrysomphali* are facultative gregarious synovigenic (i.e.
142 females mature eggs throughout their adult life), idiobionts (i.e. the host is paralyzed
143 and arrests development once parasitized) and ectoparasitoids species (Rosen &
144 DeBach, 1979). They exploit the same hosts and both feed and lay eggs on them (Pekas,
145 2010). Individuals of both species were obtained by exposing third-instar *A. aurantii*
146 reared on lemons to parasitism by the foraging adult females. The parasitoids were
147 maintained in the laboratory at 26 ± 1 °C, 60 ± 5% RH and 16:8 h (L:D) photoperiod.
148 Cultures of *A. melinus* and *A. chrysomphali* were initiated in 2008 and 2013,
149 respectively, from parasitized *A. aurantii* scales collected from citrus fields, Valencia,
150 Spain. Both cultures are renewed yearly with field collected parasitoids.

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Between five and ten late-stage pupae of both parasitoid species were removed from parasitized scales and separated into 8 mm in diameter and 35 mm long crystal vials and stoppered with a cotton plug. At emergence, *A. melinus* were sexed, and males and females were held together for a 24-hour period in order to obtain mated females [*A. chrysomphali* reproduces parthenogenetically and hence for this species this step was not performed (Gottlieb *et al.*, 1998)]. Twenty-four hours after emergence, females were again isolated in crystal vials. One *A. aurantii* female body was introduced daily to allow host feeding before their use 2-3 days later in behavioural assays (Heimpel *et al.*, 1997). A drop of honey was added on the inside wall of each vial as adults. *Aphytis* would die within three days in the absence of a carbohydrate source (Heimpel *et al.*, 1997), and sugars are not gained from host feeding (Tena *et al.*, 2013). Vials were stored in a climatic chamber (SANYO MLR- 350; Sanyo, Japan) at $25 \pm 1^{\circ}\text{C}$, 50-70% RH and 14:10 h (L:D) photoperiod.

2.2 Experimental arena.

All behavioral observations were conducted on colonised lemon fruit previously described. Second- (0.5-0.7 mm²) or third-instar scale (0.8-1 mm²) were measured and selected (Luck & Podoler, 1985; Opp & Luck, 1986; Pekas *et al.*, 2010) under a dissecting microscope with a Leica EC 3 3.1 megapixel digital color camera (Leica Microsystems GmbH, Spain). Images were processed with Leica LAS EX imaging software for Windows (Leica Microsystems GmbH, Spain) and the area of the scales (mm²) were measured with ImageJ, a public-domain Java Image-processing program (Rasband, 2015). The selected scale was mapped, and the remaining scales were removed using an entomological needle and a paper moistened with water.

2.3 Adult female behaviour.

Female parasitoids were introduced individually into a glass petri dish (4 cm in diameter, height 1.5 cm) and the petri dish placed over the individual selected scale. The behavior of *A. melinus* and *A. chrysomphali* was observed and recorded under two

different conditions for each of the two *A. aurantii* nymphal instars (second or third instar). In the control treatment, a single female parasitoid was introduced into the experimental arena and the behaviour observed. In the competition treatment, *A. melinus* and *A. chrysomphali* were introduced sequentially in both possible orders; the female of the first species introduced to the arena was allowed to forage freely until oviposition occurred and then removed, the female of the second species was then introduced 2 h after parasitism and the behaviour recorded. Both treatments for each of the host life history stages used were replicated 30 times.

All behavioural observations were continuously recorded under microscopy at 10x to 50x magnification and a cool fibre light to illuminate the arena. Behavioural recording began when the female recognized the hosts, this was determined by antennal and forefoot drumming of the scale and by positioning herself on the scale cover, moving from the centre to the edge and tapping the cover with the antennae and sometimes mouthparts (van Lenteren, 1994). Observations terminated when the female left the scale or resting behaviour continued for more than 2 min. Three separate behavioural components were identified, timed and recorded: (1) rejection, (2) oviposition and (3) host feeding. After drumming the scale with the antennae, the female might investigate the host further by probing. The female drills the scale cover with the ovipositor to explore the cavity between the scale body and cover and then pierces the body to explore the body (Casas *et al.*, 2004). The parasitoid may leave the host at any time during this process (hereinafter referred to as rejection), accept the host (oviposition) or consume the scale's body fluids (host-feeding) (Casas *et al.*, 2004). The time spent probing the host was also recorded, but probes that ended in oviposition were not included. Oviposition was identified when female abdominal vibrations were observed during probing behaviour and viscous droplets were seen exuded from the tip of the ovipositor. The ovipositor is then withdrawn and the parasitoid may leave the scale or stay to lay another egg (Pina, 2007).

205 Once the behavioural assays were complete, the parasitoid was removed from
206 the experimental arena and the lemon moved to a plastic container (14 x 14 x 8 cm)
207 covered with muslin to determine the outcome of the encounters. The containers were
208 kept in climatic chamber under the same environmental conditions ($25 \pm 1^{\circ}\text{C}$, 50-70%
209 RH and 14:10 h (L:D) photoperiod) until developing parasitoids reached the pupal
210 stage.

211 Finally, the cost of competition was determined by testing whether
212 multiparasitism imposed a time cost for either species. To achieve this, host handling
213 time in unparasitized versus heterospecific parasitized hosts was compared.

214 **Effect of host instar (size) on host discrimination**

215 The discriminatory ability of either parasitoid species between unparasitized
216 and parasitized hosts of second and third instar was determined by comparing female
217 behaviour. The following behaviours were quantified for this purpose; i) acceptance
218 rates, ii) clutch size (=number of eggs laid per host), iii) handling time and iv) duration
219 of probing. Host handling time was defined as the sum of probing duration and
220 oviposition.

221

222 **Effect of host instar (size) on immature mortality, brood size and sex ratio** 223 **in multiparasitized hosts.**

224 To evaluate whether a cost was incurred to the surviving parasitoid larvae due
225 to juvenile competition, parasitized and multiparasitized scales of both instars were
226 reared to the pupal stage (10 to 12 days). The outcome of competition was measured by
227 i) immature mortality, ii) brood size (=number of emerging parasitoid per host) and iii)
228 sex ratio. The cover of the scales was removed carefully with an entomological needle
229 under microscopy. The species was identified and number and sex of parasitoid pupae
230 were recorded. *Aphytis chrysomphali* pupae are identified by the presence of a

231 longitudinal black line on the mesosternum which is not present in *A. melinus* (Rosen &
232 DeBach, 1979).

233 **2.4 Statistical analyses.**

234 Treatments (host instar and parasitoid species) were compared using two-way
235 ANOVAs. Normality was assumed for handling time and probing duration, assumption
236 was assessed using Shapiro's test, and homoscedasticity assumption was assessed with
237 the Levene test. Initially binomial error of variance for proportional data (host
238 acceptance, immature mortality and sex ratio) and a Poisson error variance for count
239 data (clutch and brood size) was assumed. The assumed error structures were assessed
240 by a heterogeneity factor equal to the residual deviance divided by the residual degrees
241 of freedom. If over- or under-dispersion was detected, the statistical model was rescaled
242 by a Pearson's chi-square divided by the residual degrees of freedom and the
243 significance of the explanatory variables re-assessed using an *F* test (Crawley, 2007).
244 The means of untransformed proportion and count data is presented (in preference to
245 less intuitive statistics such as the back-transformed means of logit transformed data).
246 All statistical analyses were performed with R studio (Version 0.98.501 – © 2009-2013
247 RStudio, Inc) (Ihaka & Gentleman, 1996; <https://www.rstudio.com>).

248

249 **3. RESULTS**

250 **3.1 Effect of host instar (size) on host discrimination**

251 3.1.1 Acceptance

252 ~~According to the percentage of accepted hosts, *Aphytis melinus* females~~
253 ~~accepted significantly more third instar hosts (0.77 ± 0.06) than second instar hosts~~
254 ~~(acceptance ratio: 0.55 ± 0.07) ($F_{1,117} = 6.11$; $P = 0.02$) (Table 1). However, there were~~
255 ~~not significant differences between the acceptance of~~ ~~were found to discriminate~~

256 ~~between second and third instar hosts ($F_{1,117} = 6.11$; $P = 0.02$) but not between~~
 257 ~~heterospecific hosts~~ parasitized by the competitor (*A. chrysomphali*) (-0.6 ± 0.06) and
 258 unparasitized ~~hosts~~ hosts (0.72 ± 0.06) ($F_{1,117} = 1.86$; $P = 0.18$) (Table 1). The
 259 interaction between host instar and competition was not significant ($F_{1,116} = 1.36$; $P =$
 260 0.25).

261 ~~According to the percentage of accepted hosts, *Aphytis* *chrysomphali*, on the~~
 262 other hand, did not display ~~significant differences a discriminatory ability~~ between ~~the~~
 263 ~~acceptance of second third instar hosts (0.47 ± 0.07) and second third instar hosts (0.53~~
 264 ~~± 0.07) ($F_{1,117} = 0.52$; $P = 0.47$), and, between hosts parasitized by the competitor (*A.*~~
 265 ~~*melinus*) (0.42 ± 0.06), and unparasitized ~~host~~hosts (0.58 ± 0.06) ($F_{1,117} = 3.28$; $P =$~~
 266 0.72) (Table 2). The interaction between host instar and competition was not significant
 267 ($F_{1,116} = 1.13$; $P = 0.72$).

268 3.1.2 Clutch size

269 ~~According to clutch size, *A. phytis melinus* females laid significantly larger~~
 270 ~~clutches in third instar hosts (1.37 ± 0.09 eggs) than in second instar hosts ($1.06 \pm$~~
 271 ~~0.04) again discriminated between second and third instar ($F_{1,73} = 8.04$; $P < 0.01$) (Fig.~~
 272 ~~1A). However, there were not significant differences between the clutches laid in but~~
 273 ~~not between~~ hosts parasitized by the competitor (*A. chrysomphali*) (1.28 ± 0.57) and
 274 unparasitized hosts (1.2 ± 0.07) ($F_{1,73} = 0.1$; $P = 0.75$) (Fig. 1A). The interaction
 275 between host instar and competition was not significant ($F_{1,72} = 0.001$; $P = 0.98$).

276 ~~In regards to clutch size, *Aphytis* *chrysomphali* females laid significant larger~~
 277 ~~clutches in third instar hosts (1.19 ± 0.07 eggs) than in second instar host (1.04 ± 0.04)~~
 278 ~~discriminated between second and third instar ($F_{1,57} = 6.86$; $P = 0.011$) (Fig. 1B). On~~
 279 ~~the other hand, the clutches laid in), (Fig. 1B), as well as between~~ hosts parasitized by
 280 the competitor (*A. melinus*) (1 ± 0 always laid one egg) were smaller than in) and
 281 unparasitized hosts (1.2 ± 0.07) ($F_{1,57} = 4.15$; $P = 0.046$) (Fig. 1B). The interaction
 282 between host instar and competition was not significant ($F_{1,56} = 2.47$; $P = 0.12$).

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283 3.1.3 Handling time

284 ~~According to the time spent parasitizing, *Aphytis melinus* females spent~~
285 ~~significantly more time parasitizing again discriminated between second and third instar~~
286 ~~hosts (267.05 ± 15.97 sec) than second instar hosts (203.58 ± 19.07) ($F_{1,73} = 5.94$; $P =$~~
287 ~~0.017), but they spent the same time in but they did not discriminate between~~ hosts
288 ~~parasitized by the competitor (*A. chrysomphali*) (253.44 ± 20.65) and unparasitized~~
289 ~~ones hosts (226.94 ± 15.47) ($F_{1,73} = 1.16$; $P = 0.28$) (Fig. 2A). The interaction between~~
290 ~~host instar and competition was not significant ($F_{1,72} = 4.03$; $P = 0.05$).~~

291 ~~According to the time spent parasitizing, *Aphytis chrysomphali* females spent~~
292 ~~more time parasitizing third instar hosts (332 ± 18.33 sec) than second instar hosts~~
293 ~~(190.28 ± 11.74) discriminated between second and third instar ($F_{1,57} = 39.02$; $P <$~~
294 ~~0.0001) but they did not discriminate between spent the same time in hosts parasitized~~
295 ~~by the competitor (*A. melinus*) (262.8 ± 25.4) heterospecific parasitized and~~
296 ~~unparasitized hosts (268.06 ± 17.15) ($F_{1,57} = 0.05$; $P = 0.82$) (Fig. 2B). The interaction~~
297 ~~between host instar and competition was not significant ($F_{1,56} = 2.17$; $P = 0.15$).~~

298 3.1.4. Probing time

299 ~~According to the time spent probing, *A. phytis melinus* females spent more time~~
300 ~~probing in discriminated between second and third instar hosts (141.93 ± 21.42 sec)~~
301 ~~than in second instar hosts (32.79 ± 10.24) ($F_{1,76} = 15.44$; $P < 0.001$) and also, in~~
302 ~~between hosts parasitized by the competitor (*A. chrysomphali*) (137.81 ± 27.07) and~~
303 ~~than in unparasitized hosts (61.62 ± 11.82) ($F_{1,76} = 8.84$; $P = 0.004$) (Fig. 2A). The~~
304 ~~interaction between host instar and competition was not significant ($F_{1,75} = 2.3$; $P =$~~
305 ~~0.13).~~

306 ~~According to the time spent probing, *Aphytis chrysomphali* females spent more~~
307 ~~time probing in third instar hosts (162.41 ± 14.02 sec) than in second instar hosts (53.52~~
308 ~~± 6.36) discriminated between second and third instar ($F_{1,54} = 41.15$; $P < 0.0001$) (Fig.~~

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2B). However, but they spent the same time probing in did not discriminate between
 hosts parasitized by the competitor (*A. melinus*) (116.56 ± 18.4) and unparasitized hosts
 (113.16 ± 13.08) ($F_{1,54} = 0.04$; $P = 0.84$) (Fig. 2B). The interaction between host instar
 and competition was not significant ($F_{1,53} = 0.063$; $P = 0.80$).

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3.2 Effect of host instar (size) on immature mortality, brood size and sex ratio in multiparasitized hosts

3.2.1 Immature mortality

Immature mortality was independent of host instar (*A. melinus*: $F_{1,76} = 3.89$; $P = 0.052$; *A. chrysomphali*: $F_{1,58} = 0.60$; $P = 0.44$) but was dependent of competition as immature mortality of *A. melinus* and *A. chrysomphali* increased significantly with multiparasitism (*A. melinus*: $F_{1,76} = 9.96$; $P < 0.001$; *A. chrysomphali*: $F_{1,58} = 12.96$; $P < 0.001$) (Table 3 and 4). The interaction between host instar and competition was not significant for any species (*A. melinus*: $F_{1,75} = 0.79$; $P = 0.78$; *A. chrysomphali*: $F_{1,57} = 0.06$; $P = 0.80$).

3.2.2 Brood size

In the case where at least one parasitoid emerged, the brood size of *A. melinus* and *A. chrysomphali* was independent of host instar (*A. melinus*: $F_{1,57} = 2.63$; $P = 0.11$; *A. chrysomphali*: $F_{1,33} = 2.12$; $P = 0.15$) and competition (*A. melinus*: $F_{1,57} = 0.29$; $P = 0.59$; *A. chrysomphali*: $F_{1,33} = 0.85$; $P = 0.36$) (Table 3 and 4). The interaction between host instar and competition was not significant for either parasitoid species (*A. melinus*: $F_{1,56} = 0.25$; $P = 0.62$; *A. chrysomphali*: $F_{1,32} = 0.49$; $P = 0.49$). In the second instar, both parasitoid species behaved as solitary.

3.2.3 Sex ratio

Secondary sex ratio of *A. melinus* was dependent of host instar ($F_{1,54} = 12.60$; $P < 0.001$) but it was not affected by competence ($F_{1,54} = 0.50$; $P = 0.48$) (Table 3). The

sex ratio of *A. melinus* in the second instar was male-biased, whereas it was female-biased in the third instar. The interaction between host instar and competition was not significant ($F_{1,53} = 0.011$; $P = 0.92$). *Aphytis chrysomphali* reproduced parthenogenetically as expected, and only females were recovered.

4. DISCUSSION

Effect of host instar (size) on host discrimination

Host discrimination by *A. melinus* and *A. chrysomphali* was found to be host instar (size) dependent. *Aphytis melinus* and *A. chrysomphali* were able to discriminate between unparasitized and heterospecific parasitized hosts when they encountered a third instar host (larger size) but this discriminatory ability was not observed in the second instar (smaller size) hosts. Rejection of the lower quality second instar hosts might occur under a greater range of physiological and ecological constraints (e.g., nutritional status, egg load and experience) than those under which third instar rejection occurs (Heimpel *et al.*, 1996; Fourrier *et al.*, 2004; Goubault *et al.*, 2005; Boivin & Brodeur, 2006; Hopper, 2013). As far as we are aware, intraspecific instar dependent host discrimination has not previously been reported. Goubault *et al.* (2004), however, tested the impact of host size on female decisions with two host species which differ greatly in size. Contrary to our results, the parasitoid *Pachycrepoideus vindemmiae* Rondani (Hymenoptera: Pteromalidae) was found to discriminate between parasitized and unparasitized hosts in the smaller species *Drosophila melanogaster* (Diptera: Drosophilidae) but not in the larger host species *Delia radicum* L. (Diptera: Anthomyiidae). This was attributed to the relative difficulty to find cues on bigger hosts (Goubault *et al.*, 2004).

Previous studies have documented that some *Aphytis* species are able to discriminate conspecifics parasitized hosts (Abdelrahman, 1974; Rosen & DeBach,

1979; van Lenteren & DeBach, 1981). However, heterospecific host discrimination in *Aphytis* parasitoids had not previously been investigated. Here, second instar (smaller) hosts are rejected by both *A. melinus* and *A. chrysomphali* after investigation it by antennal drumming and without inserting their ovipositor. This result reinforces Morgan and Hare (1997) observations, which considered that the primary physical cue derived from the scale cover is probably cover diameter and a kairomone, *O-caffeoyltyrosine*, in the cover for initial assessment of host quality (Morgan & Hare, 1997). Hosts rejection of heterospecific parasitized third instar hosts however, occurred significantly more frequently after females had inserted the ovipositor into the host. This suggests that the recognition of heterospecific parasitized hosts occurs in response to internal cues or physiological changes in the host after oviposition. A recent study of van Lenteren (2015) show that the neurons present in the sensillum of the ovipositor tip of *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) are used for host discrimination between parasitized and unparasitized larvae of *Drosophila* spp. (Diptera: Drosophilidae), and also to discriminate between hosts with different number of parasitoid eggs.

The behavioural strategy adopted during parasitism of a heterospecific-parasitized host was found to vary between species. *Aphytis chrysomphali* reduced its clutch size in heterospecific parasitized hosts, whereas *A. melinus* laid the same number of eggs in healthy and heterospecific parasitized hosts. When we compared the time spent parasitizing, *A. melinus* tended to probe heterospecific-parasitized hosts for longer than healthy ones, whereas there were not differences for *A. chrysomphali*. A reduction of clutch size has been widely cited in cases of superparasitism (Ikawa & Suzuki, 1982; van Dijken & Waage, 1987; Tena *et al.*, 2008), but has been documented only one in the case of heterospecific parasitism (Magdaraog *et al.*, 2013). Regarding the increased time spent probing in heterospecific-parasitized hosts, it is likely that *A. melinus* females were searching for the first clutch of eggs to commit ovicide and eliminate

387 competitors for their offspring, as suggested in the companion manuscript (Cebolla *et*
388 *al.*, 2017).

389 Finally, time costs may affect female propensity to multiparasitize, at least in
390 species that are time-limited (Strand & Godfray, 1989; Van Alphen & Visser, 1990).
391 *Aphytis* parasitoids, however, are egg-limited (Heimpel *et al.*, 1998; Casas & Nisbet,
392 2000; Casas & Mccauley, 2012; Tena *et al.*, 2015) and the same time is needed to
393 multiparasitize as to parasitize a healthy host, independently of host instar. Therefore,
394 time costs are negligible for both parasitoids when they multiparasitize.

395 **Effect of host instar (size) on immature mortality, brood size and sex ratio**
396 **in multiparasitized hosts**

397 A high cost in terms of immature mortality was incurred under multiparasitism
398 for both parasitoids, independent of host instar. When multiparasitism occurs, the
399 quality of the host may be disrupted by multiple regulatory factors as the injection of
400 polydnviruses and virus-like particles by the female parasitoid during oviposition
401 (Pennacchio & Strand, 2006; Beckage & Drezen, 2012) with consequences for the
402 developing larvae. In the most severe cases, hosts die as consequence of the attacks and
403 with them the larvae of both parasitoid species (Godwin & Odell, 1984; Lashomb *et al.*,
404 1987; Desneux *et al.*, 2009; Asgari & Rivers, 2011; Abram *et al.*, 2016). Larval
405 mortality under multiparasitism occurred in $37.5 \pm 10.1\%$ and $42.9 \pm 8.5\%$ of the
406 second and third instar hosts that were multiparasitized, respectively. Ovicide and
407 competition between immatures may have also affected offspring mortality (Cebolla *et*
408 *al.*, 2017).

409 Interspecific larval competition can also impact the development of immature
410 parasitoids and the fitness of the emerging offspring (Harvey, Poelman & Tanaka, 2013;
411 Cusumano *et al.*, 2013). Here, multiparasitism did not affect the secondary brood size
412 and sex ratio in either parasitoid species, independent of host instar. Although the sex
413 ratio of *A. melinus* was significantly higher (male-biased) in the second instar than in

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the third, it was not altered by multiparasitism. Similarly to results presented here, the secondary sex ratio was not affected by multiparasitism in a study of two egg parasitoids of *Nezara viridula* (L.) (Hemiptera: Pentatomidae), *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) and *Trissolcus basalis* (Wollaston) (Hymenoptera: Platygasteridae) (Cusumano *et al.*, 2013). Finally, the results obtained herein, together with those of Cebolla *et al.* (2017) provide new insights into the importance of host size in the competition between the sympatric parasitoids *A. melinus* and *A. chrysomphali*. That will help us to understand better the niche differentiation and the intrinsic competition that might contribute to their coexistence and/or the displacement of *A. chrysomphali* by *A. melinus*.

Acknowledgments

The authors would like to thank A. Mockford and two anonymous reviewers for her detailed review of the manuscript, Dr. A. Pekas for his comments in a previous version of the manuscript and P. García Meri (IVIA) for technical assistance with experiments. R. Cebolla received a predoctoral fellowship from the Spanish Ministry of Science and Innovation (BES-2012-058937). A. Tena received postdoctoral fellowships from INIA and IVIA. This work was partially funded through the Spanish Ministry of Science and Innovation (AGL2011-30538-C03-02) and the Conselleria d'Agricultura, Pesca i Alimentació de la Generalitat Valenciana.

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655 **Table 1.** Ratio of accepted (parasitized), rejected (before or after probing) and host-fed host (mean ± SE) when an unparasitized (control) and heterospecific
656 parasitized second- (N2) and third-instar (N3) *Aonidiella aurantii* were exposed to *Aphytis melinus*. Different upper-case and lower-case letters denote
657 significant differences at $P < 0.05$ between host instars and host status (unparasitized vs heterospecific parasitized) respectively.

HOST		Acceptance (Parasitized)	Rejected before probing	Rejected after probing	Host-fed
Instar	State				
N2	Parasitized by <i>A. chrysomphali</i>	0.43 ± 0.09 ^{Ba}	0.07 ± 0.05	0.1 ± 0.06	0.4 ± 0.09
	Unparasitized	0.67 ± 0.09 ^{Ba}	0.1 ± 0.06	0.1 ± 0.06	0.13 ± 0.06
N3	Parasitized by <i>A. chrysomphali</i>	0.77 ± 0.0 ^{Aa}	0.00	0.17 ± 0.07	0.07 ± 0.05
	Unparasitized	0.77 ± 0.08 ^{Aa}	0.07 ± 0.05	0.13 ± 0.06	0.03 ± 0.03

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660 **Table 2.** Ratio of accepted (parasitized), rejected (before or after probing) and host-fed host (mean $\pm$ SE) when an unparasitized (control) and heterospecific
 661 parasitized second- (N2) and third-instar (N3) *Aonidiella aurantii* were exposed to *A. chrysomphali*. Different upper-case and lower-case letters denote
 662 significant differences at $P < 0.05$ between instars and host status (unparasitized vs heterospecific parasitized) respectively.

HOST		Acceptance (Parasitized)	Rejected before probing	Rejected after probing	Host-fed
Instar	State				
N2	Parasitized by <i>A. melinus</i>	0.4 $\pm$ 0.09 ^{Aa}	0.27 $\pm$ 0.09	0.17 $\pm$ 0.06	0.17 $\pm$ 0.03
	Unparasitized	0.53 $\pm$ 0.09 ^{Aa}	0.33 $\pm$ 0.09	0.1 $\pm$ 0.07	0.03 $\pm$ 0.07
N3	Parasitized by <i>A. melinus</i>	0.43 $\pm$ 0.09 ^{Aa}	0.2 $\pm$ 0.03	0.33 $\pm$ 0.08	0.03 $\pm$ 0.03
	Unparasitized	0.67 $\pm$ 0.09 ^{Aa}	0.03 $\pm$ 0.07	0.27 $\pm$ 0.09	0.03 $\pm$ 0.03

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666 **Table 3.** Effect of host instar on immature mortality, secondary brood size (emerged larvae) and sex ratio (% males) (mean ± SE) -of *Aphytis melinus* when
667 females encountered an unparasitized (control) and heterospecific parasitized second- (N2) and third-instar (N3) *Aonidiella aurantii*. Different upper-case and
668 lower-case letters denote significant differences at $P < 0.05$ between instars and host status (unparasitized vs heterospecific parasitized) respectively.

Factor	Treatment	N2	N3
Immature mortality	Control	0.10 ± 0.07 (20) ^{Ab}	0.15 ± 0.07 (23) ^{Ab}
	Multiparasitism	0.27 ± 0.12 (13) ^{Aa}	0.57 ± 0.10 (13) ^{Aa}
2nd Brood size	Control	1 (18) ^{Aa}	1.10 ± 0.07 (21) ^{Aa}
	Multiparasitism	1 (10) ^{Aa}	1.18 ± 0.18 (11) ^{Aa}
Sex Ratio	Control	0.59 ± 0.12 (18) ^{Aa}	0.13 ± 0.07 (20) ^{Ba}
	Multiparasitism	0.70 ± 0.02 (10) ^{Aa}	0.20 ± 0.09 (10) ^{Ba}

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Table 4. Effect of host instar on immature mortality and secondary brood size (emerged larvae) (mean $\pm$ SE) of *Aphytis chrysomphali* when females encountered an unparasitized (control) and heterospecific parasitized second- (N2) and third-instar (N3) *Aonidiella aurantii*. Different upper-case and lower-case letters denote significant differences at $P < 0.05$ between instars and host status (unparasitized vs heterospecific parasitized) respectively.

Factor	Treatment	N2	N3
Immature mortality	Control	0.31 $\pm$ 0.12 (16) ^{Ab}	0.13 $\pm$ 0.06 (19) ^{Ab}
	Multiparasitism	0.75 $\pm$ 0.13 (12) ^{Aa}	0.69 $\pm$ 0.13 (13) ^{Aa}
2nd Brood size	Control	1 (11) ^{Aa}	1.16 $\pm$ 0.08 (19) ^{Aa}
	Multiparasitism	1 (3) ^{Aa}	1 (4) ^{Aa}

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680 **Figure legends.**

681 **Fig. 1.** Clutch size (mean $\pm$ SE) of *Aphytis melinus* (Fig. 1A) and *A. chrysomphali* (Fig.
682 1B) when they accepted an unparasitized (control) and heterospecific parasitized
683 second- (N2) and third-instar (N3) of *Aonidiella aurantii*. Different upper-case and
684 lower-case letters beside bars denote significant differences at $P < 0.05$ between instars
685 and host status (unparasitized vs heterospecific parasitized) respectively.

686 **Fig. 2.** Host handling time (mean $\pm$ SE) (time spent probing + time spent parasitizing)
687 of *Aphytis melinus* (Fig. 2A) and *A. chrysomphali* (Fig. 2B) when they accepted an
688 unparasitized (control) and heterospecific parasitized second- (N2) and third-instar (N3)
689 *Aonidiella aurantii*. Different letters inside and besides the bars denote significant
690 differences at $P < 0.05$ in the time spent probing and time spent parasitizing
691 respectively. Differences between host instars are represented in different upper-case
692 letters and between host status (unparasitized vs heterospecific parasitized) in lower-
693 case letters.

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Fig. 1A.

Fig. 1B.

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708 **Fig. 2A**

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712 **Fig. 2B**