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## PhD THESIS

Effect of competition between *Aphytis chrysomphali* (Mercet) and *A. melinus* DeBach (Hymenoptera: Aphelinidae), on their coexistence and efficacy as biological control agents of *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae)



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**“Effect of competition between *Aphytis chrysomphali* (Mercet) and *A. melinus* DeBach (Hymenoptera: Aphelinidae), on their coexistence and efficacy as biological control agents of *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae)”.**

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## SUMMARY

The California red scale *Aonidiella aurantii* is considered a key citrus pest. Parasitoids of genus *Aphytis* are the most important natural enemies of *A. aurantii*. In the Mediterranean basin, the native *A. chrysomphali* and the introduced *A. melinus* are the most abundant parasitoids of *A. aurantii*. The introduced has completely displaced the native *A. chrysomphali* in the south, whereas they coexist in the north-east of the Iberian Peninsula. We have used this well-known host-parasitoid system to investigate some gaps on the behavioral ecology of hymenopteran parasitoids, which have been exposed in the introduction. This knowledge will contribute to improve their use in biological control programs, especially in the case of *A. aurantii* in the Mediterranean basin.

In chapter 3, we showed that females of *A. melinus* and *A. chrysomphali*, after stinging their hosts, tended to reject their host without ovipositing or feeding on it (overstinging). Although overstinging is commonly observed in parasitoids, its frequency of occurrence and consequences for host physiology have been generally disregarded in hymenopteran literature. We evaluated its occurrence in both parasitoids and we found that it was even more common than host-feeding. We also determined the effect of overstinging on *A. aurantii*. The virulence of overstinging depended on the host instar attacked and the parasitoid species. Most young-instar hosts (second-instar) died when they were overstung, whereas ~50% of the adults (third-instar) survived. Our results also showed that *A. melinus* was more aggressive than *A. chrysomphali*, as the former killed more adult hosts when overstung and, moreover, reduced the fecundity of the surviving females. With all this said,

overstinging should be considered in the future selection of parasitoids for biological control; and its lethal and sub-lethal effects should be incorporated in the models due to this behavior might affect stability in host-parasitoid population models.

Host discrimination is the ability of parasitoids to discriminate between healthy hosts and hosts previously parasitized. In chapter 4, we evaluated whether the ability to discriminate between unparasitized and heterospecific parasitized hosts depends on host instar. *Aphytis* parasitoids were able to discriminate between unparasitized and heterospecifically parasitized hosts but their ability was host instar mediated. They were able to discriminate when they found third-instar hosts (larger size) but this ability was not observed in the second-instar hosts (smaller size). To our knowledge, this is the first reference that demonstrates that the ability of parasitoids to discriminate between healthy and parasitize hosts depends on the instar of the host and should be considered in further research.

In chapter 5, we evaluated how direct competition affects the coexistence of *A. melinus* and *A. chrysomphali*. In a recent study, Pekas et al. (2016) determined that one factor that could explain the coexistence of *A. chrysomphali* and *A. melinus* in some Mediterranean areas could be the plasticity of *A. chrysomphali* in exploiting host of poor quality (second instar) when *A. melinus* density is high. Another non-exclusive reason could be that *A. chrysomphali* is a better competitor when both species compete for the second instar but not for the third. Our behavioral assay shows, however, that *A. melinus* emerged at higher rates independently of host size (instar) and sequence of attack in multiparasitized hosts. These results corroborate the hypothesis of Pekas et al. (2016) and suggest that *A. chrysomphali* detects, quantifies and avoids the presence of its competitor. The superiority of *A. melinus*

was explained by i) higher acceptance rates and ii) lower immature mortality; and, iii) tendency to kill the eggs of *A. chrysomphali* (i.e. ovicide). Overall, our results in this chapter show that interference competition will contribute to the displacement of the native *A. chrysomphali* by *A. melinus*.

One of the causes that can explain the coexistence of *A. chrysomphali* and *A. melinus* in the citrus Mediterranean areas is the variation on their relative proportion depending on the spatiotemporal weather conditions and seasonal changes (Sorribas, 2010). Mainly, hot summer temperatures affect more negatively *A. chrysomphali* than *A. melinus*. Taking it into consideration and that an increase of 3°C is expected in summer due to Global warming (IPCC, 2014), we hypostasized that the superiority of *A. melinus* under these conditions might be accentuated leading ultimately to the *displacement* of *A. chrysomphali*. Therefore, in chapter 6, we evaluated how global warming and direct competition can affect the coexistence of *A. melinus* and *A. chrysomphali* and the biological control of *A. aurantii*. Our results show that the increase of temperature in summer will not affect the population of *A. chrysomphali* but its populations will be reduced by half due to direct competition. On the other hand, we observed that the increase of temperature will hinder the potential of *A. melinus* as biological control agent of *A. aurantii*, as its  $R_0$  will be reduced by half with the temperature increase. The reduction was mostly due to the decrease in the proportion of females in the *A. melinus* progeny. The negative effect of the increase of temperature in *A. melinus* populations will be mitigated by the presence of *A. chrysomphali*. Under the expected summer temperatures, the introduced parasitoid had a higher  $R_0$  when females compete with *A. chrysomphali* than when they searched alone in a patch. Overall, our study highlights the importance of considering competition to predict the consequences of global warming in any further research about biological control.



## RESUMEN

El piojo rojo de California, *Aonidiella aurantii*, se considera una de las plagas de los cítricos más importante a nivel mundial. Los principales enemigos naturales de *A. aurantii* son los parasitoides del género *Aphytis*, siendo los más abundantes en la cuenca mediterránea el nativo *Aphytis chrysomphali* y el introducido *A. melinus*. En las zonas del sur de la Península Ibérica, *A. melinus* ha desplazado completamente al nativo *A. chrysomphali*, mientras que en el noreste ambos parasitoides coexisten. Durante el desarrollo de esta tesis, hemos utilizado este conocido sistema de parasitoides-hospedante para investigar algunos aspectos poco conocidos en la ecología del comportamiento de los parasitoides himenópteros, los cuáles han sido mencionados en la introducción de la tesis. Este conocimiento contribuirá a mejorar su uso en programas de control biológico, especialmente en el caso de *A. aurantii* en la cuenca mediterránea.

Las hembras de *A. chrysomphali* y *A. melinus*, después de picar a sus hospedantes, pueden rechazarlo sin realizar la puesta ni alimentarse de éste (“overstinging”). Aunque este comportamiento se había observado que era común en parasitoides, la frecuencia de su ocurrencia, así como las consecuencias de éste en la fisiología del hospedante ha sido poco estudiada en la literatura de himenópteros. En el capítulo 3 se observó que la ocurrencia de este comportamiento fue incluso más común que las picaduras de alimentación. Además, su virulencia dependió del estadio del hospedante atacado y de la especie de parasitoide. La mayoría de los hospedantes en estadio joven (segundo estadio) murieron al ser picados, mientras que ~50% de los adultos (tercer estadio) sobrevivieron. Nuestros resultados también mostraron que *A. melinus* fue más agresivo que *A. chrysomphali*, ya que el primero mató más hospedantes adultos

y, además, redujo la fecundidad de las hembras que sobrevivieron. Dicho todo esto, nuestros resultados recalcan que este comportamiento debería considerarse en la futura selección de parasitoides para el control biológico y, sus efectos letales y sub-letales sobre el hospedante, deberían ser incorporados en los modelos poblacionales parasitoides-hospedante ya que este comportamiento puede afectar a la estabilidad de sus poblaciones.

La discriminación del hospedante es la capacidad de algunos parasitoides en diferenciar entre hospedantes sanos y aquellos que ya han sido previamente parasitados. En el capítulo 4, se determinó que las hembras de *Aphytis* fueron capaces de discriminar entre hospedadores sanos y aquellos heteroespecíficamente parasitados y que esta capacidad estuvo influenciada por el estadio del hospedante. Las hembras de *Aphytis* fueron capaces de discriminar cuando se encontraron hospedantes de tercer estadio (tamaño más grande) pero no en los hospedantes del segundo estadio (tamaño más pequeño). Hasta donde sabemos, esta es la primera referencia que demuestra que la capacidad de los parasitoides para discriminar entre hospedadores sanos y parasitarios depende del estadio del hospedante y por ello, debe considerarse en futuras investigaciones.

En el capítulo 5 se evaluó cómo la competencia directa afecta la coexistencia de *A. chrysomphali* y *A. melinus*. En un estudio reciente, Pekas et al. (2016) determinó que un factor que podría explicar la coexistencia de ambos parasitoides en algunas áreas del Mediterráneo podría ser la plasticidad de *A. chrysomphali* de utilizar hospedantes de segundo estadio (baja calidad) cuando la densidad del competidor superior *A. melinus* era alta. Otra razón, no exclusiva de la anterior, podría ser que *A. chrysomphali* fuera un mejor competidor cuando ambas especies compiten por el segundo estadio pero no por el tercero. Nuestro ensayo mostró, sin embargo, que

*A. melinus* fue el parasitoide con mayores tasa de emergencia en aquellos hospedantes multiparasitados por ambas especies independientemente del estadio del hospedante y de la secuencia de ataque. Estos resultados corroboran la hipótesis de Pekas et al. (2016) y sugieren que *A. chrysomphali* es capaz de detectar, cuantificar y evitar la presencia de su competidor. La superioridad de *A. melinus* se explicó por i) sus mayores tasas de aceptación y ii) menor mortalidad de inmaduros; y, iii) su tendencia a matar los huevos de *A. chrysomphali* en aquellos hospedantes previamente parasitados por éste (es decir, realizar ovicidio). En general, nuestros resultados en este capítulo muestran que la competencia de interferencia será un factor que contribuirá en el desplazamiento del parasitoide nativo *A. chrysomphali* por *A. melinus*.

Otra de las causas que puede explicar la coexistencia de *A. chrysomphali* y *A. melinus* en las zonas citrícolas del noreste de la Península Ibérica, es la variación de su proporción relativa en función de las condiciones meteorológicas espacio-temporales y los cambios estacionales (Sorribas et al., 2010). Principalmente, las temperaturas altas del verano afectan más negativamente a *A. chrysomphali* que a *A. melinus*. Teniendo esto en cuenta, y que se espera un aumento de 3 °C en verano para finales del siglo XXI debido al Calentamiento Global (IPCC, 2014), la superioridad de *A. melinus* podría verse acentuada desplazando finalmente a *A. chrysomphali* de estas áreas dónde ambos parasitoides aún coexisten. En el capítulo 6 se evaluó cómo el calentamiento global afectará a la coexistencia de ambos parasitoides y al control biológico de *A. aurantii*. Nuestros resultados mostraron que el aumento de temperatura estimado no afectará a *A. chrysomphali*, pero su tasa reproductiva neta ( $R_0$ ), y por tanto su población, será reducida a la mitad debido a la competencia directa con *A. melinus*. Por otro lado, se observó que el aumento de la temperatura si afectará negativamente al potencial de *A. melinus* como agente de control biológico de *A. aurantii*, ya que su  $R_0$  se reducirá a la mitad con el aumento de la temperatura. Esta reducción se debió principalmente

a la disminución de la proporción de hembras en la descendencia de *A. melinus* con el aumento de temperaturas. Por otro lado, se observó que la competencia mitigará este efecto negativo de las temperaturas sobre las poblaciones de *A. melinus*. Esto se pudo observar ya que *A. melinus* tuvo un  $R_0$  más alto cuando las hembras competían con *A. chrysomphali* en condiciones de temperatura de verano estimadas que cuando no estaban compitiendo. En general, nuestro estudio destaca la importancia de considerar la competencia para predecir las consecuencias del calentamiento global en cualquier investigación adicional sobre el control biológico.

## RESUM

El poll roig de Califòrnia, *Aonidiella aurantii*, és considerada una de les plagues de cítrics més important a nivell mundial. Els principals enemics naturals de *A. aurantii* són els parasitoids del gènere *Aphytis*, sent els més abundants en la conca mediterrània el natiu *Aphytis chrysomphali* i l'introduït *A. melinus*. A les zones del sud de la Península Ibèrica, *A. melinus* ha desplaçat completament al natiu *A. chrysomphali*, mentre que en el nord-est tots dos parasitoids coexisteixen. Durant el desenvolupament d'aquesta tesi, hem utilitzat aquest conegut sistema de parasitoids-hoste per investigar alguns aspectes poc coneguts en l'ecologia del comportament dels parasitoids himenòpters. Aquest coneixement contribuirà a millorar el seu ús en programes de control biològic, especialment en el cas d'*A. aurantii* en la conca mediterrània.

Les femelles d'*A. melinus* i *A. chrysomphali*, després de picar als seus hostes, poden rebutjar-lo sense dur a terme la posta ni alimentar-se d'aquest ("overstinging"). Encara que aquest comportament s'havia observat que era comú en els parasitoids, la freqüència de la seva ocurrència, així com les conseqüències d'aquest en la fisiologia de l'hoste ha sigut poc estudiat en la literatura d'himenòpters. En el capítol 3 es va observar que l'ocurrència d'aquest comportament va ser fins i tot més comú que les picades d'alimentació. A més, la seva virulència va dependre de l'estadi de l'hoste atacat i de l'espècie de parasitoid. La majoria dels hostes en estadi jove (segon estadi) va morir en ser picats, mentre que ~50% dels adults (tercer estadi) van sobreviure. Els nostres resultats també van mostrar que *A. melinus* va ser més agressiu que *A. chrysomphali*, ja que el primer va matar més hostes adults i, a més, va reduir la fecunditat de les femelles que van sobreviure. Dit tot això, els nostres resultats remarquen que aquest comportament hauria de considerar-se en la futura selecció de parasitoids per al control biològic i, els seus efectes letals i sub-letals,

haurien de ser incorporats en els models poblacionals parasitoids-hoste ja que aquest comportament pot afectar a l'estabilitat de les seues poblacions.

La discriminació de l'hoste és la capacitat d'alguns parasitoids a diferenciar entre hostes sans i hostes prèviament parasitats. En el capítol 4 es va determinar que les femelles d'*Aphytis* eren capaces de discriminar entre hostes sans i aquells parasitats per l'altra espècie i que aquesta capacitat va dependre de l'estadi de l'hoste. Les femelles d'*Aphytis* van poder discriminar quan es van trobar hostes de tercer estadi (major grandària) però aquesta capacitat no es va observar en els hostes del segon estadi (menor grandària). Fins on sabem, aquesta és la primera referència que demostra que la capacitat dels parasitoids per discriminar entre hostes sans i parasitats depèn de l'estadi del hoste i per això, ha de considerar-se en futures investigacions.

En el capítol 5 es va avaluar com la competència directa afecta la coexistència d'*A. chrysomphali* i *A. melinus*. En un estudi recent, Pekas et al. (2016) va determinar que un factor que podria explicar la coexistència de tots dos parasitoids en algunes àrees del Mediterrani podria ser la plasticitat d'*A. chrysomphali* d'utilitzar hostes de segon estadi (baixa qualitat) quan les densitat del competidor superior *A. melinus* era alta. Una altra raó, no exclusiva de l'anterior, podria ser que *A. chrysomphali* fos un millor competidor quan ambdues espècies competeixen pel segon estadi però no pel tercer. El nostre assaig va mostrar, no obstant això, que *A. melinus* va ser el parasitoid amb major taxa d'emergència en aquells hostes multiparasitats per ambdues espècies independentment de l'estadi del hoste i de la seqüència d'atac. Aquests resultats corroboren la hipòtesi de Pekas et al. (2016) i suggereixen que *A. chrysomphali* és capaç de detectar, quantificar i evitar la presència del seu competidor. La superioritat d'*A. melinus* es va explicar per i) les seves majors taxes d'acceptació; ii) menor mortalitat d'imadurs; i, iii) la seva tendència a matar els ous d'*A. chrysomphali*

en aquells hostes prèviament parasitats (és a dir, realitzar ovicidi). En general, els resultats d'aquest capítol mostren que la competència d'interferència serà un factor que contribuirà al desplaçament del parasitoid natiu *A. chrysomphali* per *A. melinus*.

Una altra de les causes que pot explicar la coexistència d'*A. chrysomphali* i *A. melinus* a les zones citrícoles del nord-est de la Península Ibèrica, és la variació de la seva proporció relativa en funció de les condicions meteorològiques espai-temporals i els canvis estacionals (Sorribas et al., 2010). Principalment, les temperatures altes de l'estiu afecten més negativament a *A. chrysomphali* que a *A. melinus*. Tenint això en compte, i que s'espera un augment de 3 °C a l'estiu per a finals del segle XXI a causa de l'Escalfament Global (IPCC, 2014), la superioritat d'*A. melinus* podria veure's accentuada, desplaçant finalment a *A. chrysomphali* d'aquestes àrees on tots dos parasitoids encara coexisteixen. En el capítol 6 es va avaluar com l'escalfament global afectarà a la coexistència de tots dos parasitoides i al control biològic d'*A. aurantii*. Els nostres resultats van mostrar que l'augment de temperatura estimat no afectarà a *A. chrysomphali*, però la seva taxa reproductiva neta ( $R_0$ ), i per tant la seva població, serà reduïda a la meitat a causa de la competència directa amb *A. melinus*. D'altra banda, es va observar que l'augment de la temperatura sí afectarà negativament al potencial de *A. melinus* com a agent de control biològic d'*A. aurantii*, ja que la seva  $R_0$  es reduirà a la meitat amb l'augment de la temperatura. Aquesta reducció es va deure principalment a la disminució de la proporció de femelles en la descendència de *A. melinus* amb l'augment de temperatures. D'altra banda, es va observar que la competència mitigarà aquest efecte negatiu de les temperatures sobre les poblacions d'*A. melinus*. Això es va poder observar perquè *A. melinus* va tenir un  $R_0$  més alt quan les femelles competien amb *A. chrysomphali* en condicions de temperatura d'estiu estimades que quan no estaven competint. En general, el nostre estudi destaca la importància de considerar la competència per predir les conseqüències de l'escalfament global en qualsevol recerca addicional sobre el control biològic.



Chapter 1

# GENERAL INTRODUCTION





# 1. GENERAL INTRODUCTION

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## 1.1. Parasitoids as biological control agents

Parasitoids are insects whose adult females lay their eggs in or on other invertebrates, and whose larvae develop by feeding on the host body, resulting in its death (Godfray, 1994; Jervis, 2005). Based on egg allocation and larval developing system, there are different types of parasitoids. Endoparasitoids develop within the host body, as opposed to ectoparasitoids, which develop outside the host body. Koinobiont parasitoids allow hosts to continue to grow in size after parasitism, whereas idiobionts prevent further development of the host after initial parasitization. Parasitoids that develop alone on a host are known as solitary parasitoids, as opposed to gregarious parasitoids, which develop as multiple larvae together on a single host. Based on the adult female biology, parasitoids may be univoltine, when they complete one generation with one generation of the host, or multivoltine, when they complete two or more generations within one generation of the host. When females emerge with a reduced number of ovarian eggs but more eggs are produced through their life span, they are termed as synovigenic, whereas females of proovigenic parasitoids emerge with all their mature eggs (Godfray, 1994; Quicke, 1997). Currently, the ovigeny index, defined as the proportion of the potential lifetime complement of eggs that is mature at female emergence, is used to measure the degree to which egg production is concentrated into early adult life (Jervis et al., 2001, 2008).

It is estimated that, on a worldwide basis, about 68,000 species of parasitoids are described (Godfray, 1994). Most parasitoids belong to two orders: Diptera (the true

flies) and Hymenoptera, which contain about 15,000 and 50,000 known species of parasitoids, respectively (Godfray, 1994; Quicke, 1997; Jayashankar et al., 2016).

Hymenopteran parasitoids are the most successful group of natural enemies used in the biological control of pest populations (Godfray, 1994; Jervis, 2005; Mills & Wajnberg, 2008). The success of various major families of natural pest enemies have been recently reviewed (Heimpel & Mills, 2017). According to this review, the family Aphelinidae, which is the object of this thesis, is the most successful in terms of pest management (Heimpel & Mills, 2017).

There are three broad categories that describe how parasitoids can be used in biological control: classical, augmentation, and conservation (Mills & Wajnberg, 2008). In **classical biological control**, a natural enemy is imported and established from the region of origin of an exotic pest to provide long-term control (Debach, 1964; Hoddle, 2004; Mills & Wajnberg, 2008; Heimpel & Mills, 2017). The import and introduction of natural enemies have, however, decreased in the last few decades due to concerns about their potential non-target impacts and the sovereign rights over genetic resources (Follett & Duan, 2000; Bigler et al., 2006; van Lenteren et al., 2006; Orr, 2009; Cock et al., 2010). The hazards related to non-targets impacts involve the possibility of a global or local extinction of a native species, substantial reduction in either the distribution or abundance of native organisms, interference in the efficacy of native natural enemies of pests via intraguild interactions or competitive displacement, vectoring of pathogens harmful to native organisms, loss of biodiversity and identity of native species via hybridization between close relatives, and, in general, any major shifts in the balance of native species via direct or indirect mechanisms (van Lenteren et al., 2006; Heimpel & Mills, 2017). To solve these issues, strict regulations based on scientific methods are used to evaluate the risks before

exotic natural enemies are released (van Lenteren et al., 2003; Bigler et al., 2006; Hajek et al., 2016; Heimpel & Mills, 2017). Unfortunately, the increasing number of guidelines and regulations results in delayed implementation of classical biological control (van Lenteren et al., 2010). Moreover, under the Convention of Biological Diversity (CBD, 1993), countries have sovereign rights over their genetic resources, and the access to these resources and the sharing of the benefits arising from their use need to be established between involved parties (i.e., Access and Benefit Sharing) (Cock et al., 2010). This also applies to collection and export of natural enemies, and its principles have made classical biological control difficult or impossible, disturbing the biological control research in several countries (van Lenteren et al., 2017).

In **augmentative** biological control, natural enemies are mass-reared for release in large numbers, either to achieve immediate control of pests in crops with a short production cycle (inundative biological control) or for control of pests during several generations in crops with a long production cycle (seasonal inoculative biological control) (Cock et al., 2010; Lorito et al., 2010; van Lenteren, 2012; Parnell et al., 2016; van Lenteren et al., 2017). Nowadays, 350 invertebrate species are used as biological control agents in pest management and there are about 500 commercial producers worldwide (van Lenteren et al., 2017). Augmentation has been applied with success for more than 100 years in several cropping systems (Gurr & Wratten, 2000; Cock et al., 2010). In greenhouses, the use of generalist predators in augmentative biological control has almost displaced the use of parasitoids. Some examples include the complete replacement of chemical pesticides by predators (predatory mites and predatory bugs) to control thrips and whiteflies on sweet peppers in greenhouses in Spain (Calvo et al., 2012), and the use of predatory mirids to manage tomato key pests such as the South American pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Urbaneja et al., 2012b).

Finally, **conservation biological** control focuses on the better use of existing parasitoids to improve natural biological control through habitat manipulation and the reduction of pesticide-induced harm to the natural enemies (Heimpel & Mills, 2017). Various measures are implemented to enhance the abundance or activity of the natural enemies, including manipulation of the crop microclimate, creation of overwintering refuges, increasing the availability of alternative hosts and prey, and providing essential food resources such as pollen or nectar (Gurr et al., 2000, 2017; Wäckers, 2003; Lee & Heimpel, 2008; Winkler et al., 2009; Tena et al., 2016). Under the current scenario, with limited access to the import of exotic biological control agents, and growing social demand to increase ecological infrastructures and reduce the use of pesticides, conservation and augmentation biological control should be the basis of most crop-protection programs, which should provide sufficient invertebrate biological control agents to manage the agricultural pests.

## 1.2. Behavioral ecology of hymenopteran parasitoids

### 1.2.1. Foraging and oviposition behavior

For each of the categories explained above, the success of the parasitoid depends upon the behavioral decisions made by females when they search for and find a host (Mills & Wajnberg, 2008). The division of successful parasitism into the hierarchical process of host **habitat location**, **host location**, and **host acceptance** (oviposition) has been immensely influential and has been adopted by nearly all authors reviewing the subject (Flanders, 1953; Doutt, 1959; Vinson, 1976, 1998; van Alphen & Vet, 1986; Godfray, 1994; Heimpel & Casas, 2008; Mills & Wajnberg, 2008).

During the **habitat location process**, females find and explore a great variety of stimuli, among which chemical (infochemicals), olfactory, and visual cues play a relevant role (Vinson, 1976, 1998; Vet & Dicke, 1992; Godfray, 1994; Wäckers & Lewis, 1994; Fellowes et al., 2005; Bai et al., 2011; Wajnberg & Colazza, 2013). These cues elicit a series of directed responses by the female, which serve to reduce and restrict the area and habitats searched, and the species of host thus located (Vinson, 1976).

Infochemicals are divided into pheromones, which act intraspecifically, and allelochemicals, which act interspecifically. Allelochemicals are themselves subdivided into synomones (when they evoke in the receiver a response that is adaptively favorable to both the receiver and the emitter), kairomones (when the receiver response is adaptively favorable only to the receiver), and allomones (when the receiver response is adaptively favorable only to the emitter (Dicke & Sabelis, 1988)). The majority of parasitoids respond to volatile kairomones or synomones in the long-distance location of their hosts. These chemicals may originate from: i) the host itself, e.g., from frass, during molting and feeding, sex pheromones, and aggregation pheromones, ii) from the host plant, or iii) from some interaction between the host and the food plant, e.g., feeding damage.

When a parasitoid locates a potential host habitat, it begins the next phase in the **search for hosts**. Species of parasitoids attacking the same host may differ in the way they search a patch, and at least three different searching modes exist (van Alphen & Vet, 1986): i) **ovipositor search** (probe the microhabitat with their ovipositor until they contact a host, ii) **vibrotaxis** (perceive vibrations in the microhabitat caused by movements of the host and use these cues to orient themselves to the host), iii) **antennal search** (through drumming the surface of the microhabitat with their

antennae until they contact a host). Often, the searching behavior of a parasitoid comprises a combination of search modes, as the insect responds to different cues while locating a host (Jervis, 2005). While parasitoids can respond to **visual, tactile, and chemical cues to locate their hosts**, chemical cues play the predominant role and are the best known and studied ones (Vinson, 1976, 1991, 1998; Stubbs, 1980; Carter & Dixon, 1984; Vet & Dicke, 1992; Godfray, 1994; Colazza et al., 2009; Peri et al., 2013). Often, insects show arrestment behavior in response to contact with the kairomones of low volatility deposited by their hosts on the substratum. Material containing such kairomones (also called contact chemicals) have been shown to include host salivary gland or mandibular gland secretions, host frass, and hemipteran honeydew, and cuticular secretions (Jervis, 2005). Some parasitoid species are known to leave chemical marks on the surfaces they have searched (van Alphen & Galis, 1983; Sheehan et al., 1993), and this marking behavior can have a number of functions. By leaving a trace mark on the substratum, a parasitoid can avoid wasting time and energy in searching already visited areas. Moreover, the frequency with which a female encounters the marks can be used to determine how well it has searched the patch and to decide when to leave the patch. When marks are encountered by conspecific or heterospecific competitors, sometimes they induce the competitor to leave the area. However, leaving the patch may not always be in the interest of the competitor and it can decide to stay and super/multiparasitize.

**Host acceptance**, upon its detection, has also been divided into hierarchical processes: recognition, external examination, probing (internal examination), and oviposition (Vinson, 1976; Godfray, 1994). Different host-associated stimuli are necessary for the recognition and acceptance of a prospective host for oviposition. The kairomones present on the host play a very important role in **host recognition** by parasitoids ((Strand & Vinson, 1982). Many species, especially those attacking

immobile hosts, spend a great deal of time externally **examining** the host, often by stroking or drumming with their antennae (Godfray, 1994). Chemical cues perceived through receptors in the antennae and tarsi are undoubtedly of great importance in host acceptance. Many of the chemicals used in host location are also likely to be involved in host acceptance. In addition, nonvolatile chemicals present on the surface of the host have also been shown to be the stimuli for oviposition by several parasitoid species ((Bénédet et al., 1999; Battaglia et al., 2000; Calatayud et al., 2001; Conti et al., 2003; Takasu & Lewis, 2003). Chemical cues may act in conjunction with shape, size, and texture (Hare & Luck, 1994). After external examination, many parasitoids insert their ovipositor into the host to obtain additional information about its suitability. Parasitoid may assess the suitability of the host using chemical cues, or possibly by detecting the heartbeat of a healthy host (Fisher, 1971). Sometimes, after internal examination, females reject the host (Heimpel & Collier, 1996; Heimpel et al., 1998; Tena et al., 2008; Hopper et al., 2013). This behavior is common in hymenopteran parasitoids and is known as “mutilation”, “probe/sting and rejection”, or “overstinging”. In the few studied cases, the consequences of overstinging vary from reduced fitness of the wounded hosts (mutilation) to host death (Abdelrahman, 1974b; Jones, 1985; Jones et al., 1986; Brown & Kainoh, 1992). However, despite its prevalence and the damage caused to the hosts, this behavior has been largely disregarded in the parasitoid literature, and overstinging should be an important trait in the selection of parasitoids for biological control programs.

### 1.2.2. Host quality and host utilization

Acceptance of a prospective host for oviposition depends on the quality of the located host. Variation in **host quality often depends on the age and size of the hosts**, which are generally correlated (Luck & Podoloe, 1985; Pekas et al., 2010;

Kapranas & Tena, 2015) **and host status** (previous parasitization by competitors). Host size is especially critical for idiobiont parasitoids, which kill or immobilize the host at parasitism. Small hosts may not provide adequate amounts of resources for the developing progeny, and when the hosts are very small, resources for parasitoid development may be so scarce that the parasitoids fail to mature and die. Even when successful developmental progeny is possible in small hosts, the offspring are small, and therefore, oviposition constitutes a low fitness gain. In koinobiont hosts, parasitoids continue to grow after parasitism, although their final size may be related to their size at parasitization (Godfray, 1994; Harvey et al., 1994, 1999). The importance of host size in influencing the survival and adult size has been demonstrated numerous times (Opp & Luck, 1986; Yu, 1986; Reeve, 1987; Rosenheim & Rosen, 1991; Pina 2007). Parasitoid body size determines components of fitness such as fecundity, longevity, and searching efficiency (Charnov et al., 1988; Luck et al., 1982; Luck & Podoler, 1985; Opp & Luck, 1986; Reeve, 1987; Rosenheim & Rosen, 1991; Godfray, 1994; Jervis, 2005; Kapranas et al, 2009; Pekas et al., 2010). Parasitoids should choose to obviously attack the best hosts and ignore any host with a low probability of successful larval development.

Apart from oviposition, hosts may also be used as a source of food. Many parasitoids, and particularly idiobionts, which are often synovigenic, need to feed as adults on the host hemolymph in order to gain nutrients (Bartlett, 1964). **Host-feeding** is achieved through a hole made by the parasitoid's ovipositor. Host-feeding can be divided into two types: concurrent (parasitoid feeds from the same host as it oviposits in) and non-concurrent (parasitoid feeds from a different individual) (Jervis & Kidd, 1986). Concurrent host-feeding must be non-destructive. In contrast, non-concurrent host-feeding need not leave a viable host, and is frequently, though not always, destructive (Quicke, 1997). **If parasitoids are given a choice of different-sizes hosts, they often**

**choose to feed from relatively small hosts (lower quality) and lay eggs on relatively larger hosts (higher quality)**, to increase the fitness of the progeny (Abdelhraman, 1974b; van Alphen, 1980. Kapranas & Tena, 2015).

The sex of the eggs laid by most hymenopteran parasitoids is under direct behavioral control of the mother (Godfray, 1994). Most parasitoids are arrhenotokous haplodiploids; this genetic system means that diploid females develop from fertilized eggs and haploid males develop from unfertilized eggs (Flanders, 1953; Rosen & DeBach, 1979; Heimpel & Boer, 2008). Models based on sex allocation theory predict that when the fitness gains from larger size differ between male and female offspring, mothers should produce the sex that will offer the greatest investment return (Trivers & Willard, 1973; West, 2009). It has long been observed that some Hymenopteran parasitoids respond to varying host size (i.e., host quality) by producing female offspring in large-sized (high-quality) hosts and males in small-sized (lower-quality) hosts (Chewyreu, 1913; Clausen, 1939; Charnov, 1982; King, 1987; Godfray, 1994; Beltra et al., 2014). Charnov et al. (1988) were the first to explain how this common sex allocation pattern in parasitoids may be adaptive. They argued that females would benefit more than males from developing in large hosts. The size of the adult is strongly correlated with the size of the host in which it develops, and the fecundity, and hence, fitness of female wasps is usually strongly correlated with adult size (Charnov et al., 1988; Jones, 1982; King, 1988; Heinz, 1991; Ode et al., 1996; Ueno, 1999; van den Assem et al., 1989). Male wasps may also benefit from being large, but small size probably impairs mating less than it impairs oviposition (Godfray, 1994).

Additional decisions have to be made by **gregarious** parasitoids, as females need to decide how many eggs to lay in a single host (clutch size). This clutch size is strongly affected by the host size. Generally, **parasitoids lay more eggs on larger hosts, and**

**smaller clutches are predicted on smaller hosts**, as they provide fewer resources for the parasitoid larvae. Lack's original hypothesis has been generalized by affirming that a mother should lay the number of eggs that maximizes her gain in fitness from the whole clutch (Godfray, 1987, 1994). The combined fitness for the surviving brood represents a dynamic trade-off between the number of offspring produced from a clutch and the size of the offspring (Charnov & Skinner, 1984; Waage & Godfray, 1985; Godfray, 1994). The greater the clutch size, greater is the degree of exploitative competition between the developing parasitoid larvae and smaller are the resulting offspring adults (e.g., Takagi, 1985; Hardy et al., 1992; Vet et al., 1994; Zaviezo & Mills, 2000). As female size and fitness are positively correlated in parasitoids, this means that the size of the offspring produced in a clutch will affect the subsequent fitness gain of the parent female (Visser, 1994; Petersen & Hardy 1996; Zaviezo & Mills, 2000).

Apart from host size, **host acceptance also depends upon whether the host is already parasitized**. It could either contain eggs from a different parasitoid species, from the same species, or even from the female itself. A parasitized host is considered of lower quality compared with a healthy host (Tena et al., 2008; Harvey et al., 2009, 2013; Cusumano et al., 2015). Parasitized hosts have a reduced amount of food because some portion of the limited host resources has already been ingested by the larvae that first occupied the host. Moreover, the mechanical damage inflicted on host tissues by the ovipositor movement of the female during probing may also reduce host quality for other females, such that further exploration inside the host makes it less valuable (Netting & Hunter, 2000).

Finally, it is worth mentioning that host acceptance may be altered over time because these decisions are affected by 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 (van Alphen & Visser, 1990; Harvey et al., 2013).

### 1.2.3. Intrinsic competition between parasitoids and ovicide

Immature parasitoids do not immediately consume host resources, and thus, these parasitized hosts remain *in situ* vulnerable to encounter by other foraging females (van Alphen & Visser, 1990; Godfray, 1994; Tena et al., 2008; Harvey et al., 2013). When a female parasitoid finds a parasitized host, it can either reject it and look for a host of higher quality for their progeny, or accept it and lay its own egg or clutch of eggs. If further eggs are laid on the host by the same species of parasitoid, **superparasitism** is said to occur. If a second female of a different species lays its eggs on the host, **multiparasitism** occurs (Godfray, 1994). It was thought that parasitoids could not discriminate between healthy and parasitized hosts, and therefore, they tend to superparasitize/multiparasitize, despite the apparent non-adaptive nature of superparasitism/multiparasitism behavior (van Alphen & Visser, 1990). However, it has been demonstrated that some parasitoids can **discriminate between parasitized and unparasitized hosts**, and that, at least superparasitism can be advantageous for the developing immature offspring (van Alphen & Visser, 1990; Tena et al., 2008).

Conspecific host discrimination is the discrimination between unparasitized hosts and those parasitized by a female of the same species, whereas heterospecific host discrimination involves hosts parasitized by a female of another species (Turlings et al., 1985; Collier et al., 2007; Yang et al., 2012). Here, it is important to highlight that host discrimination might be affected by host size (instar) because, as explain above,

host quality is also affected by host size. The **effect of host size on heterospecific host discrimination** has been, however, poorly explored in parasitoid literature.

Once both females have laid their eggs in the same host, there is potential for antagonistic interactions among the immature stages, seeking to monopolize the host resources. In solitary species, only one parasitoid can develop; supernumerary larvae are usually eliminated and the surviving larva then exploits the entire resource. It is, thus, a contest competition (Boivin & Brodeur, 2006) that can be mediated by physical attack and/or by physiological suppression (Mackauer, 1990; Quicke, 1997; Jervis, 2005; Pexton et al., 2009; Cusumano et al., 2012). Alternatively, in gregarious parasitoids, the host can sustain the development of several parasitoids that must share the same resources (scramble competition), in which both species emerge but with the cost of reduced adult size (Boivin & Brodeur, 2006). When the resources are insufficient for the survival of all larvae, all or part of the brood cannot complete its development. Although the majority of larvae of gregarious species do not have functional mandibles, they have nonetheless evolved competitive adaptations, and become aggressive to eliminate supernumerary larvae within a host via physical attacks (Boivin & van Baaren, 2000; Jarjees & Merritt, 2004; Pexton & Mayhew, 2004; Heslin & Merritt, 2005; Tena et al., 2009). The outcome of the intrinsic competition between the immature individuals can be affected by differences in their developmental time, clutch size, order in which oviposition occurs, and time interval between the ovipositions (van Strien-van Liempt, 1982; Mackauer, 1990; Tillman & Powell, 1992). It has been hypothesized that the decision of the second female depends on the probability of its egg or clutch winning the competition with the one already present (Netting & Hunter, 2000). However, the second female can also tip the balance and kill the progeny of the first female before laying its own egg. This

behavior is known as ovicide and can alter the outcome of competition (Netting & Hunter, 2000; Infante et al., 2001; Pérez-Lachaud et al., 2004; Tena et al., 2008).

#### 1.2.4. Effect of temperature increase on parasitoid-host interaction

Climate is expected to change rapidly in the upcoming decades. Mean temperatures have risen by about 0.8 °C since the early twentieth century, and a further increase of about 3 °C is predicted by the end of the twenty-first century in the Mediterranean Basin (IPCC, 2014). An increase in temperature can affect the capacity of adult parasitoids to locate and evaluate their host (van Baaren et al., 2010). Indeed, an increase in temperature may induce a number of physiological changes, whose cost can be expressed by a reduction in reproductive output, decrease in the growth of immature stages, and reduced lifespan, and/or changes in mating behavior (Omer et al., 1996; Hance et al., 2007; Angilletta, 2009; Amiri et al., 2010).

Such behavioral changes can have important consequences on interspecific relationships and biological control. They could affect species distributions, life histories, community composition, and ecosystem function (Bale et al., 2002; Hance et al., 2007; Northfield & Ives, 2013; Le Lann et al., 2014; Sentis et al., 2014). As a consequence, increases in the temperature may alter trait divergence among co-occurring species sharing the same resources, ultimately affecting their ability to coexist.

### 1.3. *Aonidiella aurantii* as citrus pest

In the Mediterranean Basin, the California red scale *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) became a key citrus pest at the end of the last century,

and it was rapidly parasitized by the native parasitoid *Aphytis chrysomphali* (Mercet) (Hymenoptera: Aphelinidae). Later, its coevolved parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) was introduced in a classical biological control program, and it has since displaced *A. chrysomphali* in most areas, while both species coexist in sympatry in eastern part of Spain (Pekas et al., 2010, 2016; Sorribas et al., 2010). All of these factors make this a highly suitable model system for study in this thesis.

*Aonidiella aurantii* belongs to the Diaspididae family, also known as armored scales. It is a large family, with one of the major citrus-infesting insect fauna in the majority of citrus-growing regions of the world (Ebeling, 1959).

### 1.3.1. Origin and distribution

*Aonidiella aurantii* is native to south-eastern Asia, an area between India and south-eastern China (Bodenheimer, 1951). Nowadays, *A. aurantii* **is extensively distributed worldwide**, in all tropical and subtropical regions where citrus is cultivated. The pest has been recorded in the Mediterranean Basin, South Africa, the tropical and subtropical zone of North and South America, Australia, New Zealand, Pacific islands, Indian peninsula, Philippines, Middle East, and Japan (Ebeling, 1959). In Spain, *A. aurantii* has been present since the beginning of the 20th century. It was cited for first time in Valencia by García Mercet in 1910 (Pina, 2007).

### 1.3.2. Importance of *Aonidiella aurantii* as a citrus pest

*Aonidiella aurantii* is a **polyphagous insect**, attacking a wide variety of plants belonging to at least 77 plant families (Borchsenius, 1966). Nonetheless, *A. aurantii* preferentially attacks citrus, and is considered one of the **most important citrus pests**

**worldwide** (Rosen & DeBach, 1979; Murdoch et al., 2006; Jacas & Urbaneja, 2010; Tena & Garcia-Marí, 2011). Despite being described at the beginning of the 20<sup>th</sup> century in Spain, it did not reach pest status until 1985, when a focus was detected in Alzira (Valencia) (Rodrigo & Garcia-Marí, 1990, 1992; Alfaro et al., 1999). Currently, *A. aurantii* is present in all the citrus-growing areas of Spain (García-Marí et al., 2003; González-García, 2009; Campos-Rivela et al., 2012; Boyero et al., 2014).

*Aonidiella aurantii* attacks all aerial parts of the citrus tree, including twigs, leaves, fruits (Fig. 1), and branches (Fig. 2), by sucking the sap on the plant tissue with their long, filamentous mouthparts. The presence of this insect weakens the infested organ and the plant itself, thereafter causing deformations by the action of toxic saliva (Beardsley & Gonzalez, 1975; Washington & Walker, 1990). The main economic impact of *A. aurantii*, however, is that **its presence in fruit depreciates its value, thus** downgrading the fruit. In fact, current regulations for fresh fruit only tolerate between three and ten scales, depending on the variety and the region (Vacas et al., 2012; Vanaclocha et al., 2012).



Fig. 1 Infested by *Aonidiella aurantii*



Fig. 2 Infested branches by *Aonidiella aurantii*

### 1.3.3. Morphology and development

Armored scale insects are hidden under an “armor” or “**scale**” that protects the insect body from physical aggressions and adverse climatic conditions (Dickson, 1951; Ebeling, 1959; Foldi, 1990). In *A. aurantii*, the cover is reddish-orange colored and is almost circular in females, whereas it is elongated in males (Fig. 3 and 4). The cover consists of wax secreted by the glands of pygidium and exuviae that are incorporated during the molt (Dickson, 1951; Ebeling, 1959). There is also a ventral cover that comprises secretions of ventral wax glands along with incorporated ventral exuvial residues (Dickson, 1951). The adults of diaspidids present a marked **sexual dimorphism**. Adult females have no wings or legs and are sessile. On the other hand, the adult diaspidid males are yellow-orange, approximately 0.6–0.8 mm long, and mobile, with well-developed antennae, front wings, and legs (Ebeling, 1959; Beardsley & González, 1975).

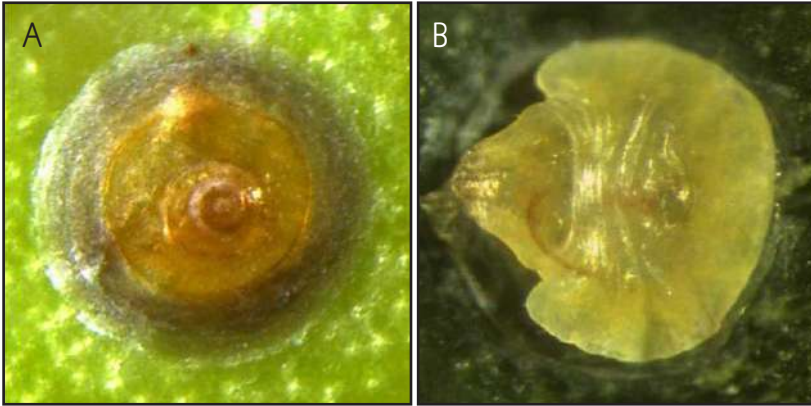


Fig. 3 Cover (A) and body (right) of a young female stage (or virgin third instar) of *Aonidiella aurantii*

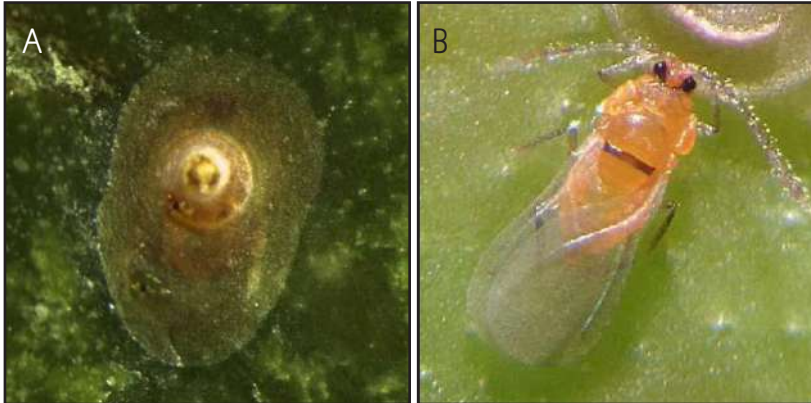


Fig. 4 Cover (A) and adult (B) male stage of *Aonidiella aurantii*

The **developmental stages** of *A. aurantii* also differ between the two sexes. Females pass through three instars (two nymphal instars and one state adult), whereas males pass through five (two nymphal instars, two pupal instars: prepupa and pupa, and one adult). During the molt stage, the body becomes orange and cannot be separated from the cover. Great differences may be found in the average size of the scale cover, depending on the instar of *A. aurantii*, plant substrate upon which the insect feeds, geographic location, time of the year, and probably nutritional status of the host plant (Ebeling, 1959; Carroll & Luck, 1984; Luck & Podoler, 1985; Yu, 1986; Reeve, 1987;

Walde et al., 1989; Hare et al., 1990; Hare & Luck, 1991, 1994; Hare & Morgan, 2000). A detailed description of *A. aurantii* life history was provided during the mid-twentieth century by Quayle (1941), Bodenheimer (1951), and Ebeling (1959), and the complete biological life cycle is represented in the figure above (Fig. 5).

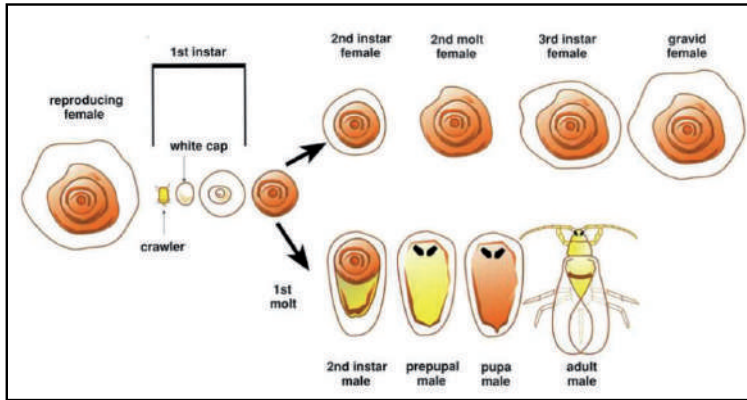


Fig. 5 Life cycle of *Aonidiella aurantii* (Pina, 2007)

#### 1.3.4. Biology and ecology

Like the majority of armored scales, *A. aurantii* reproduces sexually, which makes necessary the presence of males for female fertilization (Quayle, 1911; Nel, 1933) as well as the synchronization of development for both sexes. Thus, the male prepupa and pupa instars coincide with the second female molt, as the adult males with the **young third-instar females** (Fig. 6). Virgin third instar females release a pheromone that attracts the males. After insemination, the pygidium of the scale is withdrawn past the thoracic lobes and mating can no longer occur (Tashiro & Moffitt, 1968). At this stage, the female is called **mature or gravid**. From this point onwards, the scale cover cannot be detached from the body of the mature female. The insect stops feeding and is sealed inside the cover during the molt.

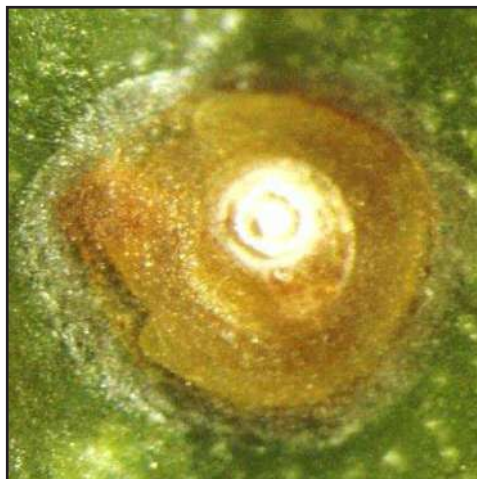


Fig. 6 Young third-instar female (virgin) of *A. aurantii* with the pidium extended

The **developmental cycle and longevity** of *A. aurantii* have been studied across different climatic zones, and therefore, under numerous humidity and temperature conditions, which can be consulted in the following references: Munger & Cressman (1948), Quayle (1932), Tashiro & Beavers (1968), Willard (1972), and Yu (1986). Interestingly, Yu and Luck (1988) found that developmental time was the same under constant lab temperatures and fluctuating temperatures in the field. Moreover, due to the high temperatures in summer, *A. aurantii* suffers a decrease in the body size with serious implications for its biological control (Yu, 1986; Yu & Luck, 1988; Hare et al., 1990).

The **number of annual generations** ranges from two to six generations per year, according to the geographical area and climatic conditions (Beardsley & Gonzalez, 1975; Carroll & Luck, 1984; Smith et al., 1997; Bedford, 1998). In general, the number of generations observed is higher in zones with low humidity and relatively high temperatures (Bodenheimer, 1951). In the Mediterranean basin, *A. aurantii* completes between three and five generations (Avidov & Harpaz, 1969; Habib et al., 1972; Delucchi, 1965; Alexandrakakis, 1983; Orphanides, 1984; Tumminelli et al.,

1996). In Spain, in the Valencia Community, *A. aurantii* completes three generations per year. The first peak of crawlers (Fig. 7) is observed around the end of May, the second at the end of August, and the third around November, depending on the climatic conditions. A forth peak can be observed in fall in the warm years (Ripollés, 1990; Rodrigo & García-Marí, 1990, 1992; Rodrigo, 1993; Vanaclocha, 2012; Urbaneja et al., 2012a).

Moreover, temperature also affects the fecundity of female *A. aurantii*. Several authors point out that the highest **fecundity** rate of *A. aurantii* is obtained in the temperature range between 25 and 35°C (Nel, 1933; Tashiro & Moffitt, 1968, Willard, 1972). A maximum average of 267 nymphs per female were obtained at 30°C and a minimum of 46 nymphs at 15°C by Willard (1972). Similarly, Wentzel (1970) obtained 266 nymphs at 30°C and 123 nymphs at 20°C.



Fig. 7 Reproducing female of *A. aurantii* with crawlers

### 1.3.5. Management of *A. aurantii* in the Mediterranean Basin

Integrated pest management (IPM) is an ecosystem-based strategy that focuses on long-term control of pests or their damage through a combination of techniques such as chemical, biotechnological, cultural, and biological approaches. Under IPM, chemical control is the last technique to be used and pesticides should be used only when pest populations reach the economic thresholds. Moreover, pesticides are selected and applied in a manner that minimizes the risks to human health, beneficial and non-target organisms, and the environment (Urbaneja et al., 2012a).

Nowadays, different techniques are used in combination to control *A. aurantii* in Valencian citrus.

- **Chemical control** using selective pesticides with a reduced impact on beneficial arthropods as those authorized in the IPM program: petroleum spray oils and spirotetramad (RD 1201/2002; DOCV 8046/23.05.2017; Urbaneja, 2012a; Vanaclocha, 2013).
- **Biotechnological methods**, including a successful mating disruption technique that has been established for *A. aurantii* (Vacas et al. 2009, 2010).
- **Cultural methods such as pruning** of trees, which improves aeration within the canopy to increase the mortality of young instars in summer;
- **Augmentative and conservation biological control**, through releases of insectary-reared *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) (Urbaneja et al., 2012a; Vacas et al., 2012; Tena et al., 2013a, b, 2015).

Generally, in organic citrus orchards, where pesticides are not sprayed, the biological control by the natural enemies of *A. aurantii* is able to maintain a low pest population level (Urbaneja et al., 2012a). Only in the areas where the pest population reaches economical thresholds, pesticides such as mineral oils that are allowed in organic agriculture (Reglamento (UE) n°354/2014) are required (Urbaneja et al., 2012a).

#### 1.4. *Aphytis* as natural enemies of *Aonidiella aurantii*

The ectoparasitoids of genus *Aphytis* Howard (Hymenoptera: Aphelinidae) (Rosen & DeBach, 1979) are the most efficient natural enemies of *A. aurantii* (Fig. 8) and of armored scales in general (DeBach & Rosen, 1976, 1991; Rosen & DeBach, 1979; Sorribas & Garcia-Mari, 2010; Pekas, 2010; Sorribas, 2011).



Fig. 8 Adult female of *A. melinus* parasitizing *A. aurantii*

Several **endoparasitoids**, like *Encarsia* (= *Prospaltella*) *perniciosa* (Tower) (Hymenoptera: Aphelinidae) and *Comperiella bifasciata* Howard (Hymenoptera: Encyrtidae) have also been described as attacking *A. aurantii*, and they have been introduced in various regions worldwide to complement the biological control

of *A. aurantii*. However, the role of these endoparasitoids can be considered as complementary to *Aphytis*, since they can attack other scales instars that ectoparasitoids are not able to parasitize (DeBach, 1969; Yu et al., 1990; DeBach & Rosen, 1991; Rosen, 1994; Pina, 2007).

The most commonly described **predators** of *A. aurantii* are *Rhyzobius* (= *Lindorus*) *lophanthae* Blaisdell and *Chilocorus bipustulatus* (L.) (Coleoptera: Coccinellidae), *Lestodiplosis aonidiellae* Harris (Diptera: Cecidomyiidae), *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), and the mites of genus *HemisarcOPTES* Meyer (Astigmata: HemisarcOPTES) (Meyer, 1962; DeBach, 1969; Ben-Dov & Rosen, 1969; Luck, 1995; Foster & Luck, 1996; Siscaro et al., 1999; Erler & Tunç, 2001; Urbaneja et al., 2005). Predators can play an important role in reducing *A. aurantii* populations that cannot be controlled by parasitoids, as some of them are able to feed on mature female instars, which are not parasitized by *Aphytis* (DeBach, 1969; Samways, 1985; Siscaro et al., 1999).

Many of these species have been introduced into classical biological control programs and subsequently through seasonal increases in different countries facing economic damage caused by *A. aurantii* (Bedford & Cilliers, 1994). In Spain, the most abundant parasitoids are *Aphytis chrysomphali* (Mercet) and *A. melinus* (Rodrigo & García-Marí, 1990; Pina & Verdú, 2007; Sorribas et al., 2008; Vanaclocha et al., 2012; Boyero et al., 2014).

*Aphytis chrysomphali* (Fig. 9A) is a native species of the Mediterranean basin that used to originally parasitize *Chrysomphalus dictyospermi* (Morgan) (Hemiptera: Diaspididae), and later began parasitizing *A. aurantii*, when it appeared in this region (Rosen & DeBach, 1979). *Aphytis melinus* (Fig. 9B) is an exotic species for Spanish

fauna that has been established perfectly since its introduction in the Valencian Community for the control of *C. dictyospermi*, as part of a classic biological control program (Jacas et al., 2006; Pina, 2007). *Aphytis melinus* is considered the most successful and widespread biological control agent of the *Aphytis* genus (Murdoch et al. 1989, DeBach & Rosen, 1991, Forster et al., 1995; Dreistadt, 2012).



Fig. 9 Pupa of *A. chrysomphali* (A) and *A. melinus* (B). *Aphytis chrysomphali* pupae are identified by the presence of a longitudinal black line on the mesosternum, which is not present in *A. melinus*

#### 1.4.1. Morphology and development of parasitoids of the genus *Aphytis*

The genus *Aphytis* belongs to the family Aphelinidae, within the Superfamily Chalcidoidea. It is a cosmopolitan and very large group of small (usually less than 1 mm in length) yellowish or grayish hymenopterans that develop exclusively as primary ectoparasitoids of diaspidids (Flanders, 1953, Rosen & DeBach, 1979). *Aphytis* are holometabolous and their development includes the following stages: egg (Fig. 10), larvae (Fig. 11A), prepupae (Fig. 11B), pupae (Fig. 12), and adult (Fig. 13), which were described in detail by Rosen & Eliraz (1978) and Rosen & DeBach (1979).



Fig. 10 Egg of *Aphytis* laid in *A. aurantii*



Fig. 11 Larvae (A) and prepupa (B) of *Aphytis*. *Aphytis* have three larval instars and toward the end of the larval period, the third-instar larva enters a short prepupal stage. The prepupal stage cannot be considered a distinct instar since no apolysis neither ecdysis take place.

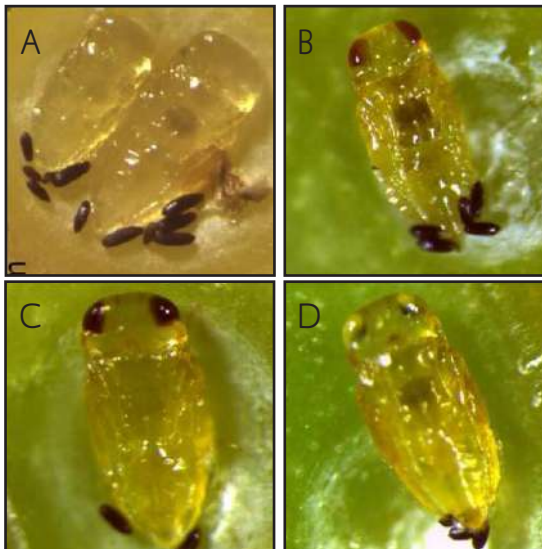


Fig. 12 Pupae of *Aphytis melinus* with different colour of eyes. The eyes of the developing pupa gradually turn from colourless (A) to red (B), Brown (C) and to green (D) providing an indication of the age of the pupa.



Fig. 13 Adult of *Aphytis*

1.4.2. Biology and ecology of parasitoids of the genus *Aphytis*

The majority of *Aphytis* species are **biparental and reproduce sexually**. Female *Aphytis* are essentially monogamous. They mate only once and the sperm is stored in the spermatheca for egg fertilization. On the other hand, males are polygamous, capable of mating with several females (Rosen & DeBach, 1979). Females control the sex of their offspring at oviposition via haplodiploidy, producing male offspring from unfertilized and female offspring from fertilized eggs (Flanders, 1953; Rosen & DeBach, 1979). However, there are some species, in which the existence of the male is unknown or occurs in such small numbers that they are considered **uniparental** (Rosen & DeBach, 1979; Viggiani, 1984). These adults exhibit thelytokous parthenogenesis as a consequence of infestation with the symbiotic bacteria *Wolbachia*, i.e., unfertilized eggs develop into females. *Wolbachia* has been detected in *A. chilensis* Howard, *A. yanonensis* DeBach, *A. diaspidis* (Howard), in the uniparental line of *A. lingnanensis* Compere, and is native to the Mediterranean *A. chrysomphali* (Zchori-Fein et al., 1994, 1995; Werren et al., 1995; Gottlieb et al., 1998; Pina, 2007). More recently, it has been also detected in *A. melinus*, and is known to cause complete cytoplasmic incompatibility (Vasquez, 2011). This means that the sperm from an infected male is unable to properly fertilize the egg of an uninfected female or a female that is infected with a different *Wolbachia* strain (Werren et al., 2008)

*Aphytis* parasitoids are considered **synovigenic**, i.e., they emerge with zero or few eggs and continue maturing new eggs throughout their life (Rosen & DeBach, 1979; Viggiani, 1984; Opp & Luck, 1986). Females require proteins for egg maturation, which are obtained by **host-feeding** (Fig. 14) or a diet rich in protein (Rosen & DeBach, 1979; Heimpel et al., 1994; Collier, 1995; Vanaclocha et al., 2014). The number of eggs with which they emerge, the time it takes for the first eggs to mature,

and the maximum number of eggs they are able to accumulate and produce vary with species (Opp & Luck, 1986). These characteristics indicate the reproductive strategies of parasitoids (Ellers, 2000, Jervis et al., 2001, Jervis & Ferns, 2004). The mean number of eggs with which *A. melinus* emerge is less than one (Opp & Luck, 1986; Collier, 1995), whereas *A. chrysomphali* emerge with zero eggs (Pina, 2007). *Aphytis melinus* can mature up to 13 eggs per day (Opp & Luck, 1986), whereas *A. chrysomphali* can mature only seven (Pina, 2007).



Fig. 14 Adult *Aphytis* feeding on its host *A. aurantii*

### 1.4.3. Behavior and efficacy of *Aphytis* parasitoids

#### 1.4.3.1. Effect of host quality on *Aphytis* behavior and efficacy

**The most reliable cue of host quality for *Aphytis* parasitoid is probably host size/instar** (Fig. 15) (Hare & Luck, 1991). As *Aphytis* are idiobionts, the female paralyzes the host by inserting venom through its ovipositor (van Lenteren, 1994) and the host stops growing (Fischer, 1952). Therefore, its size represents the food available for the parasitoid offspring at the moment of oviposition. Host size has a positive correlation

with the size of the adult *Aphytis* males and females (Opp & Luck, 1986; Yu, 1986; Reeve, 1987; Rosenheim & Rosen, 1991; Pina, 2007). Adult size may influence fitness by affecting longevity, fecundity (females), or searching capacity (Godfray, 1994). In addition, the number of mature eggs and adult size is related positively with host size (Opp & Luck, 1986; Pina, 2007).



Fig. 15 *Aphytis* female searching on a patch of different host sizes/instars

Generally, when an *Aphytis* female parasitoid encounters a host, it either lays an egg and/or feeds on the hemolymph of the host, using it to produce additional eggs (i.e., host-feeding). High quality hosts (larger hosts) are more frequently used for oviposition than lower quality hosts (smaller hosts) that are usually used for host-feeding (Flanders, 1951; Abdelrahman, 1974b; Rosenheim & Rosen, 1991). Sometimes, after the female inserts its ovipositor to obtain information about the suitability of a potential host, it rejects the host without oviposition or feeding (Heimpel & Collier, 1996; Heimpel et al., 1998; Hopper et al., 2013). Generally, this behavior is more common in egg-limited parasitoids (with low eggs and high life expectancies) than in time-limited parasitoids, as the former would reject more hosts

that have a low suitability for their progeny (Heimpel & Collier, 1996; Heimpel et al., 1998; Hopper et al., 2013). This behavior is common in *Aphytis* parasitoids (Abdelrahman, 1974b; Casas et al., 2004), but despite its prevalence, the effect of this behavior on hosts has been largely disregarded in the parasitoid literature. **In the first objective of this thesis (chapter 3), the frequency of rejection of a host after ovipositor insertion (overstinging) has been evaluated and compared to test whether it varies with parasitoid species and host size (instar) (Fig. 16).**



Fig. 16 A host of *A. aurantii* healthy (A), host-fed (B) and overstung (C)

If an *Aphytis* female decides that the host is suitable, it can lay more than one egg per host, as *Aphytis* species are facultatively gregarious (Fig. 17). Larger clutches are laid in larger hosts (high quality), as larger hosts provide more food for the developing progeny. Host size also affects sex allocation decisions of *Aphytis* species that reproduce parthenogenetically. For example, *A. melinus* allocates male eggs mostly to hosts smaller than  $0.39 \text{ mm}^2$  (in body area of CRS) and female eggs mostly to hosts larger than  $0.39 \text{ mm}^2$  (Luck & Podoler, 1985; Yu, 1986; Pekas et al., 2010).

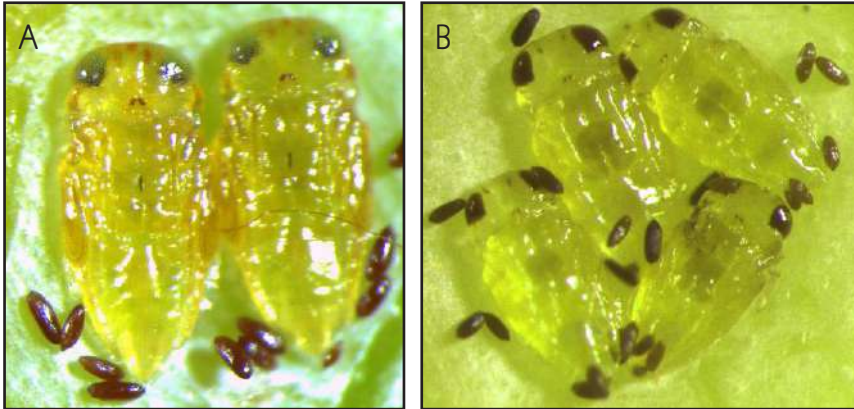


Fig. 17 Two *A. chrysomphali* (left) and four *A. melinus* (right) pupae developed in the same host

Apart from host size, as mentioned above, host acceptance also depends upon whether the host is already parasitized (Fig. 18). **The ability to discriminate between hosts heterospecifically parasitized and unparasitized by *Aphytis* parasitoids has been evaluated in the second objective of this thesis (chapter 4).** Moreover, the effect of host size on heterospecific host discrimination has been poorly explored in parasitoid literature. In this chapter, it is also tested **whether a female parasitoid is more willing to accept heterospecific parasitized hosts of a larger size than of a smaller size.**

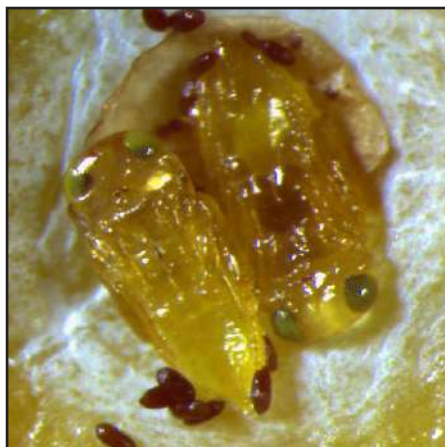


Fig. 18 *Aonidiella aurantii* multiparasitized by *A. chrysomphali* and *A. melinus*

#### 1.4.3.2. Interspecific competition between *Aphytis* parasitoids

*Aphytis* parasitoids suffer strong resource competition between parasitoid species (Borer et al., 2004). In most citrus-production areas, the main parasitoids of *A. aurantii* populations are *A. melinus*, *A. chrysomphali*, and *A. lingnanensis*. The former has displaced other *Aphytis* species in the field (DeBach & Sundby, 1963; Luck & Podoler, 1985; Murdoch et al., 1996; Smith et al., 1997; Sorribas et al., 2010). In Spanish citrus, *A. melinus* has completely displaced the native *A. chrysomphali* in the south due to its better adaptation to dry and hot climates (Sorribas, 2010; Boyero et al., 2014), superior intrinsic biological and physiological capabilities, and higher capacity of dispersion than *A. chrysomphali* (Abdelrahman 1974a, b). However, in other areas, they both coexist in sympatry. Their coexistence has been attributed to fluctuating environmental conditions, seasonal variation in parasitoid abundance (Pina, 2007; Sorribas et al., 2010; Boyero et al., 2014), and more recently, to the plasticity of *A. chrysomphali* to exploit smaller host instars (poorer quality) when the density of *A. melinus* is high and it exploits the third instar (higher quality) (Pekas et al., 2016). Direct competition between parasitoid species can play an important role in this displacement/coexistence, and these mechanisms have not been extensively studied in *Aphytis* parasitoids. **In view of this, and the hypotheses discussed above, in the third objective (chapter 5), we test whether the outcome of interspecific competition can be affected by host size (instar), which could explain this conditional patch partitioning explored in the field study by Pekas et al. (2016).**

#### 1.4.3.3. *Effect of global warming on Aphytis competition and efficacy as biological control agents*

Extreme temperatures are the highest natural mortality factor for *Aphytis* (Rosen & DeBach, 1979). **The expected temperature increase during summer due to climate change may affect interspecific competition between *Aphytis* species and the biological control of *A. aurantii*.** As commented above, *A. melinus* is better adapted to dry and hot climates where citrus is cultivated (Abdelrahman, 1974a; Rosen & DeBach, 1979). In fact, the relative proportion of *A. melinus* is higher during the warm months, and the abundance of *A. chrysomphali* increases from south to north, with higher relative abundances in the cooler northern areas. Therefore, the superiority of *A. melinus* might be accentuated with increasing temperature, leading ultimately to the *displacement* of the weaker *competitor*. **In the fourth objective of this thesis (chapter 6), the effects of the expected increase in temperature with respect to the competition and efficacy of *A. melinus* and *A. chrysomphali* as biological control agents of *A. aurantii* have been evaluated.**

## Chapter 2

# RESEARCH OBJECTIVES





## 2. RESEARCH OBJECTIVES

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Parasitoids of genus *Aphytis* are the most important natural enemies of *Aonidiella aurantii*. In the Mediterranean basin, the native *A. chrysomphali* and the introduced *A. melinus* are the most abundant parasitoids of *A. aurantii*. The introduction of *A. aurantii* has completely displaced the native *A. chrysomphali* in the south, whereas they coexist in the north-east. We have used this well-known host-parasitoid system to investigate some gaps on the behavioral ecology of hymenopteran parasitoids, which have been discussed in the introduction. This knowledge will contribute to improve their use in biological control programs, especially in the case of *A. aurantii* in the Mediterranean basin. In this context, the main objectives of this thesis are:

- i. To determine the **effect of overstinging** (host rejection after sting) **on host fitness** and to compare whether it varies with parasitoid species and host instar. Chapter 3.
- ii. To determine whether **host instar affects the ability to discriminate** between unparasitized and heterospecifically parasitized hosts. Chapter 4.
- iii. To determine whether **host instar affects the outcome of interspecific competition** between parasitoids, and whether it can explain the coexistence of *A. chrysomphali* and *A. melinus* in north-eastern Spain. Chapter 5.
- iv. To determine the **effects of global warming and direct competition** between *A. chrysomphali* and *A. melinus* on their efficacy as biological control agents of *A. aurantii*. Chapter 6.



## Chapter 3

# OVERSTINGING BY HYMENOPTERAN PARASITIDS CAUSES MUTILATION AND SURPLUS KILLING OF HOSTS





### 3. OVERSTINGING BY HYMENOPTERAN PARASITIDS CAUSES MUTILATION AND SURPLUS KILLING OF HOSTS

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#### Abstract

An appraisal of the regulatory role of natural enemies on target pests requires the identification of the mechanisms/traits that enhance the ability of an organism to control the density of its prey/host. After stinging herbivore hosts with their ovipositor, hymenopteran parasitoids tend to reject them without ovipositing or host-feeding. Termed pseudoparasitism, the frequency and consequences of this type of attack (hereafter overstinging) have been largely disregarded in the hymenopteran parasitoid literature. We choose the parasitoids *Aphytis melinus* and *A. chrysomphali* and their common host *Aonidiella aurantii* as a model system to study this behavior. Using field and laboratory observations, we showed that overstinging is a common behavior in the wild. Under controlled conditions, overstinging occurred more frequently than host-feeding, a behavioral trait that is used to evaluate the potential of parasitoids as biological control agents. Overstinging reduced the fecundity and survival of the herbivore host. When we compared between parasitoid species that attack the same host species, the virulence and frequency of this behavior depended on parasitoid species. These results demonstrate that overstinging should be incorporated in the models of host-parasitoid interactions to analyze population dynamics as well as in the future selection of parasitoids for biological control.

Keywords *Aphytis* • *Aonidiella aurantii* • Behavioral Ecology • Biological Control • Host-feeding • Overkilling • Physiological Entomology

### 3.1. Introduction

Entomologists and ecologists interested in biological control have long sought insights to guide the selection of effective natural enemies because many natural enemies have important limitations as potential regulators of herbivorous pests (Jervis, 2005). However, an appraisal of the regulatory role of natural enemies requires the identification of the mechanisms/traits that enhance the ability of an organism to control the density of its prey/host. Parasitoids are the most important and successful group of natural enemies used in the biological control of insect pests (Godfray, 1994; Jervis, 2005), and their efficacy depends on the behavioral decisions of females when they search for and find a host (Mills & Wajnberg, 2008). Generally, when a female parasitoid encounters a host, she either (1) lays eggs in/on the host and the larvae then feed on the host, and/or (2) she feeds on the hemolymph of the host and uses it to produce additional eggs (i.e., host-feeding); both behaviors eventually kill the host. To obtain information about the suitability of a potential host, the female parasitoid inserts her ovipositor and, in some cases, (3) then rejects the host (Heimpel & Collier, 1996; Heimpel et al., 1998; Hopper et al., 2013). This behavior is common in hymenopteran parasitoids and is known as “probe/sting and rejection” or “overstinging.” However, despite its prevalence, the effect of this behavior on hosts has been largely disregarded in the parasitoid literature, but it might be an important trait in the selection of parasitoids for biological control programs.

In the few studied cases, the consequences of overstinging vary from reduced fitness of the wounded hosts (mutilation) to host death (Abdelrahman, 1974b; Jones, 1985; Jones et al., 1986; Brown & Kainoh, 1992). This variability might depend on the stage of the host being stung by the female parasitoids; older and larger hosts may be more resistant to overstagings than younger and smaller hosts (Salt, 1968; Vinson,

1976; Beekage & Gelman, 2004). Therefore, as most species of parasitoids attack hosts of different sizes and even instars, we hypothesize that small hosts will be more likely to die after these attacks. If these hosts die, the stings represent a case of surplus killing or overkilling as the female parasitoid will be killing more hosts than needed for parasitism or host-feeding. Surplus killing by parasitoids might be another useful trait in the identification and evaluation of their potential as biocontrol agents, as it is for predators (Johnson et al., 1975).

The frequency of these attacks (overstings) has also been poorly researched, and it might vary among parasitoid species. Generally, parasitoids with low egg loads and high life expectancies (i.e., egg limited) might oversting more frequently than species with high egg loads and low life expectancy (i.e., time limited) because the former will reject more hosts as having low suitability for their progeny (Heimpel & Collier, 1996; Heimpel et al., 1998; Hopper et al., 2013). The probability of overstinging may also depend on the geographical origin of the species involved. In this sense, overstinging might be more frequent in noncoevolved parasitoids when compared with coevolved parasitoids because host evaluation by the parasitoid might be decoupled from the suitability of the host species for the immatures, as a result of a lack of shared evolutionary history (sensu Schlaepfer et al., 2005).

Here, we chose the parasitoids of the genus *Aphytis* (Hymenoptera: Aphelinidae), which attack the California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), in citrus, as a model system to evaluate and compare i) the occurrence and frequency of overstinging in two parasitoids under laboratory and field conditions and ii) the differences in the effects of overstinging on different instars of their common host. In the Mediterranean Basin, California red scale became a key citrus pest at the end of the last century, and it was rapidly parasitized by the native parasitoid

*Aphytis chrysomphali* (Mercet) (Hymenoptera: Aphelinidae). Later, its coevolved parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) was introduced in a classical biological control program, and it has since displaced *A. chrysomphali* in most areas (Sorribas et al., 2010). Both parasitoids tend to reject hosts after stinging according to laboratory observations (Abdelrahman, 1974b; Casas et al., 2004), but the consequences of these stings on the hosts have never been examined. All of these factors make this a highly suitable model system to study the frequency and effect of overstinging by hymenopteran parasitoids; and determine whether overstinging should be considered when evaluating the efficacy of parasitoids as biological control agents as well as modeling the population dynamics of parasitoids and hosts.

### 3.2. Materials and methods

#### 3.2.1. Experimental insects

*Aonidiella aurantii* were reared on lemons from a laboratory colony at the Instituto Valenciano de Investigaciones Agrarias, IVIA (Montcada, Valencia, Spain). This colony was initiated in 1999 from scales collected from citrus fields in Alzira (Valencia, Spain) and renewed every 2–3 years with scales from the field (Tena et al., 2013a). We followed the methodology described in (Pina, 2007) for rearing *A. aurantii*. Briefly, ~ 2/3 of the surface of each lemon was covered with red paraffin around the mid-section to retard desiccation; the red paraffin was prepared with a mixture of 1 kg of paraffin pearls (Parafina USP Perlas; Guinama S.L., Alboraya, Spain) and 1 g of red pigment (Sudan III; Panreac Química S.A., Castellar del Vallés, Spain). The remaining surface area (approx. 24 cm<sup>2</sup>) of the lemons was infested by exposure to gravid female scales in the *A. aurantii* colony for 48 h. Once infested, the lemons were

maintained at  $26 \pm 1^\circ\text{C}$ ,  $70 \pm 5\%$  RH and darkness until the female scales reached the second (9–11 days) and third (19–22 days) nymphal instars.

*Aphytis melinus* and *A. chrysomphali* were obtained by exposing third-instar *A. aurantii* on lemons to parasitism by insectary-reared adult wasps maintained in the laboratory at  $26 \pm 1^\circ\text{C}$ ,  $60 \pm 5\%$  RH and LD 16:8 h. The *A. melinus* colony was initiated in 2008 and the *A. chrysomphali* colony was initiated in 2013 from scales collected in citrus fields from Alzira and Moncada (Valencia, Spain), respectively. Both colonies are renewed yearly with parasitoids collected in the field.

Between five and ten late-stage pupae of both parasitoids were removed from parasitized scales and held separately in 8-mm-diameter and 35-mm-long crystal vials. At emergence, parasitoids were sexed and held in these vials for one day to obtain mated females of *A. melinus* (*A. chrysomphali* reproduces parthenogenetically) (Gottlieb et al., 1998). One day after their emergence, the females were isolated in the same vials as above and used 2–3 days later. A drop of honey was added to the inside wall of each vial, which were stoppered with a cotton plug. Vials were stored in a climatic chamber (SANYO MLR- 350; Sanyo, Japan) at  $25 \pm 1^\circ\text{C}$ , 50–70% RH and LD 14:10 h.

### 3.2.2. Arena

The arena consisted of a lemon with an approximately 24-cm<sup>2</sup> surface area covered with a transparent cardboard ring that 5.5 cm in diameter and 4 cm high to prevent the parasitoids from escaping. We used a dissecting microscope with a micrometer to select ten scales from the surface of the lemon, and we removed the rest using an insect pin and a paper towel that had been moistened with water. The selected scales

were 9–11 days old and  $0.55 \pm 0.05 \text{ mm}^2$  for the second instar and 19–22 days old and  $0.85 \pm 0.05 \text{ mm}^2$  for the third instar. To estimate their sizes, photographs of the scales were taken with a Leica EC 3 3.1-megapixel digital color camera (Leica Microsystems GmbH, Spain), and the images were processed with Leica LAS EX imaging software for Windows (Leica Microsystems GmbH, Spain). Measurements from all of the pictures were made with ImageJ, a public-domain Java Image processing program (Rasband, 2016). All of the scales were mapped and numbered before the observations began.

### 3.2.3. Behavioral observations

In each replicate, we continuously observed female behavior using a dissecting microscope at 109 to 509 magnification and used a cool fiber light to illuminate the arena. An observation began when a single female of one of the two species was placed in the arena with the ten host scales, and each female parasitoid was observed until she rested for more than 10 min.

We recorded sequences of behavioral interactions with all of the hosts including behaviors that took place within the host body. Thus, three separate behavioral events on a host were identified, timed and recorded: (1) overstinging, (2) ovipositing and (3) host-feeding. After drumming the scale with its antenna, a female parasitoid may investigate a host by stinging, which includes using the ovipositor to drill through the scale cover, explore the cavity between the scale body and cover, and pierce the body and explore the hemocoel. The parasitoid may leave the host at any time during this process (hereafter termed overstinging) and/or may proceed to oviposit or consume its body fluids (host feed). Vibration of the ovipositor during stinging indicates that an egg has been laid, and host-feeding is recognized by the female parasitoid lowering

its head and positioning its mouthparts over the sting immediately after probing (Casas et al., 2004). Additionally, we also mapped the ovipositor insertion points during host stinging. In detail, we distinguished between ovipositor insertions in the center of the scale cover (molt rings) and those ones made in the scale edge (gray skirt).

#### 3.2.4. *Aonidiella aurantii* fitness and survival

Once the observations ended, the parasitoid was removed, and each lemon was kept in a plastic container ( $14 \times 14 \times 8$  cm) along with another lemon infested with male and female scales of the same age. Thus, males from this second lemon could mate with the experimental female scales. The plastic container was covered with a piece of muslin fixed in place with a rubber band and kept in the same climatic chamber as above.

To determine the effect of the behavior of each parasitoid on the survival and fecundity of *A. aurantii*, scales were mapped, observed and measured as described above. We considered a scale to be dead when it did not grow, and this was confirmed by removing the scale cover and inspecting the turgency of the body. Hereafter, we use the term “surplus killing” to refer to the mortality caused by the overstings; to our knowledge, there is no existing term in the parasitoid literature to refer to this type of mortality. This term, as well as “overkilling”, is used when predators kill more prey than they eat, so in parasitoids, this term describes females killing more hosts than they eat or parasitize.

To measure the fecundity of the surviving scales, these individuals were isolated with a double-sided sticky plastic ring (3MScotch<sup>R</sup>; Cergy Pontoise Cedex, France) to

trap the crawlers produced by each female following the methodology proposed by Vanaclocha (2012). Sticky plastic rings were placed 21 days after the observation period and replaced weekly for four weeks. The number of crawlers stuck in the rings was then counted under the binocular. To compare the survival and fecundity of the probed females with those of the unattacked females, we repeated this procedure for two unattacked females on each lemon.

### 3.2.5. Field observations

We conducted a field assay to determine whether the presence of punctures/scars in field scales was correlated with *Aphytis* and predator activity as well as with climatic variables (mean temperature, maximum temperature, mean wind, maximum gust of wind, accumulated rain and maximum rain in one day), in three commercial citrus groves (Almenara, La Pobla de Vallbona and Betera) located in eastern Spain. Almenara (39°45'02.71"N; 0°12'10.09"W) consisted of 9-year-old clementine (*Citrus reticulata* Blanco) 'Oronules' trees (9 years old) grafted on Citrange Carrizo [*Poncirus trifoliata* (L.) Rafinesque-Schmaltz × *Citrus sinensis* (L.) Osbeck] with an extension of 0.2 ha. La Pobla de Vallbona (39°38'05.68"N; 0°30'51.30"W) consisted of 5-year-old clementine 'Esval' trees grafted on Citrange Carrizo with an extension of 0.2 ha, and Betera (39°35'10.13"N; 0°24'39.14"W) consisted of clementine 'Clemenules' trees (5–10-year-old) grafted on Citrange Carrizo with an extension of 1 ha. Standard agronomic practices for citrus cropping were performed, but insecticides were not sprayed during the assay.

Populations of *A. aurantii* were monitored weekly or every other week from April to November 2007 depending on their phenology (weekly from the beginning of the new generation until the sum of the first- and second-instar hosts represented 60% of the

*A. aurantii* population). In each orchard, young shoots infested with *A. aurantii* were collected at random and transferred to the laboratory in plastic bags, and a maximum of ten hosts per shoot were collected to count the number of alive, dead, predated, parasitized or punctured *A. aurantii* of each instar using a stereoscopic microscope. Observations ended when 80 second- and third-instar hosts were counted or when a total of 500 scales were counted per sample. Individuals were considered alive if they were turgid and dead if they were dry and dark (Fig. 1); scales were considered predated when their body had been partially consumed; and scales were considered parasitized if immature parasitoids were found. *Aonidiella aurantii* individuals were noted as being overstung when brown punctures were found on their bodies and they remained alive (turgid) (Fig. 1).

### 3.2.6. Statistical analysis

We applied generalized linear modeling (GLM) techniques assuming Poisson error variance for the count data (number of behavioral events per patch, number of stings per host) and binomial error variance for the proportional data (proportion of scales with punctures in the field, mortality). We assessed significance according to the change in deviance when a variable was removed from the model using a Likelihood Ratio Test with Poisson or binomial errors. Significant values are provided in the text for the minimal model, and the nonsignificant values are those that were obtained before we deleted the variable from the initial model. We assessed the assumed error structures using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If we detected an over- or underdispersion, we re-evaluated the significance of the explanatory variables using an F test after rescaling the statistical model by a Pearson's chi square divided by the residual degrees of freedom (Crawley, 2007). We present the means of the untransformed proportion

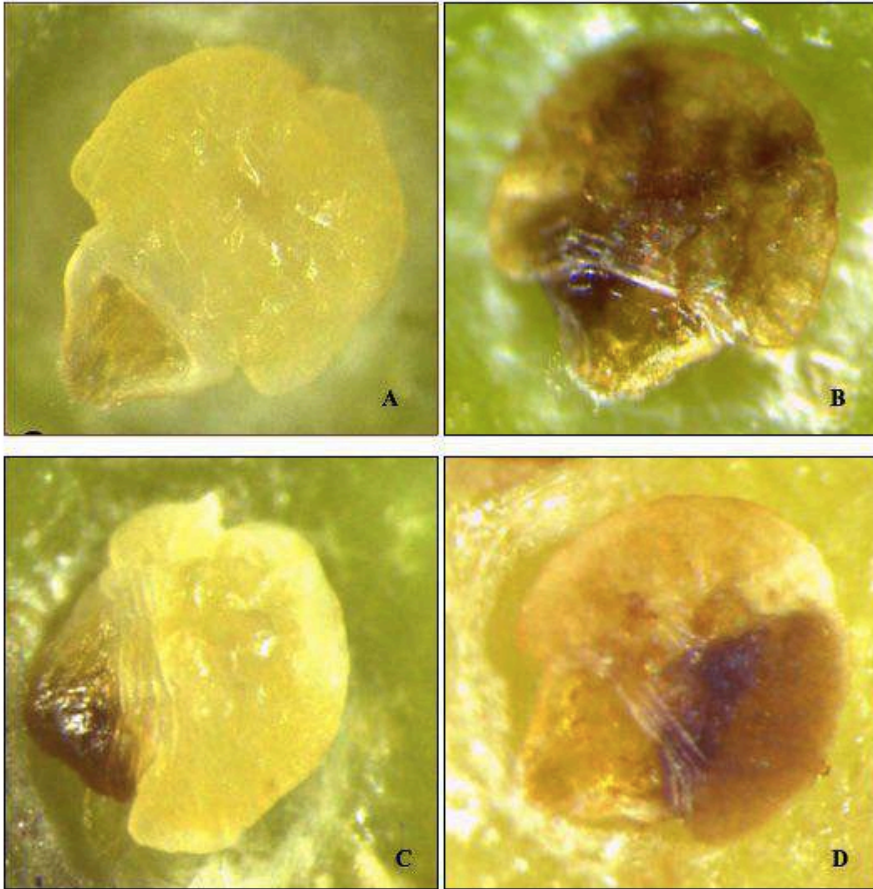


Fig. 1 Body of a third-instar *Aonidiella aurantii* when: a healthy and alive, b dead by host-feeding, and c alive and d dead 3 days after being overstung by *Aphytis* parasitoids

and count data (in preference to less intuitive statistics such as the back-transformed means of logit-transformed data). This results in the standard errors being presented as symmetrical, which results in symmetrical standard errors that did not yield impossible values such as a mortality of less than 0.

To determine whether the number of scales with punctures was correlated with the number of parasitoids, number of predated scales, mean temperature, maximum

temperature, mean wind, maximum gust of wind, accumulated rain and maximum rain in one day, we created a multiple logistic regression. In the multiple logistic regression between the proportion of scales with punctures and the multiple predictor variables, we first created a correlation matrix between all variables. If two variables were correlated ( $P < 0.05$ ), one of them was removed following biological significance (“Annex 1”). We compared *A. aurantii* fecundity and time using ANOVAs. The normality assumption was assessed using Shapiro’s test, and the homoscedasticity assumption was assessed with Levene’s test. All of the data analyses were performed with the R freeware statistical package (<http://www.R-project.org/>) except the correlation matrix that was performed with Statgraphics.

### 3.3. Results

#### 3.3.1. Frequency of overstinging

Overall, we observed 28 and 35 *A. melinus* females foraging in patches with either second- or third-instar *A. aurantii*, respectively. These females parasitized 37 second-instar and 79 third-instar scales, host fed on 27 second-instar and 16 third-instar hosts, and overstung (rejected after stinging) 20 second-instar and 42 third-instar hosts. For *A. chrysomphali*, we observed 20 and 24 females foraging in patches with either second- and third-instar *A. aurantii*, respectively. These females parasitized 19 second-instar and ten third-instar scales, host fed on 18 second-instar and one third-instar hosts; and overstung 22 second-instar and 37 third-instar hosts.

*Aphytis melinus* and *A. chrysomphali* overstung between 0.8 and 1.6 hosts out of a total of 10 hosts per patch (Fig. 2a, b). The number of hosts overstung per patch depended on host instar (second vs third instar:  $F_{1,104} = 5.59$ ;  $P = 0.02$ ), but it was independent

of the parasitoid species (*A. melinus* vs *A. chrysomphali*:  $F_{1,104} = 3.58$ ;  $P = 0.062$ ) (Fig. 2a, b). The interaction between host instar and parasitoid species was not significant ( $F_{1,103} = 0.0091$ ;  $P = 0.92$ ).

Moreover, the number of hosts overstung by *A. melinus* and *A. chrysomphali* per patch was similar to those parasitized or host fed when both parasitoids searched patches with second-instar hosts (*A. melinus*:  $F_{2,81} = 1.89$ ;  $P = 0.16$ ; *A. chrysomphali*:  $F_{2,57} = 0.31$ ;  $P = 0.74$ ) (Fig. 2a). Patch use changed when *A. melinus* and *A. chrysomphali* females searched patches with third-instar hosts (Fig. 2b). *Aphytis melinus* females parasitized significantly more hosts than they overstung or host fed ( $F_{2,102} = 23.69$ ;  $P < 0.0001$ ; uppercase letters in Fig. 2b), whereas *A. chrysomphali* females overstung significantly more hosts than they parasitized or host fed (lowercase letters in Fig. 2b;  $F_{2,69} = 28.84$ ;  $P < 0.0001$ ).

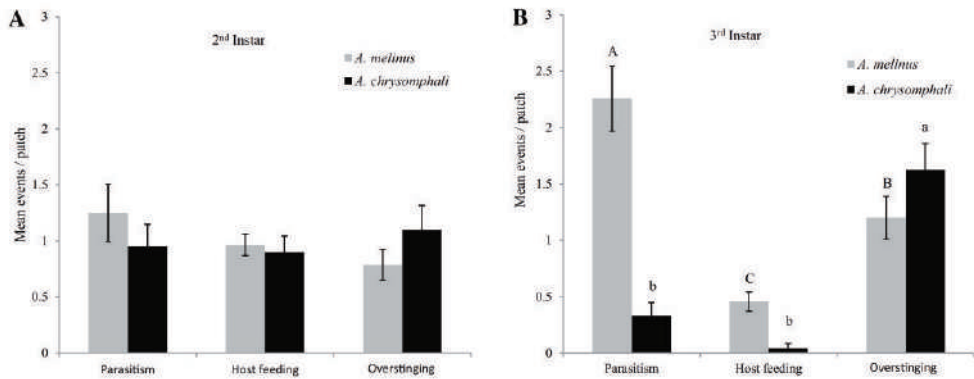


Fig. 2 Behavioral events (mean  $\pm$  SE) of the parasitoids *Aphytis melinus* and *A. chrysomphali* in patches with ten second- (a) and third-instar (b) *Aonidiella aurantii*. Different uppercase letters above the columns denote significant differences between the occurrence of the different behaviors for *A. melinus*, and lowercase letters denote differences for *A. chrysomphali*

### 3.3.2. Effect of overstinging on host fitness

#### 3.3.2.1. Lethal effect

Respectively,  $95 \pm 9\%$  and  $91 \pm 6\%$  of the second-instar hosts overstung by *A. melinus* ( $n = 20$ ) and *A. chrysomphali* ( $n = 22$ ) died (Fig. 3), but these figures changed when both parasitoids overstung the third instar. *Aphytis melinus* ( $n = 42$ ) caused  $55 \pm 8\%$  mortality in this instar vs  $22 \pm 7\%$  caused by *A. chrysomphali* ( $n = 37$ ). Thus, the mortality caused by the overstings depended on the host instar (second *vs* third instar:  $F_{1,70} = 24.92$ ;  $P < 0.001$ ) and the parasitoid species (*A. melinus vs A. chrysomphali*:  $F_{1,70} = 5.71$ ;  $P = 0.02$ ). However, the interaction between host instar and parasitoid species was not significant ( $F_{1,69} = 1.48$ ;  $P = 0.23$ ). As expected, all of the parasitized and host-fed hosts died, whereas all of the unattacked hosts survived.

The probability that the third instar of *A. aurantii* died after being overstung by both parasitoids was positively correlated with the duration of the stings, and it was independent of the sting site, the sequence of visited hosts and number of stings (Table 1, Fig. 4).

#### 3.3.2.2. Sublethal effects: fecundity of surviving hosts

The fecundity (number of crawlers per week) of the surviving hosts that were overstung by *A. melinus* ( $18.23 \pm 3.56$ ) was significantly lower ( $\sim 38\%$ ) than those overstung by *A. chrysomphali* ( $25.92 \pm 1.23$ ) and the unattacked hosts ( $26.45 \pm 0.97$ ) ( $F_{2,91} = 5.6$ ;  $P = 0.005$ ).

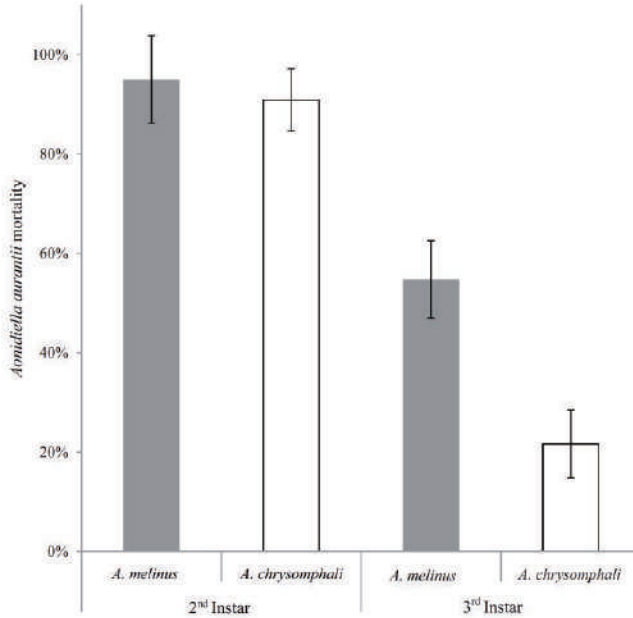


Fig. 3 *Aonidiella aurantii* mortality caused by the parasitoids *Aphytis melinus* and *A. chrysomphali* when they probed and rejected different scale instars

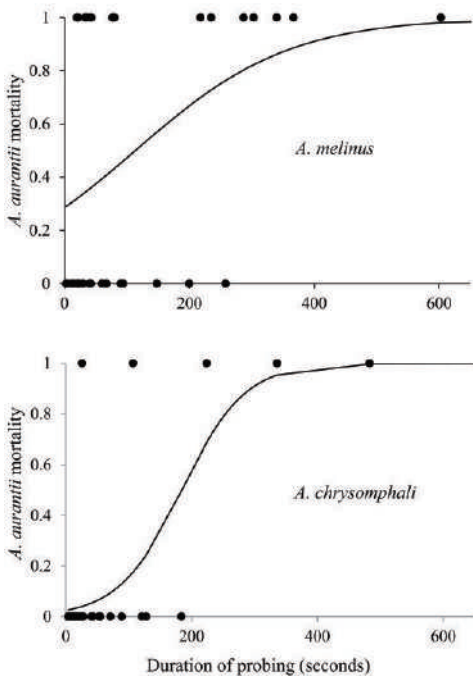


Fig. 4 Effect of oversting duration (in seconds) by *Aphytis melinus* and *A. chrysomphali* on the mortality of *Aonidiella aurantii* (statistics in Table 1). The curve represents the fitted values from the logistic regression model of the proportion of dying hosts. Points at the bottom and top of the figure represent the actual data from alive and dead hosts

**Table 1** Influence of several parasitoid behaviors on *Aonidiella aurantii* mortality when the scale was rejected by either *Aphytis melinus* or *A. chrysomphali* after stinging (overstinging)

Variable	Parameter estimate	F	P
Host overstung by <i>A. melinus</i>			
Intercept	-0.92	-1.96	0.058
Sting duration	0.0081	2.21	<b>0.034</b>
Sting site	1.99	1.9	0.07
Order	-0.62	-1.064	0.3
Number of stings	-0.66	-0.71	0.49
Host overstung by <i>A. chrysomphali</i>			
Intercept	-2.89	-3.47	0.0015
Sting duration	0.016	2.2	<b>0.035</b>
Sting site	0.24	0.14	0.89
Order	0.33	0.58	0.56
Number of stings	0.43	1.06	0.3

Host mortality was analyzed with a GLM based on quasi-binomial distribution with sting duration, sting site, host encounter sequence (order) and number of stings as factors

Significant *P*-values are presented in bold

### 3.3.3. Surplus killing

The number of *A. aurantii* killed by *A. melinus* and *A. chrysomphali* without being used for egg laying or hostfeeding (surplus killing) depended on the host instar (second vs third instar:  $F_{1,118} = 32$ ;  $P < 0.005$ ) and the parasitoid species (*A. melinus* vs *A. chrysomphali*:  $F_{1,118} = 7.34$ ;  $P = 0.008$ ). The interaction between host instar and parasitoid species was not significant ( $F_{1,117} = 1.72$ ;  $P = 0.19$ ) (Fig. 5).

The number of second-instar *A. aurantii* killed by *A. melinus* without being used for egg laying or host-feeding (surplus killing) was similar to the number of hosts killed for host-feeding, but it was significantly lower than the number of parasitized hosts ( $F_{2,78} = 4.68$ ;  $P = 0.012$ ) (Fig. 5). For *A. chrysomphali*, the number of surplus-killed hosts was similar to the number of hosts killed by hostfeeding and parasitism ( $F_{2,57} = 0.071$ ;  $P = 0.93$ ).

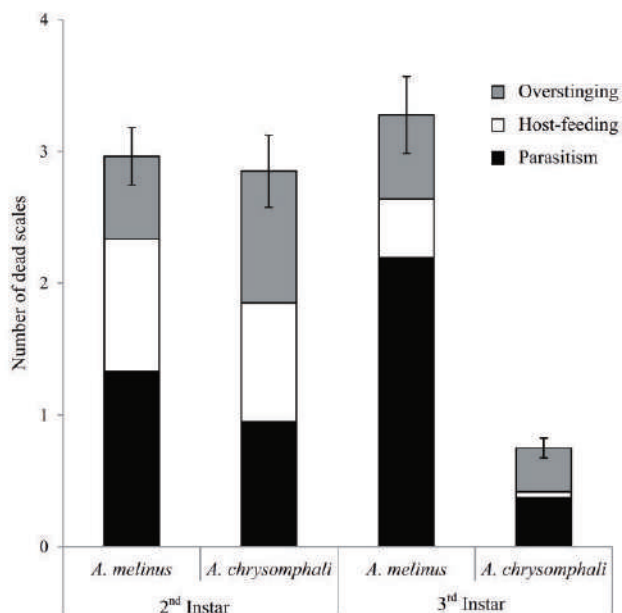


Fig. 5 Mean number of *Aonidiella aurantii* ( $\pm$  SE) dead by parasitism, host-feeding and overstinging when the parasitoids *Aphytis melinus* and *A. chrysomphali* found second- and third-instar scales

The number of third-instar *A. aurantii* killed by *A. melinus* without being used for egg laying or host-feeding (surplus killing) was similar to the number of hosts killed for host-feeding, but it was significantly lower than the number of parasitized hosts ( $F_{2,105} = 26.7$ ;  $P < 0.001$ ) (Fig. 5). For *A. chrysomphali*, the number of surplus-killed hosts was similar to the number of hosts killed by parasitism but higher than those killed by host-feeding ( $F_{2,69} = 4.39$ ;  $P = 0.016$ ).

### 3.3.4. Field observations

Overall, we observed 1933 second- and third-instar *A. aurantii* in the three citrus orchards from April to November. A total of 1079 (55.82%) were alive; 654 (33.83%) were parasitized; and 200 (10.35%) were alive but had brown punctures or scars

(Fig. 6). When we distinguished between instars, only 8 out of the 607 (1.32%) second-instar scales were alive with punctures, whereas 192 out of the 1134 (14.48%) third-instar scales were alive with punctures. The percentage of total live scales with punctures per orchard was significantly higher in the third instar than in the second ( $F_{1,4} = 1.2$ ;  $P < 0.001$ ).

The proportion of live hosts with punctures or scars was positively correlated with the number of immature *Aphytis* ( $\chi^2_1 = 36.81$ ;  $P < 0.001$ ; Fig. 7) and preyed scales ( $\chi^2_1 = 32.6$ ;  $P < 0.001$ ); negatively correlated with the accumulated rain ( $\chi^2_1 = 4.68$ ;  $P = 0.03$ ) and the maximum gust of wind ( $\chi^2_1 = 5.71$ ;  $P = 0.017$ ); and varied among the three sampled orchards ( $\chi^2_2 = 0.0028$ ;  $P = 0.03$ ). There was also a significant interaction between the number of immature *Aphytis* and predated scales ( $\chi^2_1 = 4.43$ ;  $P = 0.035$ ). However, no relationship was discerned between proportion of live hosts with punctures and maximum temperature ( $\chi^2_1 = 0.002$ ;  $P = 0.97$ ).

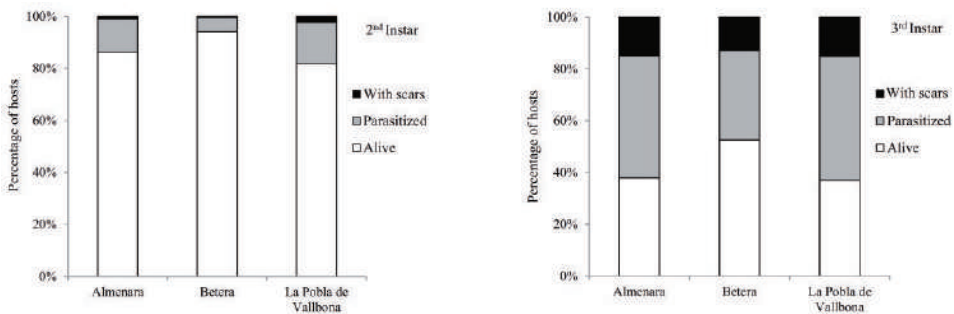


Fig. 6 Percentage of *Aonidiella aurantii* alive, parasitized by *Aphytis*, and likely overstung by *Aphytis* parasitoids in three citrus orchards (Almenara, Bétera and La Poble de Vallbona) from April to November

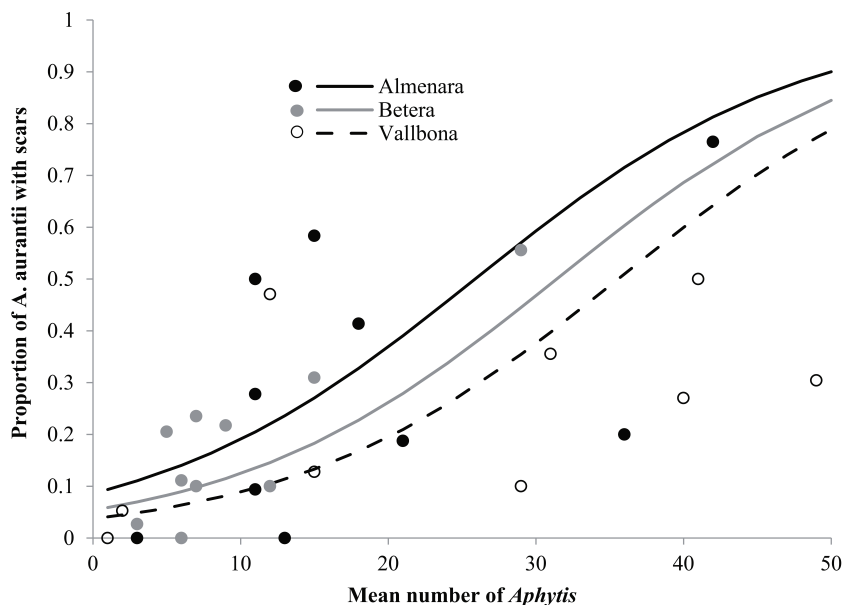


Fig. 7 Relationship between the proportion of *Aurantii aurantii* with scars and the number of *Aphytis* observed in three citrus orchards (Almenara, Bétera and La Pobla de Vallbona) from April to November. Each line represents the relation in each orchard when: number of predated scales = 7.19, max gust of wing = 22.5 km/h; accumulated rain = 27.9 mm; interaction between predated host and *Aphytis* = 115.7. Proportion of *A. aurantii* with scars =  $1/[1 + (1/\{\exp[(0.07 \times \text{number of } Aphytis) + \text{intercept}]\})]$ ;  $P < 0.0001$ ; 52.3% deviance explained)

### 3.4. Discussion

Overall, our results showed that the rejection of a host after stinging (overstinging) is a common behavior in *Aphytis* parasitoids, and it causes the mortality or mutilation of their common host, *A. aurantii*. The virulence of these stings depended on the host instar being attacked and the parasitoid species. Therefore, this behavior might be an important trait to consider in the selection of parasitoids for biological control programs.

*Aphytis* females rejected approximately 30% of the *A. aurantii* hosts they encountered after stinging them with their ovipositor, meaning they did not oviposit on the scale or consume its body fluids. This behavior was as common as parasitism or host-feeding when *A. melinus* and *A. chrysomphali* searched in patches with second-instar hosts (low quality) and even more common than host-feeding in patches with third-instar hosts (high quality). In a previous study, Casas et al. (2004) also found that *A. melinus* tended to oversting approximately 12 and 50% of the second and third instars, respectively, in patches with hosts of different instars. Overstinging seems to also be common in the field, where we recorded many *A. aurantii* scales with punctures that were likely produced by *Aphytis* stings and predators. The number of wounded scales was correlated with parasitoid activity, and most of them were third-instar individuals. This result matches our laboratory observations because the second-instars died after being stung and so could not be recorded in the field samples. Casas et al. (2004) also observed that *Aphytis* tend to sting and reject hosts in the field at even higher rates than in the laboratory when they tracked females for several hours. Therefore, overstinging seems to be a common behavior in the field and not only under experimental laboratory conditions, but its measurement is difficult if the hosts die as occurred with the young *A. aurantii* instars. Although overstinging is commonly observed in parasitoids [see references in Vinson (1976)], its frequency of occurrence and consequences for host physiology have generally been overlooked in the parasitoid literature.

Overstinging affected host survival and fecundity, and its virulence depended on the host instar being attacked. Most immature instars (second-instar hosts) died when overstung by *Aphytis*, whereas ~50% of the adults survived being attacked. Our results supported our initial hypothesis that young hosts are likely more vulnerable to this parasitoid because their immune defences are possibly weaker. Through the insertion

of the ovipositor, parasitoids can inject biochemical compounds as well as cause mechanical injury to host tissues that can lead to increased premature mortality of young hosts (Vinson, 1976; Strand, 1986; van Driesche et al., 1987; Beckage, 2008). In two different systems, the parasitoids of mealybugs (Hemiptera: Pseudococcidae) and leaf miners (Lepidoptera: Gracillariidae) also cause higher mortality rates in younger instars when they reject the host after stinging (Neuenschwander et al., 1986; van Driesche et al., 1987; Barrett & Brunner, 1990).

Parasitoid species also affected the virulence of the overstinging when *A. melinus* and *A. chrysomphali* attacked the third instar. The former parasitoid killed more adult hosts than *A. chrysomphali* and, moreover, reduced the fecundity of the surviving females, which demonstrates the superiority of *A. melinus* as a biological control agent of *A. aurantii* compared with *A. chrysomphali* (DeBach & Sisojevic, 1960; Rosen & DeBach, 1979; Pekas et al., 2010, 2016; Boyero et al., 2014; Cebolla et al., 2017b). The mortality caused by *A. melinus* was fourfold greater than that caused by *A. chrysomphali* when considering the three behaviors measured in this assay (parasitism, host-feeding and overstinging). Van Driesche et al. (1987) also compared the mortality caused by two parasitoids of the mealybug *Phenacoccus herreni* Cox and Williams (Hemiptera: Pseudococcidae) and observed that the mortality caused by *Epidinocarsis diversicornis* (Howard) was almost twice that of *Acerophagus coccois* Smith (Hymenoptera: Encyrtidae) when both reject their common host after stinging. Both results confirm the importance of measuring the frequency of occurrence and the consequences of overstinging on host physiology.

The mortality caused by *Aphytis* parasitoids depended on the duration of the stings when third-instar hosts were encountered, and it is likely that the mechanical damage as well as the potential amount of venom proteins (Asgari & Rivers, 2011)

and polynadvirus (Beckage, 2008) injected by the parasitoids increased with the time spent stinging. In fact, more than the 80% of the hosts died when the ovipositor was inside for more than 240 s. Keinan et al. (2012) studied the fitness implications of multiple stinging events and found that all of the hosts died after 4–5 stings, but this study included mortality induced by parasitoid oviposition. In our study, we did not find a correlation between mortality and the number of stings. Regardless, *A. melinus* spent more time than *A. chrysomphali* overstinging its host, which might partially explain the differences in the virulence of both parasitoids.

Overstinging by *Aphytis melinus* also reduced the fecundity of the surviving host, whereas this effect was not observed with *A. chrysomphali*. Previous studies have reported detrimental fitness costs, such as the suppression of gonad development in the host after being stung (Reedlarsen & Brown, 1990; Brown & Kainoh, 1992; Münster-Swendsen, 1994; Tagashira & Tanaka, 1998; Digilio et al., 2000; Barratt & Johnstone, 2001). These studies are based on hymenopteran parasitoids attacking lepidopteran hosts in the egg or larval stages, but the authors could not determine whether the female parasitoid laid an egg or just stung its host. Therefore, the damage could be caused by the sting or the immature parasitoid. Generally, these attacks end with the castration of the young instars (Baudoin, 1975). Adult host castration is uncommon and rarely complete, and fecundity is generally only slightly reduced (Spencer, 1926; Beard, 1940; Schlinger & Hall, 1960) as occurred when *A. melinus* attacked adult *A. aurantii*. This is because gonadal tissues are generally well formed by the time the host reaches the adult instar (Reed-larsen & Brown, 1990).

From a biological control point of view, our result supports the idea that overstinging should be considered when evaluating the efficacy of parasitoids as biological control agents, as has also been recently suggested for other cases of parasitoid-induced

mortality (Abram et al., 2016). In this sense, it is important to highlight the differences between parasitoids and predators. In the literature considering natural predators, surplus killing or overkilling is generally taken into consideration when describing predator behavior and the potential for use as a biological control agent (Pekár, 2005; Monzó et al., 2009; Pérez-Hedo & Urbaneja, 2015). The importance of overstinging and its consequences for the host (mortality and mutilation) is far from being a phenomenon isolated to this system as this behavior has been widely described in numerous parasitoids (Vinson, 1976). One of the best-known cases of overstinging and its consequences on the host was described by Münster-Swendsen (1994, 2002). He demonstrated that the parasitoid *Apanteles tedellae* Nix. (Hymenoptera: Braconidae) caused the sterilization of its host *Epinotia tedella* (Cl.) (Lepidoptera: Tortricidae) when parasitoids are disturbed before depositing an egg. This effect was later included in several models to analyze the dynamics of the host and detect the causes of population cycles (Münster-Swendsen, 2002; Münster-Swendsen & Berryman, 2005). These authors demonstrated that is the total combined impact of parasitism on mortality and fecundity that apparently provides the strong negative feedback needed to drive population cycles in all species of this community. The population dynamics of *A. aurantii*-*Aphytis* has been also analyzed (Murdoch et al., 1995, 1996, 2005), but the frequency and consequences of overstinging have not been included. Further research should consider them and, likely, also parasitoid state because the frequency of overstinging might depend on parasitoid state (i.e., number of mature eggs, age or nutritional state), which also affects stability in insect host–parasitoid population models (Shea et al., 1996; Murdoch et al., 1997)".

## Acknowledgements

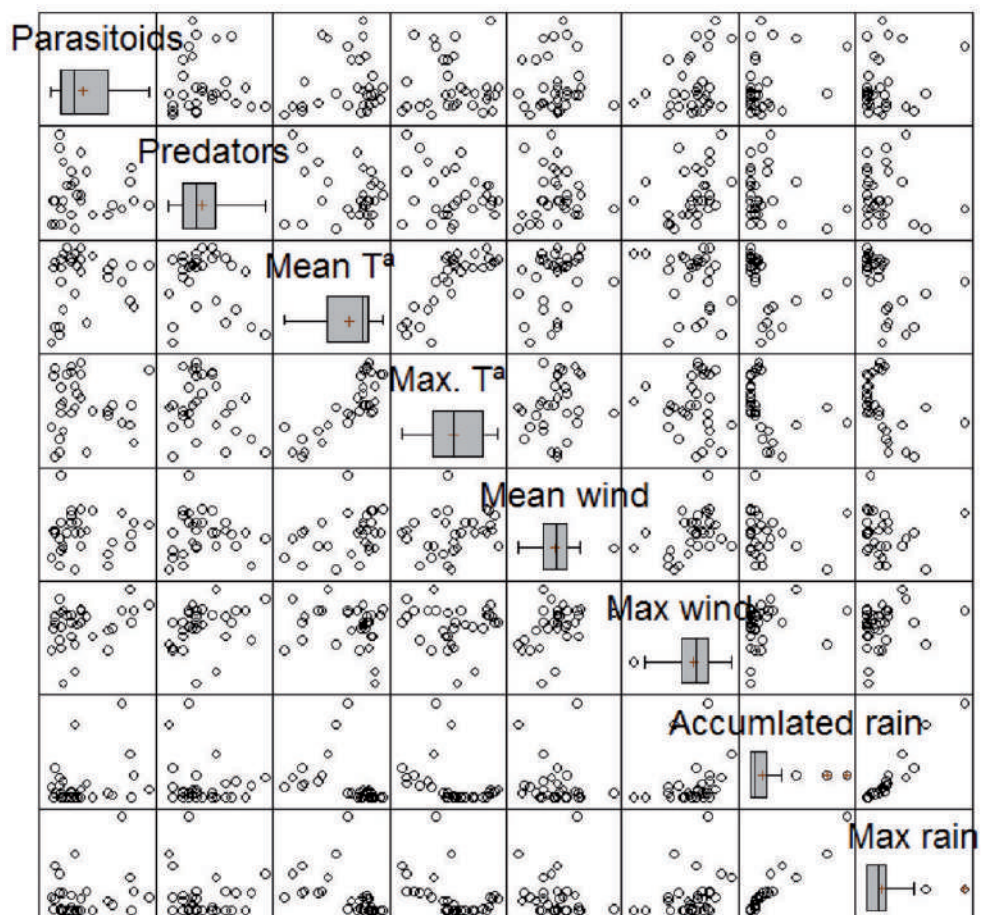
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## Annex

Annex 1 Correlation matrix between all variables to select them for the multiple correlation analysis

		Parasitoids	Predators	Mean T <sup>a</sup>	Max. T <sup>a</sup>	Mean wind	Max wind	Accumulated rain	Max rain
Parasitoids	R <sup>2</sup>		-0.04	0.11	-0.06	0.02	0.31	0.26	0.20
	P		0.82	0.54	0.77	0.90	0.09	0.16	0.27
Predators	R <sup>2</sup>	-0.04		-0.22	-0.22	-0.03	0.30	-0.16	-0.12
	P	0.82		0.23	0.23	0.85	0.10	0.38	0.54
Mean T <sup>a</sup>	R <sup>2</sup>	0.11	-0.22		<b>0.82</b>	0.32	-0.20	-0.38	<b>-0.49</b>
	P	0.54	0.23		<b>0.00</b>	0.08	0.28	0.03	<b>0.01</b>
Max. T <sup>a</sup>	R <sup>2</sup>	-0.06	-0.22	<b>0.82</b>		0.23	-0.06	-0.37	<b>-0.44</b>
	P	0.77	0.23	<b>0.00</b>		0.22	0.76	0.04	<b>0.01</b>
Mean wind	R <sup>2</sup>	0.02	-0.03	0.32	0.23		<b>0.40</b>	-0.14	-0.13
	P	0.90	0.85	0.08	0.22		<b>0.02</b>	0.47	0.49
Max wind	R <sup>2</sup>	0.31	0.30	-0.20	-0.06	<b>0.40</b>		0.22	0.26
	P	0.09	0.10	0.28	0.76	<b>0.02</b>		0.23	0.16
Accumulated rain	R <sup>2</sup>	0.26	-0.16	-0.38	-0.37	-0.14	0.22		<b>0.96</b>
	P	0.16	0.38	0.03	0.04	0.47	0.23		<b>0.00</b>
Max rain	R <sup>2</sup>	0.20	-0.12	<b>-0.49</b>	<b>-0.44</b>	-0.13	0.26	<b>0.96</b>	
	P	0.27	0.54	<b>0.01</b>	<b>0.01</b>	0.49	0.16	<b>0.00</b>	

Significant *P*-values are presented in bold





## Chapter 4

# EFFECT OF HOST INSTAR ON HOST DISCRIMINATION OF HETEROSPECIFIC- PARASITISED HOSTS BY SYMPATRIC PARASITOIDS





## 4. EFFECT OF HOST INSTAR ON HOST DISCRIMINATION OF HETEROSPECIFIC-PARASITISED HOSTS BY SYMPATRIC PARASITOIDS \_\_\_\_\_

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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 unparasitised hosts and hosts parasitised by another parasitoid species (i.e. heterospecific host discrimination). However, discriminatory ability might be affected by host instar.
2. This study reports the first results on whether host instar can influence the use of heterospecific-parasitised hosts by sympatric parasitoids of the genus *Aphytis* (Hymenoptera: Aphelinidae).
3. *Aphytis melinus* and *Aphytis chrysomphali* discriminated between unparasitised and heterospecific-parasitised hosts when they found a third-instar host (high quality), with a tendency to multi-parasitise. However, this discrimination was not observed in the second instar (lower size).
4. The behavioural strategies adopted towards multi-parasitise third-instar hosts varied between both species. *Aphytis chrysomphali* reduced its clutch size in heterospecific-parasitised hosts, whereas *A. melinus* tended to probe them for longer than healthy hosts.
5. Overall, our results highlight the importance of host instar in the study of intrinsic competition between parasitoids.

Keywords *Aphytis* • *Aonidiella aurantii* • Behavioural Ecology • Interspecific Competition • Intrinsic Competition • Multi-parasitism

#### 4.1. Introduction

Interspecific competition among hymenopteran parasitoids has shaped their behavioural strategies with regard to host resource exploitation (Connell, 1980; Hawkins, 2000). Contrary to prey eaten by predators, immature parasitoids do not immediately consume host resources. Parasitised hosts remain *in situ* and are vulnerable to attack by other foraging females (van Alphen & Visser, 1990; Godfray, 1994; Wajnberg et al., 2008). When encountering parasitised 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 parasitised hosts (i.e. multi-parasitise them) (Goubault et al., 2004; Boivin & Brodeur, 2006; Hopper et al., 2013). The decision implies that the female is able to distinguish between unparasitised hosts and hosts parasitised 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 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 have detected that the located host is already parasitised, 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 whose larvae are superior competitors are more likely to multi-parasitise than those whose larvae are inferior competitors (van Alebeek et al., 1993). Under these circumstances, the latter is expected to benefit from host discrimination, as this prevents wastage of their eggs in hosts parasitised by the superior parasitoid (Pedata et al., 2002; Wang & Messing, 2004). Thus, the female decision to multi-parasitise will depend on the probability of a second egg or clutch winning the competition against the first (Netting & Hunter, 2000). In contrast to unparasitised hosts, larvae in multi-parasitised hosts develop under conditions that are unfavourable in both quantity and quality (Harvey et al., 2009, 2013). The limited host resources have to be shared with the competitor (Cusumano et al., 2015, 2016). Furthermore, the quality of the available resources can be altered by the injection of regulatory factors by the female parasitoid during oviposition (Pennacchio & Strand, 2006; Beckage, 2012). Therefore, intrinsic competition can negatively affect fitness-related life-history traits such as immature mortality, sex ratio, developmental time, or the size of the offspring (Collier et al., 2007; Cingolani et al., 2013; Cusumano et al., 2013, 2015).

The response to heterospecific-parasitised hosts might also be affected by host size/instar. Generally, within a host species, large hosts (i.e. older instars) are considered higher quality for parasitoid development, as they provide more food for the developing progeny than small hosts. A larger host may benefit 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 heterospecific-parasitised hosts might therefore exist and affect host acceptance rates. Previous experimental assays designed to test this hypothesis have not, to our knowledge, been reported. Therefore, the aim of this study was to determine whether

a female is more willing to accept heterospecific parasitised third-instar hosts (large size) than second-instar hosts (small size).

#### 4.1.1. Parasitoid-host system

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

## 4.2. Material and methods

### 4.2.1. Insect rearing

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

*Aphytis melinus* and *A. chrysomphali* are facultative gregarious synovigenic (i.e. females mature eggs throughout their adult lives), idiobibiont (i.e. the host is paralysed and arrests development once parasitised) and ectoparasitoid species (Rosen & DeBach, 1979). They exploit the same hosts and both feed and lay eggs on them (Pekas et al., 2010). Individuals of both species were obtained by exposing third-instar *A. aurantii* reared on lemons to parasitism by the foraging adult females. The parasitoids were maintained in the laboratory at  $26 \pm 1$  °C,  $60 \pm 5\%$  RH and LD 16:8 h. Cultures of *A. melinus* and *A. chrysomphali* were initiated in 2008 and 2013, respectively,

from parasitised *A. aurantii* scales collected from citrus fields, Valencia, Spain. Both cultures are renewed yearly with field-collected parasitoids.

Between five and 10 late-stage pupae of both parasitoid species were removed from parasitised scales and separated into crystal vials (diameter 8 mm, length 35 mm) and stoppered with a cotton plug. At emergence, *A. melinus* were sexed, and males and females were held together for a 24-h 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 3 days in the absence of a carbohydrate source (Heimpel et al., 1997), and sugars are not gained from host feeding (Tena et al., 2013a). Vials were stored in a climatic chamber (SANYO MLR-350; Sanyo, Japan) at  $25 \pm 1$  °C, 50–70% RH and LD 14:10 h.

#### 4.2.2. Experimental arena

All behavioural observations were conducted on colonised lemon fruit as previously described. Second- (0.5–0.7 mm<sup>2</sup>) or third-instar scales (0.8–1 mm<sup>2</sup>) 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 colour camera (Leica Microsystems GmbH, Barcelona, Spain). Images were processed with Leica LAS EX imaging software for Windows and the area of the scales (mm<sup>2</sup>) was measured with IMAGEJ, a public-domain Java image-processing program (Rasband, 2016).

The selected scale was mapped, and the remaining scales were removed using an entomological needle and a paper moistened with water.

#### 4.2.3. Adult female behaviour

Female parasitoids were introduced individually into a glass Petri dish (diameter 4 cm, height 1.5 cm) and the Petri dish was 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 was 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 10× to 50× magnification with a cool fibre light to illuminate the arena. Behavioural recording began when the female recognised the hosts, which 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 the mouthparts (van Lenteren, 1994). Observations terminated when the female left the scale or when resting behaviour continued for more than 2 min. Three separate behavioural components were identified, timed and recorded: (i) rejection; (ii) oviposition; and (iii) 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 it (Casas et al., 2004). The parasitoid may leave the host at any time during this process (hereafter 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).

Once the behavioural assays were complete, the parasitoid was removed from the experimental arena and the lemon moved to a plastic container (14 × 14 × 8 cm) covered with muslin to determine the outcome of the encounters. The containers were kept in climatic chamber under the same environmental conditions (25 ± 1 °C, 50–70% RH, LD 14:10 h) until developing parasitoids reached the pupal stage.

Finally, the cost of competition was determined by testing whether multi-parasitism imposed a time cost for either species. To achieve this, host handling times in unparasitised versus heterospecific-parasitised hosts were compared.

#### 4.2.4. Effect of host instar (size) on host discrimination

The discriminatory ability of either parasitoid species between unparasitised and parasitised hosts of the second and third instars was determined by comparing female behaviours. The following behaviours were quantified for this purpose: acceptance rates, clutch size (i.e. number of eggs laid per host), handling time and duration

of probing. Host handling time was defined as the sum of probing duration and oviposition.

#### 4.2.5. Effect of host instar (size) on immature mortality, brood size, and sex ratio in multi-parasitised hosts

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

#### 4.2.6. Statistical analyses

Treatments (host instar and parasitoid species) were compared using two-way ANOVA. Normality was assumed for handling time and probing duration, assumption 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 Pearson's  $\chi^2$  divided by the residual degrees of freedom and the

significance of the explanatory variables reassessed using an  $F$ -test (Crawley, 2007). The means of untransformed proportion and count data are 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>).

### 4.3. Results

#### 4.3.1. Effect of host instar (size) on host discrimination

**Acceptance.** *Aphytis melinus* females accepted significantly more third-instar hosts ( $0.77 \pm 0.06$ ) than second-instar hosts (acceptance ratio =  $0.55 \pm 0.07$ ) ( $F_{1,117} = 6.11$ ,  $P = 0.02$ ) (Table 1). However, there were no significant differences between the acceptance of hosts parasitised by the competitor (*A. chrysomphali*) ( $0.6 \pm 0.06$ ) and the acceptance of unparasitised hosts ( $0.72 \pm 0.06$ ) ( $F_{1,117} = 1.86$ ,  $P = 0.18$ ) (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 ( $0.47 \pm 0.07$ ) and that of second-instar hosts ( $0.53 \pm 0.07$ ) ( $F_{1,117} = 0.52$ ,  $P = 0.47$ ), and between hosts parasitised by the competitor (*A. melinus*) ( $0.42 \pm 0.06$ ) and unparasitised hosts ( $0.58 \pm 0.06$ ) ( $F_{1,117} = 3.28$ ,  $P = 0.72$ ) (Table 2). The interaction between host instar and competition was not significant ( $F_{1,116} = 1.13$ ,  $P = 0.72$ ).

**Table 1** Ratio of accepted (parasitised), rejected (before or after probing), and host-fed hosts (mean  $\pm$  SE) when unparasitised (control) and heterospecific-parasitised second- (N2) and third-instar (N3) *Aonidiella aurantii* were exposed to *Aphytis melinus*.

Host					
Instar	State	Acceptance (parasitised)	Rejected before probing	Rejected after probing	Host-fed
N2	Parasitised by <i>Aphytis chrysomphali</i>	0.43 $\pm$ 0.09 <sup>Ba</sup>	0.07 $\pm$ 0.05	0.1 $\pm$ 0.06	0.4 $\pm$ 0.09
	Unparasitised	0.67 $\pm$ 0.09 <sup>Ba</sup>	0.1 $\pm$ 0.06	0.1 $\pm$ 0.06	0.13 $\pm$ 0.06
N3	Parasitised by <i>A. chrysomphali</i>	0.77 $\pm$ 0.0 <sup>Aa</sup>	0.00	0.17 $\pm$ 0.07	0.07 $\pm$ 0.05
	Unparasitised	0.77 $\pm$ 0.08 <sup>Aa</sup>	0.07 $\pm$ 0.05	0.13 $\pm$ 0.06	0.03 $\pm$ 0.03

Different upper-case and lower-case letters denote significant differences at  $P < 0.05$  between host instars and in host status (unparasitised versus heterospecific-parasitised), respectively.

**Table 2** Ratio of accepted (parasitised), rejected (before or after probing), and host-fed hosts (mean  $\pm$  SE) when unparasitised (control) and heterospecific-parasitised second- (N2) and third-instar (N3) *Aonidiella aurantii* were exposed to *Aphytis chrysomphali*.

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

Different upper-case and lower-case letters denote significant differences at  $P < 0.05$  between host instars and in host status (unparasitised versus heterospecific-parasitised), respectively.

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

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

**Handling time.** *Aphytis melinus* females spent significantly more time parasitising third-instar hosts ( $267.05 \pm 15.97$  s) than second-instar hosts ( $203.58 \pm 19.07$ ) ( $F_{1,73} = 5.94, P = 0.017$ ), but they spent the same time in hosts parasitised by the competitor (*A. chrysomphali*) ( $253.44 \pm 20.65$ ) and in unparasitised hosts ( $226.94 \pm 15.47$ ) ( $F_{1,73} = 1.16, P = 0.28$ ) (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 parasitising third-instar hosts ( $332 \pm 18.33$  s) than parasitising second-instar hosts ( $190.28 \pm 11.74$ ) ( $F_{1,57} = 39.02, P < 0.0001$ ) but they spent the same time in hosts parasitised by the competitor (*A. melinus*) ( $262.8 \pm 25.4$ ) and in unparasitised hosts ( $268.06 \pm 17.15$ ) ( $F_{1,57} = 0.05, P = 0.82$ ) (Fig. 2b). The interaction between host instar and competition was not significant ( $F_{1,56} = 2.17, P = 0.15$ ).

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

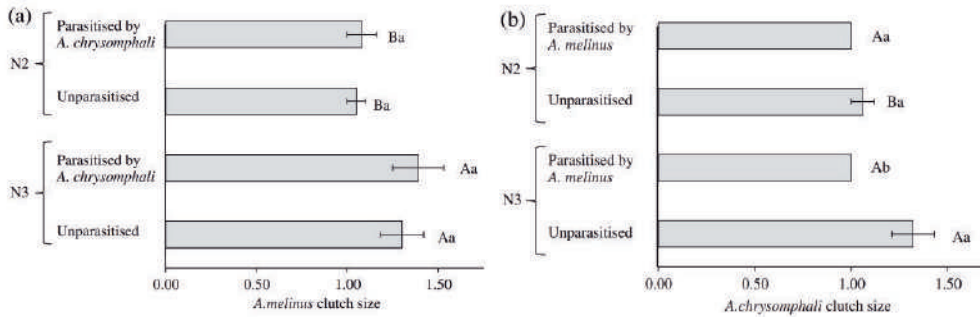


Fig. 1 Clutch size (mean  $\pm$  SE) of *Aphytis melinus* (a) and *Aphytis chrysomphali* (b) when they accepted an unparasitised (control) and heterospecific-parasitised second- (N2) and third-instar (N3) *Aonidiella aurantii*. Different upper-case and lower-case letters beside the bars denote significant differences at  $P < 0.05$  between instars and in host status (unparasitised versus heterospecific-parasitised), respectively.

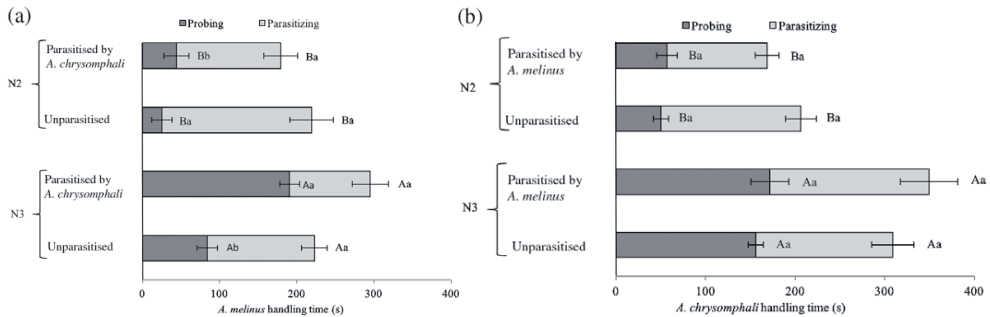


Fig. 2 Host handling time (mean  $\pm$  SE) (time spent probing + time spent parasitising) of *Aphytis melinus* (a) and *Aphytis chrysomphali* (b) when they accepted an unparasitised (control) and heterospecific-parasitised second- (N2) and third-instar (N3) *Aonidiella aurantii*. Different letters inside and beside the bars denote significant differences at  $P < 0.05$  in the time spent probing and the time spent parasitising, respectively. Differences between host instars are represented by different upper-case letters and differences in host status (unparasitised versus heterospecific parasitised) by lower-case letters.

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

### 4.3.2. Effect of host instar (size) on immature mortality, brood size, and sex ratio in multi-parasitised hosts

**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 on competition, as immature mortality of *A. melinus* and *A. chrysomphali* increased significantly with multi-parasitism (*A. melinus*,  $F_{1,76} = 9.96$ ,  $P < 0.001$ ; *A. chrysomphali*,  $F_{1,58} = 12.96$ ,  $P < 0.001$ ) (Tables 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*,  $F_{1,57} = 0.06$ ,  $P = 0.80$ ).

**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 unparasitised (control) and heterospecific-parasitised second- (N2) and third-instar (N3) *Aonidiella aurantii*.

Factor	Treatment	N2	N3
Immature mortality	Control	0.10 $\pm$ 0.07 (20) <sup>Ab</sup>	0.15 $\pm$ 0.07 (23) <sup>Ab</sup>
	Multi-parasitism	0.27 $\pm$ 0.12 (13) <sup>Aa</sup>	0.57 $\pm$ 0.10 (13) <sup>Aa</sup>
Second brood size	Control	1 (18) <sup>Aa</sup>	1.10 $\pm$ 0.07 (21) <sup>Aa</sup>
	Multi-parasitism	1 (10) <sup>Aa</sup>	1.18 $\pm$ 0.18 (11) <sup>Aa</sup>
Sex ratio	Control	0.59 $\pm$ 0.12 (18) <sup>Aa</sup>	0.13 $\pm$ 0.07 (20) <sup>Ba</sup>
	Multi-parasitism	0.70 $\pm$ 0.02 (10) <sup>Aa</sup>	0.20 $\pm$ 0.09 (10) <sup>Ba</sup>

Different upper-case and lower-case letters denote significant differences at  $P < 0.05$  between instars and in host status (unparasitised versus heterospecific parasitised), respectively.

**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 unparasitised (control) and heterospecific parasitised second- (N2) and third-instar (N3) *Aonidiella aurantii*.

Factor	Treatment	N2	N3
Immature mortality	Control	0.31 $\pm$ 0.12 (16) <sup>Ab</sup>	0.13 $\pm$ 0.06 (19) <sup>Ab</sup>
	Multi-parasitism	0.75 $\pm$ 0.13 (12) <sup>Aa</sup>	0.69 $\pm$ 0.13 (13) <sup>Aa</sup>
Second brood size	Control	1 (11) <sup>Aa</sup>	1.16 $\pm$ 0.08 (19) <sup>Aa</sup>
	Multi-parasitism	1 (3) <sup>Aa</sup>	1 (4) <sup>Aa</sup>

Different upper-case and lower-case letters denote significant differences at  $P < 0.05$  between instars and in host status (unparasitised versus heterospecific parasitised), respectively.

**Brood size.** In the case where at least one parasitoid emerged, the brood sizes of *A. melinus* and *A. chrysomphali* were 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*,  $F_{1,33} = 0.85$ ,  $P = 0.36$ ) (Tables 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.

**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 in the third instar it was female-biased. 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.

#### 4.4. Discussion

##### 4.4.1. 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 unparasitised and heterospecific-parasitised 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; Goubault et al., 2004; Goubault et

al., 2005; Boivin & Brodeur, 2006; Hopper et al., 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. In contrast with our results, the parasitoid *Pachycrepoideus vindemmiae* Rondani (Hymenoptera: Pteromalidae) was found to discriminate between parasitised and unparasitised 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 of finding cues on bigger hosts (Goubault et al., 2004).

Previous studies have documented that some *Aphytis* species are able to discriminate hosts parasitised by conspecifics (Abdelrahman, 1974b; 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's (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). Host rejection of heterospecific parasitised third-instar hosts, however, occurred significantly more frequently after females had inserted the ovipositor into the host. This suggests that the recognition of heterospecific-parasitised hosts occurs in response to internal cues or physiological changes in the host after oviposition. A recent study by Ruschioni et al. (2015) showed that the neurons present in the sensillum of the ovipositor tip of *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) are used for host discrimination between parasitised and unparasitised larvae of *Drosophila* spp.

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

The behavioural strategy adopted during parasitism of a heterospecific-parasitised host was found to vary between species. *Aphytis chrysomphali* reduced its clutch size in heterospecific-parasitised hosts, whereas *A. melinus* laid the same number of eggs in healthy and heterospecific-parasitised hosts. When we compared the time spent parasitising, *A. melinus* tended to probe heterospecific-parasitised hosts for longer than healthy ones, whereas there were no 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 once in the case of heterospecific parasitism (Magdaraog et al., 2013). Regarding the increased time spent probing in heterospecific-parasitised 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., 2017a).

Finally, time costs may affect female propensity to multi-parasitise, at least in species that are time-limited (Strand & Godfray, 1989; van Alphen & Visser, 1990). *Aphytis* parasitoids, however, are egg-limited (Heimpel et al., 1996; Casas & Nisbet, 2000; Casas & Mccauley, 2012; Tena et al., 2015) and the same time is needed to multi-parasitise as to parasitise a healthy host, independent of host instar. Therefore, time costs are negligible for both parasitoids when they multi-parasitise.

#### 4.4.2. Effect of host instar (size) on immature mortality, brood size and sex ratio in multi-parasitised hosts

A high cost in terms of immature mortality was incurred under multi-parasitism for both parasitoids, independent of host instar. When multi-parasitism occurs, the quality of the host may be disrupted by multiple regulatory factors such as the injection of polydnaviruses and virus-like particles by the female parasitoid during oviposition (Pennacchio & Strand, 2006; Beckage, 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 multi-parasitism occurred in  $37.5 \pm 10.1\%$  and  $42.9 \pm 8.5\%$  of the second- and third-instar hosts that were multi-parasitised, respectively. Ovide and competition between immatures may also have affected offspring mortality (Cebolla et al., 2017a).

Interspecific larval competition can also impact the development of immature parasitoids and the fitness of the emerging offspring (Harvey et al., 2013; Cusumano et al., 2013). Here, multi-parasitism 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 multi-parasitism. Similar to the results presented here, the secondary sex ratio was not affected by multi-parasitism 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. (2017a), provide new

insights into the importance of host size in the competition between the sympatric parasitoids *A. melinus* and *A. chrysomphali*. These 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*.

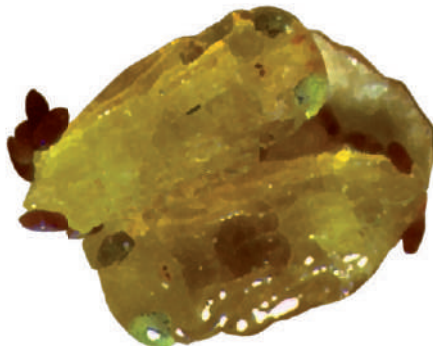
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## Chapter 5

# DOES HOST QUALITY DICTATE THE OUTCOME OF INTERFERENCE COMPETITION BETWEEN SYMPATRIC PARASITOIDS? EFFECTS ON THEIR COEXISTENCE





## 5. DOES HOST QUALITY DICTATE THE OUTCOME OF INTERFERENCE COMPETITION BETWEEN SYMPATRIC PARASITOIDS? EFFECTS ON THEIR COEXISTENCE \_\_\_\_

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### Abstract

The suitability and quality of herbivorous insect hosts for hymenopteran parasitoids is dynamic, varying with host development. Generally, within a host species, large hosts (i.e. older instars) are considered of higher quality for parasitoid development. Studies of interspecific competition between parasitoids have considered the effect of host instar on indirect competition but its effect on interference competition remains unknown. Here, we report the first results on whether the quality of host instars might dictate the outcome of interference competition between sympatric parasitoids of the genus *Aphytis* (Hymenoptera: Aphelinidae) when they attack low-quality (second) and high-quality (third) instars of their common host *Aonidiella aurantii* (Hemiptera: Diaspididae). Oviposition behaviour (host acceptance and clutch size) in low- and high-quality host instars was similar for both *Aphytis* species in the absence of competition. When they found heterospecific parasitized hosts of high quality, *Aphytis melinus* laid more eggs and accepted significantly more hosts than *Aphytis chrysomphali*, whereas there were no significant differences in the low-quality instar. This result suggests that interference competition is mediated by host quality. However, the progeny proportion of both parasitoids in multiparasitized hosts (outcome of competition) was independent of host quality and *A. melinus* always emerged at higher rates. Therefore, the result of interference competition between these sympatric parasitoids was not affected by host quality and this competition will contribute to the displacement of the native *A. chrysomphali* by the introduced *A. melinus*, which has been observed in some areas of the Mediterranean basin.

**Keywords** *Aphytis* • *Aonidiella aurantii* • Armoured scales • Competitive Exclusion • Infanticide • Interspecific Competition • Intrinsic Competition • Size-mediated Interactions

### 5.1. Introduction

Interspecific competition drives community structure and function (Morin, 2011). In extreme cases, stronger competitors can drive weaker competitors to extinction by monopolizing resources (Chesson, 2000). Herbivorous insects are frequently attacked by several hymenopteran parasitoid species whose larvae engage in interspecific competition (Godfray, 1994). Host suitability and quality vary during development and can dictate the outcome of competition among developing parasitoids sharing a host (Price, 1972; Harvey et al., 2013). Generally, parasitoid species that find and parasitize younger hosts have an exploitative advantage over their antagonists because they can use their host earlier in the sea son and also because they have a head start in intrinsic competition (i.e. competition among immature stages (eggs or larvae) of parasitoids on or in hosts). This competition among free-living adult parasitoids searching for and using hosts of different instars/sizes is a type of interference competition and has been documented in the field (Luck & Podoler, 1985; Bográn et al., 2002) as well as in theoretical studies (Briggs, 1993; Murdoch et al., 1996; Harvey et al., 2013). However, the effect of the host instar/stage on interference competition has never been tested, and we hypothesized that the host instar might facilitate the coexistence of ecological homologue parasitoids when the outcome of competition depends on the parasitized instar/stage.

Several mechanisms related to the behaviour of the mother and/ or competition between larvae might explain the apparent instarrelated reduction in competitive advantage (Collier et al., 2007; Harvey et al., 2013; Cusumano et al., 2016). First, the mother can provide an advantage to its own progeny by killing immature individuals of the competing species or by laying a larger clutch (Tena et al., 2008; Cusumano et al., 2016). We expected this behaviour to vary depending on host suitability and

quality, i.e. instar (Hopper et al., 2013). We thus hypothesized that a mother would be less willing to expend energy and time killing progeny of a competitor species in a heterospecific-parasitized host of low quality, i.e. small or young instar. Second, competition between immature parasitoids through either physical contests or a scramble for host resources may also depend on the host instar. For example, parasitoid species with long embryonic development times might have a higher probability of surviving in adult hosts than in young/small hosts in which resources are scarcer. Finally, the outcome might depend on a combination of maternal behaviour and offspring competition.

Here, we studied whether the host instar/stage dictates the outcome of interference competition between parasitoids of the genus *Aphytis* (Hymenoptera: Aphelinidae) and facilitates their coexistence in sympatry in Mediterranean citrus (Sorribas et al., 2010; Pekas et al., 2016). The introduced species *Aphytis melinus* is a superior competitor to the native *Aphytis chrysomphali* as a parasitoid of *Aonidiella aurantii* (Hemiptera: Diaspididae). Their coexistence has been attributed to fluctuating environmental conditions, seasonal variation in parasitoid abundance (Pina, 2007; Sorribas et al., 2010; Boyero et al., 2014) and, more recently, the plasticity of *A. chrysomphali* in exploiting different host instars depending on the *A. melinus* density (Pekas et al., 2016). The latter field study showed that *A. chrysomphali* are recovered in greatest numbers from second-instar hosts, which are poorer quality hosts, when the *A. melinus* density is high and exploits the third instar, a higher quality host (Pekas et al., 2016). However, we hypothesized that this conditional patch partitioning might reflect the fact that *A. melinus* is a superior competitor when both parasitoids parasitize third-instar hosts (high quality), but carries less advantage in second-instar hosts (low quality).

To test our hypothesis and the mechanisms underlying it, we first observed female parasitoids to directly investigate whether females can provide an advantage to their own progeny by laying a larger clutch or killing the progeny of the competitor, depending on the host instar. Then, we analysed the intrinsic competition between parasitoid species to test whether the outcome depends on host instar and/or order of attack (generally, the offspring of the first female have an advantage). Finally, we provide an explanation for the coexistence of *A. melinus* and *A. chrysomphali* in terms of the results obtained here and in a field study (Pekas et al., 2016).

## 5.2. Methods

### 5.2.1. System

Parasitoids of the genus *Aphytis* are the most successful and widespread biological control agents of *A. aurantii* in citrus (DeBach & Rosen, 1991; Foster & Luck, 1996; Murdoch et al., 2005). These specialist parasitoids can reduce their shared host to levels nearly 200 times lower than the average density observed in their absence (DeBach et al., 1971), suggesting strong resource competition between parasitoid species (Borer et al., 2004). In fact, species of the genus *Aphytis* represent one of the best-known cases of competitive displacement in insects (Luck et al., 1982; Luck & Podoler, 1985; Luck & Nunney, 1999; Sorribas et al., 2010; Pekas et al., 2016). *Aphytis melinus* displaced *Aphytis lingnanensis* (Hare & Luck, 1991) in interior California (Podoler, 1981; Luck et al., 1982; Luck & Podoler, 1985) because the former uses smaller hosts for production of female progeny such that it exploits its hosts before they reach a size suitable for the production of female *A. lingnanensis* (Luck & Podoler, 1985; Luck & Nunney, 1999; Hudak, 2003). Thus, host size represents a resource that is available for the developing parasitoid and is probably the most reliable cue of host quality for

*Aphytis* (Hare & Luck, 1991; Pekas et al., 2010). In fact, larger adults of *A. melinus* and *A. chrysomphali* emerge from third-instar hosts than from the second instar; they prefer the third instar when both instars are available, and the immature mortality is slightly lower in the third than in the second instar (Hare & Luck, 1991; Pekas et al., 2010; Pina, 2007; Pekas et al., 2016; Table 2). In the Mediterranean basin, *A. melinus* has displaced *A. chrysomphali* in some areas, whereas the species coexist in other areas (Sorribas et al., 2010). Although *A. chrysomphali* reproduces parthenogenetically and produces only females when it is infested with the bacterium *Wolbachia* (Pina, 2007), *A. melinus* is considered a superior competitor in the field because it has a higher capacity for dispersion (McLaren, 1976) and is better adapted to climates where citrus is cultivated (Abdelrahman, 1974a; Rosen & DeBach, 1979).

### 5.2.2. Insects

The host herbivore *A. aurantii* was reared on lemons, *Citrus limon*, from a laboratory colony at the Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain. This colony was initiated in 1999 from scales collected from citrus fields in Alzira in Valencia, Spain, and renewed every 2-3 years with field-collected scales (Tena et al., 2013a). Approximately two-thirds of the surface of each lemon was covered with red paraffin around the mid-section to retard its desiccation. The red paraffin was prepared with a mixture of 1 kg paraffin pearls (Parafina USP Perlas; Guinama S.L., Alboraya, Spain) and 1 g red pigment (Sudan III; Panreac Química S.A., Castellar del Vallés, Spain). The remaining surface (approximately 24 cm<sup>2</sup> area) of the lemons was infested by exposure for 48 h to gravid female scales from the *A. aurantii* colony. Once they were infested, lemons were maintained at  $27 \pm 1$  °C at  $70 \pm 5\%$  relative humidity and darkness until female scales reached the second

(9–11 days) and third (19–22 days) nymphal instars, both of which were used in these assays.

*Aphytis melinus* and *A. chrysomphali* were obtained by exposing third-instar *A. aurantii* on lemons to parasitism by insectary-reared adult wasps maintained in the laboratory at  $26 \pm 1$  °C,  $60 \pm 5\%$  relative humidity and 16:8 h light:dark cycle. The colonies of *A. melinus* and *A. chrysomphali* were initiated in 2008 and 2013, respectively, from *A. aurantii* scales collected in citrus fields located in the Valencia region of Spain. Both colonies are renewed yearly with field-collected parasitoids.

Between five and 10 late-stage pupae of both parasitoids were removed from parasitized scales and held separately in crystal vials that were 8 mm in diameter and 35 mm long. At emergence, parasitoids were sexed and held in these vials for 1 day to obtain mated females of *A. melinus*. One day after their emergence, females were isolated in the same vials described above, and one *A. aurantii* female body was introduced daily to allow them to feed on a host until they were used 2–3 days later (Heimpel et al., 1997). Since *Aphytis* do not obtain sugars from host feeding (Tena et al., 2013b) and adults die within 3 days without a carbohydrate source (Heimpel et al., 1997), a drop of honey was added on the inside wall of each vial, which was stoppered with a cotton plug. The vials were stored in a climatic chamber (SANYO MLR-350; Sanyo, Japan) at  $25 \pm 1$  °C, 50–70% relative humidity and 14:10 h light:dark.

### 5.2.3. Experimental Arena

We conducted behavioural observations on a lemon from the colony, where we measured and selected a second-instar scale, of 0.5–0.7 mm<sup>2</sup>, or a third-instar scale, of 0.8–1.0 mm<sup>2</sup> (Luck & Podoler, 1985; Opp & Luck, 1986; Pekas et al., 2010). To

measure the surface of each scale, we used a dissecting microscope with a Leica EC 3 3.1 megapixel digital colour camera (Leica Microsystems GmbH, Spain). Images were processed with Leica LAS EX imaging software for Windows (Leica Microsystems GmbH, Spain) and the areas of the scales ( $\text{mm}^2$ ) were measured with ImageJ, a publicdomain Java Image-processing program (Rasband, 2016). The selected scale was mapped, and the remaining scales were removed using an entomological needle and a piece of paper moistened with water.

#### 5.2.4. Adult Female Behaviour

Female parasitoids were introduced individually into a glass petri dish (diameter 4 cm, height 1.5 cm), and the petri dish was placed over an individual scale on a lemon. The behaviours of *A. melinus* and *A. chrysomphali* were observed and recorded under four different conditions for each *A. aurantii* nymphal instar (second or third instar; Table 1). In ‘control treatments’, parasitoids were introduced individually into the experimental arenas described above. In the ‘competition treatments’, *A. melinus* and *A. chrysomphali* were introduced sequentially in both possible orders: the first female was observed until she oviposited in the host and was then removed; the female of the other species was introduced 2 h later and observed. Each treatment was replicated 30 times (Table 1).

In each replicate, we continuously observed the behaviour of the second female using a dissecting microscope at 10× to 50× magnification and a cool fibre light to illuminate the arena. We initiated behavioural recording when the female recognized the host, i.e. she drummed the scale by positioning herself on the scale cover and moving from the centre to the edge while tapping the cover with her antennae and sometimes her mouthparts (van Lenteren, 1994). Each observation terminated when the female left

the scale or rested 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 her antennae, the female might investigate the host by probing, a behaviour set that includes using her ovipositor to drill through the scale cover, explore the cavity between the scale body and cover, pierce the body and explore the haemocoel (Casas et al., 2004). The parasitoid may leave the host at any time during this process (rejection), may accept the host (oviposition) or may consume the scale's body fluids (host feeding; Casas et al., 2004). The time spent probing the host was recorded (not including probes that ended in oviposition). We identified oviposition as occurring when female abdominal vibrations were observed during probing, with exudation of a viscous substance from the ovipositor tip. The ovipositor was then withdrawn, and the female either left the scale or retracted the ovipositor to puncture the scale cover again, to lay another egg (Pina, 2007). We recorded additional behavioural data during the observations: (1) the position and number of ovipositions per scale and (2) the position and number of probes.

**Table 1** Number of replicates used per treatment and per variable in the experiment

	Second-instar <i>A. aurantii</i>				Third-instar <i>A. aurantii</i>			
	Control		Multiparasitism		Control		Multiparasitism	
	<i>A. melinus</i>	<i>A. chrysomphali</i>	<i>A. melinus</i>	<i>A. chrysomphali</i>	<i>A. melinus</i>	<i>A. chrysomphali</i>	<i>A. melinus</i>	<i>A. chrysomphali</i>
Behavioural observations	30	30	30	30	30	30	30	30
Clutch size	20	16	13	12	23	19	23	13
Immature mortality	20	16	13	12	23	19	23	13
Brood size	18	11	10	3	21	19	11	4
Sex ratio	18	<sup>a</sup>	10	<sup>a</sup>	20	<sup>a</sup>	10	<sup>a</sup>

<sup>a</sup> *Aphytis chrysomphali* reproduced parthenogenetically and only females were recovered.

We used the acceptance rates and clutch size to test whether *A. melinus* females accept and lay more eggs than *A. chrysomphali* in the third but not the second instar when both instars were parasitized by the competitor.

After the observation period ended, the parasitoid was removed and each lemon was kept in a plastic container (14 × 14 cm and 8 cm high) covered with a piece of muslin. The containers were kept in the same climatic chamber described above (25 ± 1 °C, 50-70% relative humidity and 14:10 h light:dark) to determine the outcomes of these encounters.

#### 5.2.5. Possible Heterospecific Ovicide

To determine whether *A. melinus* and *A. chrysomphali* are able to detect and kill the eggs of the competing female (heterospecific ovicide), we first determined and compared the duration of probing in healthy and parasitized hosts and then determined whether the second female probed the scale in the direction of the clutch laid by the first female (Netting & Hunter, 2000). Later, we checked whether these second probes were lethal. We considered only ovicidal behaviour in third-instar hosts because it was difficult to determine the outcome in the second instar. Both parasitoid species probed close to the eggs because of the small size of this instar.

#### 5.2.6. Outcome of Competition

To determine the outcome of competition between *A. melinus* and *A. chrysomphali*, we observed parasitized and multiparasitized scales between 10 and 12 days after the original observations (the time needed to reach the pupal stage under our conditions). The covers of the scales were removed carefully with an entomological needle under

a binocular lens. Then, we identified the species and recorded the sex and number of parasitoid pupae. To differentiate between species, we checked the conspicuous longitudinal black line that is present in the mesosternum of *A. chrysomphali* pupae but not in *A. melinus* (Rosen & DeBach, 1979). These data were used to determine and compare the outcome of competition measured as the immature mortality, brood size, sex ratio and proportion of emergence, depending on the host instar. We expected that the proportion of emergent *A. melinus* would be higher than that of *A. chrysomphali* in the third-instar host, but not in the second, independent of the order of attack.

#### 5.2.7. Statistical Analysis

We compared the probing duration across treatments using ANOVA. The normality assumption was assessed using Shapiro's test, and homoscedasticity was assessed by the Levene test. Proportional and count data were analysed with generalized linear models. Initially, we assumed a binomial error variance for proportional data (host acceptance, probing in the direction of the first clutch, progeny proportion, sex ratio) and a Poisson error variance for count data (clutch and brood size). We assessed the assumed error structures using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If we detected over- or underdispersion, we re-evaluated the significance of the explanatory variables using an  $F$  test after rescaling the statistical model by a Pearson chi-square divided by the residual degrees of freedom (Crawley, 2007). We present the means of the untransformed proportion and count data (in preference to less intuitive statistics, such as the back-transformed means of logittransformed data). All statistical analyses were performed with R studio (Version 0.98.501, RStudio, Inc., <https://www.rstudio.com>; Ihaka & Gentleman, 1996).

### 5.3. Results

#### 5.3.1. Behaviour of Adult Females

##### 5.3.1.1. Host acceptance

In the absence of competition, *A. melinus* and *A. chrysomphali* displayed similar rates of acceptance for second-instar hosts (acceptance of healthy host by *A. melinus*: mean  $\pm$  SE  $0.67 \pm 0.09$ ; *A. chrysomphali*:  $0.53 \pm 0.09$ ;  $F_{1,58} = 1.08$ ,  $P = 0.30$ ) and third-instar hosts (*A. melinus*:  $0.77 \pm 0.08$ ; *A. chrysomphali*:  $0.67 \pm 0.09$ ;  $F_{1,58} = 1.23$ ,  $P = 0.27$ ; Fig. 1). When the scales had been previously parasitized by their competitor, *A. melinus* accepted heterospecificparasitized third-instar *A. aurantii* more frequently ( $0.77 \pm 0.08$ ) than did *A. chrysomphali* ( $0.43 \pm 0.09$ ;  $F_{1,58} = 6.87$ ,  $P = 0.011$ ). However, the two parasitoids accepted second-instar hosts at similar rates (*A. melinus*:  $0.43 \pm 0.09$ ; *A. chrysomphali*:  $0.40 \pm 0.09$ ;  $F_{1,58} = 0.07$ ,  $P = 0.8$ ), as predicted by our hypothesis (superiority diminishes in the second instar).

##### 5.3.1.2. Clutch size

In the absence of competition, *A. melinus* and *A. chrysomphali* laid similar-sized clutches of eggs in second-instar hosts (eggs laid in healthy hosts by *A. melinus*: mean  $\pm$  SE =  $1.05 \pm 0.05$ ; *A. chrysomphali*:  $1.06 \pm 0.06$ ;  $F_{1,34} = 0.03$ ,  $P = 0.88$ ) and third-instar hosts (*A. melinus*:  $1.3 \pm 0.12$ ; *A. chrysomphali*:  $1.32 \pm 0.11$ ;  $F_{1,37} = 0.04$ ,  $P = 0.84$ ; Fig. 2).

When the scales had been previously parasitized by their competitor, *A. melinus* laid a significantly larger clutch ( $1.39 \pm 0.14$ ) than did *A. chrysomphali* (1) on third-instar

hosts ( $F_{1,34} = 5.25$ ,  $P = 0.028$ ). However, the two parasitoids laid similar numbers of eggs in the second instar (*A. melinus*:  $1.08 \pm 0.08$ ; *A. chrysomphali*: 1;  $F_{1,23} = 0.95$ ,  $P = 0.34$ ), as predicted by our hypothesis (the advantage diminishes in the second instar).

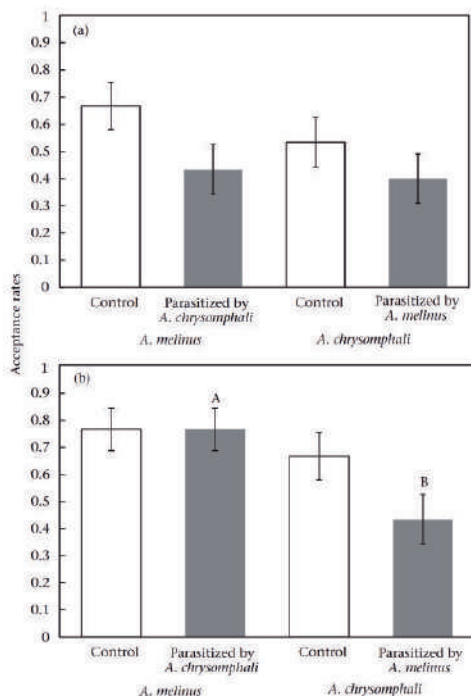


Fig. 1 Acceptance rates (mean  $\pm$  SE) of healthy (control) and heterospecific-parasitized *A. aurantii* by *A. melinus* and *A. chrysomphali* when they encountered (a) second-instar and (b) third-instar hosts. Different letters above columns denote significant differences between parasitoids at  $P < 0.05$ .

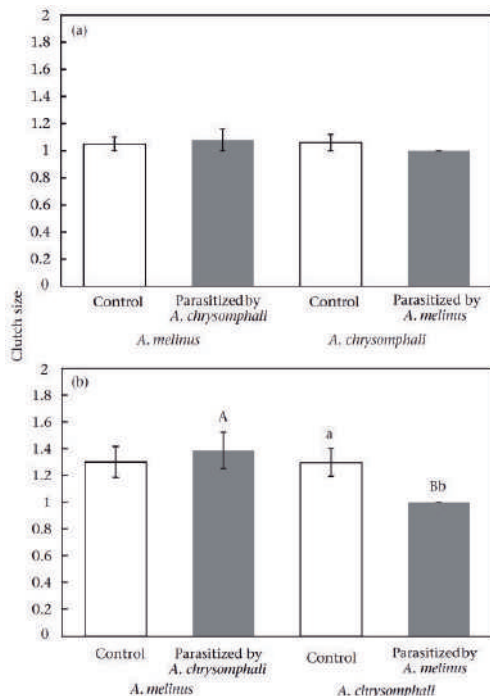


Fig. 2 Clutch size (mean number of eggs laid per host  $\pm$  SE) of *A. melinus* and *A. chrysomphali* when they accepted healthy (control) and heterospecific-parasitized (a) second and (b) third instars of *A. aurantii*. Different uppercase letters above columns denote significant differences between parasitoid species at  $P < 0.05$ . Different lowercase letters above columns denote significant differences between treatments (healthy versus heterospecific-parasitized) within a parasitoid species at  $P < 0.05$ .

### 5.3.1.3. Probing time, site and potential heterospecific ovicide

When the scales had been previously parasitized by their competitor, the time spent probing the second-instar host was similar for both parasitoid species (*A. melinus*: mean  $\pm$  SE =  $44.2 \pm 16.1$  s; *A. chrysomphali*:  $57 \pm 11.4$  s;  $F_{1,23} = 0.41$ ,  $P = 0.53$ ). The time spent probing the third-instar host increased, but there were no significant differences between parasitoid species (*A. melinus*:  $190.7 \pm 12.7$  s; *A. chrysomphali*:  $171.5 \pm 21.2$  s;  $F_{1,34} = 1.13$ ,  $P = 0.72$ ). However, *A. melinus* tended to probe the scale in the direction of the first clutch (ratio:  $0.65 \pm 0.10$ ) more frequently than did *A. chrysomphali* (ratio:  $0.33 \pm 0.13$ ;  $\chi^2 = 42.7$ ;  $N = 36$ ,  $P = 0.049$ ) when they encountered heterospecific-parasitized thirdinstar hosts. Finally, no *A. chrysomphali* emerged from the nine hosts in which *A. melinus* had probed in the direction of the first clutch.

### 5.3.1.4. Outcome of Competition

Given the acceptance ratios, clutch size laid, ovicide and immature competition (next section), *A. melinus* produced a greater proportion of progeny (ca. 0.7) than did *A. chrysomphali* (ca. 0.3), independent of the host instar ( $F_{1,57} = 0.085$ ,  $P = 0.77$ ) and order of exposure ( $F_{1,57} = 0.02$ ,  $P = 0.89$ ; Fig. 3). The interaction between the host instar and order of exposure was not significant ( $F_{1,56} = 0.01$ ,  $P = 0.91$ ). According to these results, and contrary to our hypothesis, *A. melinus* was a superior competitor in both instars.

### 5.3.1.5. Immature Mortality

In the absence of competition, the immature mortality rates of *A. melinus* and *A. chrysomphali* were similar in second-instar ( $F_{1,34} = 2.44$ ,  $P = 0.13$ ) and third-

instar hosts ( $F_{1,41} = 0.09$ ,  $P = 0.77$ ; Table 2). In multiparasitized hosts, the immature mortalities of *A. melinus* and *A. chrysomphali* were similar in third-instar hosts ( $F_{1,34} = 0.56$ ;  $P = 0.46$ ), but mortality was significantly higher for *A. chrysomphali* than for *A. melinus* in the second instar ( $F_{1,23} = 5.89$ ;  $P = 0.02$ ; Table 2).

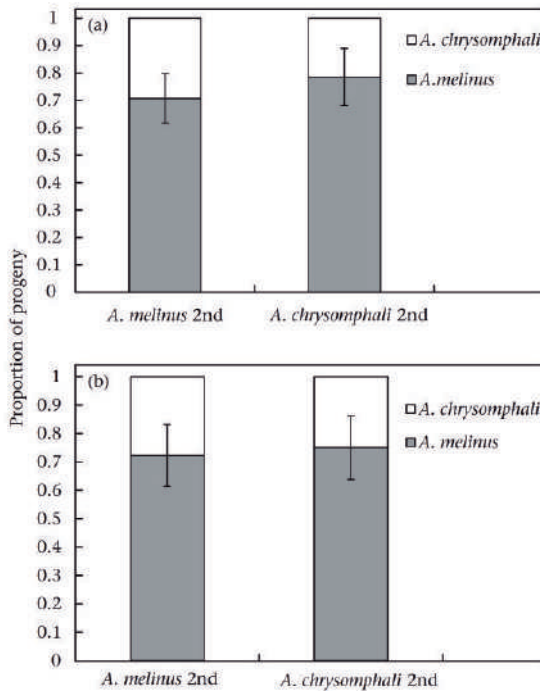


Fig. 3 Effect of order of attack on the outcome of competition between *A. melinus* and *A. chrysomphali* in (a) multiparasitized second-instar *A. aurantii* and (b) multiparasitized third-instar *A. aurantii*, represented as the proportion of total progeny (mean  $\pm$  SE) produced by each species

Table 2 Mean  $\pm$  SE immature mortality, secondary brood size (emerged adults) and sex ratio (% males) of *A. melinus* and *A. chrysomphali* that had accepted healthy (control) and heterospecific-parasitized second and third instars of *A. aurantii*

		<i>A. melinus</i>		<i>A. chrysomphali</i>	
		Control	Multiparasitism	Control	Multiparasitism
Immature mortality	N2	0.1 $\pm$ 0.07	0.27 $\pm$ 0.12	0.31 $\pm$ 0.12	0.75 $\pm$ 0.13
	N3	0.15 $\pm$ 0.07	0.52 $\pm$ 0.1	0.13 $\pm$ 0.06	0.69 $\pm$ 0.13
Brood size	N2	1	1	1	1
	N3	1.1 $\pm$ 0.07	1.18 $\pm$ 0.18	1.16 $\pm$ 0.08	1
Sex ratio	N2	0.59 $\pm$ 0.12	0.7 $\pm$ 0.02	0	0
	N3	0.13 $\pm$ 0.07	0.2 $\pm$ 0.09	0	0

#### 5.3.1.6. Brood Size

In the absence of competition, the brood sizes of *A. melinus* and *A. chrysomphali* were similar when they emerged from second-instar (one parasitoid always emerged) and third-instar hosts ( $F_{1,37} = 0.43$ ,  $P = 0.52$ ; Table 2). Similarly, in multiparasitized hosts, the brood sizes of *A. melinus* and *A. chrysomphali* were similar when they emerged from second-instar (always one parasitoid) and third-instar hosts ( $F_{1,13} = 0.37$ ,  $P = 0.55$ ; Table 2).

#### 5.3.1.7. Sex Ratio

In the absence of competition, the secondary sex ratio of *A. melinus* was male-biased in second-instar hosts and became female-biased in the third-instar (Table 2). *Aphytis melinus* sex ratio followed the same pattern in multiparasitized hosts. As expected, all emerging *A. chrysomphali* were females and, therefore, we could not compare the sex ratios between parasitoid species.

### 5.4. Discussion

#### 5.4.1. Effect of Host Instar on Interference Competition

Our results contradict our initial hypothesis that *A. melinus* is a superior competitor when both parasitoids parasitize the third instar (high quality) but that this advantage diminishes in the second instar. Given adult female behaviour and immature competition, the introduced parasitoid *A. melinus* was a superior competitor to the native *A. chrysomphali*, independent of the host instar (low- versus high-quality hosts) and sequence of attack. The superiority of *A. melinus* is explained by the

higher aggressiveness of the mother on encountering third-instar hosts (accepting more hosts, laying more eggs and high rates of ovicide) and the higher mortality of *A. chrysomphali* larvae when they develop in multiparasitized second-instar hosts. The higher mortality of *A. chrysomphali* than *A. melinus* larvae might be the result of physical and physiological mechanisms employed by dominant parasitoids to suppress their competitors (Harvey et al., 2013). These mechanisms have never been studied in *Aphytis*, but their larvae have mandibles (Eliraz & Rosen, 1978; Rosen & DeBach, 1979) that can be used to kill the eggs or larvae of competitors in different larval instars, as occurs in other parasitoid species (Tena et al., 2008). It has generally been assumed that species with shorter developmental times are at an advantage because they can ingest resources earlier. However, the developmental time of *A. chrysomphali* is shorter than that of *A. melinus* at 25 °C (the conditions of the experiment; Abdelrahman, 1974a).

#### 5.4.2. Effect of Interference Competition on Parasitoid Coexistence

Theoretical studies have highlighted the role of interspecific competition in structuring parasitoid communities (May & Hassell, 1981; Murdoch et al., 1996; Borer, 2002; Borer et al., 2003). At present, however, there is relatively little information explaining how parasitoids with broadly overlapping host niches coexist in nature (but see Tscharncke, 1992; Bográn et al., 2002; Borer et al., 2004; Snyder et al., 2005). In some areas of the Mediterranean basin, *A. melinus* and *A. chrysomphali* coexist as parasitoids of *A. aurantii*. Pekas et al. (2016) showed that *A. chrysomphali* is recovered mostly from second-instar hosts of poorer quality when the density of the superior competitor *A. melinus* is high in areas where they coexist. We hypothesized that this conditional patch partitioning might reflect the fact that *A. melinus* is a superior competitor when both parasitoids parasitize the third instar (high quality), but that

this advantage diminishes in the second instar. Contrary to our initial hypothesis, our results indicate that *A. melinus* is a superior competitor to native *A. chrysomphali* when they compete for the same individual host, independent of host instar. Therefore, the superior biological traits of *A. melinus* described herein, together with its higher capacities for dispersion (McLaren, 1976) and parasitism (Pekas et al., 2010), contribute to the displacement of *A. chrysomphali*, as has occurred in southeastern Spain (Sorribas et al., 2010; Boyero et al., 2014).

On the other hand, other factors may affect the intrinsic competition between these parasitoids, favouring their coexistence. For example, the facultative symbiont *Hamiltonella defensa* can reverse the outcome of competition between two parasitoids of the pea aphid *Acyrtosiphon pisum* (Hemiptera: Aphididae) (McLean & Godfray, 2016). In the case of *Aphytis*, both parasitoids are infected with the bacterium *Wolbachia* and it is unknown whether its absence might modify the competition. Another factor that can modify the outcome of competition and their coexistence is the presence of alternative hosts. The outcome of the intrinsic competition between the parasitoids *Hyposoter ebeninus* (Hymenoptera: Ichneumonidae) and *Cotesia glomerata* (Hymenoptera: Braconidae) depends on both plant and herbivore host species (Poelman et al., 2014). It is well known that *A. melinus* and *A. chrysomphali* can find and develop in alternative hosts in the Mediterranean basin ([www.nhm.ac.uk/our-science/data/chalcidoids/database](http://www.nhm.ac.uk/our-science/data/chalcidoids/database)). The presence of these alternative plants and hosts might also facilitate the parasitoids' coexistence as suggested by Pekas et al. (2016).

Finally, the results obtained herein, together with those of Pekas et al. (2016), suggest that *A. chrysomphali* is able to evaluate the density of its competitor, *A. melinus*, and alter the use of the host instar (quality) according to that density. This ability provides

*A. chrysomphali* with competition-free host resources and, together with favourable climatic conditions (Sorribas et al., 2010), permits sympatry with the dominant *A. melinus* in northeastern Spain, which is not possible elsewhere in this range. Further research is necessary to corroborate this hypothesis.

#### 5.4.3. Heterospecific Ovicide in the Genus *Aphytis*

*Aphytis melinus* females tended to probe the scale in the direction of the eggs laid by *A. chrysomphali* and probably killed them with their ovipositor, eliminating competitors for their offspring. Heterospecific ovicide has been documented in several species of ectoparasitoids (Infante et al., 2001; Pérez-Lachaud et al., 2004). Ectoparasitoids find heterospecific eggs outside the host cuticle and either eat them or stab them with their ovipositor (Collier et al., 2007). Nevertheless, ovicide might be less feasible for specialized ectoparasitoids of diaspine hosts, such as *Aphytis*, because the eggs are located under the scale cuticle and sometimes even under the host's body (Luck et al., 1982). Therefore, *Aphytis* females must pierce one or two barriers with their ovipositor to reach the first female's eggs, behaving as an endoparasitoid. With all this, it was impossible to determine whether females probed eggs through the dark scale cuticle of *A. aurantii*. Rather than observe the probes, we observed how females moved their ovipositors towards the first female's eggs. The fact that none of these supposedly probed eggs survived the attack suggests ovicide. This hypothesis was supported by two other observations. First, *A. chrysomphali* survived ( $37.5 \pm 18.3\%$ ) when it shared the scale with *A. melinus* whose mother had not tried to probe the eggs of the competitor. Second, *A. melinus* females always allocated a clutch of eggs after probing in the direction of the first female's eggs. *Aphytis melinus* also commits ovicide when females sting the eggs of competitors while trying to host feed on

scales parasitized by conspecifics (Collier personal observation cited in Collier & Hunter, 2001) and *Encarsia perniciosi* (Yu et al., 1990).

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## Chapter 6

# NEGATIVE EFFECT OF GLOBAL WARMING ON BIOLOGICAL CONTROL IS MITIGATED BY DIRECT COMPETITION BETWEEN SYMPATRIC PARASITOIDS





## 6. NEGATIVE EFFECT OF GLOBAL WARMING ON BIOLOGICAL CONTROL IS MITIGATED BY DIRECT COMPETITION BETWEEN SYMPATRIC PARASITOIDS

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### Abstract

Parasitoids are the most important and successful group of natural enemies used in the biological control of insect pests. In most systems, several parasitoid species can parasitize the same pest. The parasitoids' coexistence in agroecosystems and their efficacy as biological control agents may be disrupted by global warming. An increase of approximately 3°C is predicted by the end of the twenty-first century in the Mediterranean basin (IPCC, 2014). In this context, we compared the present and future performance of two sympatric parasitoids of the genus *Aphytis* (Hymenoptera: Aphelinidae), which control the armoured scale *Aonidiella aurantii* Hemiptera: Diaspididae) in Mediterranean citrus, either alone or in competition. The net reproductive rate ( $R_0$ ) of the introduced *Aphytis melinus* DeBach was higher than that of its competitor, the native *Aphytis chrysomphali* (Mercet), in the present conditions. The higher temperature and the competition affected both parasitoids differently. The  $R_0$  of *A. chrysomphali* decreased by 50% when both parasitoids competed in the same patch but was not affected by the temperature increase. The  $R_0$  of *A. melinus* decreased approximately 40% with the increase in temperature because the proportion of females was reduced. However, the presence of *A. chrysomphali* competing in the same patch mitigated the negative effect of the increase in temperature on *A. melinus* ( $R_0$  decreased by only 20%). Overall, our results suggest that global warming will have a negative effect on the biological control of *A. aurantii* and that this effect will be higher in areas, such as southern Spain, where *A. melinus* has displaced *A. chrysomphali*.

Keywords *Aphytis* • *Aonidiella aurantii* • Citrus • Interspecific competition • Displacement

## 6.1. INTRODUCTION

Due to global warming, average temperatures have risen by approximately  $0.8^{\circ}\text{C}$  since the early twentieth century, and a further increase of  $3^{\circ}\text{C}$  is predicted by the end of the twenty-first century for summer temperatures in the Mediterranean basin (IPCC, 2014). Because insects are ectotherms, their physiology, behaviour and fitness are directly affected by ambient temperature variation (Hance et al., 2007). The impact of global warming is likely to be more important in higher trophic levels that also depend on the capacity of the lower trophic level to adapt to these changes (Hance et al., 2007; van Baaren et al., 2010). That impact is observed for parasitoids in which immature individuals feed and develop in (endoparasitoids) or on (ectoparasitoids) hosts. Parasitoids are the most important and successful group of natural enemies used in the biological control of insect pests (Godfray, 1994; Jarvis, 2005), and their efficacy could be disrupted by changes in environmental conditions (van Baaren et al., 2010).

An increase in temperature can affect host-parasitoid relationships, mostly because they may have different thermal preferences (developmental or phenological), which can affect their synchronization (Hance et al., 2007). For parasitoids, an increase in temperature can affect their morphology (body size, wing size, wing loading), fecundity, longevity, dispersal capacity, metabolism rate, trade-offs between life-history traits, capacity to locate and evaluate host quality and the capacity of the larvae to evade or overcome the host immune response (Hance et al., 2007; Moiroux et al., 2010; van Baaren et al., 2010; Vayssade et al., 2012; Vuarin et al., 2012). Indeed, an increase in temperature may induce a number of physiological changes, the cost of which may be expressed by a reduction in reproductive output, decrease in growth of immature stages and in lifespan and/or changes in mating behaviour (Omer et

al., 1996; Hance et al., 2007; Angilletta, 2009; Řežucha et al., 2010). Understanding these characteristics is crucial for using parasitoids as biological control agents.

Because of interspecific differences in thermal responses among parasitoid species (Berg et al., 2010), global warming can also have a major influence on interspecific competition between species (Northfield & Ives, 2013). As such, global warming will have consequences for parasitoid species distributions, community compositions, and ecosystem services, i.e., biological control (Bale et al., 2002; Hance et al., 2007; Northfield & Ives, 2013; Tougeron et al., 2017). Recent studies suggest that environmental changes may tip the balance between interacting species (Northfield & Ives, 2013; Andrade et al., 2016), and to persist, species may thus have to address challenging thermal changes (Hance et al., 2007; Le Lann et al., 2014; van Baaren et al., 2010).

In this context, we investigated the influence of the expected temperature increase on the efficacy and competition of parasitoids of the genus *Aphytis* (Hymenoptera: Aphelinidae), which are the most successful and widespread biological control agents of *Aonidiella aurantii* (Hemiptera: Diaspididae) in citrus (DeBach & Rosen, 1991; Forster & Luck, 1996; Murdoch et al., 2005; Pekas et al., 2016). These specialist parasitoids can reduce their shared host to levels nearly 200 times below the average density observed in their absence (DeBach et al., 1971), suggesting strong resource competition between parasitoid species (Borer et al., 2004). Species of the genus *Aphytis* represent one of the best-known cases of competitive displacement in insects (Luck et al., 1982; Luck & Podoler, 1985; Luck & Nunney, 1999; Sorribas et al., 2010; Pekas et al., 2016). In the Mediterranean basin, *A. aurantii* became a key citrus pest at the end of the last century, and it was rapidly parasitized by the native parasitoid *Aphytis chrysomphali* (Mercet). Later, its coevolved parasitoid *Aphytis melinus* DeBach was introduced into a classical biological control programme, and it has displaced

*A. chrysomphali* in some areas, whereas both species coexist in others (Sorribas et al., 2010; Boyero et al., 2014). Although *A. chrysomphali* reproduces parthenogenetically and produces only females (Gottlieb et al. 1998; Pina, 2007), *A. melinus* is considered to be a superior competitor in the field because it has a higher capacity for dispersion (McLaren, 1976) and is better adapted to dry and hot climates where citrus is cultivated (Abdelrahman, 1974a; Rosen & DeBach, 1979). Consequently, the relative proportion of *A. melinus* is higher during the warm months, and the abundance of *A. chrysomphali* increases from south to north, being higher in the cooler northern areas. This alteration in parasitoid dominance could be one of the reasons why the more efficient parasitoid *A. melinus* has not completely displaced *A. chrysomphali* in most Valencia citrus orchards (Sorribas et al., 2010). Therefore, we hypothesize that the superiority of *A. melinus* will be accentuated when the temperature increases due to global warming, possibly leading to the extinction of the weaker competitor. To test this hypothesis, we performed laboratory experiments to evaluate how the increase in temperature in summer, when *A. chrysomphali* is a weaker competitor, will affect i) the fecundity and parasitism rate of both parasitoids when they exploit hosts alone or in competition and ii) the influence of temperature and competition on their efficacy as the biological control of *A. aurantii* by comparing their net reproductive rates ( $R_0$ ) and parasitoid-induced mortality (host mortality caused due to parasitoid activity as parasitism, host-feeding or probing).

## 6.2. MATERIALS AND METHODS

### 6.2.1. Abiotic conditions and treatments

The climate data used for rearing hosts and parasitoids and for experiments was  $26/20 \pm 1^\circ\text{C}$  (day/night) to mimic average summer temperatures in the last fifteen years

in the Valencia Region (Moncada meteorological station: <http://riegos.ivia.es/>) and  $29/23 \pm 1^\circ\text{C}$  (day/night) to mimic warmer average summer temperatures predicted by the end of the twenty-first century (IPCC, 2014) at  $70 \pm 5\%$  RH and LD 12:12.

The experiment consisted of four treatments in the two different abiotic conditions explained above for a total of eight combinations: 1) exploitation of 40 third-instar *A. aurantii* per patch by a single female of *A. melinus* (number of replicates = 42); 2) exploitation of 40 third-instar *A. aurantii* per patch by a single female of *A. chrysomphali* (n = 46); 3) exploitation of 40 third-instar *A. aurantii* per patch by *A. melinus* and *A. chrysomphali* simultaneously (n = 44); and 4) 40 third-instar *A. aurantii* (n = 37).

#### 6.2.2. Insects

The phytophagous host, *Aonidiella aurantii*, was reared on lemons from a laboratory colony at the Instituto Valenciano de Investigaciones Agrarias, IVIA (Moncada, Valencia, Spain). This colony was initiated in 1999 from scales collected in citrus fields in Alzira (Valencia, Spain) and renewed every 2-3 years with field-collected scales. Approximately 2/3 of the surface of each lemon was covered with red paraffin around the mid-section to retard its desiccation. The red paraffin was prepared with a mixture of 1 kg of paraffin pearls (Parafina USP Perlas; Guinama S.L., Alboraya, Spain) and 1 g of red pigment (Sudan III; Panreac Química S.A., Castellar del Vallés, Spain). The remaining surface (aprox. 24-cm<sup>2</sup> area) of the lemons was infested by exposing them to gravid female scales of the *A. aurantii* colony for 48 h at  $27 \pm 1^\circ\text{C}$  at  $70 \pm 5\%$  RH and LD 14:10. Once infested, lemons were kept in climate chambers (SANYO MLR- 350; Sanyo, Japan) at the two temperature conditions described above until female scales reached the third nymphal instar (21-25 days), which was later used

for rearing the parasitoids and for the experiments (Treatments 2,3 and 4) or until females become gravid (43-50 days) for the fecundity investigation (Treatment 1).

*Aphytis melinus* and *A. chrysomphali* are facultative gregarious ectoparasitoids (Rosen & DeBach, 1979). Females of both species mature eggs throughout their adult life (synovigenic) and lay between 2 and 6 eggs per day (Heimpel et al., 1997; Casas et al., 2000; Tena et al., 2015). These species are also idiobionts (i.e., the host is paralyzed and arrests development once parasitized) and feed on the haemolymph of hosts which they do not use to lay eggs. Individuals of both species were obtained by exposing third-instar *A. aurantii* on lemons to parasitism by insectary-reared adult wasps. The colonies of *A. melinus* and *A. chrysomphali* were initiated in 2008 and 2013, respectively, from *A. aurantii* scales collected in citrus fields located in the Valencia region (Valencia, Spain). Both colonies are renewed yearly with field-collected parasitoids.

For this experiment, five adults of each species were transferred to rearing cages containing third-instar *A. aurantii* on lemons reared in the two abiotic conditions (described above) and were maintained in climatic chambers at these two abiotic conditions to obtain parasitoids. Between 10-12 days later, scales were observed under binoculars and late-stage pupae of both parasitoids were removed from parasitized scales. Pupa were held in crystal vials 8 mm in diameter and 35 mm long tapped with a cotton plug and with a drop of honey on the wall. At emergence, parasitoids were held in these vials for one day to obtain mated females of *A. melinus* [*A. chrysomphali* reproduces parthenogenetically (Gottlieb et al. 1998)]. Next, parasitoids were sexed, and females were isolated in vials (same as above). One *A. aurantii* female was introduced daily to let them feed on the host until they were used 2-3 days later (Heimpel et al. 1997). Vials were stored in a climatic chamber

at the two different abiotic conditions until they were used in the experiment (Treatments 2, 3 and 4).

### 6.2.3. Experimental microcosm and measures

For all treatments, the experimental microcosm was composed of a polystyrene plastic box (10 × 14×14 cm) with a lateral hole (4 × 9 cm) covered with muslin. One lemon infested with *A. aurantii* was introduced in the box. An acrylic cylinder (5 cm diameter) was used to hold the lemons. We used a dissecting microscope to select 40 scales from the lemon surface, and we removed the rest using an entomological pin and a paper towel moistened with water. The selected scales were  $0.85 \pm 0.05$  mm<sup>2</sup>. To estimate scale sizes, photos of the scales were taken with a Leica EC 3 3.1 megapixel digital colour camera (Leica Microsystem GmbH, Spain), and the images were processed with Leica LAS EZ imaging software for Windows (Leica Microsystems GmbH, Spain).

### 6.2.4. Effect of competition and a temperature increase on parasitoid fitness and biological control potential

For treatments 1, 2 and 3, parasitoids were introduced in the box and remained in contact with hosts for 72 hours. *Aphytis* parasitoids parasitize between two to five hosts per day and per female (Heimpel et al., 1997; Pina, 2007; Cebolla et al., submitted). Therefore, microcosms contained hosts *ad libitum* in all the treatments. A drop of honey was added to the inside wall as a food source. Microcosms were held in climatic chambers at one of the two abiotic conditions. Seven days after parasitoid removal, scale covers were carefully removed with an entomological needle under a binocular lens. The numbers of unparasitized (turgent), parasitized and dead hosts

were counted. We used these data to evaluate i) the number of parasitized and ii) the dead hosts per patch. The number of dead was the sum of parasitized and hosts dead by unknown reasons (caused by the parasitoids or natural death of hosts). *Aphytis* parasitoids cause the death of their host when they host-feed and when they probe the host with their ovipositor even when they reject the host (Cebolla et al., 2017c).

The parasitoid pupae of each parasitized host were measured and then transferred to crystal vials as described above. Between one and three pupae were obtained per host. To measure the effect of temperature and parasitoid competition on parasitoid size (measured as the length of the pupae), only pupae of females that developed in solitary were considered because sex and brood size can affect pupae size (Salt, 1940; Abdelrahman, 1974b). At emergence, parasitoids were identified and sexed. *Aphytis chrysomphali* pupae are identified by the presence of a longitudinal black line on the mesosternum which is not present in *A. melinus* (Rosen & DeBach, 1979). Progeny production was calculated as the number of adults of each species that emerged per patch. Secondary sex ratio was calculated as the proportion of males of each species per host and patch.

#### 6.2.5. Effect of an increase in temperature on host fecundity

To determine the effect of an increase in temperature on host fecundity (Treatment 4), we used the same methodology described by Vanaclocha et al. (2012). In detail, infested lemons were kept in climatic chambers at the two abiotic conditions. Before the female scales began to produce crawlers (~40 days), four scales per lemon were isolated with a double-sided sticky plastic ring (3M Scotch®; CergyPontoise Cedex, France) to trap the crawlers. Sticky plastic rings were replaced twice a week and the

crawlers were counted under binoculars. The total number of progeny was calculated as the sum of each ring per female.

#### 6.2.6. Effect of an increase in temperature on parasitoid net reproductive rate ( $R_0$ )

The net reproductive rate ( $R_0$ ) during 72 hours was compared between the two parasitoid species in treatments 1, 2 (without competition) and 3 (with competition).  $R_0$  was calculated as  $R_0 = \sum l_x \cdot m_x$ .  $R_0$  represents the mean number of female offspring produced by each female (Birch, 1948; Carey, 1993), where  $x$  is the age class,  $l_x$  is the probability of survival till class  $x$ , and  $m_x$  is the fecundity of class  $x$ . Values of  $R_0 < 1$  indicate a declining population,  $R_0 > 1$  an increasing population, and  $R_0 = 1$  a stable population (Carey, 1993). To facilitate comparison of the demographic parameters, we calculated the standard errors (SE) of the demographic parameters at each temperature using a jackknife algorithm described by Meyer et al. (1986). The jackknife analysis method removes one observation at a time from the original dataset and recalculates the statistic of interest from the truncated data. The values of  $R_0$  per female and per temperature were calculated in Microsoft Excel and its numerical solver. The method can estimate  $R_0$  values with their respective jackknife variances.

#### 6.2.7. Statistical analyses

Proportional and count data were analysed with generalized linear models (GLMs). Initially, we assumed a Poisson error variance for count data (number of parasitized hosts and number of progeny per patch) and a binomial error variance for proportional data (sex ratio and hosts killed per patch). We assessed the assumed error structures by a heterogeneity factor equal to the residual deviance divided by the residual degrees of

freedom. If we detected an over- or underdispersion, we re-evaluated the significance of the explanatory variables using an F test after rescaling the statistical model by a Pearson's chi-square divided by the residual degrees of freedom (Crawley, 2007). We present the means of untransformed proportion and count data (in preference to less intuitive statistics such as the back-transformed means of logit-transformed data).

We compared pupa size of the offspring, host fecundity and  $R_0$  using ANOVAs. The normality assumption was assessed using Shapiro's test, and the homoscedasticity assumption was assessed with the Levene test. All data analyses were performed with the R freeware statistical package (<http://www.R-project.org/>).

### 6.3. RESULTS

#### 6.3.1. Effect of competition and an increase in temperature on parasitoids fitness and biological control potential

##### 6.3.1.1. Number of parasitized hosts

The number of hosts parasitized by *A. melinus* females was independent of the increase in temperature ( $F_{1,83} = 0.34$ ;  $P = 0.56$ ) and the presence of their competitor, *A. chrysomphali* ( $F_{1,83} = 0.31$ ;  $P = 0.58$ ) (Fig. 1). The interaction between temperature and competition was not significant ( $F_{1,82} = 0.31$ ;  $P = 0.58$ ).

The number of hosts parasitized by *A. chrysomphali* females was independent of temperature ( $F_{1,87} = 0.15$ ;  $P = 0.70$ ) but it decreased with the presence of *A. melinus* (interspecific competition) ( $F_{1,87} = 13.92$ ;  $P < 0.001$ ) (Fig. 1). The interaction between temperature and competition was not significant ( $F_{1,86} = 0.74$ ;  $P = 0.79$ ).

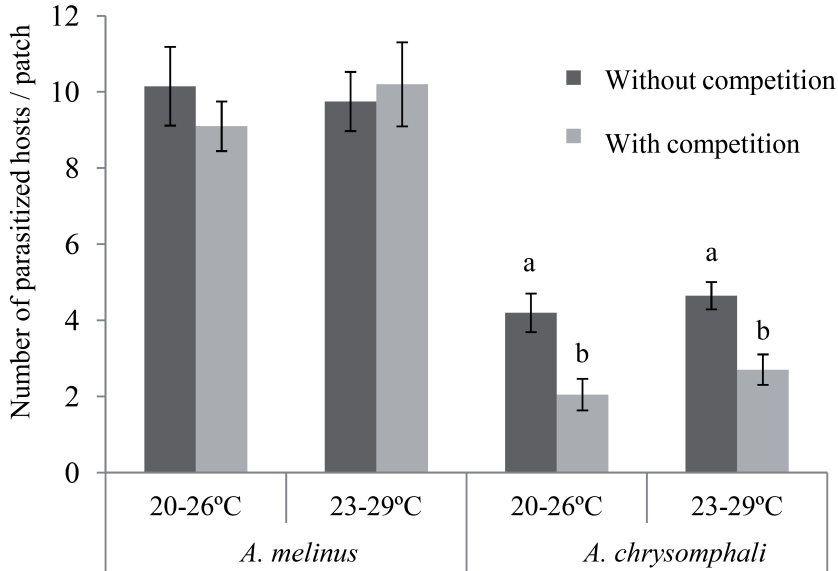


Fig. 1 Effect of temperature (20-26°C or 23-29°C) and interspecific competition on the number of hosts parasitized by the parasitoids *Aphytis melinus* and *A. chrysomphali* during 72 hours. The different temperatures represent the mean temperature of summer from 2009 to 2014 in the Mediterranean basin and the temperature predicted by the end of the twenty-first century by the IPCC. Different letters above columns denote significant differences between treatments (with and without competition) within a parasitoid species and temperature at  $P < 0.05$ .

### 6.3.1.2. Progeny production

The amount of progeny emerging from the hosts parasitized by *A. melinus* was independent of temperature ( $F_{1,83} = 0.044$ ;  $P = 0.83$ ) and competition ( $F_{1,83} = 0.55$ ;  $P = 0.46$ ) (Fig. 2). The interaction between temperature and competition was not significant ( $F_{1,82} = 0.041$ ;  $P = 0.84$ ).

The amount of progeny emerging from the hosts parasitized by *A. chrysomphali* was independent of temperature ( $F_{1,87} = 0.012$ ;  $P = 0.91$ ) but it decreased in the presence of *A. melinus* (competition) ( $F_{1,87} = 13.86$ ;  $P < 0.001$ ) (Fig. 2). The interaction between temperature and competition was not significant ( $F_{1,86} = 0.33$ ;  $P = 0.56$ ).

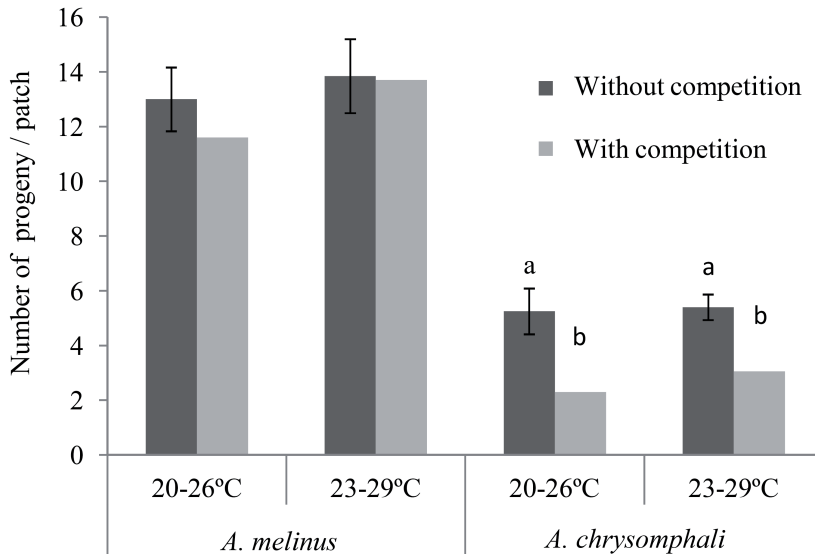


Fig. 2 Effect of temperature (20-26°C or 23-29°C) and interspecific competition on the number of progeny of the parasitoids *Aphytis melinus* and *A. chrysomphali* during 72 hours. The different temperatures represent the mean temperature of summer from 2009 to 2014 in the Mediterranean basin and the temperature predicted by the end of the twenty-first century by the IPCC. Different letters above columns denote significant differences between treatments (with and without competition) within a parasitoid species and temperature at  $P < 0.05$

### 6.3.1.3. Parasitoid size

The pupae size of *A. melinus* and *A. chrysomphali* offspring was not affected by the increase in temperature (*A. melinus*:  $F_{1,46} = 2.93$ ;  $P = 0.094$ ; *A. chrysomphali*:  $F_{1,41} = 0.26$ ;  $P = 0.61$ ) or competition (*A. melinus*:  $F_{1,46} = 0.31$ ;  $P = 0.58$ ; *A. chrysomphali*:  $F_{1,41} = 0.044$ ;  $P = 0.83$ ) (Fig. 3). The interaction between temperature and competition was not significant in either parasitoid species (*A. melinus*:  $F_{1,45} = 0.032$ ;  $P = 0.86$ ; *A. chrysomphali*:  $F_{1,40} = 1.54$ ;  $P = 0.22$ ).

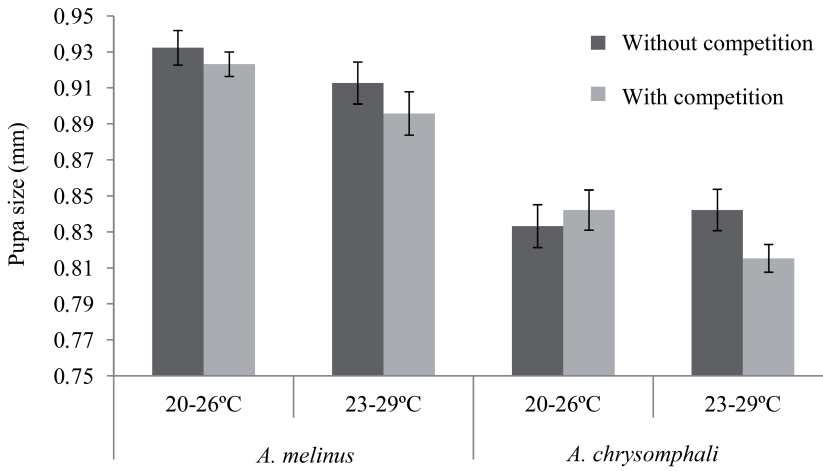


Fig. 3 Effect of temperature (20-26°C or 23-29°C) and interspecific competition on the pupa size of the parasitoids *Aphytis melinus* and *A. chrysomphali*. The different temperatures represent the mean temperature of summer from 2009 to 2014 in the Mediterranean basin and the temperature predicted by the end of the twenty-first century by the IPCC).

#### 6.3.1.4. Sex ratio

The secondary sex ratio (proportion of males out of the total emerging wasps) of *A. melinus* was affected by temperature ( $F_{1,65} = 5.37$ ;  $P = 0.024$ ). The proportion of males increased with temperature. However, sex ratio was independent of the presence of the competitor, *A. chrysomphali* ( $F_{1,65} = 1.83$ ;  $P = 0.18$ ) (Fig. 4). The interaction between temperature and competition was not significant ( $F_{1,64} = 0.049$ ;  $P = 0.83$ ). As expected, all emerging *A. chrysomphali* were females.

#### 6.3.1.5. Host mortality

In the absence of competition, the number of *A. aurantii* hosts killed by *A. melinus* and *A. chrysomphali* was not affected by the increase in temperature (*A. melinus*:

$F_{1,40} = 0.24$ ;  $P = 0.63$ ; *A. chrysomphali*:  $F_{1,44} = 0.028$ ;  $P = 0.87$ ). The same phenomenon occurred when both parasitoids were competing ( $F_{1,86} = 0.18$ ;  $P = 0.68$ ) (Fig. 5).

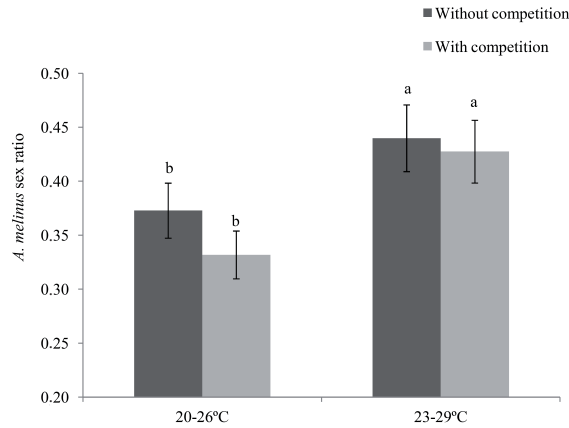


Fig. 4 Effect of temperature (20-26°C or 23-29°C) and interspecific competition on the sex ratio of the parasitoid *Aphytis melinus*. Presented as the mean proportion of males of *A. melinus* ( $\pm$  SE). The different temperatures represent the mean temperature of summer from 2009 to 2014 in the Mediterranean basin and the temperature predicted by the end of the twenty-first century by the IPCC. Different letters above columns denote significant differences between temperatures within the same competition treatment at  $P < 0.05$ .

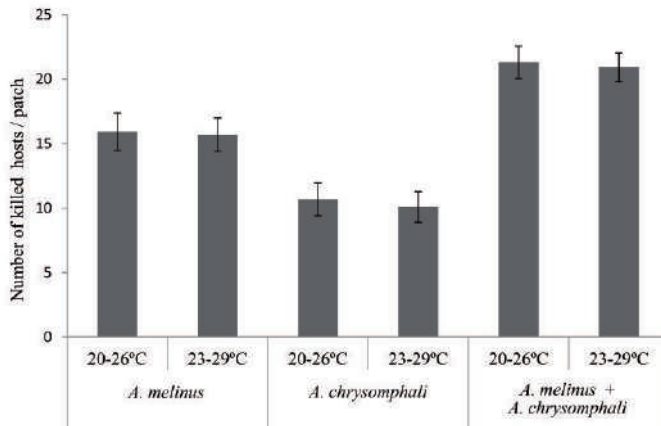


Fig. 5 Effect of parasitoid species (*Aphytis melinus* or *A. chrysomphali*), temperature (20-26°C or 23-29°C) and parasitoid competition on the mortality of their common host, *Aonidiella aurantii*. Presented as the mean number of *A. aurantii* killed ( $\pm$  SE) by the parasitoids during 72 hours. The different temperatures represent the mean temperature of summer from 2009 to 2014 in the Mediterranean basin and the temperature predicted by the end of the twenty-first century by the IPCC.

### 6.3.2. Effect of an increase in temperature on host fecundity

*Aonidiella aurantii* fecundity, measured as the total number of crawlers, was not affected by an increase in temperature (20-26°C =  $224.82 \pm 11.99$  crawlers; 23-29°C =  $242.29 \pm 23.37$  crawlers;  $F_{1,35} = 0.48$ ;  $P = 0.49$ ).

### 6.3.3. Effect of an increase in temperature on parasitoid net reproductive rate ( $R_0$ )

The net reproductive rate ( $R_0$ ) of *A. melinus* was affected by the interaction between temperature and competition ( $F_{1,88} = 279.73$ ;  $P < 0.0001$ ) (Table 1), indicating that  $R_0$  increased with competition when there is a high temperature but it decreased with competition at a low temperature. This finding showed that the negative effect of a temperature increase is reduced in the presence of an inferior competitor.

The net reproductive rate ( $R_0$ ) of *A. chrysomphali* was affected by the interaction between temperature and competition ( $F_{1,91} = 50.69$ ;  $P < 0.0001$ ) (Table 1), indicating that  $R_0$  decreased with the increase in temperature when there was no competition but it increased with the increase in temperature when there was competition. This showed that the negative effect of a temperature increase is absent in the presence of a superior competitor.

**Table 1** Net reproductive rate ( $R_0$ ) of the parasitoids *Aphytis melinus* and *A. chrysomphali* at two temperatures, with and without interspecific competition.

Parasitoid species and competition	$R_0$	
	20-26°C	23-29°C
<i>A. melinus</i>		
Without competition	8.53 ± 0.04 aA	4.64 ± 0.14 bB
Competition	7.02 ± 0.03 aB	5.93 ± 0.05 bA
<i>A. chrysomphali</i>		
Without competition	3.64 ± 0.04 aA	3.38 ± 0.03 bA
Competition	1.74 ± 0.02 bB	1.89 ± 0.02 aB

Data are presented as the mean ± SE, obtained by the jackknife method. Different lowercase letters denote significant differences between temperatures within a treatment (without competition and with competition) and parasitoid species at  $P < 0.05$ . Different uppercase letters denote significant differences between treatments (with and without competition) within a temperature and parasitoid species at  $P < 0.05$ .

## 6.4. DISCUSSION

The presence of the introduced parasitoid *Aphytis melinus* reduced the efficacy of the native *Aphytis chrysomphali* as a biological control agent of the California red scale (*Aonidiella aurantii*) by approximately 50% in the present conditions. The potential of *Aphytis* parasitoids was measured using the net reproductive rate ( $R_0$ ). The reduction in net reproductive rate was mainly due to the reduction of scales successfully parasitized by *A. chrysomphali* females when they searched in the same patch as *A. melinus* females compared to patches without competition. Several biological traits described previously in both parasitoids can explain the reduction in the number of scales successfully parasitized by *A. chrysomphali* when it shared the patch with its competitor. *Aphytis melinus* females tend to kill *A. chrysomphali* eggs (i.e., ovicide) before laying their own egg when they locating a host already parasitized by *A. chrysomphali* (Cebolla et al., 2017a, b). Moreover, *A. melinus* larvae seem to be more aggressive than those of *A. chrysomphali* (Cebolla et al., 2017a, b). Therefore, *A. melinus* might have killed some of the progeny of *A. chrysomphali*,

reducing its  $R_0$ . Another non-mutually exclusive reason that may explain the reduction of *A. chrysomphali*  $R_0$  is that females of this species might avoid using patches where *A. melinus* are searching. However, this hypothesis needs to be tested. Overall, the 50% reduction of *A. chrysomphali*  $R_0$  when it searches in the same patch as *A. melinus* represents another result that explains the displacement of *A. chrysomphali* by *A. melinus* in southern Spain (Sorribas et al., 2010; Boyero et al. 2014). Other reasons are the lower searching capacity and tolerance to hot and dry climates of *A. chrysomphali* (Abdelrahman, 1974a, b; McLaren, 1976). Both parasitoids, however, coexist in eastern Spain where the proportion of *A. chrysomphali* increases with latitude and colder temperatures (Sorribas et al., 2010; Pekas et al., 2010, 2016). Under this scenario, we expected that the predicted increase in temperature under global warming, especially in the summer, would negatively affect the net reproductive rate of *A. chrysomphali*, accelerating its displacement by *A. melinus*. Our results, however, showed that the net reproductive rate of *A. chrysomphali* was not affected by the expected increase of temperature, although it was 50% lower than that of *A. melinus*.

The expected increase in temperature in summer will hinder the efficacy of *A. melinus* as a biological control agent of *A. aurantii*, as the  $R_0$  of this parasitoid was reduced by half. The reduction of *A. melinus*  $R_0$  when temperature increased was mostly due to the reduction in the proportion of females (i.e., increase of the secondary sex ratio proportion). This result is in accordance with previous studies of hymenopteran parasitoids, which suggest that the proportion of males increases with temperature (King, 1987). The biological reasons behind the change in sex ratio are less clear. Two hypotheses might explain the increase in *A. melinus* sex ratio with temperature. First, mothers might consider high temperatures unfavourable for the development of their offspring and thus may have intentionally allocated sons, as suggested by Force & Messenger (1964) and Moiroux et al. (2014). Second, the high

temperatures used in this assay might also decrease the ability of *A. melinus* to mate, as was previously observed in parasitoids exposed to hot thermal stresses (Abram et al., 2017; Jørgensen et al., 2006; Krebs & Loeschcke, 1994; Patton & Krebs, 2001; Rohmer et al., 2004; Roux et al., 2010; Sisodia & Singh, 2006; Wilkes, 1963). In our assay, adult parasitoids were paired in small vials under the two selected abiotic conditions before the experiments started. Two out of the 24 couples exposed to the temperatures predicted by the end of the twenty-first century did not produce females, whereas all couples produced at least one female under the current summer temperatures. This result suggests that mating was negatively affected by the increase in temperature but can, together with other traits, explain the increase in sex ratio. Conversely, we do not expect that host size and/or the infection of *A. melinus* by *Wolbachia* affected the sex ratio of *A. melinus* because we used hosts of similar size in the experiment (see section 2.3 in Materials and Methods) and temperatures below 30°C do not completely cure *A. melinus* of the infection (Vasquez et al., 2011). The curation of females might have produced incompatible crosses and production of males by cured females (Vasquez et al., 2011).

The presence of *A. chrysomphali* did not reduce *A. melinus*  $R_0$  when females of both species shared the same patch. Additionally, the competition between both parasitoids mitigated the negative effect of the increase in temperature on *A. melinus*. Under the summer temperatures expected for the end of the twenty-first century, the introduced parasitoid had higher  $R_0$  when females competed with *A. chrysomphali* than when they searched alone in a patch. Generally, high temperatures affect the metabolic rate of insects and can diminish their locomotion speed and activity level (Vogt et al. 2003; Irlich et al. 2009), increasing, in the case of the hymenopteran parasitoids, the handling time per oviposition (Langer et al., 2004; Wu et al., 2011). The presence of a competitor could, however, stimulate *A. melinus* to complete oviposition faster

as occurs in the solitary egg parasitoid *Enoggera nassau* (Griault) (Hymenoptera: Pteromalidae) when it shares a patch with competitors (Mansfield, 2016). Another possibility is that the presence of a competitor provoked an aggressive behaviour in *A. melinus*. There are numerous studies linking female-female competition and aggressiveness in diverse animal taxa (Stockley & Campbell, 2013). This aggressiveness could be reflected in direct aggressive behaviours towards the competing parasitoid, which would explain the reduction of *A. chrysomphali*  $R_0$  when competing together but could also be reflected in an increase in parasitism that could compensate for the diminution of the  $R_0$  consequence of the temperature increase.

Overall, our study highlights the importance of considering competition to predict the consequences of global warming for biological control. The expected increase in temperature in the Mediterranean basin will negatively affect the introduced parasitoid *A. melinus* and, consequently, the biological control of *A. aurantii*, one of the main citrus pests (Tena & Garcia-Marí, 2011). Interestingly, in southern Spain and other areas where *A. melinus* has already displaced the native *A. chrysomphali*, the effect of global warming will be higher than in eastern Spain and other areas where both parasitoids coexist. This is because *A. chrysomphali*  $R_0$  will not be affected by the increase in temperature and competition will mitigate the negative effect of the increase in temperature on *A. melinus*. Several non-excluding hypotheses have been proposed to explain their coexistence in eastern Spain. These hypotheses include the presence of alternative hosts and climate, conditional patch partitioning and local weather conditions (Pina, 2007; Sorribas et al., 2010; Pekas et al., 2016; Cebolla et al., 2017a). If the native is finally displaced in this area, a decline in the biological control of *A. aurantii* can also be expected.

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## Chapter 7

# GENERAL DISCUSSION





## 7. GENERAL DISCUSSION

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### 7.1. Efficacy of *Aphytis* as biological control agents of *Aonidiella aurantii*

In the Mediterranean basin, as in most citrus areas worldwide, *Aonidiella aurantii* is considered a key citrus pest (Rosen & DeBach, 1979; Murdoch et al, 2005, 2006; Jacas & Urbaneja, 2010; Tena & Garcia-Marí, 2011). Parasitoids of the genus *Aphytis* are the most efficient natural enemies of this pest. The level of natural parasitism on *A. aurantii* is rarely higher than 40% (Pina, 2007; Vanaclocha et al., 2009; Pekas et al., 2010). However, in addition to parasitism, other behaviors such as host feeding, in which females feed on the scale body, causing permanent damage to their host (Heimpel & Rosenheim, 1995). In the chapter 3 we demonstrated how *Aphytis* females, after stinging their hosts, tended to reject them without ovipositing or feeding on them (**overstinging**). We evaluated this occurrence in *Aphytis* parasitoids and demonstrated that it **was as common as parasitism or host-feeding in second-instar hosts, and even more common than host-feeding in third-instar hosts**. Casas et al. (2004) also observed how this behavior was common in *A. melinus* females in laboratory conditions and even occurred at higher rates than in field conditions. In our field samples, we also found wounded hosts due to *Aphytis* overstings. Previous studies have mentioned this overstinging by *Aphytis* but the frequency has never been measured. These studies used the term “host mutilation” to refer to host feeding and overstinging together (Quednau, 1964; Abdelhraman, 1974a; Murdoch et al., 1995).

Overstinging is expected to occur more in synovigenic and long-living parasitoids than in pro-ovigenic and short-living ones because they tend to be more selective on where to lay the low number of eggs that they contain. *Aphytis* falls within this

pattern because they are synovigenic and emerge with no or few developed eggs, they generate additional eggs and increase their longevity if they have access to carbohydrates and hosts on which to feed (Heimpel & Rosnheim, 1998). Therefore, *Aphytis* may experience transient bouts of egg limitation when they use their supply of mature oocytes and must await further egg maturation (Charnov & Skinner, 1988; Heimpel & Rosnheim, 1998; Casas et al., 2000). This biologic characteristic has made *Aphytis* parasitoids more selective when choosing hosts upon which to lay eggs. The rejection of a host after ovipositor insertion has been documented, although it has not been measured in many parasitoids with different reproductive strategies [see references in Vinson (1976)]. Therefore, a quantitative meta-analysis of the rates of overstinging among parasitoid lifestyles would be useful to test this hypothesis.

**Overstinging caused lethal and sublethal effects on *A. aurantii* and its virulence depended on the host instar attacked and on the parasitoid species.** Most of the second-instar hosts attacked died after the attack, whereas ~50% of the third-instar hosts survived. The mechanical damage caused in host tissues through the insertion of the ovipositor and/or by the injection of biochemical compounds can cause death in *A. aurantii* (Vinson, 1976; Strand, 1986; van Driesche et al., 1987; Beckage, 2008; Keinan, et al., 2012). Other studies also found more mortality in young host after being overstung (Neuenschwander et al., 1986; van Driesche et al., 1987; Barret & Brunner, 1990). Therefore, within the little data currently available, it seems there is a general pattern where the first instars are more sensitive to overstinging than later instars. The weaker immune system of first hosts can explain this observed pattern (Beckage & Gelman, 2004). The lethal effect of overstinging on the first instars could also explain why we did not find live second-instar hosts with scars caused by overstinging in our field samples.

Interestingly, we also found differences in the virulence of overstinging between the two parasitoid species. *Aphytis melinus* was more aggressive than *A. chrysomphali* as the former killed more adult hosts when overstung moreover, reduced the fecundity of the surviving females. This result reinforces the higher value of *A. melinus* as biological control agent of *A. aurantii* and, more importantly, indicates that overstinging should be considered as trait to select biological control agents. To our knowledge, this trait has not yet been considered, although Campbell (1963) did use sarcophagid flies to evaluate the frequency of overstinging by several ichneumonid parasitoids of the gypsy moth *Porthetria dispar* L. (Lepidoptera: Lymantriidae).

Fecundity reduction of overstung hosts has been documented in other systems (Reed-Larsen & Brown, 1990; Brown & Kainoh, 1992; Münster-Swedensen, 1994; Tagashira & Tanaka, 1998). All these studies are based on hymenopteran endoparasitoids of the family Braconidae attacking lepidopteran hosts in the egg or larval stages. Polydnaviruses (PDVs), which are associated with endoparasitic wasps belonging to the families Braconidae (bracoviruses, BVs), together with parasitoid eggs, are injected into the host, during the host oviposition and in several host-parasitoid systems are responsible for castrating the host larvae (Asgari & Rivers, 2011; Beckage & Gelman, 2004). Therefore, it is expected that PDVs are also injected when parasitoids oversting. Although most of the studies concerned with PDVs have dealt with parasitoids that attack Lepidopteran, host fecundity regulation has also been documented in aphids (Digilio et al., 2000) and curculionid egg parasitoids (Barrat & Johnstone, 2001). Generally, these attacks result in the castration of young instars (Baudoin, 1975), whereas in adult instars castration is rare, and fecundity is generally only slightly reduced (Spencer, 1926; Beard, 1940; Schling & Hall, 1960). This might have also occurred in our system. *Aphytis* are idiobiont parasitoids that paralyze the host, likely, by inserting venom through the ovipositor (Rosen & Debach, 1979; van

Lenteren, 1994). However, the substance or substances that *Aphytis* inject into the scale body are unknown. Since host castration can be the result of physical damage to the gonad tissue by the ovipositor (Baudoin, 1975) more research is needed to determine whether the venom injected by *A. melinus* also significantly affects host gonad development. Likewise, parasitoid stings cause the suppression of the gonad development in young instars whereas the gonadal tissues are generally well formed in adults (Reed-Larsen & Brown, 1990).

Lethal and sub-lethal effects caused by overstinging, having been widely described in numerous parasitoids, is far from being an isolated phenomenon in this system. Therefore, **our results reinforce the idea that overstinging should be considered in the future selection of parasitoids for biological control** as has been recently suggested in other systems where parasitoids induce host mortality (Abram et al., 2016). Moreover, **the frequency and consequences of overstinging should be included in those models that analyse population dynamics in host-parasitoid systems** as did Münster-Swendsen (2002) and Münster-Swendsen & Berryman (2005).

## 7.2. Effect of host instar on heterospecific host discrimination

When we studied the competition between *A. chrysomphali* and *A. melinus*, we observed that both parasitoids were able to discriminate between unparasitized and previously parasitized hosts by a different species (heterospecific host discrimination). However, this ability to discriminate was host-instar mediated (Chapter 4). Females **were able to discriminate when they found third-instar hosts (larger size) but not second-instar hosts (smaller size)**. Until now, the ability to discriminate between unparasitized and parasitized hosts in *Aphytis* had only been documented in hosts

parasitized by conspecifics (Abdelhraman, 1974b; Rosen & Debach, 1979; van Lenteren & Debach, 1981). Conspecific host discrimination has been observed in more than 200 parasitoid species and has been found in most studied species (van Lenteren, 1981; van Alphen & Visser, 1990). Heterospecific host discrimination has been studied and detected less often among parasitoid species (Turlings et al., 1985; van Baaren et al., 1994; van Baaren & Boivin, 1998; Collier et al., 2007; Yang et al., 2012). Our results suggest a **need to identify the most appropriate instar for those parasitoids which have not yet been documented to have this heterospecific host discrimination ability.**

Host discrimination is generally facilitated through external and/or internal host cues (Sugimoto et al., 1986; Hoffmeister & Roitberg, 1997). External cues for host discrimination can originate from a pheromone deposited during oviposition or from a physical mark left on the host body (Mackauer, 1990). Internal cues can be a result of substances injected by a previous parasitoid or from host quality changes due to previous parasitism (Mackauer, 1990). Some accessory glands (e.g. Dufour's gland) of parasitoids are sources of chemical pheromones which are associated with the marking of hosts (Greany & Oatman, 1972; Guillot & Vinson, 1972; Holler et al., 1994). Host physiological changes may be mediated by viruses, teratocytes and venoms that are injected into the host along with the egg(s) (Fisher & Ganesali, 1970; Vinson & Hegazi, 1998). Our results showed that host rejection of heterospecific parasitized third-instar hosts, occurred significantly more frequently after *Aphytis* females had inserted the ovipositor into the host. This suggests that the recognition occurs in response to internal cues or physiological changes in the host after ovipositor insertion. A study by Ruschioni et al, (2015) showed that the neurons present in the sensillum of the ovipositor tip are used for host discrimination between unparasitized and parasitized hosts. They also play a role in the discrimination between hosts with

different numbers of parasitoid eggs. Our results also corroborate the examination of the scale cover diameter and or the presence of a kairomone, O-caffeoyltyrosine, in the initial assesment of host quality by *Aphytis* parasitoids (Morgan & Hare, 1997). Parasitoids rejected more healthy second-instars of poorer quality than third-instars before probing the host. In other words, they were rejected after only making external examinations.

### 7.3. Effect of interference competition and ovicide on parasitoid coexistence

The plasticity of *A. chrysomphali* in exploiting hosts of smaller size (lower quality) depending on *A. melinus* density is, together with those mentioned above, another factor that could lead to the coexistence of both parasitoids in some Mediterranean areas (Pekas et al., 2016). These authors found higher parasitism rates of second-instar hosts by *A. chrysomphali* when *A. melinus* density was high. Taking the field data into consideration, these authors suggested that *A. chrysomphali* does indeed exploit hosts of poor quality (second instar) when *A. melinus* density is high. Another non-exclusive reason to explain this result could be that *A. chrysomphali* is a better competitor when both species compete for the second instar but not for the third. In other words, the superiority of each species is mediated by the available host instar. In chapter 5, we demonstrated that **in multiparasitized hosts *A. melinus* emerged at higher rates independently of host instar and attack sequence.** These results corroborate the hypothesis of Pekas et al. (2016) and suggest that ***A. chrysomphali* detects, quantifies and avoids the presence of its competitor.**

The superiority of *A. melinus* was due, in part, to its tendency to detect and kill the eggs of *A. chrysomphali* in heterospecific parasitized hosts. *Aphytis melinus* females moved their ovipositors towards the first female's eggs laid in hosts. None of the probed

eggs survived the attack, suggesting ovicide (Chapter 5). Other studies showed that *A. melinus* also commits ovicide when females sting the eggs of competitors while trying to host-feed on hosts parasitized by conspecifics (Collier personal observation cited in Collier & Hunter, 2001). *Encarsia perniciosi* was also identified to have similar behavior (Yu et al, 1990). The occurrence of ovicide may be influenced by host size. In smaller hosts the eggs from competing females may be more easily detected (Netting & Hunter, 2000; Goubault et al., 2004) and may allow for a shorter handling time (Schmidt & Smith, 1987; King, 1994). In our case, however, we observed that ***A. melinus* committed ovicide only in heterospecific parasitized third-instar hosts. In second-instar hosts, ovicide could not be confirmed** because most hosts died, most likely, due to the mechanical injury caused by the stings of both parasitoids.

#### 7.4. Effect of global warming in parasitoid coexistence

One of the causes that can explain the coexistence of *A. chrysomphali* and *A. melinus* in the citrus Mediterranean areas is the variation on their relative proportion depending on the spatiotemporal weather conditions and seasonal changes (Sorribas et al., 2010). Mainly, hot summer temperatures affect *A. chrysomphali* more negatively than *A. melinus*. In fact, in the southern areas with hot dry summers, the native *A. chrysomphali* has been completely displaced by the introduced *A. melinus*. Whereas in northern citrus areas with milder summers there is a higher proportion of *A. chrysomphali*. In the Mediterranean Basin an increase of 3°C is expected in summer due to Global warming (IPCC, 2014) Due to this we hypothesized that the superiority of *A. melinus* in these weather conditions might be accentuated leading ultimately to the complete displacement of *A. chrysomphali*. However, in chapter 6, we observed that **the increase of temperature in summer will not affect the population of *A. chrysomphali* but will hinder the potential of *A. melinus* as biological control**

**agent of *A. aurantii*, as their  $R_0$  will be reduced by half with the temperature increase. The reduction was mostly due to the decrease in the proportion of females in the *A. melinus* progeny.** This result is in accordance with previous studies of hymenopteran parasitoids, which suggest that the proportion of males in the progeny increase with the increase of temperature (King, 1987). Two hypotheses might explain that change in sex ratio at higher temperatures. Firstly, mothers might consider high temperatures unfavourable for their offspring development and thus may have intentionally allocated sons, as suggested by Force & Messenger (1964) and Moiroux et al., (2014). Secondly, the high temperatures used in this assay might also decrease the ability of *A. melinus* to mate as was previously observed in parasitoids exposed to hot thermal stresses (Wilkes, 1963; Krebs & Loeschcke, 1994; Patton & Krebs, 2001; Rohmer et al., 2004; Jørgensen et al. 2006; Sisodia & Singh, 2006; Roux et al., 2010; Abram et al., 2017).

A recent study has demonstrated that temperatures over 32.5°C result in a significant reduction in the number of copies of the symbiotic bacteria *Wolbachia* found in *A. melinus* (Vasquez et al., 2011). *A. melinus* adults are infested with *Wolbachia* and it causes complete cytoplasmic incompatibility in this species (Vasquez et al., 2011). Thus sperm from an infected male is unable to properly fertilize an egg of an uninfected female or a female that is infected with a different *Wolbachia* strain (Werren et al., 2008). Therefore, the elimination of *Wolbachia* in females might produce incompatible crosses and the production of males by cured females (Vasquez et al., 2011). In our study, we did not consider this extreme temperature. However, global warming will not only increase mean temperatures, as studied herein, but will also increase the number of days with extreme temperatures. In this situation, temperatures over 32.5°C can become more common in the coming years and affect

negatively *A. melinus*. Further studies should analyze the effect of these extreme temperatures on the biological control of *A. aurantii*.

Our data shows that **the negative effect of the increase of temperature on *A. melinus* population will be mitigated with the presence of *A. chrysomphali***. With summer temperatures expected from global warming, the introduced parasitoid had a higher  $R_0$  when females compete with *A. chrysomphali* than when they searched alone in a patch. Generally, the increase of temperatures affects the metabolic rate of insects and can diminish their locomotion speed and activity level (Vogt et al., 2003; Irlich et al., 2009), increasing, in the case of the hymenopteran parasitoids, the handling time per oviposition (Langer et al., 2004; Wu et al, 2011). The presence of a competitor could, however, stimulate *A. melinus* to complete oviposition faster as occurs in the solitary egg parasitoid *Enoffera nassau* (Griault) (Hymenoptera: Pteromalidae) when it shares a patch with competitors (Mansfield, 2016). Another possibility is that the presence of a competitor provoked an aggressive behavior in *A. melinus* as has been documented in numerous studies that link female-female competition and aggressiveness in diverse animal taxa (Stockey & Campbell, 2013). This aggressiveness could be reflected in direct aggressive behaviors towards the competing parasitoids, which would explain the reduction of *A. chrysomphali*  $R_0$  when competing.

Overall, our results corroborate that *A. melinus* is a superior competitor compared to *A. chrysomphali* and contributes to the understanding of their current and future geographical dispersion as well as their potential as biological control agents of *A. aurantii*. *A. melinus* killed more hosts than *A. chrysomphali* when it overhung (chapter 3); *A. melinus* accepted more third-instar heterospecific parasitized hosts than *A. chrysomphali* (chapter 4 and 5); *A. melinus* tended to kill the eggs previously

laid by *A. chrysomphali* (4 and 5); *A. melinus* reduced *A. chrysomphali* population by half due to direct competition (chapter 6). As a whole, **interference competition will contribute to the displacement of the native *A. chrysomphali* by *A. melinus* as has been observed in southern Spain** (Sorribas et al., 2010; Boyero et al., 2014).

## Chapter 8

# CONCLUSIONS





## 8. CONCLUSIONS

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Overstinging by hymenopteran parasitoids causes mutilation and surplus killing of hosts.

- i. Overstinging (the rejection of a host after stinging) is a common behavior in *Aphytis* parasitoids in the wild.
- ii. In laboratory conditions, this behavior is even more common than host-feeding.
- iii. The virulence of overstinging depended on the host instar attacked and the parasitoid species.
- iv. Most young-instar hosts (second-instar) attacked died when overstung, whereas ~50% of the adults (third-instar) survived.
- v. *Aphytis melinus* killed more adult hosts than *A. chrysomphali* when overstung and, moreover, reduced the fecundity of the surviving females.
- vi. Overstinging should be incorporated in the selection of parasitoids for biological control and in the models of host-parasitoid populations.

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

- i. *Aphytis* parasitoids were able to discriminate between unparasitized and heterospecific parasitized hosts when they found third-instar hosts (larger size) but not when they found second-instar hosts (smaller size).
- ii. The behavioral strategies observed 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.
- iii. Multiparasitism caused a high cost in terms of immature mortality and it was independent of the host instar. However, brood size and sex ratio was not affected.
- iv. Our results highlight the importance of study different host instars to determine whether parasitoids can discriminate between parasitized and unparasitized hosts.

### Does host quality dictate the outcome of interference competition between sympatric parasitoids? Effect on their coexistence.

- i. Oviposition behavior (host acceptance and clutch size) in young- (low-quality) and adult (high-quality) host was similar for *Aphytis melinus* and *A. chrysomphali* in the absence of competition

- ii. *Aphytis melinus* laid more eggs and accepted more hosts than *A. chrysomphali* in the heterospecific parasitized high-quality hosts. Moreover, *A. melinus* tend to kill the eggs laid by *A. chrysomphali* with the ovipositor when females found a host parasitized by *A. chrysomphali*.
- iii. *Aphytis melinus* emerged at higher rates in multiparasitized hosts independent of host quality and sequence of attack (first female or second female that laid the egg on a host).
- iv. **Interference competition contribute to the displacement of the native *A. chrysomphali* by *A. melinus*.**

Negative effect of global warming on biological control is mitigated by direct competition between sympatric parasitoids

- i. The potential of *A. chrysomphali* as biological control agent of *A. aurantii* was reduced by half when both parasitoids competed in the same patch at the current and expected temperatures.
- ii. The expected increase of temperature will reduce the population of *A. melinus* as it will affect the production of females in the *A. melinus* progeny.
- iii. Competition will mitigate the negative effect of the increase of the temperature in *A. melinus* population.
- iv. **Overall, our study highlights the importance of considering competition between natural enemies to predict the consequences of global warming in biological control.**







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