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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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TABLE OF CONTENTS

SUMMARY	xiii
RESUMEN	xvii
RESUM	xxi
Chapter 1. GENERAL INTRODUCTION.....	1
1.1. Parasitoids as biological control agents.....	3
1.2. Behavioral ecology of hymenopteran parasitoids	6
1.2.1. Foraging and oviposition behavior	6
1.2.2. Host quality and host utilization.....	9
1.2.3. Intrinsic competition between parasitoids and ovicide.....	13
1.2.4. Effect of temperature increase on parasitoid-host interaction	15
1.3. <i>Aonidiella aurantii</i> as citrus pest.....	15
1.3.1. Origin and distribution	16
1.3.2. Importance of <i>Aonidiella aurantii</i> as a citrus pest.....	16
1.3.3. Morphology and development	18
1.3.4. Biology and ecology	20
1.3.5. Management of <i>A. aurantii</i> in the Mediterranean Basin	23
1.4. <i>Aphytis</i> as natural enemies of <i>Aonidiella aurantii</i>	24
1.4.1. Morphology and development of parasitoids of the genus <i>Aphytis</i>	26
1.4.2. Biology and ecology of parasitoids of the genus <i>Aphytis</i>	28
1.4.3. Behavior and efficacy of <i>Aphytis</i> parasitoids	29
1.4.3.1. Effect of host quality on <i>Aphytis</i> behavior and efficacy	29

1.4.3.2. Interspecific competition between <i>Aphytis</i> parasitoids.....	33
1.4.3.3. Effect of global warming on <i>Aphytis</i> competition and efficacy as biological control agents.....	34
Chapter 2. RESEARCH OBJECTIVES.....	35
Chapter 3. OVERSTINGING BY HYMENOPTERAN PARASITIDS CAUSES MUTILATION AND SURPLUS KILLING OF HOSTS	39
3.1. Introduction.....	42
3.2. Materials and methods	44
3.3. Results	51
3.4. Discussion.....	58
Chapter 4. EFFECT OF HOST INSTAR ON HOST DISCRIMINATION OF HETEROSPECIFICPARASITISED HOSTS BY SYMPATRIC PARASITIDS.....	67
4.1. Introduction.....	70
4.2. Material and methods.....	73
4.3. Results	78
4.4. Discussion.....	83
Chapter 5. DOES HOST QUALITY DICTATE THE OUTCOME OF INTERFERENCE COMPETITION BETWEEN SYMPATRIC PARASITIDS? EFFECT ON THEIR COEXISTENCE.....	89
5.1. Introduction.....	92
5.2. Methods.....	94
5.3. Results	101
5.4. Discussion.....	105

Chapter 6. NEGATIVE EFFECT OF GLOBAL WARMING ON BIOLOGICAL CONTROL IS MITIGATED BY DIRECT COMPETITION BETWEEN SYMPATRIC PARASITOIDS	111
6.1. Introduction.....	114
6.2. Materials and methods	116
6.3. Results	122
6.4. Discussion.....	128
Chapter 7. GENERAL DISCUSSION	133
7.1. Efficacy of <i>Aphytis</i> as biological control agents of <i>Aonidiella aurantii</i>	135
7.2. Effect of host instar on heterospecific host discrimination	138
7.3. Effect of interference competition and ovicide on parasitoid coexistence.....	140
7.4. Effect of global warming in parasitoid coexistence.....	141
Chapter 8. CONCLUSIONS.....	145
Chapter 9. REFERENCES	151

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

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 20th 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 pedium 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; Alexandrakis, 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: HemisarcOPTidae) (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 mm^2 (in body area of CRS) and female eggs mostly to hosts larger than 0.39 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

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

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

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

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 PARASITIDS

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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

Chapter 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

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-Mari, 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. Polydnnaviruses (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

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.**

Chapter 9

REFERENCES

Overwintering by hymenopterous parasitoids causes mutilation and voracious killing of hosts

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Abstract The majority of the reproductive cycle of parasitoids in temperate regions requires the overwintering of the parasitoid in diapause. The ability of parasitoids to overwinter in diapause is a key factor in their survival. Overwintering parasitoids can cause host mortality by voracious killing of hosts. This study aimed to determine the effect of overwintering parasitoids on host mortality. The results showed that overwintering parasitoids caused host mortality by voracious killing of hosts. This study aimed to determine the effect of overwintering parasitoids on host mortality. The results showed that overwintering parasitoids caused host mortality by voracious killing of hosts.

Keywords Hymenoptera · Parasitoids · Diapause · Overwintering · Host mortality · Parasitoid mortality

Key message

- Overwintering parasitoids cause host mortality by voracious killing of hosts.
- The majority of the reproductive cycle of parasitoids in temperate regions requires the overwintering of the parasitoid in diapause.
- The ability of parasitoids to overwinter in diapause is a key factor in their survival.
- Overwintering parasitoids can cause host mortality by voracious killing of hosts.

Introduction

Parasitoids are a group of insects that live on or inside their hosts and eventually kill them. They are found in all parts of the world and are important natural enemies of many pest insects.

Effect of host instar on host discrimination of heterospecific-parasitised hosts by sympatric parasitoids

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