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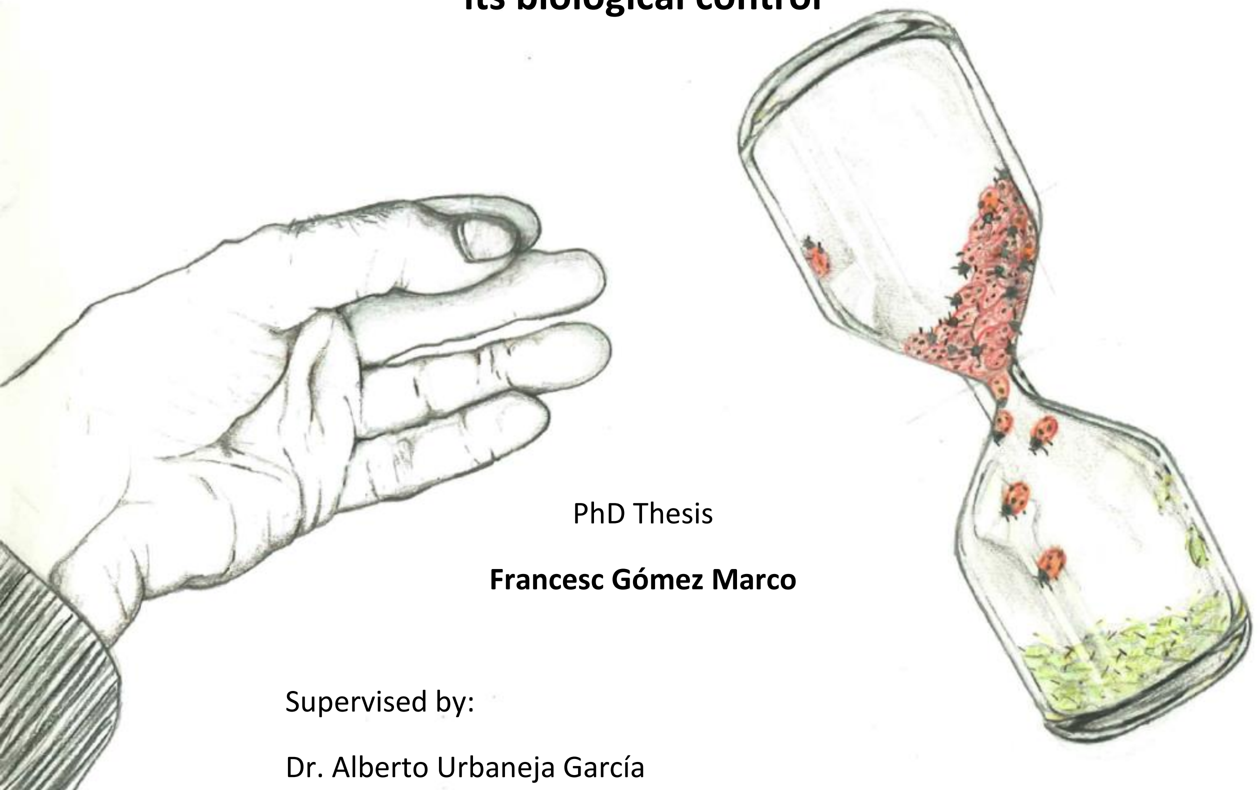
Integrated Pest Management of *Aphis spiraecola* (Hemiptera: Aphididae) in clementines: enhancing its biological control



PhD Thesis

Francesc Gómez Marco

Supervised by:

Dr. Alberto Urbaneja García

Dr. Alejandro Tena Barreda

Valencia, May 2015

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Integrated Pest Management of *Aphis spiraecola* (Hemiptera: Aphididae) in clementines: enhancing its biological control

International PhD Thesis

Francesc Gómez Marco

Supervised by:

Dr. *Alberto Urbaneja García*

Dr. *Alejandro Tena Barreda*

Illustrated by *Eva Maria Abad Madrid*

A ma mare

"la entrada del neolítico es la del abuso y la del sojuzgamiento, y en ella seguimos, inadaptados"

Félix Rodríguez de la Fuente

Agraïments

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Resum

Aphis spiraecola Patch. (Hemiptera: Aphididae) és una de les plagues clau en el cultiu de clementins de la conca Mediterrània. En primavera, este àfid colonitza les brotacions tendres dels clementins i provoca importants pèrdues econòmiques tots els anys. Actualment la gestió integrada d'*A. spiraecola* en clementins està basada en el control químic ja que es desconeix prou sobre el control biològic d'*A. spiraecola* en cítrics i els esforços realitzats fins ara s'han centrat en el us i conservació de parasitoids encara que es desconeix les causes de la seua baixa eficàcia. Per altra banda, es coneix molt bé el conjunt de depredadors d'*A. spiraecola* però el seu impacte sobre les poblacions de l'àfid no s'han documentat. Tenint en compte estos antecedents, els objectius d'esta tesis foren: i) desentrampar les raons per les quals els parasitoids no són efectius; ii) determinar quan i com els depredadors poden controlar les poblacions d'*A. spiraecola*; i finalment iii) determinar si una coberta de poàcies pot millorar el control biològic d'este àfid en clementins mitjançant la millora en el establiment del seus depredadors.

Els estudis es dugueren a terme entre els anys 2011 i 2013 en camps de clementins, tots ells emplaçats en la Província de Valencia i la Província de Castelló.

En el primer objectiu es van mostrejar setmanalment quatre parcel·les i s'identificà el complex de parasitoids i les taxes de parasitisme (i hiperparasitisme). Els percentatges de parasitisme foren baixos (menys del 5%) i *Binodoxys angelicae* Haliday (Hymenoptera: Braconidae) va ser l'únic parasitoid primari emergit de les mòmies d'*A. spiraecola*. Mitjançant mètodes clàssics es van identificar al menys sis especies d' hiperparasitoids atacant este parasitoid primari: *Syrphophagus aphidivorus* (Mayr) (Encyrtidae), *Alloxysta* sp. (Forster) (Figitidae), *Asaphes* sp. (Walker) (Pteromalidae), *Pachyneuron aphidis* (Bouché) (Pteromalidae), *Dendrocerus* sp. (Ratzeburg) (Megaspilidae) i *Phaenoglyphis villosa* (Hartig) (Figitidae). Més a més, es desenvolupà un mètode basat en la detecció de DNA amb el que es confirmà que totes les especies d'hiperparasitoids hiperparasiten *B. angelicae*. Els hiperparasitoids més abundants foren *S. aphidivorus* i *Alloxysta* sp. Tots dos dominaren aquesta xarxa tròfica i foren

abundants a l'inici de l'estació amb uns percentatges d'hiperparasitisme al voltant del 40%. Finalment s'observà que els hiperparasitoids també incrementaren la proporció de mascles en la descendència de *B. angelicae*. Per tant, l'hiperparasitisme podria explicar el baix impacte que *B. angelicae* té sobre les poblacions d'*A. spiraecola*.

Per al segon objectiu es mostrejaren tres camps de clementins on es determinà l'efecte dels depredadors en les colònies d'*A. spiraecola* i en el dany que estes generen al cultiu. Els paràmetres de vida de les colònies d'*A. spiraecola* (màxim número d'àfids, longevitat i fenologia de la colònia) variaren entre les tres parcel·les els tres anys. En totes les parcel·les i durant els tres anys els depredadors sempre atacaren un terç de les colònies estudiades sense que es trobaren diferències significatives entre les parcel·les. Per altra banda, el màxim número d'àfids i la longevitat de les colònies d'*A. spiraecola* es correlacionaren negativament amb el moment del primer atac del depredador a la colònia. Caldria destacar que el percentatge de brots ocupats per *A. spiraecola* es mantingué per baix o prop del llindar de tractament quan les colònies foren atacades abans dels ~200 graus dia (GD) des de l'inici de formació de la colònia. Estos resultats sugereixen que: i) la presència de depredadors a l'inici de la estació de l'àfid podria ser considerada per al desenvolupament de nous llindars de tractament i ii) els programes de control biològic deurien promoure l'avançament de la presència de depredadors en els camps de clementins.

Per promoure la l'avançament de la presència de depredadors en els camps de clementins, com tercer objectiu s'avaluà el maneig de cobertes vegetals basades en poàcies, com estratègia de control biològic per conservació. Amb aquest maneig es persegueix aportar preses alternatives per als enemics naturals d'*A. spiraecola*. Per això, es compararen quatre camps de clementins amb coberta vegetal front a quatre amb sol nu. En els camps amb coberta sembrada creix, junt a les Poáceas sembrades, un conjunt de plantes salvatges que podrien afectar també el control biològic d'*A. spiraecola*. S'investigà quines especies de plantes componien la coberta vegetal així com les especies d'àfids que les habitaven. Les poàcies representaren un 66% de la coberta vegetal, sent les plantes salvatges més abundants *Malva* sp. (13%), *Oxalis* sp. (5%) i *Sonchus* sp. (2%). Les poàcies i *Oxalis* sp. hostejaren respectivament àfids estenòfags de poàcies i *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae). Estes espècies d'àfid aparegueren més prompte en l'ecosistema que aquells àfids associats a cítrics. Estos àfids actuaren com preses/hostes alternatius per als enemics naturals, per lo que podrien millorar el control biològic d'*A. spiraecola*. Al contrari, *Malva* sp. i *Sonchus* sp. hostejaren especies d'àfids que podrien ser potencials plagues de cítrics com per exemple *Aphis gossypii* Glover (Hemiptera: Aphididae) i altres àfids que aparegueren simultàniament a *A. spiraecola*. Per tant, este últim grup pot atenuar l'atac dels enemics naturals a les poblacions d'*A. spiraecola* que habiten les copes, atraient-los cap a la coberta. Encara així, l'efecte total de la coberta sembrada a base de poàcies va resultar positiu per al control d'*A. spiraecola* ja que promogué la presència anticipada de depredadors en les copes dels cítrics, no així dels parasitoids associats a *A. spiraecola*. Els atacs dels depredadors a les colònies d'*A. spiraecola* es produïren abans del creixement exponencial dels àfids en les copes dels cítrics. Estos atacs resultaren en un control satisfactori de les poblacions d'*A. spiraecola* i en conseqüència les parcel·les de cítrics amb coberta vegetal tendiren a no sobrepassar el llindar de tractament.

Summary

Aphis spiraecola Patch. (Hemiptera: Aphididae) is a key pest of clementines in the Mediterranean basin. This aphid colonizes tender clementine shoots in spring and causes important economic losses. Integrated management of *A. spiraecola* in clementines is currently based in chemical control because Biological control of *A. spiraecola* is still poorly known and efforts were based on the use and conservation of parasitoids but it did not success. On the other hand, the predator complex of *A. spiraecola* is well known but its impact on populations of this aphid has not been documented. With all this said, the aims of this thesis were: i) to disentangle the reasons behind the low parasitism of *A. spiraecola*; ii) to determine when and how predators can control *A. spiraecola* populations; and, finally, iii) to evaluate whether a ground cover of Poaceae plants can enhance the biological control of this aphid in clementines by improving the establishment of its predators.

The studies were carried out in clementine orchards located in "Provincia de Valencia" and "Provincia de Castellón" from 2011 to 2013.

In the first objective we sampled four orchards and determine the parasitoid complex and parasitism (and hyper-) rates weekly. Parasitism percentages were low (below 5%) and *Binodoxys angelicae* Haliday (Hymenoptera: Braconidae) was the unique primary parasitoid emerged from mummies of *A. spiraecola*. At least six hymenopteran hyperparasitoid species were identified by classical means attacking this primary parasitoid: *Syrphophagus aphidivorus* (Mayr) (Encyrtidae), *Alloxysta* sp. (Forster) (Figitidae), *Asaphes* sp. (Walker) (Pteromalidae), *Pachyneuron aphidis* (Bouché) (Pteromalidae), *Dendrocerus* sp. (Ratzeburg) (Megaspilidae) and *Phaenoglyphis villosa* (Hartig) (Figitidae). In addition, we developed a DNA-based approach to untangle the structure of the aphid-parasitoid food web in citrus. This methodology confirmed that all six species hyperparasitized *B. angelicae*. The most abundant hyperparasitoids were *S. aphidivorus* and *Alloxysta* sp. Both dominated this food web and they were abundant from the beginning of the season, and hyperparasitism percentages remained high around 40% throughout

both seasons. Finally, hyperparasitoids also increased the secondary sex ratio of *B. angelicae*. Thus, hyperparasitism probably explains the low impact of *B. angelicae* on *A. spiraecola* populations.

For the second objective we sampled three clementine orchards to determine the effect of aphid predators on *A. spiraecola* colonies and damage over a three-year period. Life parameters of *A. spiraecola* colonies (maximum number of aphids, longevity and colony phenology) varied among the orchards over the three years. Predators attacked one third of the colonies, and it did not significantly differed among orchards any year. However, the maximum number of aphids and the longevity of *A. spiraecola* colonies were negatively correlated with the time of first attack by predators. More importantly, the percentage of shoots occupied by *A. spiraecola* (damages) remained below or close to the intervention threshold when colonies were attacked prior to ~200 degree days (DD) since the beginning of the aphid colonization. These results suggest that: i) the presence of predators at the beginning of the season should be considered to develop new intervention thresholds and ii) biological control programs should promote the early presence of predators in clementine orchards.

To promote the early presence of predators in clementine orchards, in the third objective we evaluated ground cover management, as strategy of conservation biological control. This ground cover management may provide alternative preys to natural enemies. The effect of a sown ground cover (based on *Poaceae* plants) on the biological control of *A. spiraecola* was evaluated in four orchards with ground cover management compared with four orchards with bare soil management. This sown Poaceae cover coexists with a complex of wild plants that might also affect biological control of *A. spiraecola*. Therefore, the ground cover plant composition and their inhabiting aphids were also described. Finally, we compared the presence of *A. spiraecola* and its natural enemies in these orchards. While Poaceae plants represented ~66% of the ground cover, the rest of the cover comprised mainly *Malva* sp. (13%), *Oxalis* sp. (5%) and *Sonchus* sp. (2%). Poaceae plants and *Oxalis* sp. harbored stenophagous aphids and *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae), respectively, which appeared sooner in the system than citrus aphids. These aphids serve as alternative prey/hosts for natural enemies, thus enhancing the biocontrol of *A. spiraecola*. By contrast, *Malva* sp. and *Sonchus* sp. harbored the potential citrus pest *Aphis gossypii* Glover (Hemiptera: Aphididae) and other aphids that appear simultaneously with *A. spiraecola*. Therefore, by attracting them to the cover, this latter group could relieve the attack of natural enemies on *A. spiraecola* in the canopy. Although these wild plants may act as reservoirs for *A. spiraecola* as well as other aphid species that can disrupt the biocontrol services of natural enemies, overall, the sown cover was effective in terms of biological control of *A. spiraecola* in the citrus canopy. It promoted the early presence of predators in citrus canopies but did not promote the early presence of parasitoids. Predators attacked *A. spiraecola* colonies in the canopies before their exponential increase. These attacks resulted in satisfactory aphid control, because citrus orchards with ground cover never exceeded the aphid economic threshold.

Resumen

Aphis spiraecola Patch. (Hemiptera: Aphididae) es una de las plagas claves en el cultivo de clementinos de la cuenca Mediterránea. En primavera, este pulgón coloniza las brotaciones tiernas de los clementinos y causa importantes pérdidas económicas todos los años. Actualmente la gestión integrada de *A. spiraecola* en clementinos está basada en el control químico ya que se desconoce bastante sobre el control biológico de *A. spiraecola* en cítricos. Los esfuerzos realizados hasta la fecha se han centrado en el uso y conservación de parasitoides aunque se desconocen las causas de su baja eficacia. Por otro lado, se conoce muy bien el complejo de depredadores de *A. spiraecola* pero su impacto sobre las poblaciones del pulgón no se ha documentado. Por todo ello, los objetivos de esta tesis han sido i) desentrañar las razones por las que se dan bajos niveles de parasitismo de *A. spiraecola* ii) determinar cuándo y cómo los depredadores pueden controlar las poblaciones de *A. spiraecola* y finalmente iii) determinar si una cubierta de poáceas puede mejorar el control biológico de este pulgón en clementinos mediante la mejora en el establecimiento de sus depredadores.

Los estudios se llevaron a cabo entre los años 2011 y 2013 en campos de clementinos, todos ellos emplazados en la Provincia de Valencia y la Provincia de Castellón.

En el primer objetivo se muestrearon semanalmente cuatro parcelas y se identificó el complejo de parasitoides y las tasas de parasitismo (e hiperparasitismo). Los porcentajes de parasitismo fueron bajos (~menos del 5%) y *Binodoxys angelicae* Haliday (Hymenoptera: Braconidae) fue el único parasitoide primario emergido de las momias de *A. spiraecola*. Mediante métodos clásicos se identificaron al menos seis especies de hiperparasitoides atacando este parasitoide primario: *Syrphophagus aphidivorus* (Mayr) (Encyrtidae), *Alloxysta* sp. (Forster) (Figitidae), *Asaphes* sp. (Walker) (Pteromalidae), *Pachyneuron aphidis* (Bouché) (Pteromalidae), *Dendrocerus* sp. (Ratzeburg) (Megaspilidae) y *Phaenoglyphis villosa* (Hartig) (Figitidae). Además, se desarrolló un método basado en la detección de ADN con el cual se confirmó que todas las especies de hiperparasitoides hiperparasitan *B. angelicae*. Los hiperparasitoides más abundantes fueron

S. aphidivorus y *Alloxysta* sp. Ambos dominaron esta red trófica y fueron abundantes desde el inicio de la estación con unos porcentajes de hiperparasitismo en torno al 40%. Finalmente se observó que los hiperparasitoides también incrementaron la proporción de machos en la descendencia de *B. angelicae*. De este modo, el hiperparasitismo probablemente explica el bajo impacto que *B. angelicae* tiene sobre las poblaciones de *A. spiraecola*.

Para el segundo objetivo se muestrearon tres campos de clementinos donde se determinó el efecto de los depredadores en las colonias de *A. spiraecola* y en el daño que estas generan en el cultivo. Los parámetros de vida de las colonias de *A. spiraecola* (máximo número de pulgones, longevidad y fenología de la colonia) variaron entre los diferentes cultivos los tres años. En todas las parcelas y durante los tres años los depredadores siempre atacaron un tercio de las colonias estudiadas y no se observaron diferencias entre parcelas ningún año. Sin embargo, el máximo número de pulgones y la longevidad de las colonias de *A. spiraecola* se correlacionaron negativamente con el momento del primer ataque del depredador a la colonia. Cabe destacar que el porcentaje de brotes ocupados por *A. spiraecola* permaneció por debajo o cerca del umbral de tratamiento cuando las colonias fueron atacadas antes de los 200 grados días (GD) desde el inicio de formación de la colonia. Estos resultados sugieren que: i) la presencia de depredadores al inicio de la temporada de pulgón debes ser considerado para el desarrollo de nuevos umbrales de tratamiento y ii) los programas de control biológico deben promover el adelanto de la presencia de depredadores en los campos de clementinos.

Para promover la presencia anticipada de depredadores en los campos de clementinos, como tercer objetivo se evaluó el manejo de cubiertas vegetales a base de poáceas, como estrategia de control biológico por conservación. Con este manejo se persigue aportar presas alternativas para los enemigos naturales de *A. spiraecola*. Para ello, se compararon cuatro campos de cítricos con cubierta vegetal frente a cuatro con suelo desnudo. En los campos con cubierta sembrada apareció además de las poáceas sembradas, un complejo de plantas salvajes que podrían afectar también el control biológico de *A. spiraecola*. Por ello, se investigó qué especies de plantas componían la cubierta vegetal así como las especies de pulgones que las habitaron. Las poáceas representaron un 66% de la cubierta vegetal, siendo las plantas salvajes más abundantes *Malva* sp. (13%), *Oxalis* sp. (5%) y *Sonchus* sp. (2%). Las poáceas y *Oxalis* sp. albergaron respectivamente pulgones estenófagos de plantas poáceas y *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae). Estas especies de pulgones aparecieron más pronto en el ecosistema que los pulgones de cítricos y sirvieron como presas/hospederos alternativos para los enemigos naturales, por lo que podrían mejorar el control biológico de *A. spiraecola*. Al contrario, *Malva* sp. y *Sonchus* sp. albergaron especies de pulgón que podrían ser potenciales plagas de cítricos como *Aphis gossypii* Glover (Hemiptera: Aphididae) y otros pulgones que aparecieron simultáneamente a *A. spiraecola*. Por lo tanto, este último grupo puede atenuar el ataque de los enemigos naturales sobre las poblaciones de *A. spiraecola* de las copas, atrayéndolos hacia la cubierta. A pesar de esto, el efecto total de la cubierta sembrada resultó positivo para el control de *A. spiraecola* ya que promovió la presencia anticipada de depredadores en la copa de los cítricos, no así de los parasitoides asociados al pulgón *A. spiraecola*. Los ataques de depredadores a las colonias de *A. spiraecola* se produjeron antes del crecimiento exponencial de los pulgones en la copa de los cítricos. Por lo tanto, estos ataques resultaron en un control satisfactorio de las poblaciones de *A. spiraecola* y en consecuencia las parcelas de cítricos con cubierta vegetal tendieron a no sobrepasar el umbral de tratamiento.

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CHAPTER I

Introduction

1. Introduction

1.1. Citrus

Citrus is one of the most important crops in Spain, where production has increased threefold since 1961 (FAO, 2014). Currently, Spain produces 5,501,500 tons and is the fourth major citrus-producing country worldwide (FAO, 2014). Spain has three main citrus-producing regions: Andalucía, Comunitat Valenciana and Murcia. The Comunitat Valenciana region is the major producer of clementines (80.6% of the national production) and oranges (55.3%) and is the second largest producer of lemons (28.5%).

Most of the production, 1,526,624 tons in 2011, is typically exported for fresh consume, which places Spain as the largest exporter of citrus worldwide (FAO, 2014). Because of the high quality standards for fruit as well as the quarantine requirements of the international market, citrus pest management has to provide a product without damages (including those merely cosmetic) and free of quarantine pests. Both conditions must also be achieved with minimal pesticide residue. The demands of international market together with the new European Directive 2009/128/CE that regulates the use of pesticides place the Spanish citrus industry in an unbeatable position to implementing area-wide Integrate Pest Management (IPM) programs (Urbaneja *et al.*, 2014). Indeed, the adoption of IPM programs increased fivefold in the citrus-producing areas between 2005 and 2012 in Comunitat Valenciana (Anonymous, 2014b).

1.2. Integrated Pest Management in Spanish citrus

Integrated Pest Management (IPM) is defined by the Food and Agriculture Organization of the United Nations (FAO) as follows: "the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agroecosystems and encourages natural pest control mechanisms." (FAO, 2014).

1.2.1. Primary citrus pests in Spain and their management

Spanish citrus orchards harbor an abundant and diverse community of arthropods that includes numerous phytophagous species but also a wide range of natural enemies that keep, in most cases, pest populations below economic injury levels (Jacas and Urbaneja, 2010). Some well-known examples of satisfactory biological control include the citrus red mite *Panonychus citri* McGregor (Acari: Tetranychidae) controlled by *Euseius stipulatus* Athias-Henriot (Acari: Phytoseiidae), the cottony cushion scale *Icerya purchasi* Maskell (Hemiptera: Monophlebidae) controlled by *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae) and *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) regulated by the parasitoid *Citrostichus phyllocnistoides* Narayan (Hymenoptera: Eulophidae) (Garcia-Marí *et al.*, 2004; Jacas *et al.*, 2006). However, a few phytophagous species, classified as key pests, are not well controlled by their natural enemies and thus need additional control measures, primarily chemical control.

The California red scale *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) and the medfly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) are today, as ten years ago, the key citrus pests in the Mediterranean basin, including Spain (Urbaneja *et al.*, 2008; Tena and Garcia-Marí, 2011). Additionally, *Tetranychus urticae* Koch (Prostigmata: Tetranychidae) and the aphids *Aphis spiraecola* Patch (Figure 1) and *Aphis gossypii* Glover (Hemiptera: Aphididae) are key pests on mandarin clementines *Citrus clementina* Hort. ex Tan. (Geraniales: Rutaceae) because of the specific physiological features of these cultivars: mandarin trees are vigorous and leafy with abundant spring leaf-flush. These shoots are highly tender and are a perfect niche for citrus infesting aphids in spring and *T. urticae* in summer (Hermoso de Mendoza *et al.*, 2006; 2012; Tena and Garcia-Marí, 2011).

Logically, IPM strategies in citrus should differ among areas depending on the key pests (Anonymous, 2014a). To date, Spain is privileged with respect to other citrus-producing areas because it is free of some key pests that require multiple chemical treatments throughout the year, such as *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) and *Phyllocoptruta oleivora* (Ashmead) (Acari: Eriophyidae) (CABI 2014; Vacante, 2010). Therefore, the key to successful IPM in Spanish citrus is to keep key pests below economic injury levels, without disrupting the natural and satisfactory control of the other phytophagous species. Thus, the choice of pesticide is one of the foundations of IPM in citrus (conservation biological control) because the pesticide must not (in addition to their high efficiency against the selected pests) harm the natural enemies to avoid disruption of biological control (Urbaneja *et al.* 2014; Real Decreto 1311/2012 (article 34)). Notably, most IPM strategies emphasize the combinations of control methods from chemical to biological, ignoring that informed inaction in some cases may be a better IPM option for arthropod pest management (Kogan, 1998).

1.2.2. Aphids as citrus pests

Aphids are major pests in agriculture because they obtain energy from plants by sucking on the phloem; induce deformation of plant tissues by secreting and injecting saliva, which can be toxic, into plants before ingesting plant sap; vector plant viruses; and excrete abundant honeydew. All crops are attacked by at least one species of aphid (Van Emden and Harrington, 2007).

More than 20 aphid species are recorded on citrus worldwide, but many are visitors by chance and others are of low economic importance. Among these, four species in the subfamily Aphidinae of the family Aphididae are primary citrus pests because of their direct or indirect damage (Hermoso de Mendoza *et al.*, 1997; Van Emden and Harrington, 2007; Vacante and Gerson, 2012). These aphids are *A. spiraecola*, *A. gossypii*, *Toxoptera aurantii* (Boyer de Fonscolombe) and *Toxoptera citricida* (Kirkaldy), and the relative abundance and economic importance vary among citrus-producing areas. For example, these species are classified as "minor" pests in Australia (Smith *et al.*, 1997), California (Flint, 1991; Grafton-Cardwell *et al.*, 2006) and Florida (Michaud, 1998; Powell *et al.*, 2006; Stansly *et al.*, 2012), and sprays are recommended only for young trees.

In the Mediterranean region, the most abundant and widely distributed species are *A. spiraecola* and *A. gossypii* (Tena and Garcia-Marí, 2011). Both species are pests in Italy (Albanese *et al.*, 2010), Greece (Kavallieratos *et al.*, 2002), Syria (Abou Kubaa *et al.*, 2009), Israel (Zehavi and Rosen, 1987) and Turkey (Yoldas *et al.*, 2011). *Toxoptera aurantii* is also widely distributed throughout the Mediterranean area but it is much less abundant than the other two aphids (Hermoso de Mendoza *et al.*, 1997). *Toxoptera citricida*, which is the most efficient vector of Citrus Tristeza Virus (CTV), and some years ago, invaded Portugal and northern Spain (Hermoso de Mendoza *et al.*, 2008). However, *T. citricida* has not reached the major citrus-producing areas of the Mediterranean.

Figure 1. *Aphis spiraecola* Patch (Hemiptera: Aphididae).

In Spain, as in the Mediterranean region, *A. spiraecola*, *A. gossypii* and *T. aurantii* are the most widespread and harmful aphid species, especially in clementines (Hermoso de Mendoza *et al.*, 2012), but their relative importance has changed during the last century (Gómez-Menor, 1943; Meliá, 1982; Hermoso de Mendoza *et al.*, 1986, 1997). *Aphis spiraecola* displaced the native and previously dominant species *T. aurantii* to become the most important citrus aphid species after 1960. This displacement was thought to be due to the low efficacy of the native natural enemies. In the 1980s, *A. gossypii*, which coexisted in low populations densities with *A. spiraecola* and *T. aurantii*, developed resistance against carbamate and other organophosphate pesticides (Takada and Murakami, 1968; Meliá and Blasco, 1990) and became a key citrus pest (Hermoso de Mendoza *et al.*, 1997). Currently, with the rational use of pesticides implemented in Spanish citrus, *A. spiraecola* is the most abundant and harmful aphid species (Hermoso de Mendoza *et al.*, 2012).

1.3. *Aphis spiraecola* as a citrus pest

Aphis spiraecola (Figure 1 and 2) is a polyphagous species (Pons and Lumbierres, 2004; Van Emden and Harrington, 2007; Andreev *et al.*, 2009; Yovkova *et al.*, 2013) and was considered a pest of citrus since the first quarter of the 20th century (Cole, 1925; Miller, 1929). It was referred to as *Aphis citricola* van der Goot in the literature from 1975 to 1988 because of a misidentification (Eastop and Blackman, 1988). The first reference of *A. spiraecola* as a citrus pest in the Mediterranean basin dates from the 1940s by Gómez-Menor (1943) in Spain. Afterward, the species was documented in the rest of the Mediterranean (Barbagallo, 1966; Van Emden and Harrington, 2007).

1.3.1. Origin and geographical distribution

Aphis spiraecola is native to East Asia (Blackman and Eastop, 1994; Van Emden and Harrington, 2007), and this aphid spread during the 20th century worldwide and currently has a cosmopolitan distribution (Blackman and Eastop, 1994; Van Emden and Harrington, 2007). The species was first documented in North America in 1907; in Australia and New Zealand in 1926 and 1931, respectively; in South America and Europe in the 1930s; and finally, in Africa in 1961 (Blackman and Eastop, 1994; Van Emden and Harrington, 2007). Although the pest was introduced into Europe many years ago, it became a key citrus pest near 1960.

1.3.2. Morphological description

A general morphological characterization of *A. spiraecola* is found in Blackman and Eastop (1994). *Aphis spiraecola* as a Sternorrhyncha hemipteran has the rostrum deflected back along the ventral surface of the body and inserted between the fore coxae. The species has well-developed antennae and two segmented tarsi (Blackman and Eastop, 1994). The apterous adults and nymphs are bright greenish yellow to apple green with a brown head, mainly pale legs and antennae and dark brown to black siphunculi and cauda (except nymphs, which do not have a cauda)(Figure 2). The winged stage differs from apterous forms with a dark brown head and thorax, and the abdomen has dusky lateral patches on each segment (Figure 2C). Both stages are similar in size, from 1.2 to 2.2 mm, with the largest body sizes in spring (Blackman and Eastop, 1994). The winged nymphs compared with apterous forms have wing primordia (Figure 2B). In advanced nymphal instars, the third and fourth, black wing primordia appear clearly, and the thorax turns pink-brown (Figure 2B and 3). Hermoso de Mendoza (1996) published an

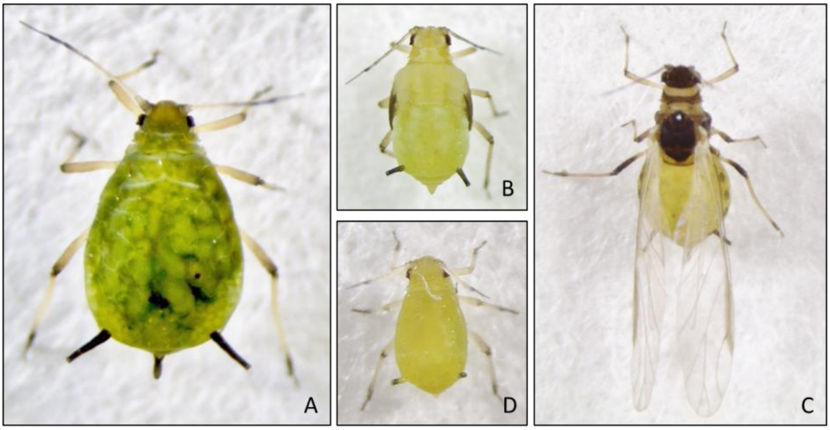

Figure 2. Visual differences between *A. spiraecola* Patch (Hemiptera: Aphididae) stages. Apterous (A), winged nymphs (B), winged adults(C), apterous nymphs (D).

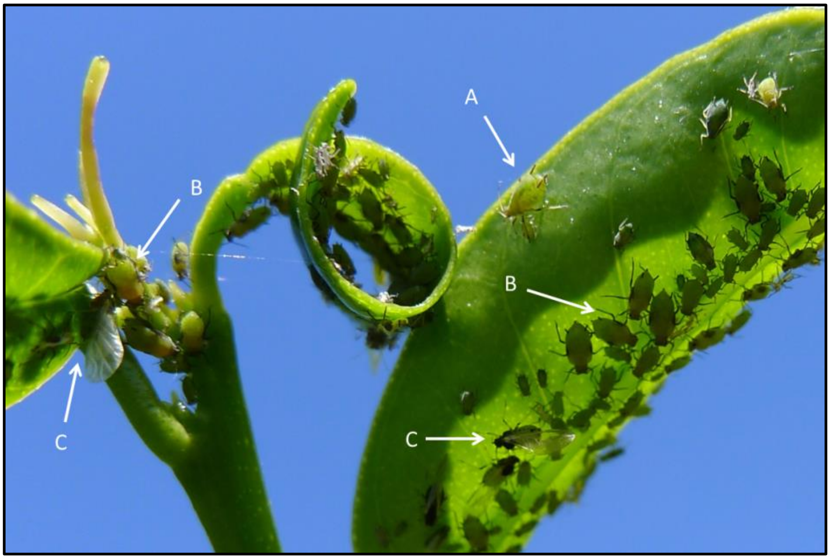

Figure 3. Visual differences in a colony between *A. spiraecola* Patch (Hemiptera: Aphididae) stages. Apterous (A), winged nymphs (B), winged adults(C).

identification key for the aphid species attacking citrus in Spain based on visual inspection in the field. *Aphis spiraecola* differs from *A. gossypii* because the former is yellow to apple green and the cauda and the siphunculi are black, whereas *A. gossypii* is variable in color, but the cauda is paler than the siphunculi. Under the microscope, *A. spiraecola* and *A. gossypii* can be distinguished by the number of setae on the cauda. *A. spiraecola* has eight to thirteen, whereas *A. gossypii* has six.

1.3.3. Life cycle

Aphis spiraecola dwells on a wide range of secondary hosts that includes more than 20 plant families, particularly Caprifoliaceae, Asteraceae, Rosaceae, Rubiaceae and Rutaceae, in addition to plants of shrubby habit. This species is anholocyclic in Spain and throughout most of the world but is heteroecious holocyclic in East Asia, North America and Brazil where it finds its primary host *Spiraea* spp. for development of the sexual phase (Blackman and Eastop, 1984; 1994). In Japan, a form with a sexual phase occurs on *Citrus unshiu* (Komazaki *et al.*, 1979), but no records of sexual generations on citrus occur outside Japan.

Komazaki (1982) studied the biological parameters of *A. spiraecola* at constant temperatures when developed on *C. unshiu* and found that the prereproductive period and longevity decreased as temperature increased to 29.5°C and 30.1°C, respectively. The maximum fecundity occurred at 19.8°C with 64.43 nymphs per female, and the minimum occurred at 30.1°C with 1.25 nymphs per female. The survival rates at 14.7°C and 30.1°C were 0.95 and 0.37, respectively. The threshold temperature for *A. spiraecola* was 7.9°C, and the optimum development rate (d^{-1}) was at approximately 27°C.

1.3.4. Seasonal abundance

Aphis spiraecola, as with the other aphid species attacking citrus in Spain, has two to three primary infestation peaks synchronized with the citrus flushing periods of spring and autumn (Shindo, 1972; Hermoso de Mendoza *et al.*, 1986; Yokomi and Oldfield, 1991). The spring flushing in clementines in this region begins in late winter and early spring (end of February) and is accompanied by a rapid increase of winged aphids. After the arrival of winged aphids, populations increase exponentially. *Aphis spiraecola* numbers decrease rapidly as the new foliage matures in late spring and with the action of natural enemies (Hermoso de Mendoza *et al.*, 1986; Yokomi and Oldfield, 1991). Occasionally, secondary peaks in trees that undergo further flushing in summer and fall are found, typically without reaching economic thresholds (Shindo, 1972; Hermoso de Mendoza *et al.*, 1986).

1.3.5. Ecology

Aphis spiraecola has the "r" strategy with extremely high growth and developmental rates and short generation times typical of many aphids (Van Emden and Harrington, 2007). Such short developmental times are possible because newborn aphids contain the embryos of their first granddaughters. This "Russian dolls" strategy results in an individual aphid that has completed two-thirds of its development before it is born (Dixon and Kundu, 1998). This strategy, combined with winged aphids' ability to fly long distances and spread rapidly throughout the orchards, classifies *A. spiraecola* a key citrus pest. *Aphis spiraecola* also increases populations on other hosts, which facilitates spread early in the season (Holman, 2009).

For the appearance of winged aphids in the colonies, the phenological stage and quality of the host plant during aphid development are the primary factors (Evans, 1938; Mittler and Dadd, 1966; Muller *et al.*, 2001; Mehrparvar *et al.*, 2013). However, severe crowding (Wadley, 1923; Johnson, 1965) or alarm pheromone signals (Dixon and Agarwala, 1999; Weisser *et al.*, 1999; Kunert *et al.*, 2005; Hatano *et al.*, 2010; Balog *et al.*, 2013) may also induce the production of winged aphids.

Aphids, as many honeydew producers, benefit from ants that collect honeydew to supply carbohydrate requirements (Rico-Gray and Oliveira, 2007). The ant attendance symbiosis provides protection from natural enemies, results in higher aphid growth rates, improves hygiene conditions and increases the transport and dispersal abilities of the pest, thus increasing the rate of infestations (Buckley, 1987; Stadler and Dixon, 2005; Calabuig *et al.*, 2014). This myrmecophilous association was described specifically for *A. spiraecola* on citrus (Shindo, 1972; Pekas *et al.*, 2011; Tena *et al.*, 2013)(Figure 4). However, when compared to other honeydew producing species, *A. spiraecola* is poorly attended (Pekas *et al.*, 2011; Tena *et al.*, 2013) largely because ants are scarce in spring and prefer to attend other hemipterans such as *Planococcus citri* Risso or *Coccus hesperidium* L. (Hemiptera: Coccidae) (Pekas *et al.*, 2011; Tena *et al.*, 2013).

Figure 4. *Aphis spiraecola* colony tended by *Lasius niger* L. (Hymenoptera: Formicidae).

Aphis spiraecola shares shoots with other citrus aphid species. Michaud (1999) found that one-third or more of *T. citricida* colonies were frequently joined by *A. spiraecola* in Florida, despite a large proportion of shoots that remained uninfested. Citrus aphid species share the same guild of predators, and advantages might exist for interspecific aggregations that offset the increased costs of competition for food (Michaud, 1999).

1.3.6. Damages and economic thresholds

As explained above, *A. spiraecola* colonizes and feeds on growing citrus shoots (Hermoso de Mendoza *et al.*, 2001; 2006). These feeding habits cause direct and indirect damages. Direct damages are caused during feeding as aphids suck sap from these tender organs and drain nutrients, reducing the vigor of the trees. Additionally, developing leaves curl as *A. spiraecola* feeds (Figure 5) with the apex of the leaves curled toward the petiole and the front toward the underside, which affects the tree growth in consecutive seasons. Other aphids infesting citrus such as *A. gossypii*, *T. citricida and* T. *aurantii* do not cause this damaging deformation (Hermoso de Mendoza *et al.*, 2001; 2006; Van Emden and Harrington, 2007).

Figure 5. Clementine flush damaged by *A. spiraecola* colony.

In addition to these direct damages, *A. spiraecola* excretes large amounts of honeydew through the cornicles (or siphunculi), which drops on leaves and fruit. This rich sugar excretion (honeydew) is colonized by sooty molds, which reduce fruit value and decrease photosynthetic activity (Wood *et al.*, 1988). Another type of indirect damage is from the transmission of viral diseases such as the causal agent of the destructive disease CTV (Cambra *et al.*, 2000; Marroquin *et al.*, 2004; Vidal *et al.*, 2012). The efficacy of *A. spiraecola* in transmitting CTV in citrus is limited when compared to other aphid species such as *T. citricida* or *A. gossypii*. Moreover, Yokomi and Garnsey (1987) found that the latter species was more efficient by a factor of three in transmission of CTV than *A. spiraecola*. Although not the most efficient vector of CTV, *A. spiraecola* is factored into studies on the spread of this disease (Norman and Grant, 1956; Powell *et al.*, 2006).

Figure 6. Ring use to track the percentage of infested shoots.

The direct and indirect damage produced by *A. spiraecola* can lead to significant economic losses, particularly in young nonbearing trees. To evaluate aphid populations in the field and to determine the pest levels that cause economic losses, sample methods and intervention thresholds were proposed and developed in the last 30 years (Cavalloro and Prota, 1980; Ortu and Prota, 1980; Meliá, 1995). The first study recommended monitoring aphid populations with weekly counts during the period of exponential growth and with the number of aphid infested and uninfested shoots within a 0.25 m^2 ring placed on the canopy surface (see 1.2.4.) The intervention threshold was established at between 5% and 15% of infested shoots within a ring (Cavalloro and Prota, 1980; Ortu and Prota, 1980) (Figure 6). Later, Hermoso de Mendoza *et al.* (2001; 2006), based on this methodology, suggested an economic threshold of 25% of infested shoots for the application of pesticides against the two primary citrus aphid species for clementines, *A. spiraecola* and *A. gossypii.*

1.4. Integrated Management of *Aphis spiraecola* in clementine citrus

The integrated management of *A. spiraecola* in clementines is currently based on chemical control. In recent years, emphasis was placed on implementing more economical and environmentally safe measures to control citrus pests in Spain (Jacas and Urbaneja, 2008; Jacas *et al.*, 2010). As a consequence, two lines of research on conservation biological control are currently being pursued. The first assesses the efficacy of insecticides and the side effects on natural enemies. The second line of research is based on the use of interline cover crops, which is a focus of this thesis.

1.4.1. Chemical control

Pesticide sprays against *A. spiraecola* in IPM programs are recommended only for the spring flushing (Urbaneja *et al.*, 2014). Generally, growers spray once or twice per season against this aphid in clementines within this period. Because of the exponential increase in populations of *A. spiraecola,* the recommendation is to spray within 24 hours after the economic threshold is exceeded (Hermoso de Mendoza *et al.*, 2006). The IPM guidelines recommended spraying only the outside of tree canopies, where aphids infest the shoots, to preserve natural enemies inside the canopy. Because *A. spiraecola* curls the leaves and the colonies are protected within the curled leaf, growers should reach the undersides of leaves when spraying or use systemic insecticides.

The selection of an appropriate insecticide is an important requirement in IPM to maximize the efficacy and to minimize the side effects on nontarget arthropods. The active ingredients recommended for Spanish citrus are as follow: the neonicotinoids acetamiprid and thiamethoxam, the acetylcholinesterase inhibitors chlorpyrifos, pirimicarb, methil-chlorpyrifos and dimethoate (only for young nonbearing trees), pymetrozine as a selective feed blocker, etofenprox as a sodium channel modulator and spirotetramat as a lipid synthesis inhibitor (Anonymous, 2014a; Urbaneja *et al.*, 2014).

These active ingredients should be rotated to avoid the development of resistance, which was previously documented in citrus infesting aphids (Takada and Murakami, 1968; Meliá and Blasco, 1990; SeungSong *et al.*, 1995; Nauen and Elbert, 2003; Li and Han, 2004; Toda *et al.*, 2004; Carletto *et al.*, 2010).

1.4.2. Cultural control

Two primary control strategies are used against *A. spiraecola* on citrus in Spain. The most common is the use of yellow (water or tangle) traps to detect the arrival of winged aphids (Meliá, 1989). Another method is to prune the excess shoots from the canopy and eliminate the basal shoots (suckers), thus decreasing the number of appropriate niches for *A. spiraecola* (Urbaneja *et al.*, 2014).

1.4.3. Biological control

Aphids are attacked with a diverse complex of aphidophagous predators and parasitoids. Citrus orchards harbor a rich naturally occurring complex of these natural enemies, which significantly reduce aphid populations in specific situations. Historically, research efforts were biased toward "rear-and-release" classical programs and away from other ecologically sound approaches to pest management such as conservation biological control. Michaud (2002) reflected that classical programs were typically employed as a reflexive response to invasive pests, often without prerelease surveys to document indigenous natural enemies. The net result is a prevailing tendency to underestimate the potential ecological resilience of established insect communities to invasive pests (Michaud, 2002) which could develop into spontaneous biological control of pest populations. Therefore, the following sections first document the most abundant species and the biology of predators and parasitoids of aphids in citrus. Then, the role of predators and parasitoids in the success and failure of the different biological control programs conducted against *A. spiraecola* are discussed.

1.4.3.1. Parasitoids

The subfamily Aphidiinae (Hymenoptera: Braconidae), with at least 50 described genera and over 600 species, is the largest group of parasitoid species of aphids (Mackauer and Starý, 1967). Additionally, all of the species of the genus *Aphelinus* and several species of *Encarsia* (Hymenoptera: Aphelinidae) also parasitize aphids. For *A. spiraecola,* the role of parasitoids for biological control is poorly known worldwide.

In the Mediterranean basin, the parasitoid complex of citrus aphids is widely studied and includes indigenous parasitoids and several exotic species that were introduced during classical biological control programs. The citrus aphids, *A. gossypii* and *T. aurantii*, have a complex of primary parasitoids that has been described throughout the Mediterranean basin in Turkey, Greece, Italy and Spain (Tremblay *et al.*, 1983; Michelena *et al.*, 1994; Michelena and Sanchís, 1997; Kavallieratos *et al.*, 2002; Kavallieratos *et al.*, 2004a; Kavallieratos *et al.*, 2004b; Michelena *et al.*, 2004; Bañol *et al.*, 2012). However, a single primary parasitoid species was found on *A. spiraecola* in the majority of the surveys (Kavallieratos *et al.*, 2002; Kavallieratos *et al.*, 2004a; Kavallieratos *et al.*, 2004b).

Figure 7. Adult of *Binodoxys angelicae*. (Hymenoptera: Braconidae).

Binodoxys angelicae (Haliday) (Hymenoptera: Braconidae) (Figure 7) is the only primary parasitoid species to parasitize and complete its development on *A. spiraecola* in Spanish citrus (Michelena and Sanchís, 1997; Michelena *et al.*, 2004). The sporadic appearance of *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) on *A. spiraecola* was reported (Michelena *et al.*, 1994; Bañol *et al.*, 2012).).

In addition to these surveys, several efforts were conducted to introduce exotic parasitoids against citrus aphids. *Lysiphlebus testaceipes* was imported from Cuba (via Czechoslovakia) to southern France in 1973 or 1974 to control *A. citricola* (*spiraecola*) and *T. aurantii* (Starý *et al.*, 1988; Jacas *et al.*, 2006). This parasitoid established in the entire west of Mediterranean

Europe in 1986 and became the predominant parasitoid of numerous indigenous pest aphids in all types of agroecosystems. In citrus, *L. testaceipes* was effective against *T. aurantii*, but unfortunately, it did not parasitize *A. spiraecola* effectively (Starý *et al.*, 1988; Jacas *et al.*, 2006). Similarly, *Aphidius colemani* (Haliday) (Hymenoptera: Braconidae) was introduced from India and Pakistan to control *A. gossypii* in 1992 (Jacas *et al.*, 2006), when this aphid became a key pest in citrus orchards (see 1.1.2). Although *A. colemani* is a polyphagous parasitoid, subsequent surveys of *A. spiraecola* parasitoids have not recovered *A. colemani*.

Binodoxys angelicae is a solitary, obligate endoparasitoid of several aphid species (Michelena and Oltra, 1987; Michelena *et al.*, 1994; Suay *et al.*, 1998; Kavallieratos *et al.*, 2001; Kavallieratos *et al.*, 2002; Michelena *et al.*, 2004). Though the biology was not studied specifically, all of the Aphidinae species are arrhenotokous (unfertilized eggs produce males, and fertilized eggs produce females) and usually deposit a single egg per aphid (Cook 1993). After eclosion of the egg, the larva first feeds on the hemolymph of the aphid (Couchman and King, 1977; Mackauer, 1986) but later feeds destructively on other tissues and kills the host (Polaszek, 1986). The parasitoid larva induces a mummification of the aphid during the feeding, and the mature larva spins a cocoon either inside or below the mummy (Figure 8). The adults feed on aphid honeydew and extrafloral nectaries. The origin and geographical distribution of *B. angelicae* remains unclear.

Figure 8. *Binodoxys angelicae* adult emerging from *A. spiraecola* mummy.

Figure 9. Two hyperparasitoid species from the subfamily Alloxystinae (Hymenoptera: Figitidae). *Alloxysta* sp. (A) and *Phaenoglyphis villosa* (B).

In Spain, *B. angelicae* was in less than 30% of the citrus orchards sampled by Michelena *et al.* (1994). Several reasons might explain this poor distribution in Spanish citrus. As occurs with *L. testaceipes* (Michelena *et al.*, 2004), *A. spiraecola* might not be an optimum host for *B. angelicae*, and the rate of immature deaths might be high during development. However, the abundant aphid hyperparasitoids in citrus agroecosystems may offer an alternative explanation.

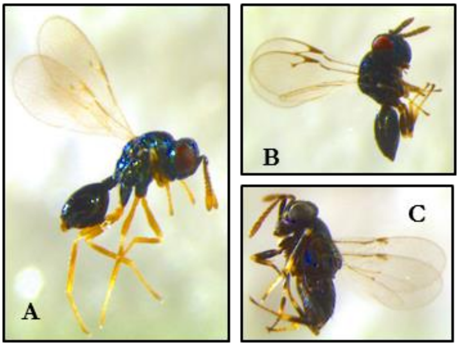

Figure 10. Hyperparasitoid species from the family Pteromalidae; *Asaphes* sp. (A), *Pachyneuron sp.* (B) and family Encyrtidae *Syrphophagus aphidivorus* (C) (Hymenoptera: Chalcidoidea).

Aphid hyperparasitoids in citrus agroecosystems are documented worldwide. Most of these hyperparasitoids are in the genera *Alloxysta* (Figure 9A), *Asaphes* (Figure 10A), *Pachyneuron* (Figure 10B), *Phaenoglyphis* (Figure 9B) and *Syrphophagus* (Figure 10C) (Yokomi and Tang, 1996; Michelena and Sanchís, 1997; Michaud, 1999; Kavallieratos *et* *al.*, 2002; Persad *et al.*, 2007). In the Mediterranean basin, Kavallieratos *et al.* (2002) found a diverse complex of hyperparasitoid species (*Alloxysta* spp., *Asaphes* spp., *Pachyneuron* spp., *Phaoneglyphis* spp. and *Syrphophagus aphidivorus* (Figure 10C)) that hyperparasitized 100% of citrus aphids at the end of the season. In Spain, Michelena and Sanchís (1997) detected high hyperparasitization rates (~30%) by these same genera (*Alloxysta* sp., *Asaphes vulgaris* Walker and *Pachyneuron aphidis* (Bouché) (Hymenoptera: Pteromalidae) and *Syrphophagus aphidivorus* (Mayr) (*Aphidencyrtus aphidivorus*) (Mayr)). These two studies did not specify the aphid-parasitoid-hyperparasitoid relations; therefore, it was not possible to establish the impact of the hyperparasitoids on *A. spiraecola*. The relation of *A. spiraecola* with this complex of hyperparasitoids was confirmed by Suay *et al.*, (1998) when they described, with classical methods, the trophic link of *A. spiraecola* as host of the primary parasitoid *B. angelicae* (*Trioxys angelicae* Haliday) and the hyperparasitoids *Alloxysta* sp., *Asaphes vulgaris* and *Pachyneuron aphidis*. Later, Bañol *et al.*, (2012) described sporadic cases of *A. spiraecola* parasitized by *L. testaceipes* from which they recovered a high number of hyperparasitoids without describing the trophic link.

1.4.3.2. Predators

Aphidophagous predators are generally euryphagous, preying on a variety of aphid species. In some families of predatory arthropods, both larvae and adults prey on aphids, whereas in other families, only the larvae are predatory. Citrus aphids have a rich complex of predators, in part because the permanent and perennial nature of the citrus crop provides an environment in which natural enemy guilds can develop (Cole, 1925; Michaud, 1999; Urbaneja *et al.*, 2000; Kavallieratos *et al.*, 2004a; Trejo-Loyo *et al.*, 2004; Yoldas *et al.*, 2011; Hermoso de Mendoza *et al.*, 2012; Romeu-Dalmau *et al.*, 2012a). This rich complex contains coleopterans, dermapterans, dipterans and neuropterans, which may control aphid populations. The relative abundance and seasonal trends of the primary predators were studied in the field (Michelena and Sanchís, 1997; Alvis *et al.*, 2002; Bru and Garcia-Marí, 2007; Hermoso de Mendoza *et al.*, 2012), as well as some aspects of the biology in the laboratory (Michaud, 2000; Belliure and Michaud, 2001; Michaud, 2001). However, the efficacy of these predators as biological control agents of *A. spiraecola* in citrus either individually or as a complex is poorly understood. The following sections describe the biology, relative abundance and seasonal trends of the primary families of aphidophagous predators. Obviously, most of the studies discussed below refer to aphid predators in general (not only those of *A. spiraecola*).

Coccinellidae

Most aphidophagous coccinellids (Coleoptera: Coccinellidae) are in the subfamilies Coccinellinae and Scymninae. Both larvae and adults feed on aphids and occur in identical habitats (Hagen, 1962; Van Emden and Harrington, 2007). Eggs are usually laid in clusters, but some species of the subfamily Scymninae may deposit eggs singly (Hagen, 1962; Van Emden and Harrington, 2007). Many species share characteristics of successful predators, such as high searching capacity, high voracity, appropriate food range and the capability

Figure 11. *Propylea quatordecimpunctata* L. (Coleoptera: Coccinellidae).

to develop on alternative foods if aphids are scarce (Hagen, 1962; Van Emden and Harrington, 2007). Moreover, most aphidophagous ladybird species are able to develop on a variety of aphids. The relative abundance and importance of the coccinellid species in citrus depends more on the region and season than on the citrus aphid species dominating the aphid complex (Smith *et al.*, 1997).

In European citrus, Franco *et al.* (1992), Magro *et al.* (1999), Longo and Benfatto (1987) and Kavallieratos *et al.* (2004a) found *Scymnus* to be the most abundant genus in Portugal, Italy and Greece. These studies were based in indirect sampling techniques (beating and trapping) and thus did not record predation on aphid colonies. By contrast, Yoldas *et al.* (2011) recorded *C. semptencpunctata* (Figure 12) as the most abundant coccinellid that preyed directly on aphid colonies in Turkey. Using traps, Panis *et al.* (1977), Michelena and Sanchís (1997) and Alvis *et al.* (2002) found that *Scymnus* sp. and *Propylea quatuordecimpunctata* L. were the most abundant coccinellids in Spain. Alvis (2004) reported positive correlations between citrus aphid populations and these coccinellid species, and Hermoso de Mendoza *et al.* (2012) recorded specific and direct predation by *Coccinella*

Figure 12. *Coccinella semptempunctata* L. (Coleoptera: Coccinellidae) preying on *A. spiraecola* colonies.

septempunctata, *P. quatuordecimpunctata*, *Scymnus subvillosus* and *Scymnus interruptus* on *A. spiraecola* colonies.

Generally, in temperate regions, coccinellid populations peak in spring or early summer and then decline (Hagen, 1962; Michelena and Sanchís, 1997; Alvis, 2004; Bru and Garcia-Marí, 2007; Hermoso de Mendoza *et al.*, 2012). Notably, Alvis (2004) recorded *Scymnus subvillosus* in March (early spring) in citrus orchards in eastern Spain. This early appearance in citrus orchards could be the key to control aphid populations before aphid population's peak and therefore this species might be used as biological control agent against *A. spiraecola* in citrus.

Syrphidae

Hoverflies (Diptera: Syrphidae) are in one of the largest dipteran families. The larvae of about one-third of the species in the subfamily Syrphinae are predators of Sternorrhyncha, typically aphids (Rotheray and Gilbert, 1989). The adults (Figure 13) are daylight active and feed on nectar and honeydew and need pollen proteins for gonads to mature. Females then oviposit up to 1,000 eggs. Normally eggs are long, reticulate and white and are deposited either singly or in small groups near aphid colonies. The larvae in this stage are ravenous and can kill up to 600 aphids. Field observations suggest that many hoverfly species are specialized to attack a range of

Figure 13. *Episyrphus balteatus* de Geer (Diptera: Syrphidae).

prey species in the field, though the larvae develop on a broader range of aphid species in the laboratory (Belliure and Michaud, 2001).

Hoverflies are abundant predators of aphids in citrus orchards of the Mediterranean basin (Michelena and Sanchís, 1997; Yoldas *et al.*, 2011; Hermoso de Mendoza *et al.*, 2012) and play an important role in reducing aphid populations worldwide (Cole, 1925; Michaud 1999, 2000; Trejo-Loyo *et al.*, 2004). The most abundant hoverfly species in citrus in Spain are *Paragus haemorrhous* Meigen *Epistrophe eligans* (Harris), *Episyrphus balteatus* (de Geer), *Syrphus vitripennij* (Meigen) (Diptera: Syrphidae) (Michelena and Sanchís, 1997) and *Eupeodes corollae* Fabricius (Diptera: Syrphidae) (Hermoso de Mendoza *et al.* 2012).

The most striking effect of syrphids on aphid populations occurs when a large number of larvae hatch before aphid populations attain a rapid growth rate (Tenhumberg and Poehling, 1995). Syrphids tend to appear in citrus early in the season (Michelena and Sanchís, 1997; Hermoso de Mendoza *et al.* 2012), and this occurrence can be supported by conservation biological control strategies because two reasons. First, because the foraging activity of syrphids adults in crops can be increased by a continuous supply of flowers (Ruppert and Molthan, 1991; Colley and Luna, 2000) and second because larva of syrphids can feed on a wide range of preys (Gomez-Polo *et al.* 2014) before the increase of aphid populations. Nonetheless, Michaud (1999) compared the relative efficiency of aphid colony elimination by predation between coccinellids and syrphids in citrus, and the beetles eliminated a significantly higher proportion of the colonies on which they were feeding.

Cecidomyiidae

In the dipteran family Cecidomyiidae, at least five predatory species of the genera *Aphidoletes* and *Monobremia* have larvae that prey exclusively on aphids (Harris, 1973), whereas the adults are nocturnal and feed on nectar and honeydew (Van Emden and Harrington, 2007; Harris, 1973). The best-known species is *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae) (Figure 14), which is commonly used in biological control programs (van Schelt and Mulder, 2000). *Aphidoletes aphidimyza* is also recognized for the impressive ability of the adults to detect oviposition sites (El Titi, 1974) using honeydew excreted by aphids as a cue (Choi *et al.*, 2004) and to choose large colonies situated on the meristems of the plants to oviposit (Jandricic *et al.*, 2013). A positive correlation was found between aphid density and oviposition of females (El Titi, 1974; Lucas and Brodeur, 1999; Choi *et al.*, 2004). The larvae of *A. aphidimyza* are orange and feed on a variety of aphids by sucking the hemolymph (Harris, 1973), followed by pupation in the soil. *Aphidoletes aphidimyza* is a "furtive predator" because the larvae prey on aphids as they simultaneously reside and remain undetected within the aphid colonies without generating alarm (Lucas and Brodeur, 2001). Additionally, larvae kill many more aphids than needed for development with "overkilling behavior", a feature that increases success in biological control programs.

Figure 14. *Aphidoletes aphidimyza* larvae preying on *A. spiraecola* colonies.

The gall midge *A. aphidimyza* is distributed worldwide (Havelka and Zemek, 1988; Yukawa *et al.*, 1998; Havelka and Zemek, 1999; Miñarro *et* *al.*, 2005; Frechette *et al.*, 2008; Frank, 2010; Jandricic *et al.*, 2013) and is found throughout Mediterranean citrus (Yoldas *et al.*, 2011; Hermoso de Mendoza *et al.*, 2012; Vacante and Gerson, 2012; Urbaneja *et al.*, 2014). Hermoso de Mendoza *et al.* (2012) found that *A. aphidimyza* was the most widely distributed predator in Spanish citrus based on direct observations, with two potential generations per year and the first appearance of adults in mid spring (Soler *et al.* 2002; Hermoso de Mendoza *et al.* 2012).

Neuroptera

Lacewings (Neuroptera: Chrysopidae and Hemerobiidae) and dusty-wings (Neuroptera: Coniopterygidae) are polyphagous predators that feed primarily on soft-bodied insects such as aphids (New, 1975). They are present in citrus worldwide, (Cole, 1925; Muma, 1959; Michaud, 1999; Souza and Carvalho, 2002) including in the Mediterranean basin (Yoldas *et al.*, 2011; Michelena and Sanchís, 1997; Hermoso de Mendoza *et al.* 2012; Vacante and Gerson, 2012; Urbaneja *et al.* 2014) (Figure 15

Figure 15. Chrysopid eggs on citrus leaf.

and 16). Bru (2007) found that 81% of the predators captured with traps in citrus in eastern Spain were neuropteran (66% Coniopterygidae and 15% Chrysopidae).

The most common Coniopterygidae species in Spanish citrus are *Semidalis aleyrodiformis* Stephens and *Conwentzia psociformis* (Curtis) (Soler *et al.*, 2002; Alvis, 2004; Urbaneja *et al.*, 2014). The highest seasonal activities of *C. psociformis* were recorded in spring and fall (Soler *et al.* 2002; Alvis, 2004). Of the Chrysopidae, *Chrysopa pallens* (Rambur) and *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (Figure 15 and 16) are the most abundant species in Mediterranean citrus (Michelena and Sanchís, 1997; Alvis, 2004; Yoldas *et al.*, 2011; Hermoso de Mendoza *et al.* 2012; Vacante and Gerson, 2012; Urbaneja *et al.* 2014), and the larvae of *Chrysoperla carnea* were recorded preying specifically on *A. spiraecola* colonies (Michelena and Sanchís, 1997; Hermoso de Mendoza *et al.* 2012).

The maximum populations of chrysopids tended to occur at the end of the spring flushing (Michelena and Sanchís, 1997; Hermoso de Mendoza *et al.* 2012), when aphid populations were in decline (Soler *et al.* 2002). However, direct predation is difficult to record in the field because of crepuscular activity and hiding behavior (Brown and Schmitt, 2001; Michaud, 2001). Despite these difficulties in measuring activity, chrysopids have the potential to

Figure 16. *Chrysoperla carnea* (Neuroptera: Chrysopidae) adult resting on *A. spiraecola* colony.

reduce aphid numbers in citrus because the larval stage is ravenous and citrus aphids are suitable prey (Michaud, 2001; Michelena and Sanchís, 1997; Hermoso de Mendoza *et al.* 2012).

Forficulidae

The dermapteran *Forficula auricularia* L. (Dermaptera: Forficulidae) (Figure 17) was documented preying on aphids in Spanish citrus (Cañellas *et al.*, 2005), including *A. spiraecola* (Romeu-Dalmau *et al.*, 2012a; Romeu-Dalmau *et al.*, 2012b). Because this species is nocturnal and hides during the day, the occurrence and efficacy of earwigs is highly dependent on the availability of a sufficient number of shelters within or in close proximity to the crop. Piñol *et al.* (2009) described the importance of polyphagous sedentary predators, such as earwigs, on aphid populations of citrus. Low densities of sedentary predators can strongly affect the final aphid density because they prey on small populations at the beginning of the aphid spring season, when the per capita effect on the aphid population is high.

Figure 17. *Forficula auricularia* L. (Dermaptera: Forficulidae).

Other predator families

Aphids are attacked by a wide variety of other generalist predators in citrus orchards, such as spiders (Llorens, 1990; Vacante and Gerson, 2012). The order Araneae and the predation activity of spiders in citrus crops were studied worldwide, including in Argentina (Avalos *et al.*, 2013), Brazil (Ott *et al.*, 2007), Mexico (Rodriguez-Almaraz and Contreras-Fernández, 1993), Montenegro (de Morais *et al.*, 2007), the USA (Florida) (Amalin *et al.*, 2001) and Spain (Alvis, 2004; Monzó *et al.*, 2009; Barrientos *et al.*, 2010; Monzó *et al.*, 2011), where spiders are abundant throughout the year (Barrientos *et al.*, 2010). Spiders are active aphid predators in citrus orchards (Monzó *et al.*, 2009). Llorens (1990) recorded *Evarcha falcata* (Clerk) (Araneae: Salticidae) preying directly on *A. spiraecola*. One of the most abundant spider families in citrus is Theridiidae (Alvis, 2004; de Morais *et al.*, 2007; Ott *et al.*, 2007; Barrientos *et al.*, 2010), with species that may prey on *A. spiraecola* (Rodriguez-Almaraz and Contreras-Fernández, 1993; de Morais *et al.*, 2007).

Among the coleopterans, not only coccinellids prey on aphids. For example, *Ragonycha* sp. (Coleoptera: Cantharidae) is commonly cited as predator of aphids in the inflorescences of Asteraceae. Day *et al.* (2006) studied the role of the beetle in suppressing populations of the green spruce aphid *Elatobium abietinum* (Hemiptera: Aphididae), but little is known about its predatory behavior in citrus orchards.

1.5. Rationale and Objectives

1.5.1. Poor performance of parasitism

The studies reviewed above (see 1.4.3.1.) on classical biological control and the poor parasitoid complex of *A. spiraecola* on citrus as well demonstrate the difficulty in finding a key parasitoid against this aphid. The effect of the unique parasitoid, *B. angelicae*, that is able to parasitize and complete its development on *A. spiraecola* appears to be low, which may be a reflection of low parasitoid abundance. Identifying those factors that affect the abundance of *B. angelicae* and other aphid parasitoids on citrus is key to assessing and potentially improving their efficacy as biological control agents of *A. spiraecola*. One of the factors may be the high abundance of hyperparasitoids in citrus agroecosystems. Despite the large number of hyperparasitoid species described in previous studies (see 1.4.3.1.), no study describes the parasitism rates of *B. angelicae* on *A. spiraecola* or the effects of the hyperparasitoids on this food web. Interactions in aphid food webs may contain up to five trophic levels, all of which may influence biocontrol success, as was previously described in other crops (Traugott *et al.*, 2008; Harvey *et al.*, 2009; Nagasaka *et al.*, 2010; Schooler *et al.*, 2011; Gagic *et al.*, 2012; Gariepy and Messing, 2012; Nofemela, 2013). Therefore, **the first objective of my thesis was to disentangle the interactions in the *A. spiraecola*-parasitoid food web on citrus**.

1.5.2. Asynchrony between aphids and predators

Despite the abundant and diverse complex of predators described above (see 1.4.3.2.), the biological control of *A. spiraecola* by predators in clementines is generally insufficient. The most plausible explanation could be the asynchrony between the aphid and its predators (Hermoso de Mendoza *et al.*, 2012; Welch and Harwood, 2014). This asynchrony occurs because aphids represent a temporally discontinuous resource that is characterized by rapid increases (Kindlmann and Dixon, 1999; Kindlmann and Dixon, 2001; Van Emden and Harrington, 2007). Whereas predators have a relatively long generation time compared with aphids, which hinders top-down regulation (Kindlmann and Dixon, 1999). Therefore, the early arrival of predators is a critical prerequisite for control of aphid populations (Chiverton, 1986; Landis and Van der Werf, 1997; Harwood *et al.*, 2004; Brown *et al.*, 2010). Determining the optimal degree of temporal synchrony between predators and aphids is not straightforward. Welch and Harwood (2014) recently posed several key questions regarding asynchrony in biological control: Do natural enemies have greater effects when in complete synchrony or when slightly out of synchrony with pests? What dynamics of the natural enemy ecology must be in synchrony with the population cycle of the pest? With which dynamics of pest ecology must the natural enemies be in synchrony? These are particularly pertinent questions to address in the biological control of *A. spiraecola* on clementines because it is unknown whether an early establishment of predators would maintain the population levels of this aphid below the economic threshold. Therefore, **the second objective of my thesis was to determine whether the early presence of predators in clementine orchards improved biological control of *A. spiraecola***. If the early presence improves biological control, then biological control strategies should be directed at enhancing favorable conditions for the early establishment of predators.

1.5.3. Cover management as conservation biological control of aphids in citrus

The aim of habitat management in conservation biological control is to create a suitable ecological infrastructure to favor natural enemies and enhance biological control in agricultural systems (Landis *et al.*, 2000). In monoculture agroecosystems, natural enemies suffer from a lack of food for adults, alternative prey or hosts and shelter from adverse conditions (Landis *et al.*, 2000; Heimpel and Jervis, 2005). In the absence of these vital resources, colonization of crops by predators and parasitoids is often much lower than colonization by herbivores (Altieri and Whitcomb, 1979; Thies and Tscharntke, 1999). An extensively researched form of habitat management to favor natural enemies in tree crops is the use of ground covers (Altieri and Whitcomb, 1979; Landis *et al.*, 2000; Danne *et al.*, 2010; Silva *et al.*, 2010; Paredes *et al.*, 2013).

Spanish citrus orchards are commonly grown on bare soil maintained with herbicides or mechanical means. This situation is not ideal, and the use of cover crops as ecological infrastructure is encouraged by IPM guidelines. In the last five years, the use of a ground cover based on Poaceae, such as the grass *Festuca arundinacea* L. (Poales: Poaceae), has been promoted in Spanish citrus for control of the two-spotted spider mite *Tetranychus urticae* Koch (Prostigmata: Tetranychidae) (Aguilar-Fenollosa *et al.*, 2011a and 2011b) and to improve the biological control of the Mediterranean fruit fly *Ceratitis capitata Wiedemann* (Diptera: Tephritidae) (Monzó *et al.*, 2011a and 2011b). **Thus, the third objective of my thesis was to analyze the effects of a sown ground cover based on Poaceae plants on the biological control of *A. spiraecola* on citrus**. I hypothesize that the sown ground cover will host alternative preys where aphid predators will be able to feed and establish before *A. spiraecola* infest the citrus canopies.

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CHAPTER II

Untangling the aphid-parasitoid food web in citrus: can hyperparasitoids disrupt biological control?

2. Untangling the aphid-parasitoid food web in citrus: can hyperparasitoids disrupt biological control?

Gómez-Marco, F., Urbaneja, A., Jaques, J. A., Rugman-Jones, P. F., Stouthamer, R., Tena, A. (2015) Untangling the aphid-parasitoid food web in citrus: can hyperparasitoids disrupt biological control? ***Biological Control*** 81: 111-121

Abstract: Molecular techniques are irreplaceable to untangle the trophic links in communities where immature entomophagous species (either in the third or fourth level) develop inside the phytophagous. This is the case of aphid-parasitoid communities. Here, we develop a DNA-based approach to untangle the structure of the aphid-parasitoid food web in citrus, where *Aphis spiraecola* Patch. (Hemiptera: Aphididae) is a key pest and *Binodoxys angelicae* Haliday (Hymenoptera: Braconidae), its dominant primary parasitoid, is attacked by a complex of hyperparasitoids. Aphid populations and parasitism were followed at weekly intervals in 2012 and 2013. Parasitism rates were low (~0.04 in the four sampled orchards). Simultaneously, colonies harboring aphid mummies were collected. Approximately half of the mummies were reared to adulthood and at least six hymenopteran hyperparasitoid species were identified by classical means: *Syrphophagus aphidivorus* (Mayr) (Encyrtidae), *Alloxysta sp.* (Forster) (Figitidae), *Asaphes* sp. (Walker) (Pteromalidae), *Pachyneuron aphidis* (Bouché) (Pteromalidae), *Dendrocerus* sp. (Ratzeburg)

(Megaspilidae) and *Phaenoglyphis villosa* (Hartig) (Figitidae). The other half was subjected to a Taqman-based multiplex PCR to investigate trophic relationships in this food web. We confirmed that all six species hyperparasitized *B. angelicae*. The most abundant hyperparasitoids were *S. aphidivorus* and *Alloxysta* sp. Both were abundant from the beginning of the season, and hyperparasitism rates remained high (~0.4) throughout the season in the two study years. Although these species could share the same mummy, *S. aphidivorus* and *Alloxysta* sp. were the most abundant species and dominated this food web. Finally, hyperparasitoids also increased the secondary sex ratio of *B. angelicae*. Thus, hyperparasitism probably explains the low impact of *B. angelicae* on *A. spiraecola* populations.

2.1. Introduction

The spirea citrus aphid *Aphis spiraecola* Patch (Hemiptera: Aphididae) and the cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) are major pests in Mediterranean citrus crops, and clementine, *Citrus clementina* Hort. ex Tan. (Geraniales: Rutaceae), is particularly susceptible to attack (Hermoso de Mendoza *et al.*, 2006; Jacas *et al.*, 2010; Tena and Garcia-Marí, 2011; Hermoso de Mendoza *et al.*, 2012; Vacante and Gerson, 2012). Both aphids feed and form colonies on young and tender clementine shoots during the spring, summer and fall leaf-flushing periods. However, they can cause serious damage when they attack the spring leaf-flush (Hermoso de Mendoza *et al.*, 2001; Hermoso de Mendoza *et al.*, 2006), through sucking sap, vectoring *Citrus tristeza virus*, excreting large amounts of honeydew and, in the case of *A. spiraecola*, curling the developing leaves as they grow (Hermoso de Mendoza *et al.*, 2001; Hermoso de Mendoza *et al.*, 2006). This damage leads to significant economic losses, especially in young non-bearing trees.

Since the 1980s, *A. spiraecola* has displaced the previously dominant species, *A. gossypii*, as the key pest, and this is thought to be due to the low efficacy of the former's natural enemies (Meliá, 1982; Hermoso de Mendoza *et al.*, 1986; Meliá and Blasco, 1990; Hermoso de Mendoza *et al.*, 2001; Hermoso de Mendoza *et al.*, 2012). Like most citrus feeding aphids, *A. spiraecola* has a rich complex of natural enemies, in part because the permanent and perennial nature of the citrus crop provides an environment in which natural enemy guilds can develop (Cole, 1925; Urbaneja *et al.*, 2000; Michaud, 2001; Kavallieratos *et al.*, 2004; Yoldas *et al.*, 2011; Hermoso de Mendoza *et al.*, 2012; Romeu-Dalmau *et al.*, 2012). The arthropod predator complex is rich, containing neuropterans, coleopterans, dipterans and dermapterans. However, only one primary parasitoid, *Binodoxys angelicae* Haliday (Hymenoptera: Braconidae), has been confirmed in surveys of the natural enemy complex in Mediterranean citrus (Michelena *et al.*, 1994; Michelena *et al.*, 2004; Bañol *et al.*, 2012). Unfortunately, the impact of *B. angelicae* on *A. spiraecola* populations appears to be small and this may be a reflection of low parasitoid abundance. *B. angelicae* was present in less than 30% of the citrus orchards sampled by Michelena *et al.* (1994). Thus, identifying factors that affect the abundance of *B. angelicae* and other aphid parasitoids in citrus is key to assessing and potentially improving its efficacy as a biological control agent.

At least three factors may contribute to the apparent low abundance of *A. spiraecola* parasitoids on citrus. First, side effects of pesticides used against aphids, mites [e.g., *Tetranychus urticae* Koch (Acari: Tetranychidae)] and armored scale [e.g., *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae)] in clementines, may hinder parasitoid performance (Jacas and Garcia-Marí, 2002; Urbaneja *et al.*, 2008; Jacas and Urbaneja, 2010). Second, aphid-attending ants might attack parasitoids or otherwise disrupt their host finding behavior. It is well-known that ants collect honeydew excreted by aphids and in return ants protect them from natural enemies (Völkl, 1992). In the case of *A. spiraecola*, ants do not seem to hinder parasitism as the intensity of this mutualism is low and most of the colonies are untended (Pekas *et al.*, 2011; Tena *et al.*, 2013). Finally, and perhaps most importantly, hyperparasitism may be a major mortality factor for primary parasitoids of aphids (Rosenheim, 1998; Sullivan and Völkl, 1999). Four species of secondary parasitoids belonging to different Hymenopteran families have been identified attacking *B. angelicae* in different Spanish agroecosystems (Suay *et al.*, 1998; Bañol *et al.*, 2012). However, the relative abundance, seasonal trend and impact on the population dynamics of *B. angelicae* remain unknown.

Disentangling aphid-parasitoid food-webs and analysis of their dynamics is difficult because the biology of the hyperparasitoids (Traugott *et al.*, 2008; Gariepy *et al.*, 2013). According to their oviposition behavior and immature development, secondary parasitoids of aphids have historically been divided into two types: those that attack the primary parasitoid inside the still living aphid (generally referred to as hyperparasitoids), and those that attack the aphid after it has been mummified by the primary parasitoid (generally referred to as mummy parasitoids) (Müller and Godfray, 1998). The latter category can also develop as facultative tertiary parasitoid if they attack an aphid mummy containing a hyperparasitoid (Müller and Godfray, 1998). Furthermore, it is known that at least one parasitoid species, *Syrphophagus aphidivorus* (Mayr) (Hymenoptera: Encyrtidae), can have hyperparasitoid and mummy parasitoid development (Sullivan and Völkl, 1999; Buitenhuis *et al.*, 2004; Gariepy and Messing, 2012) but it is unknown whether it also develops as a tertiary parasitoid. Considering that all modes of attack result in the death of the primary parasitoid, hereinafter we simply refer to all secondary parasitoids as hyperparasitoids.

Interactions in parasitoid food webs associated with *A. spiraecola* can contain up to five trophic levels composed of potential new hosts, new enemies and new competitors, all of which may influence biocontrol success. Although these interactions are important, these dynamics are impractical to determine from field samples using host collection. Nowadays molecular ecology has been utilized as a tool of choice to overcome difficulties of trophic interaction studies (Harper *et al.*, 2005; Juen and Traugott, 2007; King *et al.* 2008; Poulsen and Sapountzis, 2012;

Luong *et al.*, 2013) and analysis of aphid-parasitoid food-webs has also recently benefited from advances in molecular ecology (Traugott *et al.*, 2008; Gariepy and Messing, 2012; Varennes *et al.*, 2014). DNA based techniques provide advantages over conventional rearing and dissection methods (Gariepy *et al.*, 2007; Greenstone *et al.*, 2011). Advantages include fewer lag times, avoidance of data loss due to emergence failures (parasitoid / hyperparasitoid mortality), and the identification of immature stages which are generally difficult to distinguish based on morphology (Walton *et al.*, 1990; Gariepy *et al.*, 2008). Therefore, molecular techniques are the key to obtain efficient tracking of the complete trophic pathways and unravel the food web interactions among aphids, their parasitoids and hyperparasitoids.

In this study, we develop a DNA-based approach to untangle the structure of the *Aphis spiraecola*-parasitoid food web in citrus. This knowledge will help determine the reasons for success or failure of past and future biological control programs against aphids in citrus. Initially, we determined field parasitism rates of *A. spiraecola* in clementine orchards during spring infestation. Subsequently, the complex of species responsible for this parasitism was identified using taxonomical and molecular methods, revealing i) the relative abundance of each species; ii) seasonal trends in the abundance of each species; iii) the relationships between the primary parasitoid and each hyperparasitoid species (trophic links), and between the hyperparasitoids; and iv) rates of hyperparasitism. With these data, we discuss the implication of hyperparasitism for the efficacy of biological control of *A. spiraecola* in clementines.

2.2. Material and methods

2.2.1. Experimental site and sampling dates

Four commercial clementine orchards (*C. clementina* Hort. ex Tan. cv. clementine grafted on Carrizo citrange) were sampled during the spring leaf-flushing period in Valencia region, eastern Spain. Orchard “A” was located in Moncada (39° 35' 18.34" N - 0° 23' 57.96" W, 0.79 ha), “B” and “C” in Almenara (orchard “B”: 39° 44' 45.04" N - 0° 14' 40.73" W, 1.35 ha; orchard “C”: 39° 45' 56.79"N-0° 14' 12.69" W, 0.81 ha), and “D” in Vall d’Uixó (39° 46' 35.31"N- 0° 16' 4.14" W, 0.74 ha). These orchards followed Integrated Pest Management guidelines and were drip irrigated. Orchards “A” and “C” had bare soil (following herbicide applications), and orchards “B” and “D” had a cover crop consisting of a mix of grass plants (*Festuca arundinacea*, *Poa* sp., *Bromus* sp., etc.) and sporadic minor weeds. This cover crop was mowed twice per year; early spring and summer. No pesticides were applied during the sampling period. Samples were collected weekly from the period of aphid colonization to the period of aphid population breakdown. The sampling dates started the 27th and 12th of April and ended the 25th and 13th of May of 2012 and 2013, respectively. Orchard “D” was sampled only in 2013.

2.2.2. Field sampling

2.2.2.1. Parasitism rates

In consecutive years (2012 and 2013), 20-40 colonies of *A. spiraecola* per orchard were marked at the beginning of the spring leaf-flushing period and tracked weekly over the duration of the flush (five weeks between April and May). One shoot per tree was marked with a 1 cm diameter plastic ring. Colonies were selected if they initially contained less than 20 aphids (mean number of aphids / colony ± SE = 11.54 ± 0.45) and were settled in tender shoots (less than 5 cm. long). In each colony, the total numbers of aphids and mummies were counted. Each week, parasitism rate was calculated as the number of mummies per colony divided by the total number of aphids (both live and mummified).

2.2.2.2. Parasitoid complex

To determine the parasitoid complex of *A. spiraecola,* ten *A. spiraecola*-infested shoots with at least two mummies per shoot were collected on the same dates from the same orchards listed above. One shoot per tree was collected and taken to the lab using plastic cages (100 cm^3). Once in the lab, colonies were observed under a binocular microscope within 8 h, and mummies were labeled and randomly divided into two groups. Mummies assigned to the group 1 were placed in individual 15 x 45 mm glass vials with a mousseline cover, and maintained inside a climatic chamber (25 ± 1 °C; 60-80% H.R.; L:D 18:6) until parasitoid emergence. Emerged parasitoids were identified and sexed (Starý, 1976; Fergusson, 1986; Michelena *et al.*, 2004).

Mummies assigned to group 2 were immediately placed in 95% ethanol and stored at -20 °C for molecular identification (see below). In addition, at least two adult specimens of each parasitoid species identified from the first group were similarly stored in 95% ethanol.

2.2.3. Molecular identification

2.2.3.1. DNA sequencing of adult specimens and design of multiplex qPCR primers

To enable the identification of immature parasitoids present in the mummies, we first produced a genetic signature for several adults of each of the species identified using morphological means (group 1).

Specifically, these species were, *B. angelicae*, *S. aphidivorus*, *Alloxysta* sp. Forster (Hymenoptera: Figitidae), *Phaenoglyphis villosa* Hartig (Hymenoptera: Figitidae), *Asaphes* sp. Walker (Hymenopter: Pteromalidae), *Pachyneuron aphidis* Bouché (Hymenoptera: Pteromalidae) and *Dendrocerus* sp. Ratzeburg (Hymenoptera: Megaspilidae) (see results). DNA was extracted from 2-4 individual adult specimens of each species using the nondestructive EDNA HiSpEx Tissue Kit (Saturn Biotech, Perth, Australia), following the manufacturers protocol for 1 mm^3 tissue. This kit involves simple mixing of three proprietary solutions, no grinding of the specimen, and incubation at 95 ˚C for 30 min. Following extraction, the intact carcass of each specimen was retrieved and kept at Instituto Valenciano de Investigaciones Agrarias (IVIA) collection.

Two regions of ribosomal RNA (rRNA) were selected for amplification and sequencing, using universal primers: 28S was amplified using 28sF3633 (5'- ACCGTGAGGGAAAGTTGAAA-3') paired with 28sR4076 (5'-AGACTCCTTGGTCCGTGTTT-3') (Rugman-Jones *et al.*, 2010), and ITS2 was amplified using ITS2-forward (5'-TGTGAACTGCAGGACACATG-3'; Campbell *et al.* (1993)) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al.* (1990)). PCR was performed in 25-µl reactions containing 2 µl of DNA template (concentration not determined), 1X ThermoPol PCR Buffer (New England BioLabs (NEB), Ipswich, MA), an additional 1.5 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP, 400 µM dUTP, 10 µg BSA (NEB), 1 U Taq polymerase (NEB), and 0.2 µM each PCR primer. Reactions were performed in a Mastercycler® ep gradient S thermocycler (Eppendorf North America Inc., New York, NY) programmed for initial denaturing at 95 ˚C for 3 min; followed by 38 cycles of 94 ˚C for 45 s, 55 ˚C for 30 s, 72 ˚C for 1 min 30 s; and a final extension of 3 min at 72 ˚C.

Amplification was confirmed by standard gel electrophoresis and PCR products were purified using Wizard® PCR Preps DNA Purification System (Promega Corporation, Madison, WI) following the manufacturer's instructions, and sequenced in both directions at the Institute for Integrative Genome Biology, University of California Riverside. Representative sequences were deposited in GenBank [accession numbers: KJ624626- KJ624632]. Sequences were aligned manually in Bio-Edit version 7.2.0 (Hall, 1999.) and areas of nucleotide variation between consensus sequences were determined by visual inspection. These areas were subsequently targeted to design specific primers and probes for each parasitoid and hyperparasitoid species.

The 28S and ITS2 sequences for each species were concatenated and "Multiplexed Sets" of PCR primers and TaqMan® probes were designed using Beacon Designer 8.10 (Premier Biosoft, Palo Alto, CA). The Beacon Designer software supports multiplexing for up to four sequences and checks for cross homologies with all probes and primers, seeking to minimize competition in multiplex reactions. A limiting factor in the design of our multiplex quantitative PCR (qPCR) assay was the number of channels available to detect different fluorescent signals (in our case, four). Thus, in order to identify all 7 target species (see Results) two separate multiplex assays were required. Different combinations of the 7 target species were tested to identify the best overall combination of species for each of the two assays. We adjusted the melting temperature (Tm) in the Beacon Designer search to 55 ± 5 °C, but all other parameters were left at default settings. The suggested PCR primers were obtained for four possible sets of multiplex TaqMan®probes (data for ultimately unsuccessful primer/probes not shown) and the specificity of each primer pair was evaluated against the panel of EDNA-extracted, morphologically identified specimens (see above), to ensure that the primers amplified all individuals of the species for which they were designed, without amplifying the other species investigated. DNA from several specimens of *A. spiraecola* and *A. gossypii* were also challenged with the primers to ensure that they did not amplify host aphid DNA. Substituting in the respective species-specific primers, constituents of each PCR reaction were as described above for the universal 28S and ITS2 primers, but with the inclusion of 1X EvaGreen (a fluorescent nucleic acid binding dye; Biotium, Inc., Hayward, CA) and the exclusion of additional $MgCl_2$. Reactions were performed in a Rotor Gene 3000 (Corbett Research [now QIAGEN]) programmed for initial denaturing at 95°C for 3 min; followed by 40 cycles of 95°C for 15 s, suggested annealing temperature for 30 s, and 72°C for 30 s. Amplification was verified by measuring increase in fluorescence throughout the reaction. This resulted in the identification of two multiplexed sets of primers for which TaqMan probes were obtained (Table 1).

Set "A" contained the primers and probes for *Alloxysta* sp., *B. angelicae*, *Asaphes* spp. and *P. villosa*. Set "B" contained the primers and TaqMan probes for *S. aphidivorus*, *P. aphidis* and *Dendrocerus* sp.. The efficacy of each individual primer/probe set and each multiplexed assay (set of primers/probes; Table 1) was evaluated using qPCR against DNA from each individual target species, and against a mixture of all DNA from the species not targeted by any given set (individual or multiplexed), including DNA from the host. Reactions were performed in 20μL volumes containing 2.0 μL of DNA template, 1 X ThermoPol PCR buffer (NEB), 200 nM each PCR primer (Table 1), 0.05 μM each TaqMan Probe, 200 μM each dATP, dCTP, dGTP, 400 μM dUTP, 10

Table. 1. PCR primers and TaqMan probes (included in the name the related Set "2A" or "2B") designed for parasitoid complex of *A. spiraecola* in citrus orchards.

Target species	Biology	Primer	Primer Sequence (5'-3')	Target	TaqMan probe Sequence (5'-3')	Reporter
Binodoxys angelicae (Hymenoptera: Braconidae)	Primary parasitoid	Tr-2A-for Tr-2A-rev	TCG CTT GAT TAT AAA TTA TGC CGG TAT TAT ATA TAA AGT CAG TTC	28S	AGATAAACCCAAGTTAGCCAACAGC	FAM
***Alloxysta* sp.** (Hymenoptera: Figitidae)	Hyperparasitoid	Al-2A-for Al-2A-rev	GAG GGT CGT TTA TAA ATT AAA G GAA CGT TTC ATC AAC TTG	ITS2	AACGACGCCACAAGACACAATC	Cy5
***Asaphes* spp.** (Hymenoptera: Pteromalidae)	Hyperparasitoid	As-2A-for As-2A-rev	CGC GTA CTT TCA ACT TAC AGG TCG TCG TAA TAA AAT AAC	ITS2	CGCTTGTCTCTTACAACTGGTTGAC	JOE
Phaenoglyphis villosa (Hymenoptera: Figitidae)	Hyperparasitoid	Ph-2A-for Ph-2A-rev	CTC TCT TTC TTC TCA GTG A GAA CGT GAA ATC GAT TAT ATT ATC	ITS2	TGCTCCAGGACTCGACAACC	ROX
Syrphophagus aphidivorus (Hymenoptera: Encyrtidae)	Hyperparasitoid	Sy-2B-for Sy-2B-rev	GTG TGT GCA AAT ATG TGG GCT ACG TTC TTT TAT GCG	ITS2	AATAACGAGATCGGTCCGCCA	Cy5
Pachyneuron aphidis (Hymenoptera: Pteromalidae)	Hyperparasitoid	Pa-2B-for Pa-2B-rev	GAT CGA ATG GGG AGA TTC GTC CTA CTA GGG GAG AAG	28S	CGTTGGCTTCCGTGCTGATC	FAM
***Dendrocerus* sp.** (Hymenoptera: Megaspilidae)	Hyperparasitoid	De-2B-for De-2B-rev	GAG AGA GTT CAA GAG TAG CGA GAT CAC ACA AGC ATC	28S	CGCCGTTGACGATGAATCTTCC	ROX

μg BSA (NEB), and 1 U of Taq DNA polymerase (NEB). Reactions were performed on a Rotor Gene 3000 programed for an initial denaturing at 95 °C for 3 min; followed by 40 cycles of 95 °C for 10 s, 50 °C (set A) or 55 °C (set B) for 15 s, 72 °C for 20 s.

2.2.3.2. Multiplex qPCR assay of aphid mummies

Having developed the qPCR assay, we subsequently extracted DNA from the second group of mummies (N=880) using a simple Chelex-based method (Edwards and Hoy, 1993). Individual mummies were incubated at 55 ˚C for one hour, followed by 10 min at 99 ˚C. Subsequently, the Chelex resin was pelleted and the supernatant (containing the extracted DNA) was transferred to a new tube and stored at -20 ˚C. Multiplex qPCR was set up as described above and each run included a standard set of controls: three positive controls (a mix of DNA from the species targeted by the particular probe set), three negative controls (a mix of DNA from the species not targeted by the particular probe set), and three no-template controls. Post-PCR runs were analyzed using the Rotor-gene software (version 6.0) applying quantification analysis and linear scale for normalized fluorescence in each channel (reporter). Amplification was assessed by first manually setting a fluorescence threshold for each qPCR run to bring the dRn (baseline-corrected normalized fluorescence) to a value of twice that of the respective negative controls at cycle number 35. In the event that this number fell below 0.01, the threshold was set at 0.01 to avoid recording false positives in the fluorescence data. Thus, in order to record the presence of a parasitoid species in a mummy template required that its associated Taqman probe fluoresced at a level exceeding the baseline threshold before cycle 35. The end result was a set of presence/absence data for each parasitoid species in each mummy.

2.2.4. Secondary sex ratio

Generalized linear models were used to compare secondary sex ratios of *B. angelicae* (obtained in section 2.2.2) in colonies with (n= 56) and without hyperparasitoids (n = 53) (including also data from molecular identification). Binomial error variance was assumed and assessed using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. Since under-dispersion was detected, we re-evaluated the significance of the explanatory variables using an F-test after re-scaling the statistical model by a Pearson's χ^2 divided by the residual degrees of freedom (Crawley, 2007).

2.3. Results

2.3.1. Parasitism rate

Across the two study years, and four orchards (three in 2012), a total of 229 field *A. spiraecola* colonies (32.7 ± 2.3 colonies per orchard ± SE) were tracked over the 5-week spring leaf-flush (Fig. 1). In general, parasitism rates were low. A total of 40,590 susceptible aphids (winged nymphs, winged adults and apterous adults) were counted, with the maximum number of aphids per colony averaging 64.67 ± 4.23 (mean ± SE). From these, a mere 286 mummies were counted, with the maximum number of mummies per colony averaging 0.89 ± 0.17 (mean ± SE). In orchard "A", no aphid mummies were found in 2012 and for this reason, the first year of this orchard was excluded from the following survey. In the remaining orchards, parasitism rates increased over the five week sampling period, but remained below 0.11 in both years.

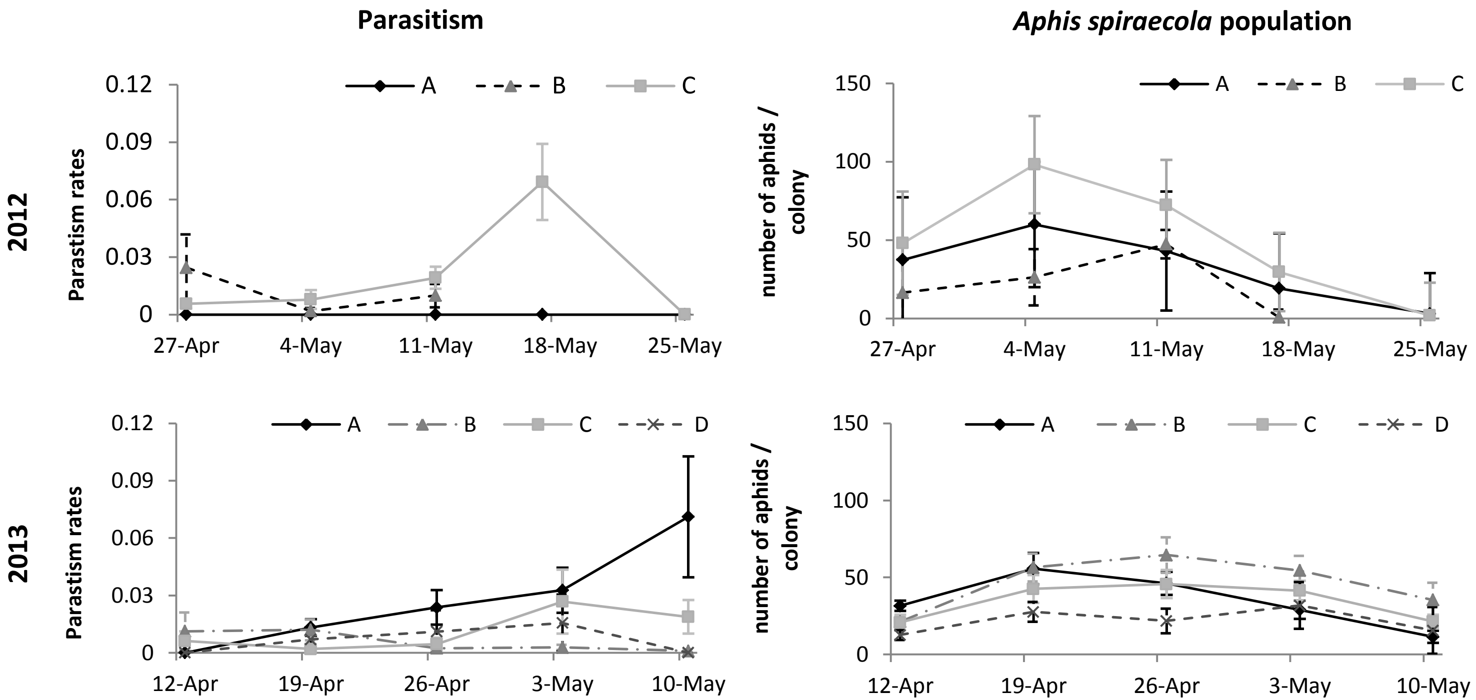

Fig. 1 Mean parasitism rates (± SE)(on the left) and aphid density (on the right) of *Aphis spiraecola* colonies tracked during the spring leaf-flushing period in three and four clementine orchards (A, B, C and D) in 2012 and 2013, respectively.

2.3.2. Primary parasitoid and hyperparasitoid complex

In the companion parasitoid survey, a total of 263 colonies of *A. spiraecola* with at least two mummies were collected from two and four orchards in 2012 and 2013, respectively. From these colonies, 1,827 aphid mummies of *A. spiraecola* were isolated for morphological (n = 947) or molecular identification (n = 880). The mean number of mummies per orchard increased during the sampling period of 2012, but remained constant in 2013 (Fig. 2).

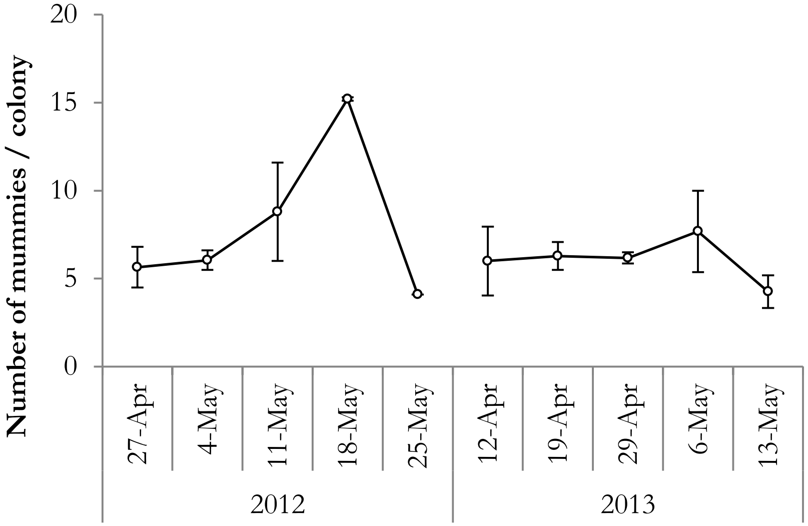

Fig. 2 Mean number of mummies per colony and orchard (±SE) collected during the spring leaf-flushing period in two and four clementine orchards in 2012 and 2013, respectively.

Table. 2. Relative and total abundance of *Aphis spiraecola* parasitoids during the spring leaf-flushing period of 2012 and 2013 in two and four clementine orchards, respectively. Parasitoids were identified using taxonomical (emerged from mummies) and molecular techniques (DNA-detected).

Biology	**Species**	**2012**			**2013**		
		Emerged	DNA-detected	Presence*	Emerged	DNA-detected	Presence*
Primary parasitoid	***Binodoxys angelicae***	18.1	46.0	2/2	45.0	68.4	4/4
Hyperparasitoids	***Syrphophagus aphidivorus***	47.5	30.8	2/2	14.7	7.9	4/4
	***Alloxysta* sp.**	21.6	10.6	2/2	26.3	11.7	4/4
	***Asaphes* sp.**	3.1	0.5	2/2	8.4	8.7	4/4
	Pachyneuron aphidis	7.2	7.2	2/2	4.2	2.3	4/4
	***Dendrocerus* sp.**	0.6	3.6	2/2	1.4	0.7	4/4
	Phaenoglyphis villosa	1.9	1.2	2/2	0.0	0.3	2/4
	Total	n = 320	n = 415		n = 429	n = 573	

(*) Number of orchards with parasitoid presence/number of sampled orchards.

2.3.2.1. Emergence and morphological identification assay

Out of the 947 mummies collected for this method, 198 (21.3%) were non-viable with no primary parasitoid or hyperparasitoid adults emerging. Among the 749 parasitoids successfully reared from the mummies, one primary parasitoid species was identified, *B. angelicae* (n = 251). In addition, at least six hyperparasitoid species were identified: *Syrphophagus aphidivorus* (n = 215), *Alloxysta* sp. (n = 182), *Asaphes* sp. (n = 46), *Pachyneuron aphidis* (n = 41), *Dendrocerus* sp. (n = 8), and *Phaenoglyphis villosa* (n = 6). The abundance of the different hyperparasitoids varied between years (Table 2). In 2012, the percentage of emerged hyperparasitoids was 81.9 % and *S. aphidivorus* was the most abundant, whereas this percentage decreased to 55 % in 2013 when *Alloxysta* sp. was the most abundant hyperparasitoid (Table 2).

2.3.2.2. Quantitative PCR assay

The multiplex qPCR assay allowed each *A. spiraecola* mummy to be screened for the primary parasitoid *B. angelicae* and all six hyperparasitoid species identified above in two separate reactions (Table 1). In the validation process, each primer/probe combination proved to be highly specific, amplifying nothing other than the target species for which it was designed. Subsequently, 880 mummies were assayed using this method. Most of them (86.0 %) tested positive for DNA from at least one of the target parasitoids. DNA of the primary parasitoid *B. angelicae* was detected in 583 mummies. In order of decreasing abundance, the DNA of the hyperparasitoids *S. aphidivorus* (n = 173), *Alloxysta* sp. (n = 111), *Asaphes* sp. (n = 52), *P. aphidis* (n = 43), *Dendrocerus* sp. (n = 19) and *P. villosa* (n = 7) was also detected. In several cases, DNA from multiple parasitoid species was detected from a single mummy. This was not surprising (since they are hyperparasitoids) and explains why the total number of detected parasitoids was higher than the number of collected mummies. Consistent with the identifications based on morphology, the abundance of the different hyperparasitoids, based on positive PCR, again varied between years (Table 2). In 2012, the percentage of detected hyperparasitoids was 53.9 % and *S. aphidivorus* was the most detected species, whereas this percentage decreased to 31.6 % in 2013 when *Alloxysta* sp. was the most abundant hyperparasitoid (Table 2).

When both primary and secondary parasitoids were detected simultaneously in a mummified aphid, detection of each of the six hyperparasitoid species was possible in association with B. angelicae (Table 3). The three most abundant hyperparasitoid species *S. aphidivorus*, *Alloxysta* sp. and *Asaphes* sp. were detected mostly with DNA of the primary parasitoid *B. angelicae* or alone. In contrast, the less abundant hyperparasitoids shared their mummies with other hyperparasitoids (*P. aphidis* shared 24 mummies with other hyperparasitoids out of the 43 in which it was detected , *Dendrocerus* sp. 17 out of 19 and *P. villosa* 4 out of 7). We also detected *B. angelicae* with multiple hyperparasitoids (in a single mummy) on four occasions, and two further cases where three hyperparasitoids species were present without *B. angelicae* DNA (Table 4).

Table. 3. Interactions between *Aphis spiraecola* parasitoids within mummies collected in clementine orchards. Each number represents the percentage of times that the parasitoid species in the row has been DNA-detected in a mummy with the parasitoid species in the column.

	Binodoxys angelicae*	***Syrphophagus aphidivorus***	***Alloxysta* sp.**	***Asaphes* sp.**	***Pachyneuron aphidis***	***Dendrocerus* sp.**	***Phaenoglyphis villosa***	**Detected alone****	**Total**
Syrphophagus aphidivorus	48.6	-	6.4	0.6	3.5	1.2	0.0	46.8	n=173
***Alloxysta* sp.**	59.5	9.9	-	6.3	5.4	1.8	1.8	33.3	n=111
***Asaphes* sp.**	53.8	1.9	13.5	-	5.8	0.0	0.0	42.3	n=52
Pachyneuron aphidis	44.2	14.0	14.0	7.0	-	25.6	0.0	23.3	n=43
***Dendrocerus* sp.**	31.6	10.5	10.5	0.0	57.9	-	10.5	10.5	n=19
Phaenoglyphis villosa	57.1	0.0	28.6	0.0	0.0	28.6	-	14.3	n=7

(*) Percent of DNA-detection of each hyperparasitoid species with the primary parasitoid *Binodoxys angelicae*.

(**) Percent of DNA-detection of each hyperparasitoid without any other parasitoid species.

Table. 4. Detection of DNA from three or four parasitoids sharing a sole mummy in field-collected *Aphis spiraecola* mummies (n = 1,827) in clementine orchards.

Primary parasitoid	Hyperparasitoids			number of cases
Binodoxys angelicae	*Alloxysta* sp.	*S. aphidivorus*	-	n = 6
		Asaphes sp.	-	n = 5
		P. aphidis	-	n = 4
		Dendrocerus sp.	-	n = 1
	P. aphidis	*S. aphidivorus*	-	n = 1
		Asaphes sp.	-	n = 1
		Dendrocerus sp.	-	n = 1
	S. aphidivorus	*Asaphes* sp.	-	n = 1
	Dendrocerus sp.	*Phaenoglyphis* sp.	-	n = 1
	S. aphidivorus	*Alloxysta* sp.	*P. aphidis*	n = 1
	Alloxysta sp.	*Asaphes* sp.	*P. aphidis*	n = 1
-	*S. aphidivorus*	*P. aphidis*	*Dendrocerus* sp.	n = 1
	Alloxysta sp.	*Dendrocerus* sp.	*Phaenoglyphis* sp.	n = 1

2.3.3. Seasonal trend

Hyperparasitoids of B. *angelicae* were abundant from the beginning of the spring leaf-flush both years and the percentage of hyperparasitism was above 25% for both methods the first sampling week.

The hyperparasitoid complex of B. *angelicae* and their abundance showed a different trend in 2012 and 2013, independently of the method used to identify them (Fig. 3). In 2012, the abundance of B. *angelicae* (in both morphological emergence and qPCR assays) decreased throughout the sampling period and became almost nil by the end of the fifth week. In contrast, the number of hyperparasitoids increased over the first four weeks, before collapsing during the last week, when most of the immature parasitoids died. Among the hyperparasitoid species, *Alloxysta* sp. was the most abundant one at the beginning of the season and *S. aphidivorus* increased and became the most abundant one by the fifth week (Fig. 4).

In 2013, the relative abundance of the primary parasitoid B. *angelicae* and its hyperparasitoids remained constant over the five week season (Fig. 3). The percentage of DNA detection for B. *angelicae* (~67 %) was higher than its percentage of emergence (~36 %) throughout the season. The percentage of immature mortality remained also constant. Although the abundance of *S. aphidivorus* increased again, *Alloxysta* sp. was the most abundant hyperparasitoid throughout this season (Fig. 4). At the start of the 2013 season, *Asaphes* sp. was as abundant as *S. aphidivorus* (Table 2) but it tended to decrease throughout the season. Finally, in both years, *P. aphidis* became relatively abundant at the end of the season.

2.3.4. Hyperparasitism effect on the primary parasitoid sex ratio

When we pooled all the data, the secondary sex ratio of *B. angelicae* was significantly higher when emerged from *A. spiraecola* colonies with at least one hyperparasitoid in the colony (0.40 ± 0.06) than without hyperparasitoids (0.28 ± 0.05) (GLM: $F_{1, 118} = 6.67$; $P < 0.011$).

Fig. 3 Find the figure in the next page. Seasonal trend of the primary parasitoid *T. angelicae* and its hyperparasitoids emerged and detected in mummies of *Aphis spiraecola* collected in two and four citrus clementine orchards in 2012 and 2013, respectively. **A** Mean percentage (± SE) of primary parasitoids and hyperparasitoids emerged and not emerged (immature mortality) from reared mummies per orchard. **B** Mean percentage (± SE) of PCR-detection of primary parasitoids alone and with one hyperparasitoids or several hyperparasitoid species (multi-hyperparasitism) per orchard.

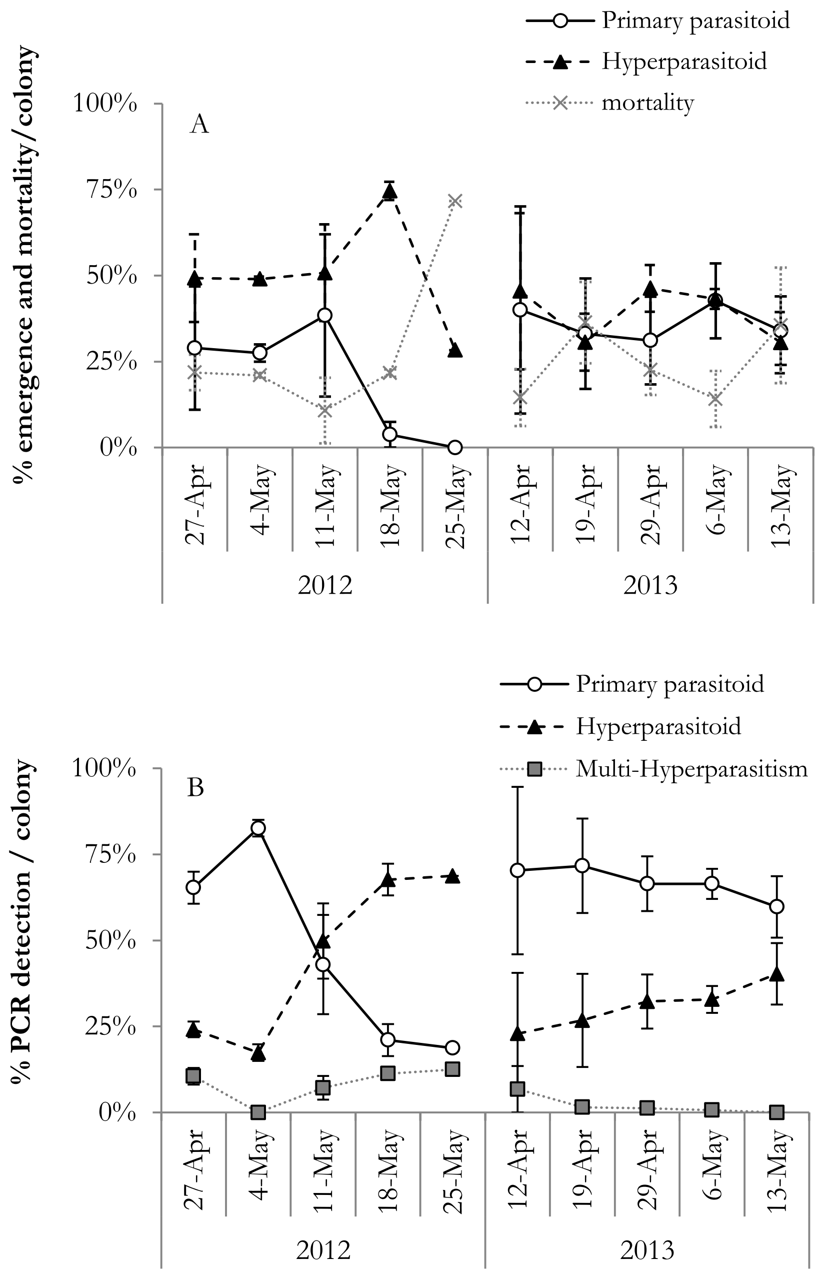

Fig. 3 Find the caption in previous page.

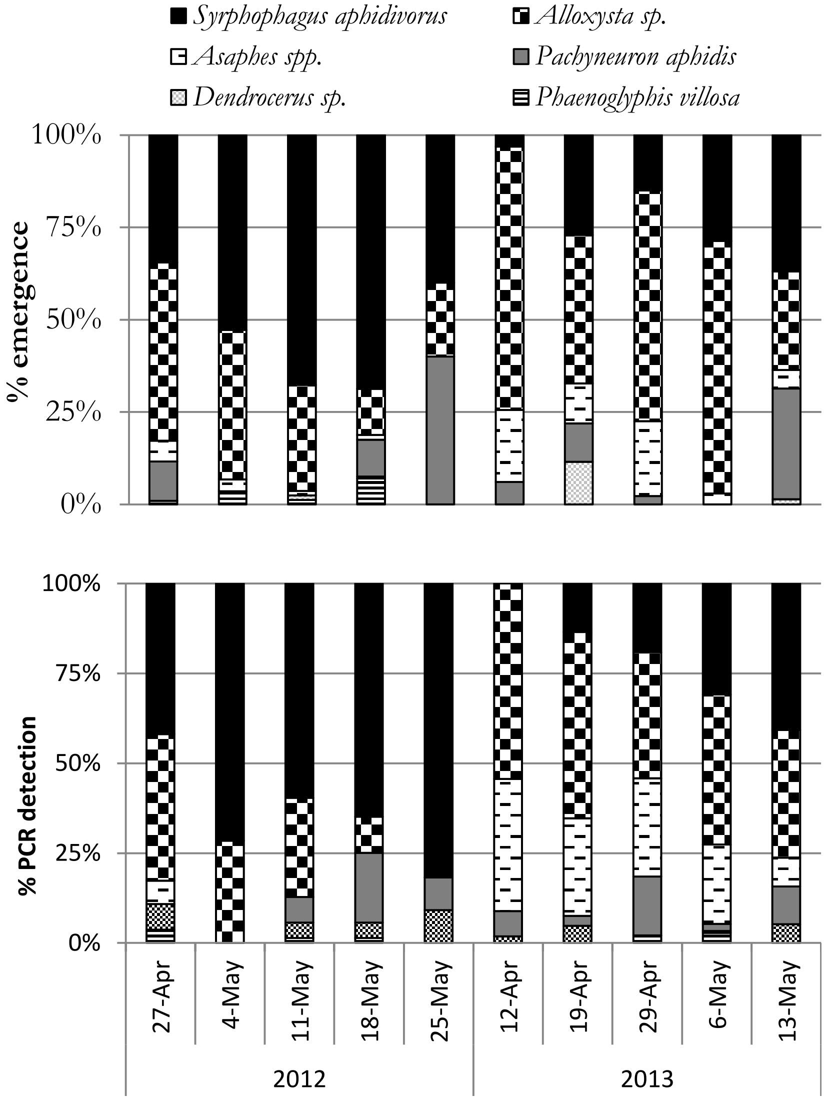

Fig. 4 Relative seasonal abundance of *A. spiraecola* hyperparasitoids collected in two and four citrus clementine orchards in 2012 and 2013, respectively. **A** Percentage of hyperparasitoids emerged from reared mummies. **B** Percentage of hyperparasitoids detected by PCR in *A. spiraecola* mummies.

2.4. Discussion

This study confirms that *B. angelicae* is likely the only primary parasitoid of *A. spiraecola* in Spanish citrus clementine. It remains unknown why other common generalist parasitoids of aphids in Mediterranean citrus do not use *A. spiraecola* as a host (Michelena *et al.*, 1994; Michelena *et al.*, 2004). One factor that is likely to influence this outcome is that the parasitoids, mostly native to Europe or America (Cole, 1925; Miller, 1929; Tang *et al.*, 1994; Tang and Yokomi, 1996) have not coevolved with *A. spiraecola*, which is native to the Far East (Blackman and Eastop, 1984). Thus *A. spiraecola* may simply not be a suitable host for "local" parasitoid development, as occurs with the parasitoid *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) (Tremblay *et al.*, 1983).

In contrast to the paucity of primary parasitoids, the hyperparasitoid complex of *A. spiraecola* was large and diverse. At least six species belonging to six genera make up its hyperparasitoid complex in citrus. These hyperparasitoids have all previously been reported as common obligate hyperparasitoids of aphid primary parasitoids (Sullivan and Völkl, 1999). Using our multiplex qPCR assay, we confirmed that all these species hyperparasitize *A. spiraecola* via *B. angelicae*. Among these hyperparasitoids, *S. aphidivorus* and *Alloxysta* sp. were the most abundant in the two seasons monitored. *Asaphes* sp. was also quite abundant in 2013, when the relative abundance of *S. aphidivorus* was reduced in comparison with 2012. Despite their abundance and diversity, references of *A. spiraecola* hyperparasitoids in citrus are scarce. *Alloxysta* sp. was recorded as the major hyperparasitoid in previous surveys of *A. spiraecola* parasitoids in citrus (Michelena and Sanchís, 1997; Suay *et al.*, 1998; Bañol *et al.*, 2012). The rest of hyperparasitoid genera identified in our study had been previously cited hyperparasitizing *A. spiraecola* in citrus.

Bañol *et al.* (2012) found *Asaphes sp.* as the most abundant hyperparasitoid of aphids in an organic citrus orchard but these authors did mention neither the aphid host nor the primary parasitoid.

Two reasons might explain the high abundance and diverse complex of hyperparasitoids of the invasive aphid *A. spiraecola.* First, *A. spiraecola* shares its niche in citrus with *A. gossypii,* which has a richer complex of primary parasitoids (Suay *et al.*, 1998; Kavallieratos *et al.*, 2002) and appears slightly earlier than *A. spiraecola* in spring (Hermoso de Mendoza *et al.*, 2012). Therefore, hyperparasitoids might increase their populations via the primary parasitoids of *A. gossypii* and then, once their populations have increased, move onto parasitized *A. spiraecola.* Alternatively, ants can provide an 'enemy-free space' for some primary parasitoids that have evolved mechanisms (for example, by cryptic behavior or chemical camouflage) to avoid ant aggression (Völkl, 1992; Mackauer and Völkl, 1993). However, *B. angelicae* has not evolved such mechanisms and it is therefore restricted to aphid colonies that are not tended by ants (Völkl, 1992). As a result, these “easily accessed” aphids parasitized by *B. angelicae* are also open to high incidence of hyperparasitism.

In both study years, hyperparasitoids were abundant from the beginning of the spring leaf-flush and hyperparasitism rates remained high throughout the season. Hyperparasitism is likely to affect the parasitoid complex, and in turn parasitism rates of *A. spiraecola.* Indeed, the early occurrence of hyperparasitoids may have disrupted aphid control by *B. angelicae.* This has important implications for biological control since female *B. angelicae* are usually more successful when attacking small and medium-sized aphid colonies (Mackauer and Völkl, 1993), i.e. those typically present at the beginning of the aphid season in citrus (Gómez-Marco *et al.*, Submitted). *B. angelicae* abandons colonies after a short time and few ovipositions irrespective of the size of the colony or the number of eggs stored in the parasitoid's ovaries and, therefore, becomes less efficient with an increase in colony size (Mackauer and Völkl, 1993).

Alloxysta sp. was the earliest detected hyperparasitoid but its relative abundance decreased throughout the season as *S. aphidivorus* became the most abundant hyperparasitoid. This seasonal trend may be explained by their different preference for a particular host stage. *Alloxysta* sp. develops as a koinobiont endohyperparasitoid that parasitizes primary parasitoid larvae within living aphids (Singh and Srivastava, 1988; Sullivan and Völkl, 1999). *S. aphidivorus* also develops as a koinobiont endohyperparasitoid that can attack primary parasitoid larvae when aphids are still alive, but prefers to attack the pre-pupae or pupae of the primary parasitoid after the aphid has been killed and mummified (Sullivan and Völkl, 1999; Buitenhuis *et al.*, 2004; Gariepy and Messing, 2012). Therefore, at the end of the season it might be harder for *Alloxysta* sp. to find a suitable host since most of the parasitized aphids has already become mummies, giving *S. aphidivorus* an advantage.

Apart from increasing the mortality of *B. angelicae*, the hyperparasitoids also increased the secondary sex ratio of *B. angelicae.* Our results show that the secondary sex ratio (proportion male) of *B. angelicae* per aphid colony was higher when at least one hyperparasitoid emerged in the colony. There are other examples of hyperparasitoids affecting the secondary sex ratio of primary parasitoids in aphids. Mackauer and Völkl (2005) found that the secondary sex ratio of *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae) was female biased in patches without hyperparasitoids, but became Fisherian (1:1) in patches with hyperparasitoids. Sequeira and Mackauer (1993) found that mortality from all sources including hyperparasitism was greater among *Aphidius* *ervi* Haliday (Hymenoptera: Braconidae) eclosing from the relatively larger mummies of *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae), which contained a higher proportion of females. Generally, haplodiploid female parasitoids deposit fertilized eggs (females) in large hosts containing more resources (Godfray, 1994), nevertheless the secondary sex ratio of the primary parasitoids will differ from the primary sex ratio if developmental mortality is greater in one sex than another (Hardy and Cook, 1995; Hardy *et al.*, 1998). Similarly, hyperparasitoids select larger mummies, which contain more female primary parasitoids, to deposit their eggs because these mummies have more resources. Therefore, the preference for large mummies by the hyperparasitoid will result in a proportionately greater mortality risk for female progeny of the primary parasitoid and, consequently increase the secondary sex ratio (Mackauer and Völkl, 2005). It is suspected that the form of the relationship is association specific, depending on the offspring- and sex-allocation strategies of both primary parasitoid and the hyperparasitoids (Mackauer and Völkl, 2005). It is hard to determine the behavioral or physiological traits behind the increase of sex ratio in our study because there are at least six species of hyperparasitoids attacking one species of primary parasitoid.

The multiplex qPCR assay allowed us to assess levels of hyperparasitism and the relation between parasitoids in the field with high efficiency. Constructing host-parasitoid-hyperparasitoid food–web assemblages and attempting to analyze their dynamics is difficult and usually involves large field collections of hosts followed by labor-intensive rearing or dissecting of the insects to evaluate the rates of parasitism, along with morphological identification of the parasitoid species (Holler *et al.*, 1993; Müller *et al.*, 1999; Tylianakis *et al.*, 2007; Van Emden and Harrington, 2007; Alhmedi *et al.*, 2011; Gagic *et al.*, 2012), and these methods can lead to an incorrect quantification of trophic links and bias food-web studies (Gariepy and Messing, 2012). In our study, comparing with the emergence protocol, we were able to detect a higher percentage of parasitoids in mummies using the multiplex qPCR. The molecular analysis elucidated both primary parasitoid-hyperparasitoid links and detected multi-hyperparasitism. Such precision is not usually possible using rearing techniques or host dissection only. In addition, with this method we confirmed that the high hyperparasitism rate is the most plausible reason to explain the inefficiency of *B. angelicae* to control *A. spiraecola*. Generally, parasitoids and hyperparasitoids fail to emerge when hyperparasitism is high, as in our study, because larvae are killed by hyperparasitoid host-feeding, or hyperparasitoids fail to complete development (Holler *et al.*, 1993). Also, the qPCR assay revealed new trophic links between aphid (hyper-) parasitoids because with the emergence method it is possible known only the "winner" specie inside the mummy. At least two previous studies have also used molecular techniques to reveal similar trophic links in aphid-parasitoid-hyperparasitoid systems (Traugott *et al.*, 2008; Gariepy and Messing, 2012). The emergence method was, however, complementary and necessary to study the effect of hyperparasitoids on a primary parasitoid, since it allowed us to i) identify the parasitoid species to design the specific primers for each species and ii) determine the outcome of the competition between the hyperparasitoids.

Unless the high efficiency obtained in DNA detection by real-time PCR, *B. angelicae* could not be detected in 23 % of the total positives. Taking into account that these hyperparasitoids species cannot develop on unparasitized aphids (Brodeur and Rosenheim, 2000), the non-detection of *B. angelicae* may be explained by: (i) hyperparasitoids ovipositing into unparasitized aphids (Brodeur and Rosenheim, 2000) and their DNA is detected from the doomed eggs, (ii) failure to detect very small quantities of DNA (we defined a high threshold level in the PCR assay to avoid false positives) and (iii) the primary parasitoid being completely consumed by the hyperparasitoid and aphid hosts containing late instar larvae, pupae or adult hyperparasitoids. Our results are consistent with findings by Traugott *et al.* (2008) and Gariepy and Messing (2012) who also found cases where hyperparasitoid DNA was detected in the absence of primary parasitoid DNA.

The multiplex real-time PCR detected guild relationships between *A. spiraecola* parasitoids. The presence of several hyperparasitoids species in a mummy was common. In detail, *S. aphidivorus*, *Alloxysta* sp. and *Asaphes* sp. were detected mostly alone or with DNA of the primary parasitoid *B. angelicae*, whereas *P. aphidis*, *Dendrocerus* sp. and *P. villosa* often shared the mummies with other hyperparasitoids. The presence of several hyperparasitoid species sharing a mummy can be explained by their different oviposition behavior and immature development (Müller and Godfray, 1998). *S. aphidivorus, Asaphes* sp., *P. aphidis*, *Dendrocerus* sp. are polyphagous mummy parasitoids and they may develop as a facultative tertiary parasitoid if they attack a mummy containing hyperparasitoids such as *Alloxysta* sp., *P. villosa* and also *S. aphidivorus,* which has the dual oviposition behavior as explained above. However, mummy parasitoids do not always win the competition with hyperparasitoids (Sullivan, 1972; Buitenhuis *et al.*, 2004). Interestingly, strict mummy hyperparasitoids were less abundant suggesting that they may be competitively inferior.

In conclusion, *B. angelicae* was the dominant primary parasitoid for *A. spiraecola* in citrus but parasitism rates were very low. Its associated complex of hyperparasitoids was large and diverse. These hyperparasitoids were abundant from the beginning of aphid season and remained high throughout the season. Apart from increasing the mortality of *B. angelicae*, these hyperparasitoids also increased its secondary sex ratio. This suggests that, biological control of *A. spiraecola* with parasitoids may be unfeasible in the Mediterranean Basin. Therefore, future research programs should concentrate their efforts on other natural enemies, as entomopathogens and, especially, predators, which are also abundant and diverse in citrus (Cole, 1925; Miller, 1929; Michelena and Sanchís, 1997; Urbaneja *et al.*, 2000; Alvis *et al.*, 2002; Kavallieratos *et al.*, 2004; Hermoso de Mendoza *et al.*, 2012; Vacante and Gerson, 2012). Aphid predators might be also causing high mortality to primary parasitoids (Brodeur and Rosenheim, 2000) but the biological control services of these predators could be improved by means of conservation techniques.

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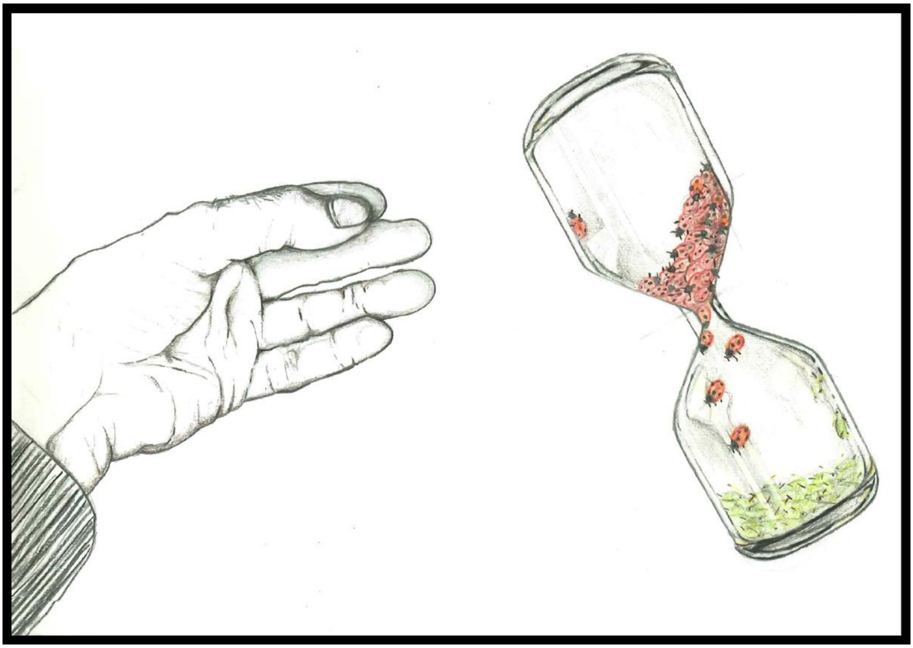
CHAPTER III

Early arrival of predatos controls *Aphis spiraecola* colonies in citrus clementine.



3. Early arrival of predators controls *Aphis spiraecola* colonies in citrus clementines.

Gómez-Marco, F., Tena, A., Jaques, J.A., Urbaneja, A. (2015) Early arrival of predators control *Aphis spiraecola* colonies in citrus clementine. ***Journal of Pest Science***. (DOI: 10.1007/s10340-015-0668-9)

Abstract

Aphis spiraecola Patch. (Hemiptera: Aphididae) is a key pest of citrus clementines. This aphid colonizes tender clementine shoots in the spring and causes important economic losses. A complex of predators prey on *A. spiraecola* colonies but does not result in satisfactory control. To disentangle the reasons for this failure, we investigated the effect of predators on *A. spiraecola* colonies and damage over a three-year period. *A. spiraecola* colonies were tracked every 48 to 72 h from the period of aphid colonization until the colony declined or disappeared. The number of aphids, their stage and the presence of predators were recorded in each colony. Different life parameters of *A. spiraecola* colonies (maximum number of aphids, longevity and colony phenology) varied among the orchards over three years. Predators attacked one-third of the colonies, and this did not significantly differ among orchards for the years studied. The maximum number of aphids and longevity of *A. spiraecola* colonies were not related to the ratio of colonies attacked by predators, but were

negatively correlated with the time of their first attack. More importantly, the percentage of shoots occupied by *A. spiraecola* remained below or close to the intervention threshold when colonies were attacked prior to ~200 degree days (DD) from the beginning of the aphid colonization. These results suggest that: i) the presence of predators at the beginning of the season should be considered to develop new intervention thresholds and ii) biological control programs should promote the early presence of predators in clementine orchards.

3.1. Introduction

The Spirea citrus aphid *Aphis spiraecola* Patch and cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) are major pests of citrus in the Mediterranean basin. Clementine mandarins, *Citrus clementina* Hort. ex Tan. (Geraniales: Rutaceae) is the citrus species most susceptible to their attack (Hermoso de Mendoza *et al.* 2006; Tena and Garcia-Marí, 2011; Hermoso de Mendoza *et al.* 2012; Vacante and Gerson, 2012). Since the 1980's, *A. spiraecola* has displaced the previously dominant species *A. gossypii* as the most abundant aphid in Spanish citrus, and this is thought to be due to the low efficacy of the former's natural enemies (Meliá, 1982; Hermoso de Mendoza *et al.*, 1986; Meliá and Blasco, 1990; Hermoso de Mendoza *et al.* 1997; Hermoso de Mendoza *et al.*, 2001; Hermoso de Mendoza *et al.*, 2012). This aphid species colonizes and feeds on young tender clementine shoots in spring (Hermoso de Mendoza *et al.* 2001; Hermoso de Mendoza *et al.* 2006). *A. spiraecola* causes economic losses because they suck sap, vector the citrus tristeza virus, excrete large amounts of honeydew and curls the developing leaves as they grow (Hermoso de Mendoza *et al.* 2001; Hermoso de Mendoza *et al.* 2006). To improve its management, Hermoso de Mendoza *et al.* (2006) established intervention thresholds. Following this recommendation, insecticides are currently used almost yearly against this aphid in citrus clementines (Tena and Garcia-Marí, 2011) to maintain aphid populations under the economic thresholds.

In recent years, emphasis has been placed on implementing more economical and environmentally safe measures to control citrus pests in Spain. As a consequence, a line of research investigating biological control is currently being developed in Spain. As a permanent and perennial crop, citrus provides an environment in which guilds of natural enemies of *A. spiraecola* effortlessly develop (Cole, 1925; Michelena and Sanchís, 1997; Alvis *et al.* 2002; Kavallieratos *et al.* 2004; Yoldas *et al.* 2011; Hermoso de Mendoza *et al.* 2012; Romeu-Dalmau *et al.* 2012a; Vacante and Gerson, 2012). These guilds consist of parasitoids and predators that naturally occur on citrus plants in spring. Unfortunately, the parasitoid complex of *A. spiraecola* is dominated by hyperparasitoids of several families, which reduces the efficacy of the unique primary parasitoid that occurs in the system *Binodoxys angelicae* (Haliday) (Hymenoptera: Braconidae) (Gómez-Marco *et al.* 2015). Therefore, the option of conservation biological control via the use of parasitoids in clementines is limited (Gómez-Marco *et al.* 2015).

Like most aphids, a rich complex of species prey on *A. spiraecola*, which may control its populations. In fact, the relative abundance and seasonal trend of its main predators have already been studied in the field (Michelena and Sanchis, 1997; Alvis *et al.* 2002; Hermoso de Mendoza *et al.* 2012), as well as some aspects of their biology under laboratory conditions (Michaud, 2000; Belliure and Michaud, 2001; Michaud, 2001). However, the efficacy of these predators as biological control agents of *A. spiraecola* in citrus either individually or as a complex is poorly understood. Citrus aphid predators belong to different orders, such as coleopterans, dipterans, dermapterans and neuropterans. In the Mediterranean basin, the most abundant coleopterans are the coccinellids *Scymnus* sp., *Coccinella septempunctata* L., *Propylea quatuordecimpunctata* L. and *Adalia bipunctata* L. (Coleoptera: Coccinellidae) (Michelena and Sanchís, 1997; Alvis *et al.* 2002; Kavallieratos *et al.* 2004; Hermoso de Mendoza *et al.* 2012); among the dipterans, the most abundant are the hoverflies *Eupeodes corollae* Fabricius (Diptera: Syrphidae) (Hermoso de Mendoza *et al.* 2012), *Paragus haemorrhous* Meigen

(Diptera: Syrphidae) (Michelena and Sanchís, 1997), and the gall midges *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae) (Hermoso de Mendoza *et al.* 2012). Finally, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) is the most abundant neuropteran (Michelena and Sanchís, 1997; Hermoso de Mendoza *et al.* 2012). The earwig *Forficula auricularia* L. (Dermaptera: Forficulidae) has recently been identified as an important generalist aphid predator in citrus (Romeu-Dalmau *et al.* 2012a; Romeu-Dalmau *et al.* 2012b). Despite this abundant and diverse complex of predators, biological control of *A. spiraecola* is generally insufficient. A possible explanation could be the asynchrony of predators with *A. spiraecola* (Hermoso de Mendoza *et al.* 2012).

The synchronized arrival of predators to the crops with the most vulnerable phenology of a pest, generally early in the season, is an important issue in designing biological control programs (Welch and Harwood 2014). In conservation biological control, it is generally assumed that the sooner the natural enemies are present the better their efficiency for biological control (Welch and Harwood 2014). This is because a crop may have the most favourable natural enemy:pest ratios at the beginning of the season when pest population densities are low (Settle *et al.* 1996; Landis and Van der Werf 1997). However, there is some evidence of this assumption (see Rutledge and O'Neil 2005; Brosius *et al.* 2007; Meihls *et al.* 2010;). Moreover, the way in which the chronology of the natural enemies affects the pest population's demographic growth remains poorly known. To avoid this asynchrony, conservation efforts are directed at enhancing favourable conditions for the establishment of predators before the arrival and seasonal increase of pests, when the expected effect of predation on the aphid populations would be greater (Southwood and Comins 1976; Carroll and Hoyt 1984;

Van Emden and Harrington 2007). In the case of clementines and *A. spiraecola,* the ability of an early establishment of predators to maintain the population levels of this aphid under the economic threshold has never been determined.

This study was conducted in three clementine orchards during three consecutive spring seasons to ascertain the importance of the arrival date of the aphid predator guild to the clementine orchard on the control of *A. spiraecola.* To achieve this goal, we tracked ~40 colonies per orchard every 48-72 h to determine i) the life parameters of *A. spiraecola* colonies (survival, maximum number of aphids and phenology) and ii) their predators (identification, attack rate and arrival time). We also measured the damages produced by *A. spiraecola* as the percentage of aphid-infested shoots on a weekly basis. With these data, we evaluated the effect of the arrival time of predators on the life parameters of and damages induced by *A. spiraecola* colonies. These results will permit the evaluation of the effect of the early arrival of predators and help design future measures of conservation biological control of *A. spiraecola.*

3.2. Materials and Methods

3.2.1. Study orchards

The assays were carried out in three commercial citrus orchards (*C. clementina* Hort. ex Tan. cv. clementine grafted on citrange Carrizo rootstock) in the Valencia region of eastern Spain. Orchard "A" was located in Moncada (39° 35' 18.34" N - 0° 23' 57.96" O, 0.79 ha) and orchards "B" and "C" were located in Almenara (orchard "B": 39° 44' 45.04" N - 0° 14' 40.73" O, 1.35 ha; orchard "C": 39° 45' 56.79"N-0° 14' 12.69"O, 0.81 ha). These orchards followed the IPM guidelines

(Urbaneja *et al.* 2014) and were drip irrigated. Orchards "A" and "C" had bare soil by means of herbicide applications. The cover crop of orchard "B" was a mix of grassy plants (*Festuca arundinacea*, *Poa* sp., *Bromus* sp., etc.) and a sporadic covering of other minor weeds. The cover crop was mowed twice per year in early spring and summer. Pesticides were not sprayed during the sampling period.

3.2.2. Aphid colonies and predators

In the present paper, the term "colony" is used for aphid nymphs and adults co-habiting a single shoot. To determine the life parameters of *A. spiraecola* colonies and identify their predators in these citrus orchards, 9-40 tender shoots per orchard containing one colony each were selected at the beginning of the flushing spring period each year. Each colony (one colony per tree) was marked with a 1 cm diameter plastic ring. The selected colonies were marked when they contained less than 20 aphids (mean aphids / colony = 11.92 ± 0.47 SE during the three years) and settled in young tender shoots (less than 5 cm. long). The aphid colonies were tracked every 48-72 h from the period of aphid colonization (6.34 ± 2.12 mean percentage of occupied shoots by *A. spiraecola* per orchard ± SE during the three years) by visual inspection until the decline of the selected colonies. The tracking periods started on the 13th, 8th and 1st of April in 2011 for orchards A, B and C, respectively, while they started on the 23rd of April for orchard A and the 17th of April for orchards B and C in 2012; the tracking periods in 2013 for all three orchards started on the 3rd of April. For each colony and date, the number of aphids (we differentiated among: nymphs, winged nymphs, apterous adults and winged adults) and predators were recorded. Predators were identified by direct observations in the field. A part from predators considered to be typically aphidophagous predators, spiders from the genus Theridion and the earwig *F. auricularia*, were considered to be predators of *A. spiraecola* because they were observed to repeatedly feed on colonies during the tracking period.

Three life parameters of the colonies were calculated with these data: the mean maximum number of aphids per colony, colony survival and colony phenology. To this end, the maximum number of aphids per colony was determined throughout their life, and colonies were considered to be alive until the last aphid died. Two parameters of predator efficacy were also calculated with these data: the ratio of colonies attacked by predators and time of first attack (when the first predator was observed in the colony). To homogenize the tracking periods between orchards, the degree days (DD) were calculated by accumulating the mean daily temperature (threshold = 0 °C) starting with the first tracking day in each orchard.

3.2.3. Damage and intervention thresholds

To improve the management of aphids in clementines, Hermoso de Mendoza *et al.* (2001, 2006) established the intervention thresholds based on the percentage of infested shoots within a 0.25 m^2 ring thrown twice on the outer canopy of trees. An insecticide application is justified when more than 25% of the shoots within the ring are infested. Following these recommendations, we calculated the percentage of infested shoots during the spring flushing. To this end, 20 trees per orchard were randomly chosen at the beginning of the sampling protocol and sampled weekly, and the numbers of infested and non-infested shoots per ring were counted.

3.2.4. Data analysis

First, the aphid colony and predator parameters were compared among orchards over the three years. The mean maximum number of aphids per colony and the mean time of first attack were compared using a one-way ANOVA followed by Bonferroni's post hoc tests for multiple comparisons. Generalized linear models were used to compare the mean number of predators per colony and the ratio of colonies attacked by predators. A Poisson error variance was assumed for the number of predators per colony (the total number of predators observed during the tracking period of the colony), and a binomial error variance was assumed for the ratio of colonies attacked by predators. The assumed error structures were then assessed using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If an over- or under-dispersion was detected, we re-evaluated the significance of the explanatory variables using an F-test after re-scaling the statistical model with a Pearson's $\chi 2$ value divided by the residual degrees of freedom (Crawley, 2007). Cox regression analyses were used to compare the colony survival among orchards.

Second, lineal models were used to analyse the effect of predator parameters (attack ratio and mean time of first attack) on the aphid colony parameters (mean maximum number of aphids per colony and mean colony longevity). We examined all possible regressions using linear, power, log, exponential and polynomial functions and selected the model with the highest coefficient of determination.

Finally, generalized linear models were used to determine the effect of the mean time of the first attack and the ratio of colonies attacked at 100 DD on the maximum percentage of infested shoots throughout the tracking period. We followed the same procedure described above assuming a binomial error variance for the maximum percentage of infested shoots. The statistical software package 'R' (http://www.R-project.org) and its packages survival and lme4 were used in our analyses.

3.3. Results

From 2011 to 2013, a total of 279 *A. spiraecola* colonies were selected and tracked in three clementine orchards (A, B and C) during the spring flushing period. In 2011, 9, 19 and 11 colonies were tracked in orchards A, B and C, respectively. In 2012 and 2013, 40 *A. spiraecola* colonies per orchard were tracked. The total numbers of aphids recorded were 8,250, 46,610 and 42,981 in 2011, 2012 and 2013, respectively.

3.3.1. Colony parameters

3.3.1.1. Maximum number of aphids per colony

The maximum number of aphids per colony differed significantly among clementine orchards in 2011 ($F_{2, 36} = 11.79$; $P < 0.001$) and 2012 ($F_{2, 117} = 16.69$; $P < 0.001$), but not in 2013 ($F_{2, 117} = 2.48$; $P = 0.088$) (Fig. 1). In 2011, the maximum number of aphids per colony was significantly lower in orchards B (24.32 ± 6.94 mean ± SE maximum aphids per colony) and C (46.82 ± 10.73) than in orchard A (124.22 ± 30.04). In 2012, the maximum number of aphids per colony was significantly lower in orchard B (25.90 ± 4.87) than in orchards A (88.60 ± 8.38) and C (117.92 ± 17.41).

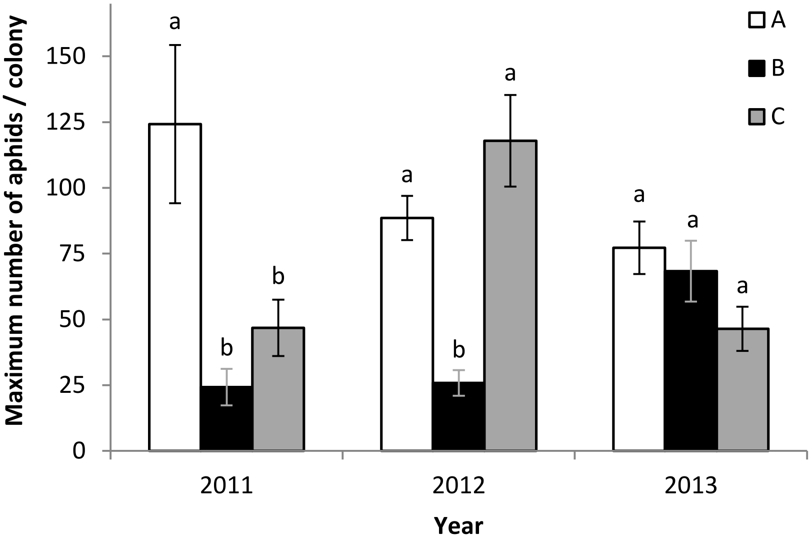


Fig. 1 Maximum number of aphids per colony (mean ± SE) tracked in three citrus orchards (A, B and C) during the spring leaf-flushing period of three consecutive years (2011-2013). Different letters indicate significant differences among the orchards each year.

3.3.1.2. Colony survival

Colony survival differed significantly among orchards over the three years (2011: Wald-statistic = 13.18, $P = 0.0014$; 2012: Wald-statistic = 50.49, $P < 0.001$; 2013: Wald-statistic = 20.97, $P < 0.001$) (Fig. 2). In orchard B, colony survival fell below 50% between 100 and 200 DD in 2011 and 2012 and was significantly lower than in orchards A and C. In the latter orchards, more than 50% of the colonies remained alive at 400 DD in these years. In 2013, the colony survival was similar in orchards B and C and significantly lower than in orchard A.

When we compared the mean longevity per orchard, we obtained similar results. In 2011 and 2012, colony longevity was significantly lower in orchard B than in orchards A and C (2011: orchard A =392.56 ± 67.18

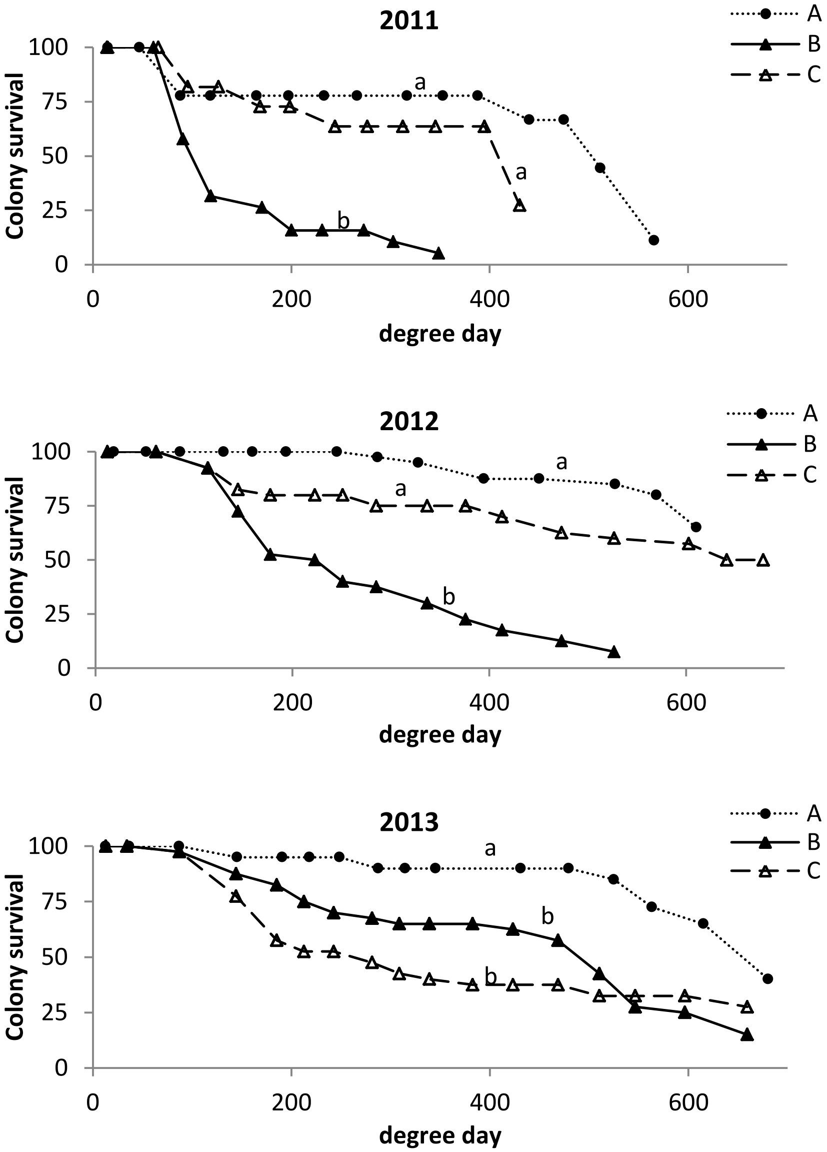


Fig. 2 Survival curves for *A. spiraecola* colonies tracked in three citrus orchards (A, B and C) during the spring leaf-flushing period of three consecutive years (2011-2013). Different letters indicate significant differences among orchards.

DD ± SE, B = 122.47 ± 20.68, C = 302.52 ±46.39, $F_{2, 36}$ = 13.07; $P <$ 0.001; 2012: orchard A = 557.75 ± 16.33, B = 238.70 ± 22.46, C = 491.43 ± 37.07, $F_{2, 117}$ = 39.63; $P < 0.001$). In 2013, colony longevity was significantly lower in orchards B (404.26 ± 31.86 DD ± SE) and C (333.48 ± 38.13) than in orchard A (574.55 ± 24.20) ($F_{2, 117}$ = 15.08; $P <$ 0.001).

3.3.1.3. Colony phenology

In 2011, the percentage of winged aphids before 250 DD was higher than ~40% of the total aphid forms only in orchard A (Fig. 3). In 2012, this percentage was reached before 250 DD in orchards A and C. Finally, in 2013, the percentage of winged aphids before 250 DD was higher than ~40% in the three orchards.

Fig. 3. Figure in the next page. Colony phenology. Percentage of *A. spiraecola* stages (nymphs, winged nymphs, apterous adults and winged adults) recorded in colonies tracked in three clementine orchards (A, B and C) during the spring leaf-flushing period of three consecutive years (2011-2013).

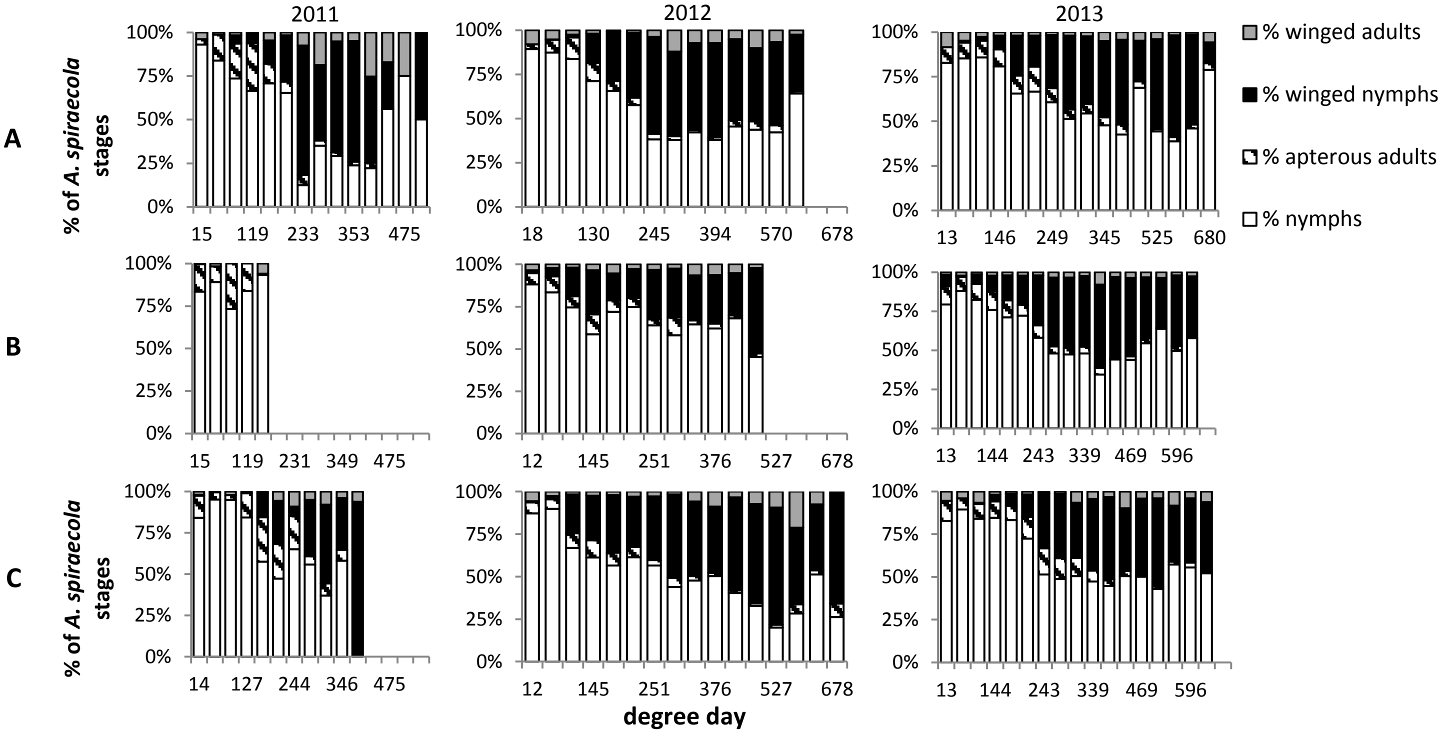


Fig. 3 Find the caption in the previous page.

3.3.2. Aphid predators

3.3.2.1. Abundance of aphid predators

A total of 88, 82 and 67 predator individuals were recorded in the *A. spiraecola* colonies tracked in 2011, 2012 and 2013, respectively. The gall midge *A. aphidimyza* was the most abundant aphid predator, followed by hoverflies and lacewings (Table 1). All of these individuals except one adult lacewing were immature and included eggs, larvae and pupae. Some of the observed eggs of these predators hatched without subsequent observation of larvae in the following tracking days. Nevertheless, predation was detected in these colonies between the observation of predator eggs and their disappearance, as the number of aphids decreased between samplings and we observed preyed aphids. Spiders of genus *Theridion* and the earwig *F. auricularia* were also observed to feed on *A. spiraecola* colonies. The earwig was present in only two orchards in 2013. When we pooled all data together, the number of predators per colony did not significantly differ by orchard in 2012 ($F_{2,\ 117} = 0.03$; $P = 0.97$) and 2013 ($F_{2,\ 117} = 1.48$; $P = 0.23$). In 2011, only one colony was attacked by a *Theridion* sp. in orchard A, and it was attacked at the end of the tracking period. Therefore, orchard A was excluded from this analysis and the following analysis in 2011. Orchards B and C did not significantly differ in 2011 ($F_{1,\ 28} = 3.95$; $P = 0.53$).

Table 1 Number of predators (mean ± SE) per colony recorded in *A. spiraecola* colonies tracked in three citrus orchards (A, B and C) during the spring leaf-flushing period of three consecutive years (2011-2013).

Predators	**2011**			**2012**			**2013**			
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**Total**
Diptera										
Aphidoletes aphidimyza	0	1.79 ± 0.93	3.27 ± 1.35	0.20 ± 0.18	0.40 ± 0.27	0.30 ± 0.12	0.03 ± 0.03	0.25 ± 0.12	0.35 ± 0.17	**6.59**
Syrphidae	0	0.16 ± 0.12	0.09 ± 0.09	0.20 ± 0.07	0.15 ± 0.06	0.08 ± 0.04	0.08 ± 0.04	0.08 ± 0.04	0.03 ± 0.03	**0.85**
Neuroptera										
Chrysopidae	0	0.11 ± 0.07	0.09 ± 0.09	0.08 ± 0.06	0.03 ± 0.03	0.20 ± 0.18	0.10 ± 0.05	0.05 ± 0.03	0.08 ± 0.04	**0.72**
Coneopterygidae	0	0	0	0	0.05 ± 0.03	0	0	0	0	**0.05**
Araneae										
Theridion sp.	0.11 ± 0.11	0.16 ± 0.08	0.09 ± 0.09	0	0.08 ± 0.04	0.05 ± 0.03	0.03 ± 0.03	0.05 ± 0.03	0.03 ± 0.03	**0.58**
Dermaptera										
Forficula auricularia	0	0	0	0	0	0	0	0.35 ± 0.28	0.13 ± 0.09	**0.48**
Coleoptera										
Coccinellidae	0	0	0	0.20 ± 0.08	0.03 ± 0.03	0.03 ± 0.03	0.08 ± 0.04	0	0	**0.33**
Ragonycha fulva Scopoli	0	0.32 ± 0.13	0	0	0	0	0	0	0	**0.32**
Total	**0.11 ± 0.11**	**2.53 ± 0.91**	**3.55 ± 1.43**	**0.68 ± 0.21**	**0.73 ± 0.28**	**0.65 ± 0.20**	**0.30 ± 0.08**	**0.78 ± 0.31**	**0.60 ± 022**	

3.3.2.2. Attack ratio and time of the first attack by aphid predators

Of the 279 *A. spiraecola* colonies tracked over the three years of study, 97 were attacked by aphid predators. Of these attacked colonies, 20 colonies were attacked by more than one group of predators. The attack ratio by predators varied between 0.30 and 0.55, but this value did not significantly differ by orchard in any year (2011: $F_{1,\,28} = 0.029$, $P = 0.86$; 2012: $F_{2,\,117} = 0.24$, $P = 0.78$; 2013: $F_{2,\,117} = 0.12$, $P = 0.88$) (Fig. 4A).

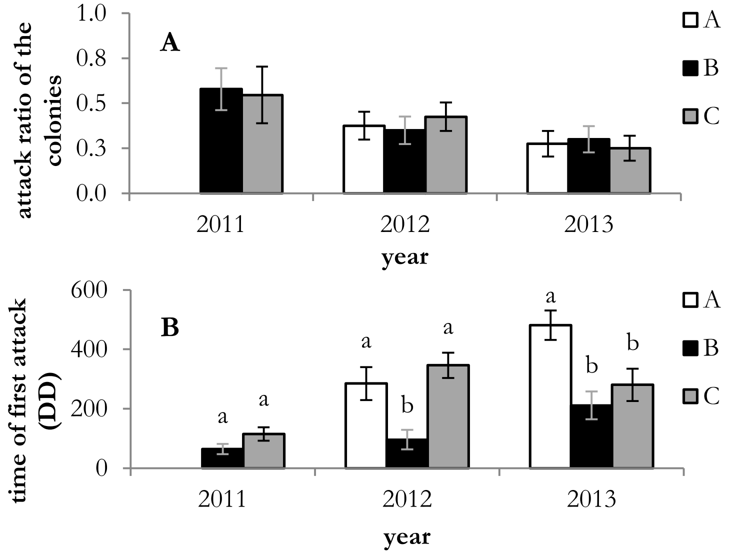


Fig. 4 Attack by predators on *A. spiraecola* colonies tracked in three citrus orchards (A, B and C) during the spring leaf-flushing period of three consecutive years (2011-2013). **A**. Attack ratio of the colonies (mean ± SE). **B**. Time of first attack (DD) (mean ± SE) for attacked colonies (2011: B = 11 colonies, C = 6; 2012: A = 15, B = 14, C = 17; and 2013: A = 11, B = 12, C =10). Different letters indicate significant differences among orchards.

Unlike the ratio of attacked colonies, the mean time of the first attack by predators differed among orchards in 2012 and 2013 when we analysed attacked colonies. First, the attack occurred significantly earlier in orchard B (~100DD) than in orchards A and C in 2012 (~300 DD) ($F_{2, 43} = 8.28$; $P < 0.001$) (Fig 4B). In 2013, the first attack occurred significantly earlier in orchards B and C (~300 DD) than in orchard A (~480 DD) ($F_{2, 30} = 8$; $P = 0.0016$). The time of attack did not significantly differ between orchards B and C in 2011 ($F_{1, 15} = 3.15$; $P = 0.096$).

3.3.3. Effect of the first attack by predators on colony parameters and damages

To estimate the effect of predators on the life parameters of *A. spiraecola* colonies, we correlated the ratio of attacked colonies and the mean time of the first attack by predators with the maximum number of aphids per colony and orchard as well as their mean longevity (DD). Both colony parameters were positively correlated with the mean time of first attack by predators (maximum number of aphids per colony and orchard: $F_{1, 7} = 14.70$; $P = 0.0064$) (colony longevity: $F_{1, 7} = 19.92$; $P = 0.0029$) (Fig. 5).

Finally, the mean time of the first attack by predators in each orchard was positively correlated with the maximum percentage of occupied shoots ($F_{1, 7} = 32.17$; $P < 0.001$) (Fig. 6). The percentage of occupied shoots exceeded the economic threshold when the first attack occurred after 200 DD. Similarly, the ratio of attacked colonies prior to 200 DD negatively correlated with the mean maximum percentage of occupied shoots per orchard ($F_{1, 7} = 6.8$; $P = 0.035$; $R^2 = 0.49$) (Fig. 6).

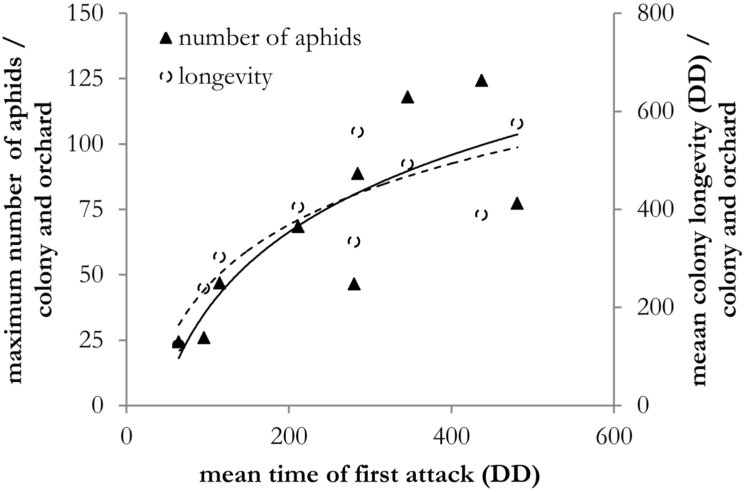


Fig. 5 Relationship between the life parameters of *A. spiraecola* colonies [maximum number of aphids and colony longevity measured in degree days (DD)] and the time of the first attack by predators (DD) in three citrus orchards (A, B and C) during the spring leaf-flushing period of three consecutive years (2011-2013). Points represent the mean for each orchard and year. Maximum number of aphids per colony = 158.92 + 42.51 * log (mean time of first attack); R^2 = 0.67; continuous line. Colony longevity = -586.62 + 180.29 * log (mean time of first attack); R^2 = 0.74; dotted line.

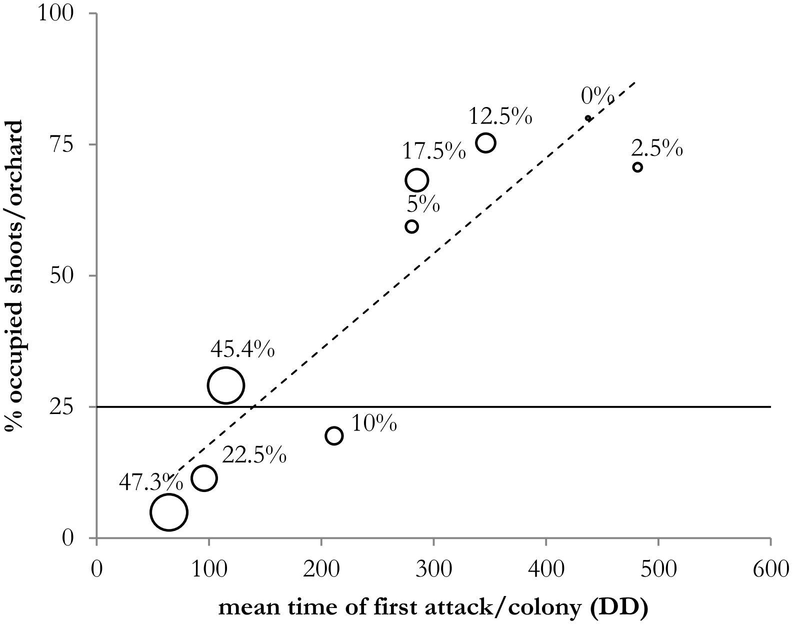


Fig. 6 Relationship between the percentages of shoots occupied by *A. spiraecola* and predator attack in three citrus orchards (A, B and C) during the spring leaf-flushing period of three consecutive years (2011-2013). Each bubble represents the maximum percentage of shoots occupied in each orchard and year and the size of the bubble (and the number above) represents the percentage of shoots attacked before 200 DD. Maximum percentage of occupied shoots per orchard *vs* mean time of first attack by predators ($R^2 = 0.67$; $F_{1,7} = 14.14$; $P < 0.001$; (spotted line) $Y = 67.15 - 1.42X$). Maximum percentage of occupied shoots per orchard *vs* percentage of *A. spiraecola* colonies attacked by predators before 200 DD ($R^2 = 0.49$; $F_{1,7} = 6.8$; $P = 0.035$; (not represented) $Y = 68.27 - 1.20X$).

3.4. Discussion

This study presents the life parameters of *A. spiraecola* colonies (maximum number of aphids, survival and colony phenology) in three clementine orchards in eastern Spain between 2011 and 2013 and relates them to the presence of predators. All of these life parameters considerably varied among orchards over the three years, and this variation depended, among other non-measured reasons, on the time that predators arrived to the aphid colony, measured herein as the time of first attack (Degree Day).

Predators attacked approximately 35% of the *A. spiraecola* colonies tracked in our study at some point of their lifespan. Interestingly, the attack ratio by predators was similar in the three orchards, except in orchard A in 2011. This orchard experienced severe chemical application until the end of 2010, which could explain the lack of predators observed in 2011. Nevertheless, the high attack ratios and their low variability among orchards were expected because a complex of predators usually attacks *A. spiraecola* after the aphid population peaks (Hermoso de Mendoza *et al.* 2012). Therefore, the low variability of the attack ratio by predators among orchards cannot explain the variability of the life parameters of the *A. spiraecola* colonies tracked in our study. For example, the attack ratio by predators in 2012 was 0.4 in the three orchards, but the maximum number of aphids per colony was four- to five-fold higher in orchards A and C than in orchard B. Similar to the attack ratio, the number of predators that attacked the colonies during their lifespan was equal among the orchards over the three years.

During our tracking, we also identified the species/genus of the predator that attacked *A. spiraecola* colonies. *A. aphidimiza* was the most abundant predator in 2011, but it appeared at the same relative abundance as the remaining predators during the following years. Therefore, we could not identify a key predator or group of predators to relate their abundance to the life parameters of *A. spiraecola* colonies. In general, we identified two groups of predators: those described as aphidophagous in numerous references and those in which its role as aphidophagous predators were poorly described, called polyphagous in this study. Among the former, *A. aphidimyza,* syrphids and chrysopids were the most abundant. These predators have been previously documented in citrus (Michelena and Sanchís, 1997; Kavallieratos *et al.* 2004; Hermoso de Mendoza *et al.* 2012), but their impact on *A. spiraecola* either alone or as a complex was unknown. Michelena and Sanchís (1997) also recorded the coccinellids *C. septempunctata* L. (Coleoptera; Coccinellidae) and *Scymnus* sp. In our orchards, coccinellids appeared only sporadically. We also identified two polyphagous predators that actively preyed on *A. spiraecola* colonies: the spiders *Theridion* sp. and the earwig *F. auricularia*. Theridion spiders surrounded *A. spiraecola* colonies with their webs and consumed the entire colony. Spiders of this genus are common in Spanish citrus (Barrientos *et al.* 2010), and they were also present in our orchards. In contrast, the earwig *F. auricularia* was only present in two orchards in 2013. Piñol *et al.* (2009) described the importance of polyphagous sedentary predators, such as earwigs, in aphid populations of citrus, despite the fact that they are not specialized natural enemies. This importance arises because low densities of sedentary predators can strongly affect the final aphid density, as they prey on small populations at the beginning of the aphid spring season, when the per capita effect on the aphid population is higher. The same effect may be attributed to *Theridion* spiders, which are present in citrus throughout the year (Barrientos *et al.* 2010).

The importance of the presence of aphidophagous predators at the beginning of aphid colonization has been documented in other crops (Tenhumberg and Poehling, 1995; Brown, 2004). Coccinellids and syrphids have evolved mechanisms to oviposit preferentially in colonies that are in the early stage of development and avoid those that are already attacked. They tend to oviposit only during this short "egg window" in aphid colonies (Ito and Iwao, 1977; Kan and Sasakawa, 1986; Chambers, 1991; Hemptinne *et al.* 1992; Hemptinne *et al.* 1993; Hemptinne *et al.* 2001). Therefore, these aphidophagous predators should be present in the field to improve the biological control of *A. spiraecola* in citrus when these "egg windows" are abundant, i.e., during the initial phase of aphid increase in the spring leaf-flush.

Our data reflect the importance of the early presence of either aphidophagous or polyphagous predators to control *A. spiraecola* in citrus clementines. Early attacks by predators affected the life parameters and the damages produced by *A. spiraecola* colonies. Specifically, the complex of predators controlled *A. spiraecola* when they attacked the colonies prior to 200 DD from the beginning of the aphid colonization (~10 *A. spiraecola* /colony and ~6% of occupied shoots per orchard). Hermoso de Mendoza *et al.* (2006) defined the intervention threshold as when more than 25% of the shoots were occupied by *A. spiraecola* colonies. Our data showed that the percentage of shoots occupied by *A. spiraecola* remained below or close to the intervention threshold when colonies were attacked by predators prior to ~200 DD. Furthermore, the percentage of colonies attacked by predators early in the aphid season (at ~200 DD) and the maximum percentage of shoots occupied by *A. spiraecola* during the season were negatively correlated. The percentage of shoots occupied by *A. spiraecola* remained below the intervention threshold when more than ~35% of the colonies had been attacked by predators prior to 200 DD from the beginning of the aphid colonization. Therefore, 200 DD of the aphid colony life is the "window of opportunity" to control *A. spiraecola* populations by predators. This presence of predators during this "window of opportunity" could be used to develop new thresholds that account for this window.

The early attacks of predators on *A. spiraecola* colonies affected not only the dynamic of the colonies (maximum and longevity) but also their phenology. *A. spiraecola* colonies that contained a low proportion of winged aphids were more abundant in orchards where predators attacked the colonies early in the season. Aphids exhibit a polymorphism whereby individual aphids are either winged or un-winged. The number of winged morphs has long been known to be associated with colony crowding (Wadley, 1923; Johnson, 1965), the nutritional status of plants (Wadley, 1923; Evans, 1938; Mittler and Dadd, 1966; Dixon and Glen, 1971), and the attack by predators on the colony (Dixon and Agarwala, 1999; Weisser *et al.* 1999). The induction of winged morphs by predators has significant implications for potential biological control strategies. Aphids respond to the presence of a predator by producing the dispersal morph (winged), which can escape by flight to colonize other plants and enable aphids to leave plants when the mortality risk is high (Weisser *et al.* 1999). Contrary to expectations, we found that orchards in our study with early attacks by predators contained *A. spiraecola* colonies with a low proportion of winged forms. We hypothesize that predators might have consumed the colony before crowding induced winged morphs or their presence might have not induced winged morphs. Interestingly, the most abundant predator in the observed colonies was *A. aphidimyza*, and this "furtive predator" does not generate alarm in aphid colonies (Lucas and

Brodeur, 2001). Furtive predators, such as the predatory midge *A. aphidimyza*, prey on aphids while simultaneously residing and remaining undetected within their colonies (Lucas and Brodeur, 2001).

Obviously, our sampling protocol may have underestimated the relative abundance of predators and their attack on the *A. spiraecola* colonies because some predator species are nocturnal and others might have attacked the colonies and departed within our 48-72 h tracking period. The relative abundances of coccinellids, syrphids and chrysopids may have been underestimated due to their higher mobility and/or nocturnal activity compared to cecidomids and Theridion spiders (Chandler 1969; Wilbert 1973; Bargen *et al.* 1998; Brown and Schmitt, 2001; Schmidt *et al.* 2008). These underestimations might explain the high variability found in the correlation between the life parameters of the *A. spiraecola* colonies (maximum number of aphids and longevity per colony) and the time of first attack by predators. For example, some colonies might have suffered undetected attacks and remained small during their lifespan. Conversely, some recorded attacks did not consume all aphids of the colony, and the colony therefore had a long lifespan.

In conclusion, the problem with citrus clementines, as in many crops, is the poor synchronization between aphids and their predators (Van Emden and Harrington, 2007). Predators should attack *A. spiraecola* colonies early in the population growth curve of the aphids. In detail, predators should attack the colonies prior to 200 DD (~7-10 days) from the onset of aphid colonization (~10 aphids /colony). To increase the number of attacks in this narrow "window of opportunity", biological control practitioners may manipulate the agricultural ecosystems. One of these manipulations could be the use of interline cover crops in citrus. This practice has been shown to have advantages in controlling aphids in other crops (Brown and Glenn, 1999; Landis *et al.* 2000; Schmidt *et al.* 2007; Danne *et al.* 2010; Schmidt *et al.* 2011). In clementine mandarins, the use of *Festuca arundinacea* Schreb. (Poales; Poaceae) as a cover crop has been recommended for several agronomic reasons (Aguilar-Fenollosa *et al.* 2011a; Aguilar-Fenollosa *et al.* 2011b; Aguilar-Fenollosa *et al.* 2011c; Monzó *et al.* 2011). This cover crop could be used to promote the presence of predators at the beginning of the season and improve aphid control if i) it harbors olyphagous aphid species that do not attack citrus; ii) these specific cover aphids are present in the orchard before the appearance of *A. spiraecola*; and iii) these aphids promote the early presence of predators in orchards and their migration to citrus spring shoots during the "window of opportunity," i.e., 200 DD.

The role of phenology and the diversity of natural enemies and their food resources in driving agroecosystems food webs are only poorly understood, despite the central role that these dynamics play (Welch and Harwood, 2014). To promote sustainable management practices in conservation and biological control, we must focus on the impacts of temporal dynamics to find the optimum "window of opportunity" for each agroecosystem and pest. In addition, researchers must contribute with support tools for the producers to identify these "windows of opportunity" and to quantify the favourable conditions of its crops, which could help to decide among pest management strategies.

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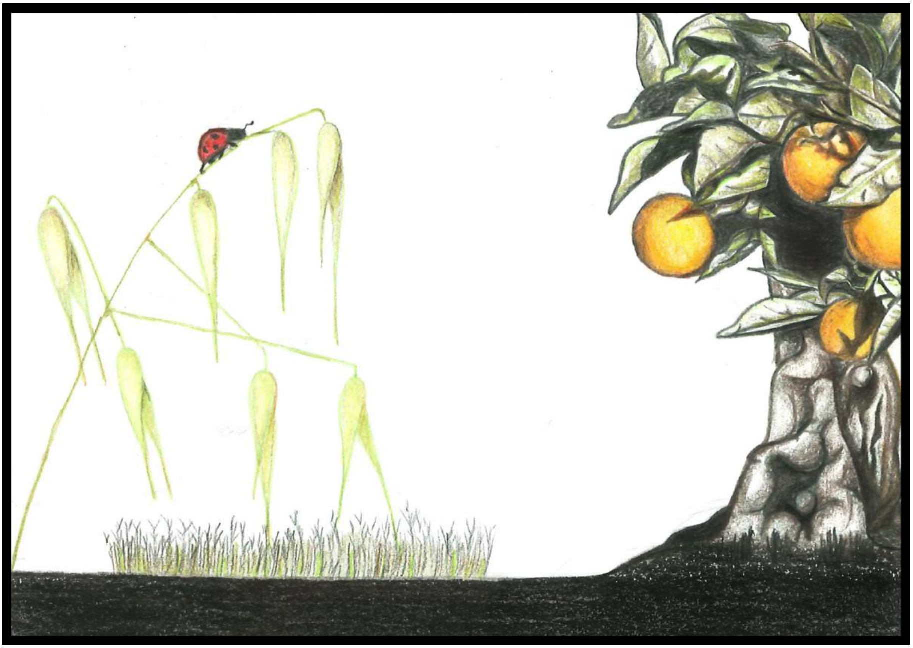
CHAPTER IV

A sown cover with wild plants improves the biological control of aphids in citrus



4. A sown cover with wild plants improves the biological control of aphids in citrus.

Gómez-Marco, F., Urbaneja A., Tena A. A sown cover with wild plants improves the biological control of aphids in citrus.

Abstract

There is increasing interest in the use of sown ground covers in agriculture to provide alternative resources to predators and parasitoids as part of conservation biological control. Nevertheless, there is limited evidence that this approach is effective in commercial orchards, where a wild complex of plants coexists with the sown plant species. In citrus orchards, ground covers with Poaceae plants were originally promoted to prevent soil erosion. Herein, we analyzed the effect of this sown ground cover on the biological control of *Aphis spiraecola* Patch (Hemiptera: Aphididae), the main aphid pest on citrus. We therefore first described the ground cover plant composition and their inhabiting aphids in four commercial citrus orchards. Second, we compared the presence of *A. spiraecola* and its natural enemies between these and four other commercial orchards with bare soil. While Poaceae plants represented ~66% of the ground cover, the rest of the cover comprised mainly *Malva* sp. (13%), *Oxalis* sp. (5%) and *Sonchus* sp. (2%). Poaceae plants and *Oxalis* sp. harbored stenophagous aphids and *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae), respectively, which appeared sooner in the system than citrus aphids. These aphids may serve as alternative

prey/hosts for natural enemies, thus could enhance the biocontrol of *A. spiraecola*. By contrast, *Malva* sp. and *Sonchus* sp. harbored the potential citrus pest *Aphis gossypii* Glover and other aphids that appear simultaneously with *A. spiraecola*. Therefore, by attracting them to the cover, this latter group could relieve the attack of natural enemies on *A. spiraecola* in the canopy. Although these wild plants may act as reservoirs for *A. spiraecola* as well as other aphid species that can disrupt the biocontrol services of natural enemies, overall, the sown cover was effective in terms of biological control of *A. spiraecola* in the citrus canopy. It promoted the early presence of predators in citrus canopies but did not promote the early presence of parasitoids. Predators attacked *A. spiraecola* colonies before their exponential increase. These attacks resulted in satisfactory aphid control, because citrus orchards with ground cover never exceeded the aphid economic threshold.

4.1. Introduction

The aim of habitat management in conservation biological control is to create a suitable ecological infrastructure to favor natural enemies and to enhance biological control in agricultural systems (Landis *et al.*, 2000; Fiedler *et al.*, 2008). In monoculture agroecosystems, natural enemies suffer from a lack of food for adults, alternative prey or hosts, and shelter against adverse conditions (Landis *et al.*, 2000; Heimpel and Jervis, 2005). In the absence of these vital resources, colonization of crops by predators and parasitoids is often much lower than colonization by herbivores (Altieri and Whitcomb, 1979; Thies and Tscharntke, 1999). An extensively researched form of habitat management that favors natural enemies in tree crops is the use of ground covers (Altieri *et al.*, 1977; Altieri and Whitcomb, 1979; Haley and Hogue, 1990; Maredia *et al.*, 1992; Liang and Huang, 1994; Smith *et al.*, 1996; Brown and Glenn, 1999; Rieux *et al.*, 1999; Landis *et al.*, 2000; Frechette *et al.*, 2008; Danne *et al.*, 2010; Silva *et al.*, 2010; Marko *et al.*, 2013). In the last ten years, ground covers based on Poaceae plants have been cultivated with citrus trees both for agronomic reasons (Fibla Queralt *et al.*, 2000; Aucejo, 2005) and because it facilitates the management of the two-spotted spider mite *Tetranychus urticae* Koch (Prostigmata: Tetranychidae), a key pest in clementines, by both bottom-up and top-down regulation mechanisms (Aguilar-Fenollosa *et al.*, 2011a, b). In addition, ground cover management could also enhance the presence of generalist ground-dwelling predators, which can prey on citrus pests inhabiting, the soil such as the Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Monzó *et al.*, 2009; Monzó *et al.*, 2011).

Aphis spiraecola Patch (Hemiptera: Aphididae) is a key pest of Clementine mandarins, *Citrus clementina* Hort. ex Tan. (Geraniales: Rutaceae), in the

Mediterranean basin (Hermoso de Mendoza *et al.*, 2006; Tena and Garcia-Marí, 2011; Hermoso de Mendoza *et al.*, 2012; Vacante and Gerson, 2012). This polyphagous aphid colonizes young, tender clementine shoots in spring (Hermoso de Mendoza *et al.*, 2006) and causes economic losses because it sucks sap, serves as a vector for Citrus tristeza virus, excretes large amounts of honeydew and curls developing leaves while the colony population is growing (Hermoso de Mendoza *et al.*, 2006). To improve the management of aphids in clementines, Hermoso de Mendoza *et al.* (2006) established intervention thresholds based on the percentage of infested shoots within a 0.25 m^2 ring throw, twice per tree, on the outer canopy of trees. An insecticide application is justified when more than 25% of the shoots are infested. Hereinafter, we refer to the time period during which the percentage of infested shoots reaches approximately 20 to 25%, as the critical period for the management of *A. spiraecola* on clementines.

Citrus, as a permanent and perennial crop, provides an environment in which numerous predators and parasitoids of *A. spiraecola* readily develop in the spring (Cole, 1925; Michelena and Sanchís, 1997; Alvis *et al.*, 2002; Alvis, 2004; Kavallieratos *et al.*, 2004; Hermoso de Mendoza *et al.*, 2012; Romeu-Dalmau *et al.*, 2012; Vacante and Gerson, 2012; Gómez-Marco *et al.*, 2015a; Gómez-Marco *et al.*, 2015b). Despite this abundant and diverse complex of natural enemies, biological control of *A. spiraecola* is generally insufficient because of the asynchrony of predators with aphid population growth (Hermoso de Mendoza *et al.*, 2012; Gómez-Marco *et al.*, 2015a) and the lack of effective parasitoids (Gómez-Marco *et al.*, 2015b). Recently, it has been demonstrated that predators can maintain aphid densities under the economic threshold if they arrive early in the season, from seven to ten days after *A. spiraecola* colonizes the spring shoots (Gómez-Marco *et al.*, 2015a). Therefore, we hypothesize that a ground cover that promotes the early establishment of natural enemies, prior to the exponential increase of the aphid (Gómez-Marco *et al.*, 2015a), might facilitate the biological control of *A. spiraecola* in citrus orchards.

To advance the presence of the natural enemies of *A. spiraecola* in citrus canopies, a ground cover based on Poaceae plants must possess certain key features. For example, the cover should harbor alternative prey or host species, such as other aphids in the appropriate time lag (Wyss, 1995; Welch and Harwood, 2014). This means at the end of winter or early spring, before *A. spiraecola* infests and damages clementine spring shoots (Gómez-Marco *et al.*, 2015a). On the other hand, this ground cover should not benefit *A. spiraecola* or other citrus pests, especially *Aphis gossypii* Glover (Hemiptera: Aphididae). In the case of a ground cover based on Poaceae plants, it is known that *A. spiraecola* does not feed on this plant species (Holman, 2009). However, sown ground covers also contains a complex of spontaneous plant species (Kruidhof *et al.*, 2008), which might reduce the efficacy of the ground cover if they harbor the target pest or reduce the use of pest aphids by natural enemies in the crop.

In this study, we first identified and quantified i) the complex of weeds that accompanied sown ground covers based on Poaceae plants as well as ii) the aphid species inhabiting these plant species in four citrus orchards with ground covers. We then tested iii) whether this aphid community enhanced the presence of natural enemies in citrus canopies before *A. spiraecola* infestation and iv) whether it reduced the damage due to aphids (Fig. 1). To do this, we compared the presence of natural enemies and the damage caused by *A. spiraecola* in orchards with and without ground cover (bare soil), the most common weed management practice in citrus orchards in Spain.

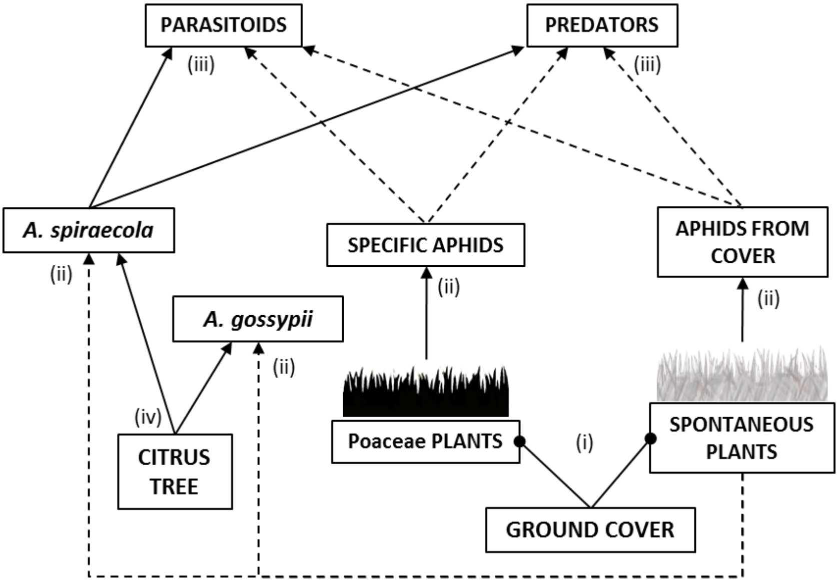


Fig. 1 The aphid food web associated with citrus orchards with a sown ground cover. The ground cover is composed by the sowed Poaceae plants and the spontaneous plants. We hypothesized that the ground cover may host aphids which act as alternative prey for parasitoids and predators. Lines with arrows indicate interaction between groups of different trophic levels. Continuous lines indicate confirmed interactions and discontinuous lines indicate unknown interactions. Letters in brackets refer to the objectives exposed in the Introduction section (page 136).

4.2. Material and methods

4.2.1. Orchards

The study was carried out in eight citrus orchards (*C. clementina* Hort. ex Tan. cv. Clementine grafted on citrange Carrizo rootstock) located in the

Valencia region of eastern Spain (Table 1). The climate of the region is classified as warm-temperate subtropical with an annual mean temperature of 16.42 °C and rainfall of 458.09 mm (average of data from 2000 to 2013) (SIAR, 2014). The orchards "PPbs", "PL", "PS" and "TA" had bare soil (following the application of herbicides since February 11/14) whereas the orchards "AU", "CD", "CU" and "PP" had a sown ground cover crop constituted by a combination of grassy plants (*Festuca arundinacea*, *Poa* sp., *Bromus* sp., etc.) and a complex of wild plants (Fig. 2). Cover crops had more than five years and they were mowed twice per year: once at the end of winter (first two weeks of February) and again in early summer (first two weeks of June). "AU" and "TA" were surrounded totally by other citrus orchards and the rest were surrounded mostly by citrus orchards and one of their sides delimited with a semi-natural habitats. No aphicides were applied during the sampling period. During the last 4 years, all orchards followed IPM guidelines (Urbaneja *et al*, 2014) and were drip irrigated. Citrus size and vigor was similar throughout the orchards with no apparent effect of inter-row cover crop. Orchards were sampled and/or tracked weekly (depending on the season) from mid-February to early May, when *A. spiraecola* populations decline at the end of the leaf-flushing period (Fig. 3).

4.2.2. Ground cover sampling

The plant and aphid complex present in the ground cover of the four orchards was estimated weekly from February 1 to March 27 and once during the following critical period. The critical period in *A. spiraecola* management (April 22) is defined below in the "*Citrus canopy sampling*" section.

To determine the percentage of ground cover coverage and describe its plant composition, a ring of 0.25 m^2 was randomly thrown 10 times on the ground cover, and the percentage of ground cover inside each ring was visually estimated. Plants were subsequently identified to genus level and the percentage of each plant genus inside the ring was also visually estimated.

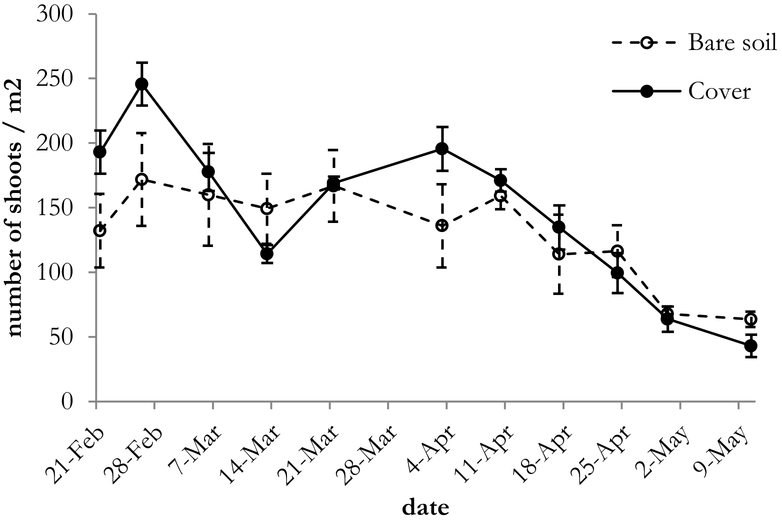


Fig. 2. Mean number of citrus shoots per m^2 (± SE) in four commercial orchards with bare soil management (discontinuous line) and four commercial orchards with cover crop management (continuous line) in the spring of 2013.

To identify and quantify the aphids present in the ground cover, 0.01 m^2 (measured with a loose-leaf ring, 8 cm in diameter) of each plant genera present within the ring was randomly selected, mowed and transported to the laboratory in a plastic bag for aphid identification. A total of 964 ground cover samples were collected (Table 1). In the lab, plants were examined in detail to separate and identify all of the aphids inhabiting the plants. Aphids were preserved in 70% ethanol, and adult aphids were identified to the species level (Blackman and Eastop, 1994; A. Hermoso de Mendoza, 1996).

To calculate the number of aphids per m^2 of ground cover for each date and sample, we considered not only the number and species of aphids recorded in our sample (0.01 m^2) but also the surface occupied by each plant genus in the ground cover. We assumed that aphids were uniformly distributed within the area occupied by each plant genus. The number of aphids per plant species was calculated as follows:

$$N = P_c * (X_a/0.01m^2)$$

Where N is the number of aphids per plant species, P_c is the percentage of ground cover occupied by each plant genus in one ring, and X_a is the number of aphids recorded in the ground cover sample (0.01 m^2) collected for each plant genus.

To analyze the seasonal trends of the aphid species identified, we divided the sampling period into three months: February, March and April. These divisions were delineated in accord with the three most important periods in citrus orchards. February is the month when the food webs related to citrus-infesting aphids may be relatively quiescent. March, is likely when aphid foods webs restart because the presence of aphids promotes trophic cascades. Finally, April is the period during which citrus-infesting aphid populations increases in size.

4.2.3. Citrus canopy sampling

Following recommendations from Hermoso de Mendoza *et al.* (2006), we calculated the percentage of the infested shoots by *A. spiraecola* weekly in the eight orchards (four with ground cover and four with bare

Table 1 Location, crop area and cover management characteristics of the eight commercial orchards included in this study. The location is referenced in the Universal Transverse Mercator coordinate system (UTM). Number of ground cover samples, *A. spiraecola* colonies counted and total number of natural enemies counted per orchard.

Orchard code	Cover	Location	Crop area	Cover samples	Canopy samples	
					Colonies counted	Natural enemies in colonies
AU	yes	39°44'45.05"N - 0°14'40.87"W	1.35 ha	281	148	146
CD	yes	39°48'40.98"N - 0°14'28.28"W	1.46 ha	313	111	21
CU	yes	39°48'45.92"N - 0°14'29.95"W	0.26 ha	214	88	11
PP	yes	39°46'35.60"N - 0°16'03.11"W	0.74 ha	156	149	38
PPbs	no	39°46'34.53"N - 0°16'08.06"W	0.28 ha	0	94	7
PL	no	39°45'52.27"N - 0°15'20.64"W	0.53 ha	0	101	34
PS	no	39°45'47.09"N - 0°16'18.82"W	0.47 ha	0	47	27
TA	no	39°45'57.56"N - 0°14'14.58"W	0.81 ha	0	91	28

soil), from February 21 until *A. spiraecola* populations decline at the end of the leaf-flushing period (May 10). Ten trees were sampled per orchard, and the number of infested and non-infested shoots per ring was counted. In the rings in which aphids were located, we counted the total number of predators (all stages) and parasitized aphids (mummies) in two of the infested shoots within the ring (n=20 per orchard and date) to determine the ratio of colonies attacked and the cumulative number of predators and parasitoids per day.

4.2.4. Statistical analysis

The ratio of attacked colonies by natural enemies was compared between soil managements in two different time periods, first, taking into account the entire sampling period (February 21 to May 10) and secondly, considering the sampling dates before the critical period for *A. spiraecola* management in 2013 (February 21 to April 18). Both comparisons were analyzed with generalized linear models nested for orchard and date. We assumed binomial error variance for the ratio of attacked colonies. The statistical software package 'R' (http://www.R-project.org) and its packages "lme4" and "phia" were used in our analyses.

As an initial approach to comparing natural enemies (predators and parasitoids) in populations associated with both soil managements, mean cumulative predators/parasitoids-days (CPreD/CParD) were calculated for each orchard. CPreD and CParD were calculated as follows:

$$\sum \Delta_t \frac{(x_1 + x_2)}{2}$$

where Σ is the summation of overall sampling dates, Δ_t is the interval between two successive sampling dates (usually 7 days in this study) and x_1 and x_2 are predators/parasitoids counts on those dates. CPreD and

CParD values were plotted against time, and linear regressions models were fitted. Once the regression lines were fitted, the analysis of covariance (ANCOVA) allowed comparison of their slopes (which represent their population growth), thus testing the null hypothesis which is the assumption of the homogeneity of regression slopes. (population growth does not depend on the interaction between treatment and time) (McDonald, 2009).

Generalized linear models were used to determine the differences in aphid damage between soil managements by the percentage of infested shoots. The statistical software package 'R' (http://www.R-project.org) and its packages "nlme" were used in our analyses.

4.3. Results

4.3.1. Ground cover characterization

The mean percentages of ground cover coverage for each orchard at the beginning of the sampling period (February) were 59.6%, 74.4%, 54.8% and 33.4%, in AU, CD, CU and PP, respectively (see forward Fig. 4). This percentage decreased in the following two weeks in orchards CD and PP because the ground cover was mowed. At the end of March, the mean percentage of cover increased in all of the orchards, reaching approximately 90%. Finally, at the beginning of the critical period, the mean cover percentages were 91%, 76.5%, 67.3% and 96.5%, in AU, CD, CU and PP, respectively.

A total of 21 plant genera belonging to 10 families were identified in the ground cover of the four orchards sampled (Fig. 2). Plants from the Poaceae family were the most abundant, representing 66.14% of ground cover plants. The most widely distributed plant genera, all of which were found in every orchards, were the Poaceae genera *Bromus* sp., *Festuca* sp. and *Hordeum* sp. as well as the genera *Malva* sp., *Oxalis* sp. and *Sonchus* sp.. *Bromus* sp. was the only plant genus that persisted in all of the orchards at all dates. Orchard "CD" had the highest number of plant genera (17 cataloged genera), whereas orchard "CU" had the lowest number of plant genera (9 genera).

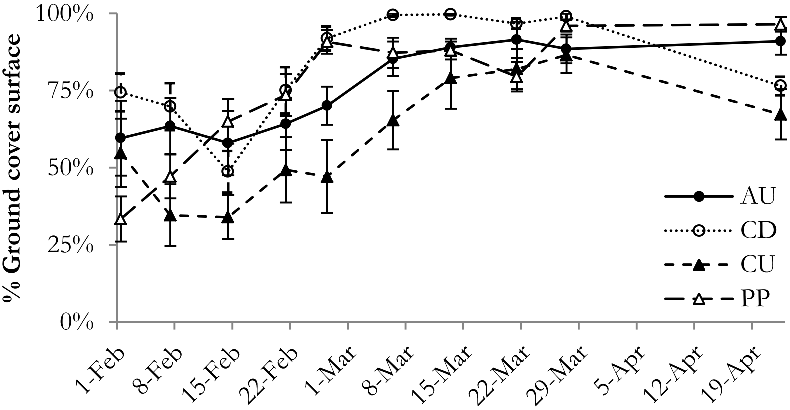


Fig. 3 Mean percentage (± SE) of soil surface covered by a ground cover in four citrus orchards before *Aphis spiraecola* infestation period.

4.3.2. Aphid community in the ground cover

4.3.2.1. Quantitative analysis

Out of the 964 ground cover samples collected, 262 (27.18%) contained aphids. 1,843 aphid specimens were extracted from these samples, with a mean of 1.91 ± 0.38 aphids per sample. The mean number of aphids per m^2 of ground cover was 14.55 ± 2.42 (aphids / m^2) in the 73.73 m^2 of ground cover analyzed. The mean number of aphids per m^2 of ground cover was constant until March 27 in the four orchards, except the two first weeks in orchard "CD", where the number of aphids was higher (Fig. 5). Finally, the number of aphids per m^2 increased in all of the orchards at the beginning of the critical period (April 17).

4.3.2.2. Qualitative analysis

Of the 1,843 aphids extracted from the ground cover plants, 237 were adults, and 158 of these were identified to the species level. In order of abundance, these species were *Aphis gossypii* Glover (n=35), *Uroleucon sonchi* L. (n=32), *Sitobion fragariae* Walker (n=28), *Hyperomyzus lactucae* L. (n=19), *Rhopalosiphum padi* L. (n=19), *Aphis spiraecola* Patch (n=14), *Macrosiphum euphorbiae* Thomas (n=7) and *Myzus persicae* Sulzer (n=4). *A. gossypii*, *H. lactucae*, *R. padi* and *S. fragariae* were present in the ground cover of the four orchards sampled (Fig. 5, Table 2). By contrast, *M. euphorbiae* and *U. sonchi* were identified in only two orchards (both in "AU" and "CD").

To analyze the seasonal trends of the aphid species identified, we divided the sampling period into three intervals: February sampling dates, March and April (Fig. 5). The aphid community collected during the sampling period differed among orchards. In February, *R. padi*, a specific aphid to monocotyledons plants (as the Poaceae family), was the most abundant species in all of the orchards except "PP", where *A. gossypii* was the only identified aphid species. In March, the number of aphid species increased in all orchards, and citrus aphids (*A. spiraecola* and *A. gossypii*) were abundant only in orchard "PP". Finally, during the critical period, citrus-infesting aphids were the most abundant in the ground cover of

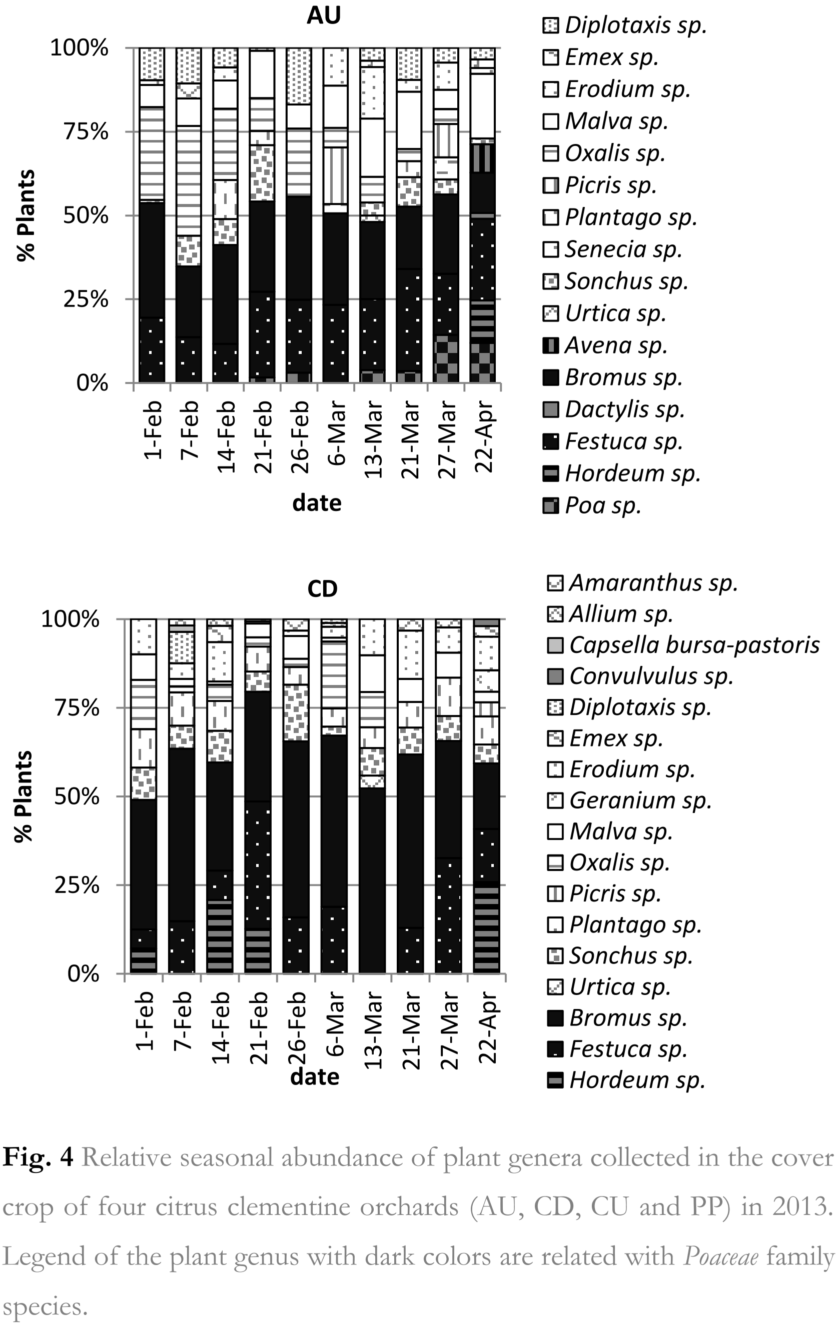
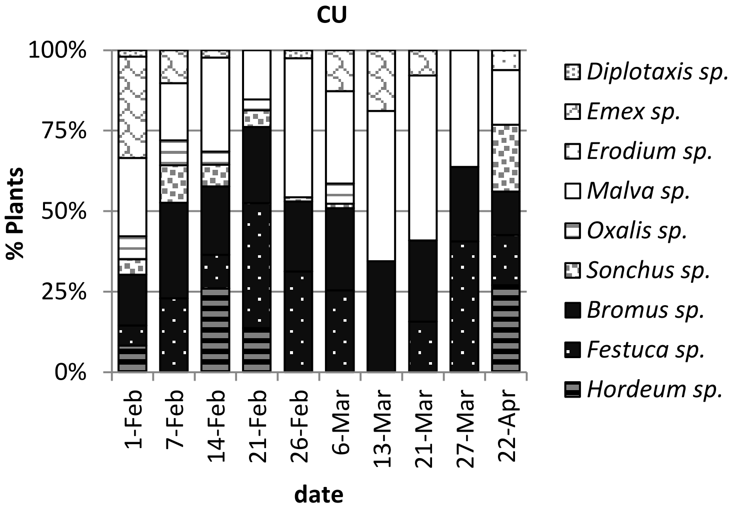
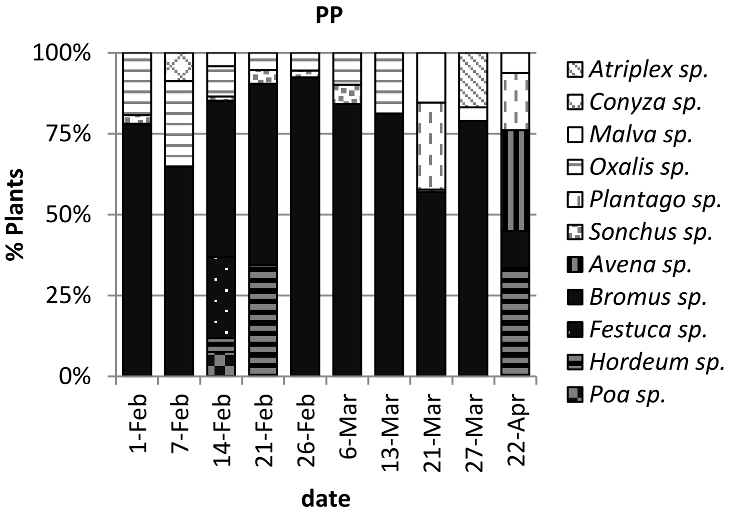


Fig. 4 Relative seasonal abundance of plant genera collected in the cover crop of four citrus clementine orchards (AU, CD, CU and PP) in 2013. Legend of the plant genus with dark colors are related with *Poaceae* family species.


CU
% Plants
100%
75%
50%
25%
0%
1-Feb
7-Feb
14-Feb
21-Feb
26-Feb
6-Mar
13-Mar
21-Mar
27-Mar
22-Apr
date
Diplotaxis sp.
Emex sp.
Erodium sp.
Malva sp.
Oxalis sp.
Sonchus sp.
Bromus sp.
Festuca sp.
Hordeum sp.


PP
% Plants
100%
75%
50%
25%
0%
1-Feb
7-Feb
14-Feb
21-Feb
26-Feb
6-Mar
13-Mar
21-Mar
27-Mar
22-Apr
date
Atriplex sp.
Conyza sp.
Malva sp.
Oxalis sp.
Plantago sp.
Sonchus sp.
Avena sp.
Bromus sp.
Festuca sp.
Hordeum sp.
Poa sp.

three orchards: AU (61.22%), CU (100%) and PP (63.81%) of the identified specimen.

Aphid species associated with ground cover plants

Among the 22 plant genera and one species identified in the ground cover of the four orchards, 16 harbored aphids, whereas genera *Amaranthus* sp., *Allium* sp., *Convolvulus* sp., *Conyza* sp., *Senecio* sp., *Urtica* sp. and *Capsella bursa-pastoris* L. (Brassicales: Brassicaceae) did not. In the order of the number of recorded aphids, *Sonchus* sp., *Erodium* sp. and *Bromus* sp. were the plant genera with the highest numbers of aphids (Table. 2). When we calculated the number of aphids per m^2 of each plant genera, *Sonchus* sp. and *Erodium* sp. contained more aphids per m^2 than the other plant genera. This was followed by a second group, composed of plants of the Poaceae family: *Bromus* sp., *Hordeum* sp., and *Poa* sp., as well as other genera belonging to different families including *Malva* sp. and *Picris* sp.. Importantly, no genus of the family Poaceae harbored *A. spiraecola*, and the genera *Festuca* sp. and *Hordeum* sp. did not harbor *A. gossypii* either. In *Bromus* sp., 0.14 (ratio of aphid species) adult aphids were identified as *A. gossypii*, 38% were winged.

Fig. 5. Seasonal trend of aphids inhabiting the cover crop of four commercial citrus orchards (AU, CD, CU and PP) represented as the mean number of aphids / m^2 of cover (± SE) and their relative abundance in February, March and April (circle graphs).

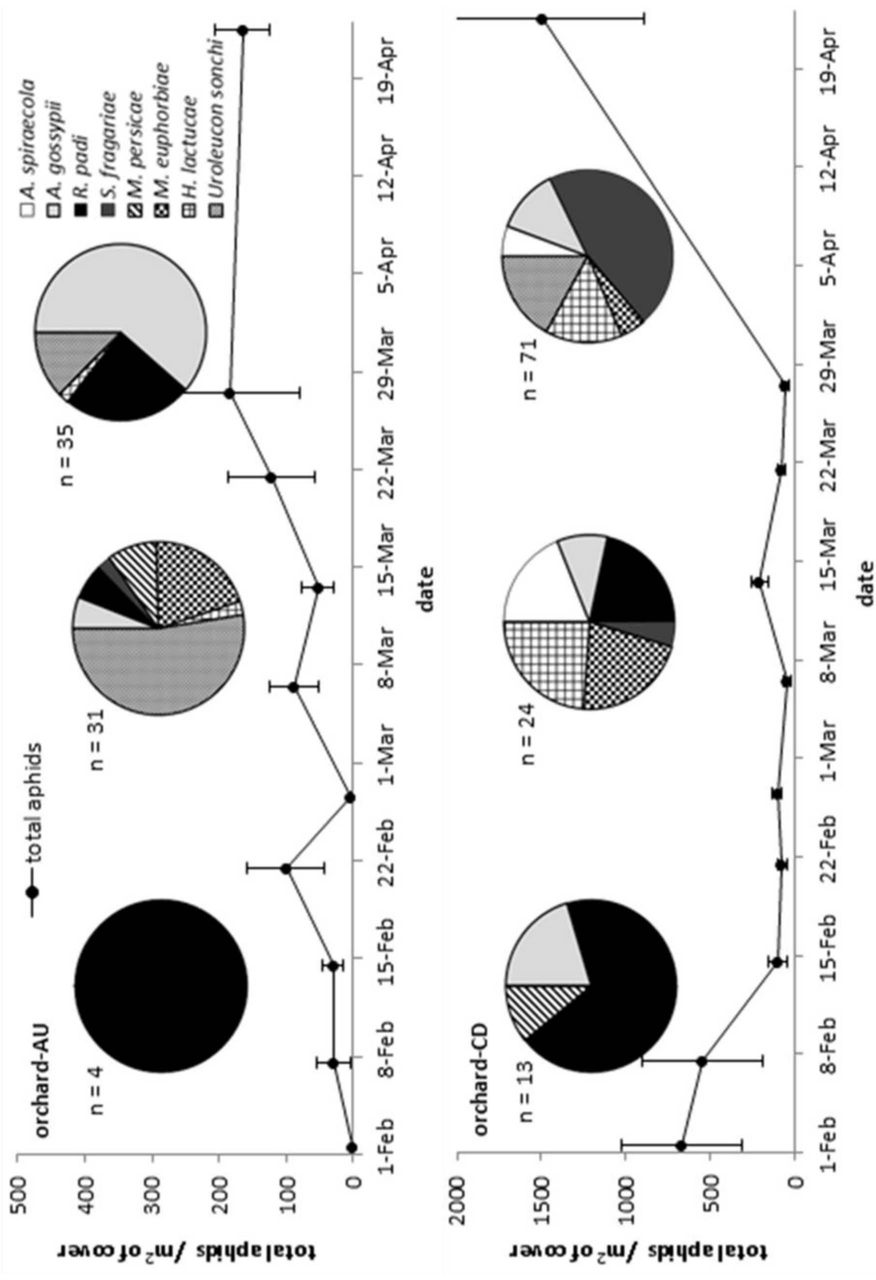


Fig. 5 Find the caption in page 143.

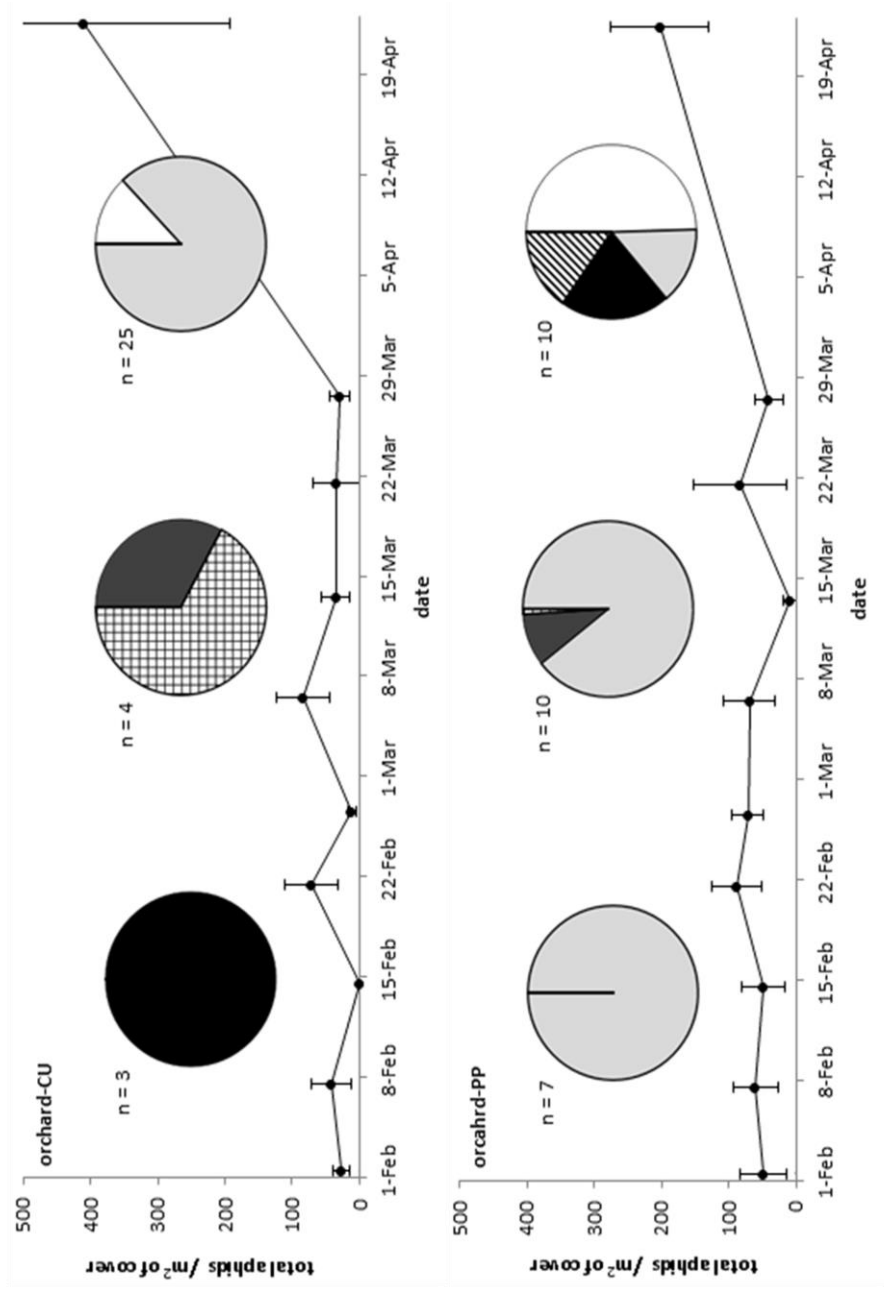


Fig. 5 Find the caption in page 143.

Table 2 Aphid community inhabiting the ground cover of four commercial clementine orchards represented as: aphids per m^2 of plant species; total number of aphids collected; total number of adult aphids; and ratio of identified aphids. ($^{\#}$) = 100% winged aphids; ($^{\#\#}$) = 38 – 50% winged aphids; bold numbers represent aphid-plant relation not referenced in previous bibliography (Holman, 2009)

				Percentage								
	Aphid/m^2	Total aphids	**Adult aphid**	***Aphis spiraecola***	***Aphis gossypii***	***Rhopalosiphum padi***	***Sitobion fragariae***	***Myzus persicae***	***Macrosiphum euphorbiae***	***Hyperomyzus lactucae***	***Uroleucon sonchi***	**Unidentified**
Amaranthaceae												
Atriplex sp.	-	1	1	0	0	0	0	0	0	0	0	1
Asteraceae												
Picris sp.	225	9	1	**1**$^{\#}$	0	0	0	0	0	0	0	0
Sonchus sp.	1997.06	679	80	0	0.05$^{\#}$	0	**0.31**	0	0	0.21	0.40	0.03
Brassicaceae												
Diplotaxis sp.	-	2	0	-	-	-	-	-	-	-	-	-
Geraniaceae												
Erodium sp.	1510	453	59	**0.03**$^{\#}$	0.05	0	0	0.02	0.02	**0.02**$^{\#}$	0	0.86
Geranium sp.	-	14	1	0	0	0	0	0	0	0	0	1
Malvaceae												
Malva sp.	127.84	124	33	**0.15**$^{\#}$	0.61	0	**0.03**	0.03	0.06	**0.03**$^{\#}$	0	0.09
Oxalidaceae												
Oxalis sp.	47.62	10	3	0	0.33	0	0	0	0.67	0	0	0
Plantaginaceae												
Plantago sp.	50	15	6	1$^{\#\#}$	0	0	0	0	0	0	0	0
Poaceae												
Avena sp.	-	2	1	0	**1**$^{\#}$	0	0	0	0	0	0	0
Bromus sp.	159.7	428	42	0	**0.14**$^{\#\#}$	0.36	0.02	0.02	0.05	0	0	0.40
Dactylis sp.	-	1	0	-	-	-	-	-	-	-	-	-
Festuca sp.	83.12	64	7	0	0	0.57	0.14	0	0	0	0	0.29
Hordeum sp.	122.73	27	3	0	0	0	0	0.33	0	0	0	0.67
Poa sp.	183.33	11	0	-	-	-	-	-	-	-	-	-
Polygonaceae												
Emex sp.	-	3	0	-	-	-	-	-	-	-	-	-

4.3.3. The effect of ground cover on aphid natural enemies in the canopy

4.3.3.1. Ratio of attacked colonies

Among the 829 *A. spiraecola* colonies sampled throughout the assay (496 colonies in orchards with ground cover and 333 in orchards with bare soil), a total of 19,693 aphids and 312 natural enemies (262 Aphididae parasitoids and 50 predators [25 Cecidomyiidae, 12 Chrysopidae, 7 Coccinellidae, 4 Syrphidae and 2 *Theridion* sp. individuals]) were counted (Table 1). There were no significant differences in the ratios of attacked colonies between soil managements during the sampling period (Feb 21 to May 9) ($\chi^2 = 0.23$, $F_{1,\,1052} = 0.23$, $P = 0.64$) (Fig. 6). However, there were significant differences in the ratio of attacked colonies between managements before the critical period for *A. spiraecola* infestation (Feb 21 to April 17) ($\chi^2 = 4.038$, $F_{1,\,683} = 3.89$, $P = 0.044$). In fact, the last

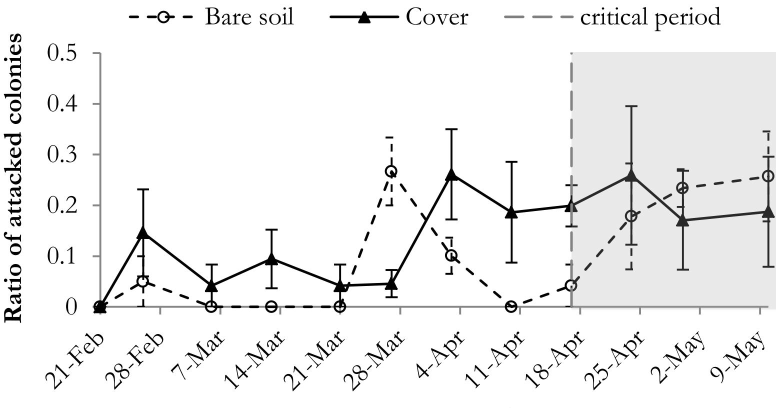


Fig. 6. Ratio of attacked colonies (mean ± SE) in four commercial orchards with bare soil management (discontinuous line) and four commercial orchards with cover crop management (continuous line) in 2013. Start day of the critical period for the management of *A. spiraecola* in 2013, represented by vertical grey discontinuous line (April 17).

sampling date before the critical period (April 10) the ratio of attacked colonies in cover orchards was 0.3 ± 0.1 and in bare soil orchards was null.

4.3.3.2. Cumulative predators and parasitoids per day

The mean cumulative predators per day (CPreD) values increased earlier and remained higher in orchards with ground cover management than in those with bare soil (Fig. 7A). The interaction between treatment and date did not affect significantly the population growth of the predators (interaction between treatment and date: $F_{1,\ 93} = 0.89$, $P = 0.35$), but there were significant differences between soil managements ($F_{1,\ 93} = 15.25$, $P < 0.001$). For parasitoids, the interaction between treatment and date affected significantly their population growth. (interaction between treatment and date: $F_{1, 93} = 10.10$, $P = 0.002$) (Fig. 7B).

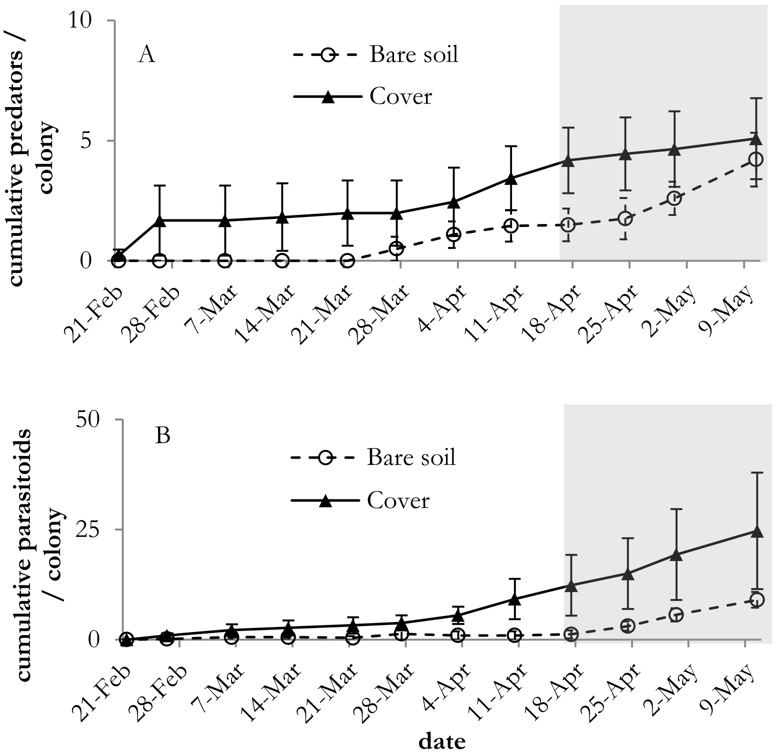


Fig. 7 Mean cumulative natural enemies (predators: A, parasitoids: B) (± SE) in *Aphis spiraecola* colonies from four commercial orchards with bare soil management (discontinuous line) and four commercial orchards with cover crop management (continuous line) in 2013.

4.3.4. Aphid damage

To estimate the effect of soil management on the amount of aphid damage, the percentage of shoots occupied by *A. spiraecola* was estimated in the eight orchards. The mean percentage of occupied shoots in orchards with ground cover never exceeded the economic threshold. In contrast, the mean percentage of occupied shoots exceeded the economic threshold around mid-April in orchards with bare soil (ANOVA with repeated measure: *F* = 6.07; df = 1, 78; *P* = 0.016) (Fig. 8).

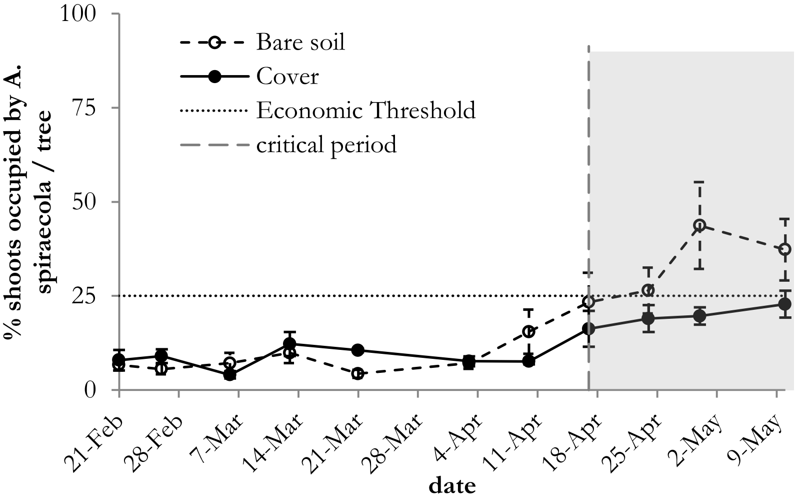


Fig. 8. Mean percentage of occupied shoots per tree in four commercial orchards with bare soil management (discontinuous line) and four commercial orchards with cover crop management (continuous line) in 2013. Economic threshold, as defined by Hermoso de Mendoza (2006) (25% of shoots occupied by *A. spiraecola*), represented by the dotted line. The critical period for the management of *A. spiraecola* in 2013, represented with vertical grey discontinuous line (April 17).

4.4. Discussion

The findings of this study illustrate the potential effect of sown ground cover on the biological control of aphids. This study differs from the majority of similar published works because it describes and analyzes the wild complex of plants that coexist with sown plant species in commercial orchards. The importance of these wild plants is reflected by our data showing that they represented approximately 40% of a sown ground cover surface of citrus orchards. Hitherto, with few exceptions (Altieri *et al.*, 1977; Altieri and Whitcomb, 1979), studies of sown ground covers have overlooked the presence of wild plants (Haley and Hogue, 1990; Liang and Huang, 1994; Landis *et al.*, 2000) or they have been conducted in experimental plots without these plants (Maredia *et al.*, 1992; Brown and Schmitt, 1996; Smith *et al.*, 1996; Brown and Glenn, 1999; Rieux *et al.*, 1999; Frechette *et al.*, 2008; Danne *et al.*, 2010; Silva *et al.*, 2010; Aguilar-Fenollosa *et al.*, 2011a; Caballero-Lopez *et al.*, 2012; Marko *et al.*, 2013). In our study, we have evaluated the effect of a sown ground cover based on Poaceae plants on the biological control of *A. spiraecola* in citrus orchards. This sown cover was selected for different agronomic reasons (Fibla Queralt *et al.*, 2000; Aucejo, 2005; Aguilar-Fenollosa *et al.*, 2011a, b, c). Given that Poaceae plants are not suitable hosts for *A. spiraecola* (Holman, 2009) they cannot be reservoirs for this pest. As expected, the ground cover was present throughout the study period (February to April), and the sown plants were the dominant plant genera. However, we also cataloged plants of 18 additional genera coexisting with the Poaceae plants, ranging from 7 (orchard "CU") to 15 (orchard "CD"). Their relative abundance varied throughout the study, but the three most abundant plants, *Malva* sp., *Oxalis* sp. and *Sonchus* sp., were always present and represented more than 50% of the wild plants. Interestingly, any of these three wild plants in the sown ground cover were not reservoir of *A. spiraecola* just before citrus trees are susceptible to their attack (March). We did not recover any *A. spiraecola* from *Sonchus* sp., the only aforementioned genus catalogued as a potential host for *A. spiraecola* (Holman, 2009). These three plant genera are suitable hosts for *A. gossypii* (Holman, 2009) and, in fact, more than 0.3 ratio of the aphids recovered from *Malva* sp. and *Oxalis* sp. were apterous *A. gossypii*. This aphid species is also a citrus pest that has been replaced by *A. spiraecola* in recent decades (Hermoso de Mendoza and Moreno, 1989; Hermoso de Mendoza *et al.*, 1997; Hermoso de Mendoza *et al.*, 2006; Hermoso de Mendoza *et al.*, 2012). Therefore, these plant genera could be reservoirs of *A. gossypii* and should be mowed to diminish the number of potential pests or, alternatively, these *A. gossypii* populations could increase predator populations, unleashing apparent competition which it may optimize the biological control of aphids (Holt, 1977; Muller and Godfray, 1997; Chailleux *et al.*, 2014; van Veen, *et al.*, 2006).

We hypothesized that the use of a sown ground cover would increase the aphid community inhabiting citrus orchards, thus enhancing the presence of natural enemies in citrus orchards before *A. spiraecola* infestation. Both the Poaceae plants and the wild complex of plants harbored a diverse community of aphids that do not attack citrus and can act as alternative prey/hosts for aphid natural enemies. As hypothesized, the Poaceae plants harbored specific aphids, primarily *R. padi,* in winter. This aphid can develop at temperatures typical of Mediterranean winter (Dean, 1974; Weibull, 1993; Gomez-Marco *et al.*, Unpublished data). Later in the season, we recovered *R. padi* and also *S. fragariae* in *Bromus* sp. and *Festuca* sp. Poaceae genera are the secondary hosts of *S. fragariae* (Blackman and Eastop, 1984). *Rhopalosiphum padi* is anholocyclic in Spain because it cannot be found in its primary host

Prunus padus (Rosales: Rosaceae), and it feeds exclusively on Poaceae plants (Van Emden and Harrington, 2007). Among the main Poaceae plants of the cover, *Bromus* sp. had the highest number of aphids (mean number of aphids / m^2).

The wild plants harbored the following community of polyphagous aphids in order of abundance: *U. sonchi*, *H. lactucae, M. euphorbiae* and *M. persicae*. Among the three most abundant wild plants, *Sonchus* sp. was the plant genus that hosted the highest number of aphids, primarily *U. sonchi* and *H. lactucae*. *Sonchus* sp. is the primary plant host of *U. sonchi* and a common secondary host of *H. lactucae* in milder climates (Blackman and Eastop, 1984). Unexpectedly, we also encountered some apterous individuals of *S. fragariae* on *Sonchus* sp. This aphid community of *Sonchus* sp. generally appeared in March but also in April when *A. spiraecola* had already infested the citrus canopy. Ergo, aphids inhabiting *Sonchus* sp. may distract the attack of predators to *A. spiraecola* colonies in citrus trees by attracting them to the cover just when the predation reduces *A. spiraecola* populations effectively (Gómez-Marco *et al.*, 2015a). This distraction might also be a long-term positive effect for biocontrol if predators use these aphids to increase their populations. Both effects will depend on the behavior and generation time of the predators (Holt, 1977; Muller and Godfray, 1997; van Veen, *et al.*, 2006; Chailleux *et al.*, 2014;). In March, *Oxalis* sp. hosted a low number of non-attacking citrus aphids but a high proportion of *M. euphorbiae*. This aphid can be an important alternative prey/host for aphid natural enemies in end winter. *M. euphorbiae* is highly polyphagous, feeding on more than 200 plant species in more than 20 plant families (Van Emden and Harrington, 2007). In fact, we recorded *M. euphorbiae* in four different plant genera from four different family plants but always during the month of March.

From this we conclude that plants of the Poaceae family and the genus *Oxalis* sp. harbor stenophagous aphids and *M. euphorbiae* that can be alternative prey/hosts for natural enemies in citrus orchards.

The sown ground cover accompanied by a wild complex of plants promoted the early presence of predators in citrus canopies. These predators attacked *A. spiraecola* colonies before they exponentially increased, which reduced the damage produced by this aphid. Orchards with this cover, therefore, never exceed the economic threshold (Fig. 8). Consequently, despite the fact that wild plants could serve as a reservoir for *A. spiraecola* as well as host aphid species that can disrupt the biocontrol services of natural enemies, the overall effect of the sown cover resulted in effective biological control of *A. spiraecola* in citrus plants. This result suggests that Poaceae plants, together with *Oxalis* sp., relieve the detrimental effect of other wild plants such as *Malva* sp. or *Sonchus* sp. Alternatively, the latter plants, in addition to the other 21 different plant genera identified in the covers, may promote overwintering habitats (Thomas *et al.*, 1991; Thomas *et al.*, 1992), thus prolonging the flowering period to provide nectar and pollen for natural enemies (Andow and Risch, 1985; Hickman and Wratten, 1996; Heimpel and Jervis, 2005; Lee *et al.*, 2006; Spellman *et al.*, 2006; Lee and Heimpel, 2008; Brown *et al.*, 2010; Gontijo *et al.*, 2013; Tena *et al.*, 2013) and/or interfere with the host-finding ability of *A. spiraecola.* It has been suggested that non-host plants may interfere with the host-finding ability of specialist herbivores (Root, 1973), resulting in the slower colonization of host plants (Smith, 1969; Horn, 1981; Vidal, 1997). Our results suggest that the wild plants can affect the host-finding ability of *A. spiraecola,* because all of the individuals recovered were winged and were settled on plant genera which are generally not suitable hosts for this aphid (Holman, 2009). Two possible mechanisms by which ground cover may disrupt aphid host-finding ability are through interference with the olfactory stimuli required for locating a host and the reduction of light reflectance at certain spectral wavebands that are less attractive than the reflectance offered by bare soil (Kennedy *et al.*, 1961).

The effect of the ground cover differed between the two groups of natural enemies. Whereas the number of predators increased early in the season in orchards with ground cover, the number of parasitoids did not increase until the critical period. This result might be due to the higher host- or habitat-specificity of parasitoids, when compared with predators (Powell, 1986; Gurr and Wratten, 1999). Therefore, it is more difficult to provide alternative hosts by managing the ground cover. In this sense, it is known that *Binodoxys angelicae* Haliday (Hymenoptera: Braconidae) is the unique primary parasitoid of *A. spiraecola* in citrus trees (Gómez-Marco *et al.*, 2015b), and it is heavily attacked by a complex of hyperparasitoids (Gómez-Marco *et al.*, 2015b) that, much like in other systems can also benefit from the ground cover (Eilers and Klein, 2009; Comério *et al.*, 2013; Paredes *et al.*, 2013). Therefore, sown ground cover improves biological control of *A. spiraecola* through an increase in predators early in the season.

Conclusions

The rationale for using Poaceae in this study was based on previous results indicating that these plants enhanced the biological control of other key pests in clementine orchards (Bugg and Waddington, 1994; Aguilar-Fenollosa *et al.*, 2011a, b). Our results demonstrate that the use of Poaceae plants, such as *F. arundinacea* and *Bromus* sp., as ground cover for citrus trees can significantly reduce *A. spiraecola* damage in the clementine trees. By contrast, bare soil resulted in higher damage to trees, which might lead to increased pesticide use and therefore higher production and environmental costs (Aguilar-Fenollosa *et al.*, 2011c). Our results provide evidence that top-down processes related to ground cover management affected aphid populations in citrus orchards, independent of the companion wild plants. The ground cover based in Poaceae, along with *Oxalis* sp., promoted the appearance of alternative aphids before the critical period for *A. spiraecola* management, leading to an increase in predators that controlled pest populations. Hence, the adoption of this biological control strategy in clementine mandarin crops is highly recommended.

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CHAPTER V

General discussion and conclusions

5. General discussion and conclusions

Integrated pest management strategies in Spanish citrus have become a solution for the citrus industry which needs to respond to international market demands, new European legislation and a society that seeks for healthier eating habits and more environmental friendly producing systems (Urbaneja *et al.*, 2014). The number of citrus growers adopting IPM systems will increase over the coming years as the number of available pesticides decreases. Therefore, it is necessary to develop new technics and knowledge to implement them. Nowadays, *A. spiraecola* is considered a key pest of clementines in the Mediterranean basin (Tena and Garcia-Marí, 2011) because economic injury level is frequently overpassed. In addition, biological control of *A. spiraecola* is still poorly known and its management is based on chemical control (Hermoso de Mendoza *et al.*, 2012; Vacante and Gerson, 2012; Urbaneja *et al.*, 2014). Several studies have tackled the potential of introduced and native parasitoids in Spanish citrus without successful results (Michelena and Sanchís, 1997; Michelena *et al.*, 2004; Jacas *et al.*, 2010) and without explaining the reason/s behind this poor performance. Other studies have described the potential complex of *A. spiraecola* predators (Soler *et al.*, 2002; Alvis, 2004; Kavallieratos *et al.*, 2004; Hermoso de Mendoza *et al.*, 2012; Vacante and Gerson, 2012), but for our knowledge the impact of these predators on *A. spiraecola* has been documented only for the *F. auricularia* (Romeu-Dalmau *et al.* 2012). Therefore, some questions need to be addressed to develop an IPM strategy against *A. spiraecola* in clementines based on biological control.

In this thesis, I first tried to understand the poor biocontrol services provided by *A. spiraecola* parasitoids, despite the efforts carried out to introduce new parasitoids (Tremblay *et al.*, 1983; Starý *et al.*, 1988; Meliá, 1993; Michelena *et al.*, 1994; Michelena and Sanchís, 1997; Michelena *et al.*, 2004; Jacas *et al.*, 2010). According to molecular and taxonomic data, ***B. angelicae*** **is likely the only primary parasitoid of** ***A. spiraecola*** but it suffers a high immature mortality due to the high pressure of hyperparasitoids. **The parasitism rates achieved by** ***B. angelicae*** **were very low likely due to the high hyperparasitism rates** recorded from the beginning of the season by a diverse complex of hyperparasitoids species. This pattern of hyperparasitism occurred in the four sampled orchards and both years suggesting that this **complex of hyperparasitoids is common and likely widely distributed in our citrus agroecosystem**.

The use of the multiplex qPCR confirmed the identification of at least six hyperparasitoids species: *Syrphophagus aphidivorus*, *Alloxysta* sp., *Asaphes* sp., *Pachyneuron aphidis*, *Dendrocerus* sp. and *Phaenoglyphis villosa*, and it allowed to confirming that all these species hyperparasitize *A. spiraecola* via *B. angelicae.* Molecular techniques are irreplaceable to untangle the trophic links in communities where immature entomophagous species (either in the third or fourth level) develop inside the phytophagous as in the case of aphid-parasitoid communities. Here, I have elucidated these links and I have also demonstrated that **these hyperparasitoids tend to multi-hyperparasitize**. From the biological control point of view, the failure of *B. angelicae* and the diverse and abundant complex of hyperparasitoids suggest that under the current situation **it is unfeasible to design biological control programs against this aphid with parasitoids**. Therefore, future research programs should concentrate their efforts on other natural enemies, especially predators, which are abundant and diverse in citrus (Cole, 1925; Miller, 1929; Michelena and

Sanchís, 1997; Urbaneja *et al.*, 2000; Alvis, 2004; Kavallieratos *et al.*, 2004; Hermoso de Mendoza *et al.*, 2012; Vacante and Gerson, 2012).

The rich complex of predators may control *A. spiraecola* in clementines but the biological control services of these predators are generally insufficient (Jacas *et al.*, 2010) and the reasons behind this variability poorly understood. For this reason, I studied the effect of early-season predators on several life parameters of *A. spiraecola* colonies as maximum aphid population, longevity, survival, phenology as well as damages, measured as percentage of infested shoots, in clementines. This chapter could be included within a recent claim of biological control researchers who have highlighted that understanding how the chronology of predators affects aphid populations is an important issue for designing conservation biological control programs (Welch and Harwood, 2014). Interestingly, I encountered that predators reduced aphid life parameters and damages when they attacked the colonies prior to ~200 degree days (DD) since the beginning of the aphid colonization. Therefore, **the early presence of either aphidophagous or polyphagous predators is key to control *A. spiraecola* in citrus clementine crops**. These data could be considered to develop future thresholds against *A. spiraecola* that will also consider the presence of predators. For example, my data suggests that if one third of the colonies are attacked by predators at the beginning of the *A. spiraecola* infestation then the percentage of occupied shoots by *A. spiraecola* will not exceed the economic threshold. Therefore, growers could discard pesticide applications under those circumstances.

During this thesis, I could not detect any key predator species. In addition to the published manuscript, where three orchards were sampled during three years, I also sampled other orchards that were not included in the final redaction of this thesis. Moreover, I also used transparent and yellow traps during the three years without obtaining any potential key predator. Taking these results into consideration, we excluded the possibility of improving biological control of *A. spiraecola* using augmentative releases of predators or improving the conservation of a particular species. Therefore, the following efforts concentrated on improving the early presence of predators in general.

To increase the number of predators early in the season, conservation biological control practitioners have used ground covers to provide alternative resources to the predators (Landis *et al.*, 2000). This ground management has demonstrated advantages to control aphids in other crops (Brown and Glenn, 1999; Landis *et al.*, 2000; Schmidt *et al.*, 2007; Danne *et al.*, 2010; Schmidt *et al.*, 2011). In citrus, the use of ground covers based on Poaceae plants has been implemented to enhance the performance of natural enemies against citrus key pest in Spain (Aguilar-Fenollosa *et al.*, 2011a; Aguilar-Fenollosa *et al.*, 2011b; Monzó *et al.*, 2011a; Monzó *et al.*, 2011b). Hitherto, in the fourth chapter of my thesis I analyzed whether **a grown ground cover based on Poaceae plants benefits the biological control of *A. spiraecola* by foreseeing the arrival of the predators**. The initial causality chain was that this sown ground cover may provide alternative prey for predators (Landis *et al.*, 2000) before *A. spiraecola* infests citrus, leading to an increase of predators that will control this aphid later. Concurrently, the ground cover should not promote the presence of *A. spiraecola* when tender shoots of clementines are scarce.

Where my thesis differs from previous studies of sown ground covers is that it also describes and analyzes the wild plants present in the cover to determine the role of these plants on the biological control of *A.*

spiraecola. The studied cover contained 18 different genera of wild plants which represented around 34% of ground cover surface. The most abundant plant genera were *Malva* sp., *Oxalis* sp. and *Sonchus* sp. Considering the sown Poaceae plants and the wild plants, the ground cover harbored a diverse community of aphids; sown Poaceae plants harbored mostly stenophagous aphids of Poaceae as *R. padi* and *S. fragariae* whereas wild plants harbored mostly generalist aphids as *M. persicae*, *M. euphorbiae*, *H. lactucae* and *U. sonchi*. These aphids may have been used by predators as alternative preys to increase or maintain their populations during the period of *A. spiraecola* scarcity (Landis *et al.*, 2000; Van Emden and Harrington, 2007). Molecular methods for gut content analysis, where the predation on the alternative prey aphid species could be confirmed, would allow for a more conclusive evaluation of the mechanism described (Waldner *et al.* 2013; Eitzinger *et al.* 2013). Anyhow, I recorded a higher number of predators in the citrus canopies early in the season in orchards with ground cover than in those with bare soil. Therefore, I provide evidence that **top-down processes related to the ground cover management affected *A. spiraecola* populations in citrus orchards and improved its biological control**. This may provide an economic benefit and potentially reduce the need for pesticide applications to control *A. spiraecola*, as well as increasing the economic and environmental sustainability of the citrus industry.

If this ground cover is promoted, we would recommend eliminating some of the wild plants that appeared in our orchards for several reasons. For example, *Sonchus* sp. harbored more aphids/m^2 than the rest of genus plants but its aphid community appeared in April when *A. spiraecola* is already infesting the citrus canopy. This may relieve the attack of predators to *A. spiraecola* colonies. *Malva* sp. harbored also a high percentage of *A. gossypii* and therefore could promote the presence of this aphid pest. Additionally, Aucejo (2005) found that *Sonchus* sp. and *Malva* sp. plants are negative for the biological control of another key pest of clementines, *T. urticae*. Therefore, these plant genera must be considered as counterproductive in IPM programs in clementines.

A part from enhancing *A. spiraecola* control, this ground cover might facilitate the establishment of natural enemies of other citrus pests in orchards. These natural enemies may encounter alternative food resources or refuge in this cover. In detail, future studies should determine the resources offered by this ground cover to key natural enemies in citrus as the parasitoids *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) and the mealybug destroyer *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae). For example, the honeydew excreted by the inhabiting aphids might be used by these natural enemies as a sugar source. Another research line to be considered in the future is to determine the reasons behind the different abundance and arrival of the predators. The main family of predators varied among years and orchards and we could not establish any pattern. Therefore, it would interesting to investigate the factors that regulate their abundance and early arrival (in addition to the plant cover) to design and evaluate other conservation biological control programs.

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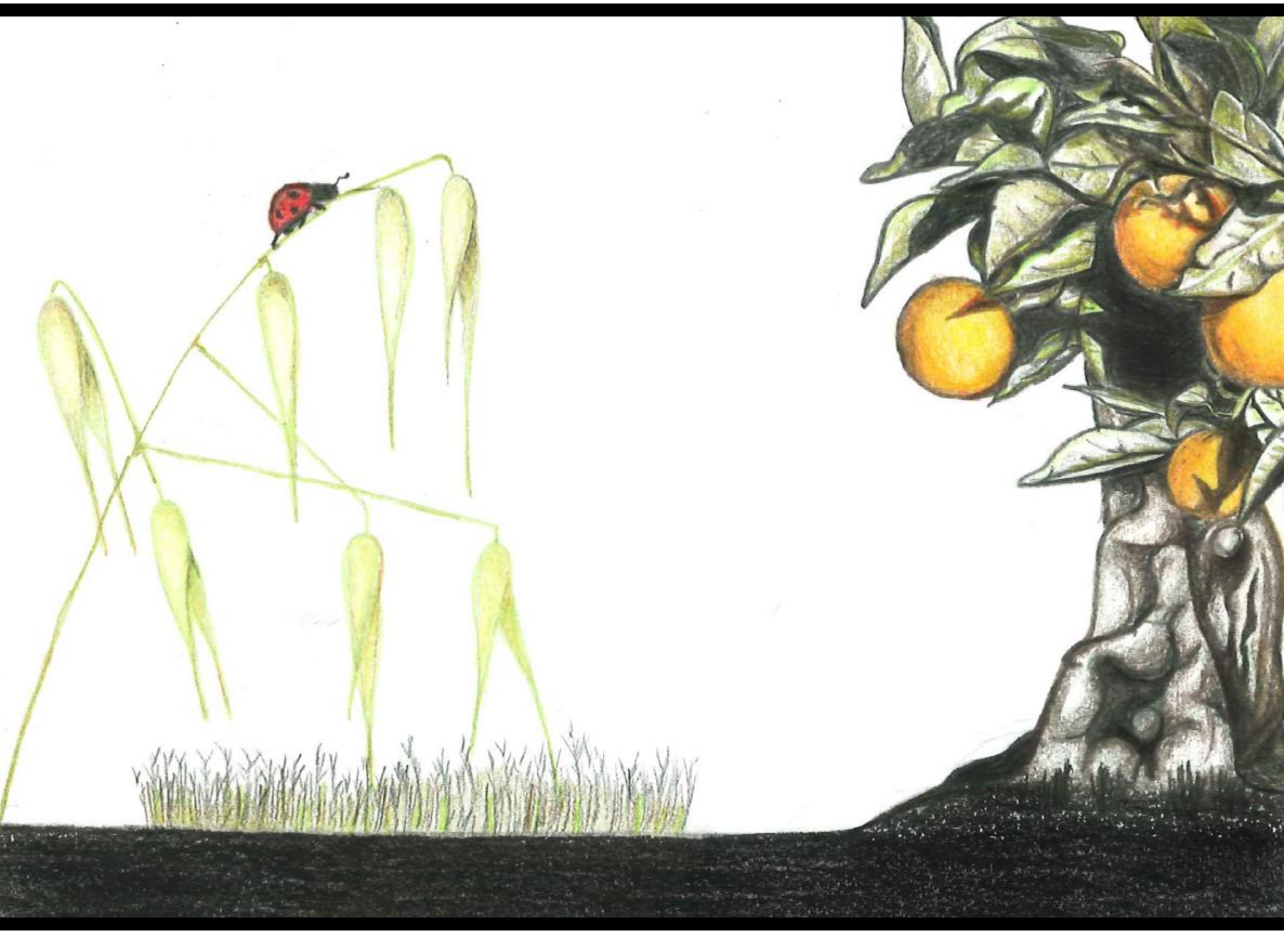

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