UNIVERSITAT POLITÈCNICA DE VALÈNCIA

50 anys

Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural

TESIS DOCTORAL

Revalorización del complejo de depredadores polífagos asociado al cultivo de los cítricos, como agentes de control biológico de plagas claves

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Directores
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Valencia, diciembre 2018

A mis padres Silvia y Raúl

"Si hay algo verdaderamente cierto es que lo ignoro todo o casi todo, y me da rabia, porque hubo un tiempo en el que una mente despierta podría haber adquirido todo el saber de la época, pero ahora ya no es posible, ya no hay más que pequeños sabios que lo saben todo de casi nada y yo soy uno de ellos"

Jean Dausset

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Resumen

Dentro de los enemigos naturales de plagas en los agroecosistemas, los depredadores han sido considerados como uno de los grupos de mayor importancia. Sin embargo, su compleja biología y comportamiento ha obstaculizado en muchos casos evaluar su verdadero papel como agentes de Control Biológico. En su mayor parte, los estudios sobre depredadores se han concentrado en las relaciones simples entre los depredadores especialistas y sus presas ya que estos sistemas son más fáciles de parametrizar. No obstante, existe un creciente interés en el control ejercido por los depredadores generalistas, debido a su capacidad de mantener las densidades de presas en equilibrios estables en formas no dependientes de la densidad de las mismas. Los estudios realizados en este trabajo se localizaron en parcelas de clementinos ubicadas en la Provincia de Valencia y Castellón. Los estudios de laboratorio se llevaron a cabo en las instalaciones del Instituto Valenciano de Investigaciones Agrarias (IVIA).

En el primer estudio presentado, se reevalúa la importancia de la depredación como factor de mortalidad biótica de una plaga clave bajo un

sistema de control biológico por conservación. Para éste, se tomó como modelo al piojo rojo de California, *Aonidiella aurantii*, en el cultivo de clementinos. Por medio de la utilización de técnicas de exclusión a campo y de detección de ADN de la presa en el contenido intestinal de los depredadores registrados, mediante la técnica de PCR, se logró dilucidar cómo un rico complejo de depredadores generalistas y estenófagos indígenas o naturalizados son los principales factores de mortalidad de la plaga en estudio.

Como segundo objetivo se planteó identificar en otro grupo de plagas claves del cultivo de clementinos, cuáles son los factores limitantes y reguladores que no permiten que las poblaciones de estos fitófagos superen el umbral de daño económico. A través de un muestreo semanal de las poblaciones de pulgones, de *A. spiraecola* y *A. gossypii*, y de sus depredadores asociados, como también de las observaciones fenológicas de los clementinos y utilizando información meteorológica de la zona, se evaluó la dinámica de los niveles de infestación producidos por pulgones. El análisis de la información obtenida nos permite considerar que la única variable que limita el desarrollo de las poblaciones de estas plagas es el estado fenológico de su huésped. También se confirma que los depredadores son un importante factor regulador en el control de las poblaciones de pulgones. Entre estos depredadores, los micrococcinélidos podrían tener el papel más significativo. Según nuestros datos podemos considerar que, para que exista un buen control de las poblaciones de pulgones en la cuenca mediterránea, deben generarse las condiciones favorables para que el hospedero limite el desarrollo de las colonias de estas plagas y que los depredadores actúen regulando sus poblaciones. Por lo tanto, una estrategia de

gestión del cultivo que favorezca la aparición de los micrococcinélidos de forma temprana y abundante, podría contribuir a que las poblaciones de estas plagas claves no excedan el umbral de daño económico y de esta manera reducir el uso de pesticidas para su control.

Debido a que se registran dos especies de pulgones como plagas claves en los clementinos (*A. spiraecola* y *A. gossypii*) y se identifican dos especies de micrococcinélidos (*Scymnus subvillosus* y *S. interruptus*) como sus depredadores más abundantes y de mayor relevancia, en un tercer objetivo se evaluó en laboratorio y campo el efecto de estas presas sobre los parámetros de desarrollo, supervivencia y reproducción de estos depredadores. La información generada en este estudio, proporciona indicios sobre las relaciones entre las especies de *Scymnus* con las especies de pulgones que se encuentran en los cultivos de clementinos de la cuenca mediterránea. Se reafirma la teoría de que la calidad de la dieta influye en los parámetros de desarrollo, fertilidad y poblacionales de los depredadores, en este caso, se considera a la especie *A. gossypii* la más adecuada para las especies de *Scymnus*.

De esta forma, la presente tesis reevalúa el papel de la depredación como factor regulador en las poblaciones de las plagas clave en los agroecosistemas de cítricos e investiga los mecanismos ecológicos y biológicos que llevan al éxito o al fracaso de este importante componente de mortalidad biótica. Los resultados presentados aquí apoyan firmemente un cambio de paradigma en los enfoques de control biológico que se está llevando a cabo en los últimos años, destacando la importancia de comprender el contexto ecológico en el que se desarrolla el

control biológico y descubriendo relaciones complejas entre todos los agentes involucrados. El conocimiento generado a través de estos estudios permitirá desarrollar estrategias de control biológico de conservación más efectivas para los agroecosistemas de cítricos.

Abstract

Predators have been considered as one of the most important groups of pest natural enemies in agroecosystems. However, their complex biology and behaviour have hindered in many cases evaluating its true role as biological control agents. Studies on predators have mostly focused on the simple relationships between specialist predators and their prey since these systems are easier to parameterize. Though, there is a growing interest in the control exerted by generalist predators owing to their ability to keep prey densities in stable equilibrium in ways not dependent on their density.

The importance of predation as a biotic mortality factor of key citrus pests in systems under conservation biological control management strategies was assessed. The California red scale, *Aonidiella aurantii*, was used as a model pest in clementine citrus groves. Through the use of field exclusion techniques and PCR detection of prey DNA in the gut content of predators, it was found that a rich complex of indigenous and naturalized generalist and stenophagous predators are the main mortality factor of this pest.

Limiting and regulating factors modulate severity of pest infestations in agroecosystems. The second objective was to identify how these variables explain the current patterns of spring aphid infestations in clementine citrus crops of the Mediterranean basin. Populations of *Aphis spiraecola* and *A. gossypii* as well as of their associated predators were sampled weekly. Phenological changes in citrus trees were also observed. Abiotic variables (temperature and precipitation) influence was also taken into account. The analysis of the relationships between all these variables showed that the only factor able to limit aphid infestations under Mediterranean conditions is the phenological state of their host. Nevertheless citrus phenology was found to be strongly mediated by temperature. Results also confirmed that predators are an important regulatory factor of aphid demography, being micrococcinellids the only natural enemies found to have a significant role. According to our study, in order to have effective aphid control in clementine citrus of the Mediterranean basin, favourable conditions must be generated so that the host limits the development of aphid colonies and predators exhibit their highest regulation potential. A crop management strategy that favours the presence of micrococcinellids early in the season could contribute to keep the populations of these key pests more frequently under their economic thresholds thus reducing the frequency of pesticide applications for their control.

Because two species of aphids are registered as key pests in clementines (*A. spiraecola* and *A. gossypii*) and two species of micrococcinellids of the same genus (*Scymnus subvillosus* and *S. interruptus*) are identified as the most abundant and important aphid predators, in a third objective it was evaluated under

laboratory and field conditions the effect of these preys on the parameters of development, survival and reproduction of these predators as well as on the predator demography. The information generated in this study provides essential clues to understand the relationships between the two *Scymnus* species and the aphid species found in clementine crops in the Mediterranean basin. The theory that the quality of the diet influences the development, fertility and population parameters of the predators was reaffirmed. In this case, *A. gossypii* is considered the most suitable for the *Scymnus* species.

In conclusion, the present thesis reassess the role of predation as a regulating factor of key pests in citrus agroecosystems and inquires into the ecological and biological mechanisms laying behind the success and failures of this important biotic mortality component of citrus phytophagous. The results presented here strongly support the change of paradigm in biological control approaches which is being carried out in the last years, by highlighting the importance of understanding the ecological context on which biological control develops and discovering complex relationships between all the agents involved. The knowledge generated through these studies will permit to develop more effective conservation biological control strategies for citrus agroecosystems.

Resum

Dins dels enemics naturals de plagues als agroecosistemes, els depredadors han sigut considerats com un dels grups de major importància. No obstant això, la seua complexa biologia i comportament ha obstaculitzat en molts casos l´avaluació del seu vertader paper com a agents de control biològic. En la seua major part, els estudis sobre depredadors s'han centrat en les relacions simples entre els depredadors especialistes i les seues preses, ja que aquests sistemes són més fàcils de parametritzar. Malgrat açò, existeix un creixent interès en el control exercit pels depredadors generalistes, a causa de la seua capacitat de mantenir les densitats de preses en equilibris estables en formes no depenents de la densitat d´aquestes. Els estudis realitzats en aquest treball es localitzaren en parcel·les de clementins situades en la Província de València i Castelló. Els estudis de laboratori es van dur a terme en les instal·lacions de l'Institut Valencià d´Investigacions Agràries (IVIA).

En el primer estudi presentat, es reevalúa la importància de la depredació com a factor de mortalitat biòtica d'una plaga clau sota un sistema de control biològic per conservació. Per a aquest, es va prendre com a model el poll roig de Califòrnia, *Aonidiella aurantii*, al cultiu de clementins. Per mitjà de la utilització de tècniques d'exclusió a camp i de detecció d'ADN de la presa en el contingut intestinal dels depredadors registrats, mitjançant la tècnica de PCR, es va aconseguir dilucidar com un ric complex de depredadors generalistes i estenófagos indígenes o naturalitzats són els principals factors de mortalitat de la plaga en estudi.

Com a segon objectiu es va plantejar identificar en un altre grup de plagues clau del cultiu de clementins, quins són els factors limitants i reguladors que no permeten que les poblacions d'aquests fitófags superen el llindar de dany econòmic. A través d'un mostreig setmanal de les poblacions de pugons, de *A. spiraecola* i *A. gossypii*, i dels seus depredadors associats, també així com de les observacions fenológiques dels clementins i utilitzant informació meteorològica de la zona, es va avaluar la dinàmica dels nivells d'infestació produïts per pugons. L'anàlisi de la informació obtinguda ens permet considerar que l'única variable que limita el desenvolupament de les poblacions d'aquestes plagues és l'estat fenològic del seu hoste. També es confirma que els depredadors són un important factor regulador en el control de les poblacions de pugons. Entre aquests depredadors, els micrococcinélids podrien tindre el paper més significatiu. Segons les nostres dades podem considerar que, perquè existisca un bon control de les poblacions de pugons en la conca mediterrània, han de generar-se les condicions favorables perquè l'hoste limite el desenvolupament

de les colònies d'aquestes plagues i que els depredadors actuen regulant les seues poblacions. Per tant, una estratègia de gestió del cultiu que afavorisca l'aparició dels micrococcinélids de forma primerenca i abundant, podria contribuir al fet que les poblacions d'aquestes plagues clau no excedisquen el llindar de dany econòmic i d'aquesta manera reduir l'ús de pesticides per al seu control.

Com que es registren dues espècies de pugons com a plagues clau en clementins (*A. spiraecola* i *A. gossypii*) i s'identifiquen dues espècies de micrococcinélids (*Scymnus subvillosus* i *S. interruptus*) com els seus depredadors més abundants i de major rellevància, en un tercer objectiu es va avaluar en laboratori i camp l'efecte d'aquestes preses sobre els paràmetres de desenvolupament, supervivència i reproducció d'aquests depredadors. La informació generada en aquest estudi, proporciona indicis sobre les relacions entre les espècies de *Scymnus* amb les espècies de pugons que es troben als cultius de clementins de la conca mediterrània. Es reafirma la teoria que la qualitat de la dieta influeix en els paràmetres de desenvolupament, fertilitat i poblacionals dels depredadors, en aquest cas, es considera a l'espècie *A. gossypii* la més adequada per a les espècies de *Scymnus*.

D'aquesta forma, la present tesi reevalúa el paper de la depredació com a factor regulador en les poblacions de les plagues clau a agroecosistemes de cítrics i investiga els mecanismes ecològics i biològics que porten a l'èxit o al fracàs d'aquest important component de mortalitat biòtica. Els resultats presentats ací recolzen fermament un canvi de paradigma en els enfocaments

de control biològic que s'està duent a terme en els últims anys, destacant la importància de comprendre el context ecològic en el qual es desenvolupa el control biològic i descobrint relacions complexes entre tots els agents involucrats. El coneixement generat a través d'aquests estudis permetrà desenvolupar estratègies de control biològic de conservació més efectives per als agroecosistemes de cítrics.

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Introducción General

Capítulo I

Introducción General

I.1. Estado actual

El creciente conocimiento sobre la trazabilidad de los productos fitosanitarios que se aplican en los cultivos para el control de plagas así como del impacto que producen sobre el medioambiente y la salud humana ha llevado al aumento de sus restricciones de uso en un mercado cada día más global [(EC) n° 1107/2009] (Bale et al., 2008; Fabroni et al., 2015). A nivel mundial, estas presiones han impulsado la implementación de enfoques de gestión de plagas sostenibles y con un uso mínimo de de plaguicidas que resulten menos agresivos. En este contexto se enmarcan las estrategias de control biológico (CB) en la agricultura (Landis et al., 2000; Jacas & Urbaneja, 2008; Tscharntke, 2012). Estas técnicas consisten en el uso de poblaciones de enemigos naturales

(parasitoides, depredadores, patógenos, antagonistas o competidores) y también otras metodologías llamadas parabiológicas (uso de feromonas, modificaciones genéticas, machos estériles, etc.), para suprimir o al menos reducir poblaciones de fitófagos plaga, haciéndolas así menos perjudiciales (DeBach & Rosen, 1991; van Driesche & Bellows, 1996). A lo largo de las últimas décadas, el uso de insecticidas selectivos, la creación de infraestructuras ecológicas y las evaluaciones sobre el papel de los enemigos naturales, se han convertido en el foco principal de las investigaciones relacionadas con la gestión de plagas (Geiger et al., 2010; Tscharntke, 2012; Rusch et al., 2017).

1.2. Control Biológico y su aplicación

Debido a que un alto porcentaje de plagas registradas en los cultivos son especies invasoras, históricamente se ha considerado que los enemigos naturales utilizados para su control, deberán provenir del mismo lugar de origen para que sean altamente efectivos (Hokkanen & Pimentel, 1984; Thomas & Reid, 2007; Van Driesche et al., 2010). Además, el efecto de la estrategia de CB seleccionada deberá ser duradero en el tiempo, aunque no logre eliminar completamente a la plaga. Este tipo de aproximación que implica la introducción de enemigos naturales exóticos se conoce como control biológico clásico y se practica desde hace más de 100 años con resultados dispares (Batra, 1982; DeBach & Rosen, 1991; Eilenberg et al., 2001; Cock, 2016). El CB clásico incluye una serie de pasos o etapas sucesivas como la correcta identificación taxonómica de la plaga, la selección del lugar donde se buscarán sus enemigos naturales autóctonos, la correcta identificación de los enemigos naturales

candidatos para la introducción, la exploración, colección y envío de éstos, el proceso de cuarentena, su cría y pruebas de seguridad, la inoculación en campo, la determinación de su establecimiento y por último, la evaluación de la eficacia del organismo introducido (Pearson & Callaway, 2005; Cullen et al., 2008; Barratt et al., 2010). Todos estos pasos y factores hacen que el proceso de introducción de enemigos naturales sea muy complejo, costoso y en consecuencia, cada vez sea menos frecuente la utilización de esta técnica (Stiling, 1993; Hill & Greathead, 2000; Michaud, 2002; Jacas et al., 2006).

Existen otras modalidades de control biológico que pueden suprimir temporalmente plagas de cultivos. Éstas se aplican por ejemplo en los cultivos anuales o estacionales, donde la utilización de enemigos naturales puede llegar a ser muy efectiva pero una vez que el cultivo se retira, éstos desaparecen y deben ser introducidos nuevamente en el siguiente ciclo de cultivo (Batra, 1982; Wissinger, 1997; Stansly et al., 2005). Esta estrategia de CB ha llevado al desarrollo y comercialización de enemigos naturales específicos para este tipo de cultivos, encontrándose actualmente en el mercado una alta disponibilidad de especies que se pueden adquirir (Naranjo et al., 2015; van Lenteren et al., 2018). Este tipo de control biológico se lo denomina aumentativo y es la tendencia que siguen los cultivos bajo cubierta y estacionales, donde han tenido mayor éxito (Urbaneja et al., 2012; Parrella & Lewis, 2017; van Lenteren et al., 2018).

En cultivos perennes la situación difiere sustancialmente, los enemigos naturales introducidos deben ser capaces de sobrevivir a las condiciones climáticas locales más inestables, la distribución espacial y los ciclos de sus

presas, como también a la disponibilidad de recursos alternativos (alimentación y refugio) (Batra, 1982; DeBach & Rosen, 1991; Barbosa, 1998; Eilenberg et al., 2001). Esto hace que el establecimiento y estabilidad de los enemigos naturales en estos sistemas de cultivo sea muy variable y poco confiable, aunque no imposible. En general, se considera que el control efectivo de las plagas en ecosistemas empobrecidos como los agroecosistemas está asociado a enemigos naturales especialistas, provenientes del mismo origen que la plaga, que operan en una red trófica altamente simplificada (Murdoch et al., 2005; Jacas & Urbaneja, 2010). En cambio, en áreas ricas en especies vegetales, el control efectivo de los fitófagos es generalmente el resultado de enlaces múltiples en redes tróficas más complejas, que incluyen enemigos naturales especialistas y generalistas (Hassell & May, 1986; Symondson et al., 2002; Snyder & Ives, 2003; Albajes & Alomar, 2008). En consecuencia, en estudios recientes se ha podido observar como la influencia de la diversificación de los agroecosistemas resulta generalmente en el incremento de oportunidades ambientales para los enemigos naturales, y consecuentemente en la mejora del control biológico de plagas (Tscharntke, 2008; Van Driesche et al., 2010). Cada vez más, se acepta que ciertos tipos de diversidad en los agroecosistemas confiere una estabilidad a largo plazo de las poblaciones de artrópodos presentes. Probablemente esto se deba a que en agroecosistemas complejos existen redes tróficas que incluyen una gran variedad de enemigos naturales capaces de regular el crecimiento potencial de las poblaciones de especies fitófagas, sin la necesidad de introducir más enemigos naturales (Symondson et al., 2002; Altieri & Nicholls 2004). Debido a esto, la conservación y el uso de los recursos por los agroecosistemas se considera la estrategia más prometedora para la gestión sostenible de plagas

en los cultivos perennes (Symondson et al., 2002; Jacas & Urbaneja, 2010; Rusch et al., 2017). La aplicación de estas estrategias, no ejerce por sí misma la acción de controlar las plagas, sino que promueve la abundancia y diversidad de los enemigos naturales ya presentes en el agroecosistema y es lo que se conoce como Control Biológico por Conservación (CBC) (DeBach & Rosen, 1991; Barbosa, 1998; Tscharntke, 2008; Woltz et al., 2012). Esta visión no es nueva, de hecho, es probablemente la forma más antigua de control biológico de plagas. Ya en el año 900 DC, citricultores chinos emplazaban nidos de la hormiga tejedora *Oecophylla smaragdina* F. en campos de cultivo de naranjos para reducir las poblaciones de insectos que se alimentaban de las hojas (DeBach & Rosen, 1991; Peng et al., 1995).

1.3. Tipos de enemigos naturales

Cuando se habla de enemigos naturales, se identifican tres tipos: (1) **parasitoides**, cuyas hembras ponen sus huevos en o sobre un huésped, las larvas eclosionan y se alimenta de dicho huésped quien muere en el proceso de desarrollo (**Figura 1.1**); (2) **depredadores**, que atacan, matan y se alimentan de más de un individuo (la presa) a lo largo de su vida (**Figura 1.2**); y (3) **entomopatógenos**, son microorganismos que viven y se alimentan de su huésped a lo largo de su vida, provocando cambios en su metabolismo que le producen una enfermedad, pudiendo matar o lesionar al huésped (**Figura 1.3**) (DeBach & Rosen, 1991; Ehler, 2006; Jacas & Urbaneja, 2008). Los primeros han sido el grupo más estudiado por los científicos, debido a la fascinación que ejercen sus ciclos biológicos, su gran diversidad biológica y su alto nivel de especificidad, algo

que ha sido considerado desde antaño como una ventaja en para los programas de control biológico (Mills & Getz, 1996; Biondi et al., 2015; Tena et al., 2016). Por otro lado, los depredadores son el grupo que ha sido mayormente subestimado debido a la dificultad de evaluar su acción (Hassell & May, 1986; Luck et al., 1988; Symondson et al., 2002; Jacas et al., 2008). Actualmente, el interés por el grupo de los depredadores ha aumentado, en particular en lo que se refiere a control biológico de conservación (Landis et al., 2000; Jacas & Urbaneja, 2010).

Figura 1.1 Parasitoide, *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae).

Dentro de los depredadores se encuentran especies de los órdenes Dermaptera, Mantodea, Hemiptera, Thysanoptera, Coleoptera, Neuroptera, Hymenoptera y Diptera, como así también especies de arañas y ácaros (**Figura 1.4**). Al necesitar más de una presa para completar su desarrollo, los depredadores suelen presentar hábitos alimenticios generalistas, aunque existen muchos ejemplos de especies para las que su rango de presas es restringido, como es el caso del coccinélido *Rodolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae), que se alimenta casi exclusivamente de la cochinilla acanalada, *Icerya purchasi* (Williston) (Homoptera: Margarodidae), o los depredadores

de pulgones que suelen ser considerados como estenófagos, es decir, que se alimentan de una diversidad limitada de presas. Como contrapartida, encontramos depredadores muy generalistas como las larvas de crisopas que pueden alimentarse de pulgones, larvas de lepidópteros, ácaros, cochinillas, trips y moscas blancas (DeBach & Rosen, 1991; Jacas & Urbaneja, 2008).

Figura 1.2 Depredador, *Scymnus interruptus* (Goeze) (Coleoptera: Coccinellidae).

Los depredadores especialistas, o al menos estenófagos, han sido tradicionalmente el objeto de las introducciones de control biológico clásico, sobre todo en el caso de plagas exóticas y cultivos exóticos (van Driesche & Bellows, 1996; Dixon & Dixon, 2000). En cambio, se consideraba que los depredadores generalistas eran más adecuados para el control de plagas nativas en plantas nativas. Esta visión lineal viene cambiando en los últimos años donde estudios más complejos de interacciones tróficas han revelado la importancia real de los depredadores generalistas en el control de plagas exóticas (Symondson et al., 2002; Sheppard & Harwood, 2005; Furlong, 2015).

Figura 1.3 Patógeno, hongo entomopatógeno sobre *Aonidiella aurantii*.

Para que un grupo de enemigos naturales sea considerado efectivo debería tener (a) una capacidad de colonización rápida para poder responder al ritmo de las fluctuaciones temporales y espaciales de sus presas; (b) persistencia temporal que permita al depredador mantener su número cuando las poblaciones de plagas disminuyen; y (c) hábitos de alimentación oportunistas que permitan al depredador explotar rápidamente un recurso alimentario, especialmente referido a los ataques de plagas que resurgen (Symondson et al., 2002). Las dos últimas características están conectadas a los depredadores generalistas en lugar de especialistas, debido a que pueden estar ya presentes, subsistiendo con presas no consideradas plaga o con otras especies de plagas que no son nuestro organismo diana (competencia aparente) (Holt & Bonsall, 2017). La supresión de las plagas en su etapa más temprana puede retrasar o prevenir la fase de crecimiento rápido que conduce a un daño significativo en el cultivo (Wiedenmann & Smith, 1997; Landis et al., 2000), o al menos puede proporcionar el tiempo necesario para la llegada de los especialistas. Además, los depredadores generalistas pueden llevar a la extinción local de las plagas sin que ello conduzca necesariamente a una disminución del número de

depredadores por disminución de su presa (Symondson et al., 2002; Harwood & Obrycki, 2005).

Figura 1.4 Coleoptera, *Scymnus subvillosus* (Goeze) (Coccinellidae).

1.4. Evaluación de la importancia de los depredadores

En general, la evaluación de la acción de los depredadores a nivel de campo es difícil, ya sea por la movilidad en la búsqueda de sus presas, por la hora del día en que realizan esta acción, como también porque su acción en muchas ocasiones no deja evidencias (Urbaneja & Jacas, 2004). Por lo cual, las observaciones directas de los depredadores suelen ser muy laboriosas, debiéndose realizar muchas veces al día visualmente o por medio de sistemas de video con su posterior análisis (Luck et al., 1988; Harwood & Obrycki, 2005). Por lo tanto, con observaciones directas solamente obtendremos información preliminar sobre la depredación, optándose de esta forma por observaciones indirectas. Los métodos indirectos para cuantificar el impacto de los depredadores sobre la población de una plaga están basados en la comparación directa de la densidad de la plaga y la abundancia de enemigos naturales en

poblaciones de la plaga con y sin los enemigos naturales de interés. El uso de este método requiere de la capacidad de establecer o crear escenarios con y sin los enemigos naturales que van a ser evaluados. Esto puede diseñarse mediante dos aproximaciones: (1) comparando la densidad de la plaga antes y después de liberar un depredador (diseño temporal) o (2) comparando la densidad de la plaga entre parcelas testigo y parcelas similares donde se ha liberado del depredador (diseño espacial). En el caso que el depredador esté presente en todas las parcelas, pueden generarse unidades de evaluación con y sin enemigos naturales mediante exclusión, ya sea con plaguicidas que afecten a los mismos y no a sus presas (diseño de exclusión con insecticidas) o con jaulas que protejan a la población plaga del ataque del enemigo natural a evaluar (diseño de jaulas de exclusión) (Grant & Shepard, 1985; Sunderland, 1988; Jacas & Urbaneja, 2008).

La forma de cuantificar el efecto de los depredadores es elaborando tablas de vida de las plagas. Éstas permiten comparar la mortalidad atribuible a un enemigo natural con otras fuentes de mortalidad que actúan sobre la plaga. Para construir tablas de vida es necesario partir de una población inicial definida y realizar su seguimiento hasta que termine una generación (Bellows et al., 1992; Jacas & Urbaneja, 2008).

Los diseños de exclusión permiten cuantificar la acción de los depredadores pero no son informativos sobre qué especies pueden estar involucradas como factor de mortalidad de la plaga que se está estudiando. Otra vez, el comportamiento críptico de los depredadores juega en su contra a la hora de identificar sus presas más frecuentes siendo de nuevo las observaciones

visuales insuficientes si se quiere determinar el complejo de depredadores asociado a un determinado fitófago. Las pruebas en laboratorio tampoco permiten extrapolar con garantías los resultados obtenidos a las condiciones de campo, ya que el comportamiento de los depredadores suele verse modificado bajo la presión de las condiciones artificiales del laboratorio. Estas pruebas son sin embargo útiles una vez que están identificadas las especies, porque permiten el desglose de las conexiones ecológicas, conductuales y tróficas dentro de las redes tróficas de invertebrados, por lo que son un buen complemento de trabajo de campo (Luck et al., 1988; Harwood & Obrycki, 2005).

La forma de asegurarnos de que un depredador se ha alimentado de una presa es mediante el análisis del contenido del tracto digestivo de dicho depredador (Agustí et al., 1999; Zaidi et al., 1999; Juen & Traugott 2005; Monzó et al., 2010). Esta información será esencial para poder entender las interacciones dentro del complejo de redes tróficas. La aplicación de estos estudios puede implicar técnicas simples como la disección e identificación visual de las presas, que ha sido practicada desde finales del siglo XIX en carábidos y coccinélidos, hasta técnicas altamente sofisticadas utilizando la identificación de ADN especifico de las presas por medio del uso de PCR o técnicas como la secuenciación masiva (Gómez-Polo et al., 2015). La detección de presas por medio de PCR involucra el diseño de cebadores específicos que permiten amplificar fragmentos del ADN de la presa de tamaños variables en función de su identidad (Sheppard & Harwood, 2005; King et al., 2008; Waldner et al., 2013). Los genes que se utilizan para esta técnica son el gen mitocondrial de la *Citocromo Oxidasa* I y II (COI y COII) y la región del espaciador transcrito interno 1

y 2 (ITS1 e ITS2) del ADN nuclear ribosomal y han demostrado ser muy útiles en los estudios de depredación en diferentes grupos de artrópodos (Hoogendoorn & Heimpel 2001; Gariepy et al., 2007; King et al., 2008; Monzó et al., 2010, 2011; González-Chang et al., 2016). Esto se debe a que son regiones conservadas del genoma, se encuentran presentes en múltiples copias y son de tamaño pequeño, por lo que no son fácilmente degradadas durante la digestión del depredador (Zaidi et al., 1999; Chen et al., 2000; Agustí et al., 2003; King et al., 2008). Por otro lado, las técnicas basadas en la amplificación masiva de fragmentos de ADN con cebadores universales y su análisis con secuenciadores de última generación (NGS) pueden ser utilizadas cuando se pretende encontrar relaciones más complejas sobre las redes tróficas, ya que son capaces de detectar las fuentes de alimentación alternativas o la depredación intragremial aportando un conocimiento más amplio sobre la dieta de los depredadores (Wirta et al., 2014; Gómez-Polo et al., 2015).

1.5. Factores que regulan y limitan las poblaciones de plagas

Los organismos vivos se ven afectados por factores ambientales o ecológicos que actúan directamente sobre los mismos, limitando su tamaño poblacional y distribución espacial y temporal. Si los factores ambientales son óptimos para el desarrollo de un ser vivo, favorecen el crecimiento de ese organismo en ese sistema, pero si alguno de esos factores impide el crecimiento de una especie, se habla de factor limitante (Berryman, 2004; Barredo et al., 2015). Dentro de los cultivos, se ha estudiado detalladamente cuales son los factores que limitan las poblaciones de las plagas, siendo los de mayor importancia los factores

climáticos (Wallner, 1987; Udell et al., 2017). Si bien los fitófagos se ven afectados por la humedad, lluvia, viento, insolación y fotoperíodo, se considera que la temperatura se destaca dentro de estos factores, ya que influye en los límites de distribución y determina el desarrollo según la cantidad de calor efectivo que requiere cada organismo (Pruess, 1983; Tsai et al., 2002). Las condiciones climáticas también pueden influir sobre las plagas en forma indirecta por su efecto sobre las plantas hospederas. La interacción entre los fitófagos y sus hospederos es, desde el punto de vista biológico, la más importante de las relaciones biológicas y por tanto, también se la considera como un factor limitante de desarrollo de las plagas (Herms, 2004). Además de las características propias de la especie o variedad vegetal, tiene gran relevancia el estado nutricional y fisiológico de la planta, que junto con las condiciones climáticas determinan el desarrollo de los diferentes estados fenológicos de las plantas. Por lo cual, la capacidad de colonización y desarrollo de una plaga dada, está relacionada con la disponibilidad de alimento que proporciona su hospedero, en consecuencia con el estado fenológico de la planta (Walker et al., 1990; Herms, 2004). Muchas especies de plagas, como pulgones, psílidos o minadores de hojas, colonizan sus hospederos cuando se encuentran en brotación y una vez que la calidad del brote disminuye, emigran hacia otros brotes del mismo o de otros hospederos (Urbaneja et al., 2018; Hermoso de Mendoza et al., 2012; Udell et al., 2017)

Una vez establecido un fitófago en una planta, son los factores reguladores quienes determinan el crecimiento o muerte de su población. Las interacciones con otros organismos de la misma o diferente especies son consideradas dentro de estos factores. En un sistema, si los recursos

son suficientemente abundantes (espacio, alimento, etc.), las poblaciones aumentarían de manera indefinida, sin embargo, los recursos suelen ser limitados y es ahí donde surgen relaciones como la competencia (Rosenheim et al., 1995; Lucas, 2005). Esta interacción juega un papel muy importante en la dinámica poblacional de los individuos que usan los mismos recursos para vivir. Pero se considera que, los factores de mortalidad que regulan mayormente la fluctuación poblacional de las plagas son los enemigos naturales, aunque existen muchas controversias sobre la efectividad de los diferentes grupos en distintas regiones del mundo (Hall et al., 1980; Jacas & Urbaneja, 2010).

El manejo del cultivo es otro factor que afecta a la dinámica poblacional de las plagas y es determinante para que el efecto que produzcan supere o no el umbral de daño económico. Un conocimiento detallado de los factores limitantes y reguladores es necesario para el diseño de las estrategias de gestión del cultivo y de esta forma mantener las poblaciones de las plagas por debajo de los niveles tolerables (Altieri & Nicholls 2004).

Figura 1.5 Piojo rojo de California *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae).

1.6. Efecto nutricional de las presas sobre los depredadores

Además del conocimiento sobre el rango de presas que tienen los depredadores, es importante conocer el efecto de las presas sobre el rendimiento de los mismos. Se conoce que la calidad y el tipo de presas juegan un rol importante en el desarrollo, supervivencia y la reproducción de estos enemigos naturales (Lucas, 2005; Schuldiner & Coll, 2017). Para poder entender el comportamiento, la biología y ecología de los insectos y desarrollar estrategias de control biológico es importante conocer de qué forma estos insectos interactúan con su alimento (Symondson et al., 2002; Harwood & Obrycki, 2005). Dentro de los depredadores, se destacan estudios sobre la alimentación de los coccinélidos, que si bien son numerosos, aun así la importancia del tema no ha disminuido (Michaud, 2000; Kalaskar & Evans, 2001; Nielsen et al., 2002). Se conoce que tanto las larvas como los adultos de la mayoría de las especies de coccinélidos son depredadores y además, en general los depredadores coccinélidos sobreviven con una "dieta mixta" compuesta de presas "esenciales" y "alternativas" (Hauge et al., 1998; Evans et al., 1999; Nielsen et al., 2002). La presa esencial apoya el desarrollo y la reproducción, mientras que la presa alternativa permite que los adultos sobrevivan cuando la presa esencial es escasa (Evans et al., 1999; Hodek et al., 2012). En general, el crecimiento, la supervivencia y la reproducción del depredador son mejores cuando se crían en presas esenciales. Además de la calidad nutricional, numerosos factores pueden influir aún más en la elección de las presas. Los mecanismos de defensa de presas (Dixon & Dixon, 2000; Agarwala & Yasuda, 2001), los compuestos adquiridos de sus plantas huéspedes (Carter et al., 1984; Clark & Messina, 1998), entre otras variables deben ser

considerados cuando se evalúan las presas asociadas a los depredadores. Aunque los estudios de laboratorio rara vez incluyen todos estos aspectos, pueden proporcionar una indicación de la preferencia de las presas y la calidad nutricional. La idoneidad de una especie de presa puede evaluarse evaluando el efecto que tiene sobre los atributos del historial de vida del depredador, como su desarrollo, supervivencia, peso adulto y reproducción. Kalushkov & Hodek (2001) sugieren que los datos cuantitativos sobre la tasa de desarrollo, supervivencia y reproducción de un depredador indican la calidad de la presa. El efecto nutricional sobre los parámetros reproductivos es considerado el más importante, debido a que requiere recursos de alta energía (Houck, 1991).

Figura 1.6 Pulgón verde, *Aphis spiraecola* Patch (Hemiptera: Aphididae).

1.7. Control biológico en cultivos de cítricos

Los servicios brindados por los enemigos naturales bajo el marco de control biológico por conservación son cada vez más utilizados en cultivos perennes y actualmente se están implementando en cultivos como el de los

cítricos (Landis et al., 2000; Jacas & Urbaneja, 2010). Si bien estos agroecosistemas son ideales para estudiar las relaciones tróficas entre las plagas y sus enemigos naturales, sin embargo, aún son escasos los conocimientos generados, sobre todo si consideramos que dentro de los países productores de cítricos en la región Mediterránea, se registran más de 140 especies de plagas, dentro de las cuales 108 incluyen insectos (Jacas & Urbaneja, 2010; Jacas et al., 2010; Tena & García-Mari, 2011). No todas estas especies son plagas claves en el cultivo, solamente algunas especies de artrópodos como la mosca de la fruta *Ceratitis capitata* (Wiedemann) (Diptera: Tephritide), el piojo rojo de California *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) (**Figura 1.5**), la araña roja *Tetranychus urticae* Koch. (Acari: Tetranychidae) en clementinos y los pulgones *Aphis spiraecola* Patch (**Figura 1.6**) y *A. gossypii* Glover (**Figura 1.7**) en clementinos y plantas jóvenes son las de mayor importancia y es sobre las que se centran la mayor parte de los estudios (Hermoso de Mendoza et al., 2012; Argolo et al., 2013; Tena et al., 2016; Gómez-Marco et al., 2016a).

Figura 1.7 Pulgón del algodón, *Aphis gossypii* Glover (Hemiptera: Aphididae).

Para un país como España, donde la mayor parte de la producción de cítricos se exporta para consumo fresco, el mayor desafío es ser capaz de proporcionar un producto sin daños (incluidos los meramente cosméticos) y libre de plagas cuarentenarias (Aznar et al., 2015). Esto junto a los altos estándares de calidad para las frutas y los requisitos de cuarentena del mercado internacional, hace que la sanidad del cultivo sea uno de los pilares dentro del gestión. Además, la gestión de plagas en estos cultivos debe proporcionar un producto con un mínimo de residuos de pesticidas, que debe ir acorde a las demandas del mercado internacional junto con la nueva Directiva Europea 2009/128 / CE que regula el uso de pesticidas. Toda esta situación, colocan a la industria citrícola española en una posición inmejorable para implementar programas de Gestión Integrada de Plagas (GIP) en toda la zona (Urbaneja et al., 2014), y es lo que ha llevado a que la adopción de programas de GIP se quintuplicara en las áreas productoras de cítricos entre 2005 y 2012 en la Comunidad Valenciana (Anonymous, 2014).

1.8. Justificación y objetivos

Históricamente, cuando se habla de control biológico se considera que las redes tróficas entre los herbívoros plagas y sus enemigos naturales en los agroecosistemas son generalmente simples, muchas veces lineales, en la que su éxito o fracaso se atribuye principalmente a unos pocos enemigos naturales clave. En los últimos años, los estudios que evidencian interacciones complejas entre diferentes niveles tróficos son cada vez más importantes y nos permiten plantearnos nuevas estrategias de manejo en los cultivos. Por ello, el **primer objetivo** de esta tesis doctoral será estudiar las relaciones tróficas vinculadas entre una plaga clave y sus depredadores, para comprender la contribución ejercida por estos enemigos naturales en el control biológico como un factor regulador demográfico. Utilizamos como modelo de fitófago plaga al piojo rojo de California, *A. aurantii,* y a sus depredares asociados en el agroecosistema de clementinos en la Comunidad Valenciana.

Uno de los fitófagos clave del cultivo de los cítricos, y en especial en clementinos, son los pulgones. Numerosos estudios sobre este grupo de fitófagos plaga nos permiten saber que la densidad de sus poblaciones está fuertemente modulada por variables ambientales, como la temperatura, así como la abundancia y calidad de los brotes de sus huéspedes y los enemigos naturales asociados. Sin embargo, no hay información sobre cómo todos estos factores limitantes y reguladores influyen específicamente en el grado y el potencial de las infestaciones de los pulgones en los cítricos en la cuenca Mediterránea. En este contexto, el **segundo objetivo** pretende evaluar la dinámica de los

pulgones en relación con su planta hospedera, en este caso las plantaciones de clementinos. Además, se estudiará la relación de los pulgones con los distintos grupos de depredadores asociados a sus colonias, determinando los grupos más relevantes y su dinámica poblacional.

Finalmente, para poder comprender mejor la relación de los depredadores con sus presas dentro de las redes tróficas, es importante saber cómo la calidad y el tipo de presas que consumen afectan su fitness. Debido a que las presas esenciales apoyan el desarrollo y la reproducción, en el **tercer objetivo** se evaluará en laboratorio el efecto de las dos especies de pulgones más abundantes en clementinos de la Comunidad Valenciana, *Aphis spiraecola* y *A. gossypii*, y la mezcla de ambas, sobre los parámetros de desarrollo, supervivencia y reproducción de dos especies de coccinélidos, *Scymnus subvillosus* y *S. interruptus*, que son las especies de coccinélidos afidiófagos más abundantes en la cuenca Mediterránea.

capítulo 2

Understanding the demographic regulation of herbivorous key pests in perennial agroecosystems

Capítulo 2

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Understanding the demographic regulation of herbivorous key pests in perennial agroecosystems

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Abstract

Biological control has historically given a simplified trophic view of relationships between herbivores and their natural enemies in agroecosystems. In recent decades even though studies illustrating complex interactions between distinct trophic levels have become more important, a reductionist vision of biological control, in which its success or failure is mostly attributed to a few key

natural enemies, is still preponderant. Species-specific trophic links between phytophagous key pests and their natural enemies was studied to understand the contribution of biological control as an herbivorous demographic regulating factor. Regulation of California red scale, *Aonidiella aurantii* (Maskel), populations in citrus agroecosystems was used as a model. The contribution of predation as the *A. aurantii* mortality factor in citrus groves was assessed through natural enemies-exclusion experiments. The relevance of most of the predator species was later estimated using gut-content analysis with prey specific DNA molecular markers.

Predation emerged as a major factor for *A. aurantii* mortality. Corrected mortality ranged between 51% and 97% during the three pest generations of the season. *Aonidiella aurantii* DNA was detected in gut contents of 11 species of predators. Generalist and stenophagous predators, mainly associated to other citrus pests such as aphids, proved to be the most relevant biological control agents of this pest, especially during its first generation which unveiled an apparent competition between key citrus pests. Specialist predators only appeared to significantly contribute to *A. aurantii* regulation at the end of the season. Results herein presented illustrate how regulation of key herbivores in perennial crops is mediated through complex trophic interactions between them and their natural enemies. This information may help to change the current reductionist paradigm of biological control of key pests.

Keywords Conservation biological control, exclusion, DNA prey detection, PCR, predator assemblages, integrated pest management, apparent competition, citrus, California Red Scale

2.1. Introduction

In recent years pesticide use has become increasing limited due to new international regulations (Regulation (EC) n° 1107/2009), market restrictions, environmental and human health concerns. Globally, these pressures have driven the implementation of less aggressive pest management approaches, such as biological control (BC) strategies in agriculture (Tscharntke et al., 2012). Conservation and use of resources offered by agroecosystems is currently considered to be the most promising strategy for sustainable pest management in perennial crops (Rusch et al., 2017). For example, habitat management strategies are used to favor natural enemies and improve their BC services (DeBach & Rosen, 1991; Woltz et al., 2012). Throughout recent decades the use of selective insecticides, creation of ecological infrastructures, and evaluations of the role of natural enemies have become the main focus of pest management research (Tscharntke et al., 2012; Rusch et al., 2017).

Predators have always been highly valued as natural enemies in agriculture. However, their complex biology and ecology has frequently hindered assessment of their true role as BC agents (Riechert & Lockley, 1984; Symondson et al., 2002). Theoretical developments were, at first, mostly concentrated on specialist predators, since these systems are easier to parameterize (Hassell & May, 1986; Van Driesche et al., 2010). Nevertheless, in recent decades, there is growing interest in the control exerted by associations of mainly indigenous and naturalized generalist predators who are recognized

to have the ability to keep prey densities at stable equilibriums in non-density-dependent ways (Symondson et al., 2002; Harwood & Obrycki, 2005).

Direct observations of the evaluation of predatory activity are often very laborious and time consuming (Luck et al., 1988); traits such as the high mobility of predators and their cryptic activity makes this task highly difficult. Interference with the observed phenomenon is also a risk associated with the observation approach (Grant & Shepard, 1985; Symondson et al., 2002; Hodek et al., 2012). *In-situ* post-evaluations usually underestimate the importance of predation as a biotic mortality factor since frequently no prey remains are left after predation occurs (Sorribas & García-Marí, 2010).

Indirect methods offer the opportunity for assessing the impact of predators on the population of a particular pest both quantitative and qualitative ways without directly interfering with the event. Quantifications are mostly based on comparisons between pest densities in the presence or absence of the predators (Grant & Shepard, 1985; Harwood & Obrycki, 2005). Exclusion of natural enemies have been achieved with pesticides that selectively affect them and not the target pest (pesticide exclusion design) and with experimental units (cages) that physically protect the pest from natural enemy attack (exclusion cage design) (Luck et al., 1988; Monzó et al., 2014). Predator gut-content studies permit the identification of components of the predator guild that are actually contributing to the predation of the target pest (Grant & Shepard, 1985; Symondson et al., 2002). Among all the existing *post-mortem* techniques, prey DNA detection by polymerase chain reaction

(PCR), using species specific primers that amplify multicopy DNA gene regions such as mitochondrial cytochrome oxidase I (COI) or Internal transcribed spacer 1 (ITS1), has proved to be a powerful tool. With PCR one can obtain important information on trophic interactions that may otherwise be challenging to acquire (Agustí et al., 2003; Sheppard et al., 2005; King et al., 2008; Monzó et al., 2011).

Citrus are perennial crops with high potentials to maintain agroecosystem functions and services. Not surprisingly, modern biological control research in agriculture began in this crop (Debach & Rosen, 1991). The high stability of citrus agroecosystems allows the harboring of rich complexes of natural enemies with the potential to keep most of the associated phytophagous species under economic injury levels, probably through complex ecological interactions (Ciancio & Mukerji, 2010). Despite this, there are few research projects aimed at disentangling the trophic relationships between these phytophages and their potential natural enemies (Gómez-Marco et al., 2016; Pérez-Sayas et al., 2015). The success or failure of BC has commonly been attributed to the role of a reduced number of specialist natural enemies (Murdoch et al., 2005; Pekas, 2010; Sorribas & García-Marí, 2010).

There is a minority group of phytophagous species associated with citrus that can escape the satisfactory regulation exerted by their natural enemies; whose control then relies mostly on the use of pesticides (Gómez-Marco et al., 2016; Tena et al., 2011). In the western Mediterranean citrus region, the California Red Scale, *Aonidiella aurantii* (Maskell) (Hemiptera:

Diaspididae) is one such pest and consequently is considered to be a key pest in the crop (Tena et al., 2011). BC of this pest is mostly attributed to specialist scale parasitoids of *Aphytis* genus (Hymenoptera: Aphelinidae) and to some specialist coccinellid species such as *Chilocorus bipustulatus* (Linnaeus, 1758), *Rhyzobius lophanthae* (Fabricius, 1787), and *Coccidophilus citricola* (Brèthes, 1905) (Hattingh & Samways, 1992; Murdoch et al., 2005; Sorribas & García-Marí, 2010). Conservation and augmentative strategies for these natural enemies have been developed and implemented in numerous citrus growing areas of the world (Vanaclocha et al., 2011; Dao et al., 2017). Some indigenous generalist predators such neuropterans have also been cited as potential natural enemies of this pest (Alvis, 2003). However, little is known of their impact; thus their role as natural enemies is probably underestimated.

The aim of this study was to unravel the role of predation in key pests associated with perennial citrus crops by quantifying this BC service and determining the importance of species-specific trophic links employing among others, molecular techniques. BC exerted by predators upon *A. aurantii* in citrus was chosen as an empirical model. Specifically, i) we weighted the contribution of predation as a biotic mortality factor of *A. aurantii* using exclusion techniques, and ii) assessed the relative importance of its potential predators through *post-mortem* evaluations based on prey DNA detection in the gut content of predators.

2.2. Materials and methods

2.2.1. Contribution of predation as *A. aurantii* mortality factor

2.2.1.1. Location and experimental conditions

Experiments were conducted in two citrus orchards in the region of Valencia, Spain; one in Moncada (39°35'17.43"N / 0°23'53.28"O; 0.18 ha) and one in Algimia (39°42'55.11"N / 0°18'57.46"O; 0.25 ha), during the 2014, 2015, 2016 seasons. Blocks contained Clementine mandarins (*Citrus clementine* Hort. Ex Tan.), all approximately 15 years old. Trees were drip-irrigated and not treated with insecticides for at least two years before the experimental preparations and during the course of the study.

2.2.1.2. Insects

The *Aonidiella aurantii* individuals used in the experiments were taken from a laboratory colony reared on lemons at the Instituto Valenciano de Investigaciones Agrarias, IVIA (Valencia, Spain). This colony was initiated in 1999 from scale insects collected in citrus fields of Alzira (Valencia, Spain). New individuals from the fields surrounding IVIA were added every 2–3 years to ensure genetic diversity of the population.

2.2.1.3. Exclusion studies

The contribution of predation as a mortality factor of *A. aurantii* in citrus was assessed through exclusion techniques. Experiments were replicated twelve times: in each orchard, one at each of the three annual *A. aurantii* field generations for two seasons. *Aonidiella aurantii* generations were predicted using the degree-day model proposed by Asplanato & García-Marí (2001) and Carot et al. (2003) with the data obtained from http://gipcitricos.ivia.es. In each experiment, *A. aurantii* cohorts were artificially initiated on leaves (1st, 2nd and 3rd generations) and fruit (2nd and 3rd generations only, as fruit is not yet present during the 1st generation). Females from the IVIA colony were synchronized by infesting between 100-125 lemons 50 days prior to the field experiments; during this period the females were maintained at 27° C and 75% HR. Under these conditions, females were expected to reach maturity just before the onset of each experiment. In each orchard, 10-13 trees with no *A. aurantii* present were randomly selected and four fruit and/or leaves, one per cardinal orientation were chosen to initiate the artificial cohorts. Lemons hosting gravid females from the IVIA colony were attached to the field selected fruit and/or leaves with adhesive tape and left for three days to facilitate first instar nymph (crawlers) infestation. In each orchard 45 colonies per plant organ (fruit or leaves), containing between 10 and 30 recently adhered *A. aurantii* first instars, were selected and randomly assigned to the following treatments: i) 'predation' (colonies exposed to natural enemies), ii) 'exclusion' (colonies protected from natural enemies with a 45 x 20 cm muslin bag), and iii) 'semi-exclusion' (colonies covered with a muslin bag opened in one end to allow

access of natural enemies and at the same time, mimic the same environment as the exclusion treatment).

Scale growth within cohorts was checked weekly and treatments were left in the field until females reached adult stage (42-60 days). At this point, leaves and/or fruit hosting the cohorts were removed from trees and taken to the laboratory. The number of scales present at each colony as well as their developmental stage, evidence of predation, parasitoid host-feeding and parasitized scales were verified with stereoscope microscopy and compared to the initial number of attached first instars.

2.2.2. *A. aurantii* predatory agents and their relevance

2.2.2.1. Sampling for predators

To identify the arthropod predatory species associated with *A. aurantii* biotic mortality and to estimate their relevance, the species present in the study citrus groves in each *A. aurantii* generation were monitored weekly by stem-tap sampling from the beginning to end of the exclusion experiments. Stem-taps consisted of striking eight randomly selected branches (two per cardinal orientation) of 10 to 13 trees adjacent to those chosen for the exclusion trials with a PVC pipe three times. Arthropod predatory specimens collected in a 45 x 30 cm plastic tray placed under the stricken branches were rapidly moved to dram containers by individually suctioning them using an entomological aspirator (pooter). The containers were then filled with ethanol

(80%) for specimen preservation. Collected material was immediately taken to the laboratory and identified to the species level with stereoscope microscopy. Specimens of interest for the gut-content studies were carefully rinsed first with water and then with ethanol (80%). Cleaned specimens were individually stored in Eppendorf tubes containing 1 ml of ethanol (80%) and kept in the freezer at -20°C for posterior gut-content examination using molecular analyses.

2.2.2.2. *Aonidiella aurantii* specific primer design

Total DNA was extracted from three *A. aurantii* individuals of IVIA's colony following a modified Salting Out protocol (Monzó et al., 2011). DNA was then amplified by PCR (Polymerase Chain Reactions) using the universal primers for the mitochondrial gene cytochrome c oxidase subunit 1 (COI) LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al., *1994*). PCR amplifications were performed in 20 µl reaction volumes containing: 1 µl of DNA from the *A. aurantii* ´s extraction, 2 µl buffer (10x Standard Reaction, Biotools), 0.7 µl $MgCl_2$ (Biotools), 0.4 µl dNTPs (10 mM each 1 ml, Thermo Scientific), 0.4 µl of each primer, 1 µl of Taq DNA polymerase (1000 units - 1 U/µl, Biotools) and 14.10 µl free water, in a thermal cycler (Eppendorf Mastercycler) for 35 cycles at: 95°C, 1 min; 45°C, 1 min; 72°C, 1.3 min. Firstly, the denaturation cycle was carried out at 94°C for 2 min, and lastly the extension cycle at 72°C for 10 min. Double-stranded PCR product was purified (NucleoSpin® Gel and PCR Clean-up; Macherey-Nagel) and sent to Institute of Molecular and Cellular Biology of Plants (IBMCP, Valencia, Spain) for sequencing. *Aonidiella aurantii*

species specific primers were later designed on these sequences with Primer3web 4.0.0 software (http://primer3.ut.ee). The oligo selected were later contrasted through BLAST on the GenBank website (https://blast.ncbi.nlm.nih.gov/), with that of other organisms to evaluate their specificity.

2.2.2.3. Amplification conditions

Primers were employed at a final volume of 20 µL containing 10 ng total extracted DNA, 1x Standard Reaction buffer with $MgCl_2$ (Biotools B&M labs S.A.), 200 nM dNTPs (Thermo Fisher Scientific, Inc.), 0.4 µl of each primer, 1 u Taq DNA polymerase (Biotools B&M labs S.A.). The amplification profile was as follows; an initial denaturation step at 94 °C for 4 min; 35 cycles of 92 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s; and a final extension at 72 °C for 4 min. Amplifications were performed in a Mastercycler® ep gradient-S thermal cycler (Eppendorf).

2.2.2.4. Test for cross-reactivity and sensibility

Primer specificity was tested by assaying CRSCOIF1 – CRSCOIR1 primers with total DNA extracted from several individuals of two strains of *A. aurantii* (laboratory colony and field strain), several closely related species and a wide range of arthropods (prey and predators) potentially present in the same citrus agroecosystem. Two PCRs per sample were run; in any case of conflicting results a third PCR was therefore conducted. Sensitivity was determined by assaying

primers with ten-fold dilutions of *A. aurantii* total DNA starting with 10 ng and progressing to a 1:10,000 dilution.

2.2.2.5. Field samples

Stored material from the exclusion studies (see section '*Sampling of predators*') was used for the *post-mortem* evaluations. The most abundant predator species and *A. aurantii* specialist predator species cited in the literature were selected (Hattingh & Samways, 1992; Vanaclocha et al., 2011). Prior to the DNA extraction, to reduce the proportion of predator/prey DNA in each tested specimen and to minimize risks of topical prey DNA contamination, the selected specimens were dissected under a stereoscope microscope. Heads, thoraxes and legs were removed; thus only abdomens were used for the DNA extraction (Monzó et al., 2011). DNA was later extracted following modified Salting Out Protocol and each sample was tested in duplicate by PCR using CRSCOI primers. For any case with contradictory results a third PCR was also conducted.

2.2.3. Statistical analysis

Aonidiella aurantii recovered at the end of each experiment were compared to the initial number of adhered first instars in each cohort, in protected ('exclusion'), semi-exposed ('semi-exclusion') or exposed ('predation') cohorts to calculate the reductions in their numbers. Parasitized scales and host-feeding damage were taken into account to discriminate between parasitism and predation components within *A. aurantii* biotic mortality.

Differences in *A. aurantii* mortality between treatments ('predation', 'semi-exclusion' and 'exclusion') were studied using generalized linear mixed model (GLMM) analysis (Wolfinger & O'connell, 1993). Normal distribution of the variable was assumed based on Akaike and Bayesian information criteria. Mortality in the 'exclusion' treatment, in which cohorts were protected from natural enemies, was considered to be the natural mortality. Differences in *A. aurantii* mortality (dependent variable) between seasons (year 1 and 2), locations (Moncada and Algimia) and plant organs (fruit and leaves) (fixed effects) in the 'exclusion' treatment (natural mortality) was also compared using GLMM analysis. Poisson distribution of the variable was assumed. Percentage mortality in exposed cohorts corrected for mortality in protected cohorts was estimated using the Henderson and Tilton formula (Henderson & Tilton, 1955). Corrected mortality was therefore the one attributed to predators (Monzó et al., 2014). Differences between *A. aurantii* generations (1st, 2nd and 3rd), seasons, locations and plant organs (fixed effects) on *A. aurantii* corrected mortality (dependent variable) were studied using GLMM analysis. The interaction between the variables 'season' and 'generation' was also included in the model. Normal distribution of the variable was assumed.

Influence of *A. aurantii* generation, season and location (fixed effects) on the seasonal-activity of the citrus predatory complex and on each predatory group (Araneae, Coleoptera, Dermaptera, Hemiptera, and Neuroptera) (dependent variables), monitored through stem-tap sampling, were evaluated using GLMM analyses. Negative binomial distribution of the variable was selected in all the cases. Interactions between all fixed effects were also included

in each model. The cases in which interactions were not significant were later removed from their respective models.

The relevance of each collected predatory species as BC agent of *A. aurantii* was estimated by multiplying the proportion of specimens testing positive in the PCR analyses in each generation, with the number of specimens collected through stem-tap sampling. Relevance of each predatory group and of the whole *A. aurantii* predatory assemblage was calculated by adding up the estimated relevance of all the species included in each group. Changes in the relevance of the *A. aurantii* predatory assemblage and of each predatory group between generations were studied through GLMM analyses where 'relevance' was the dependent variable of 'generation' (fixed effect). Negative binomial distribution of the variable was selected in all the cases based on Akaike and Bayesian information criteria.

Two GLMM were used to determine if changes in *A. aurantii* mortality attributed to predation could be explained by the predator abundance and/or relevance. In the first model *A. aurantii* corrected mortality was dependent on predator abundance (fixed factor), whereas in the second model corrected mortality was dependent on predator relevance (fixed factor). In both models 'generation' was also included as a fixed factor. Normal distribution of the variables was assumed based on Akaike and Bayesian information criteria. Goodness-of-fit of each model was used to check whether 'relevance' was able to explain better *A. aurantii* mortality than 'abundance'.

Kenward and Roger Satterthwaite approximation for degrees of freedom was included in all the models of this study (Kenward & Roger, 1997). Post-hoc t-test (Tukey) comparisons were made in each case having a significant effect ($P < 0.05$) for all analyses.

2.3. Results

2.3.1. Contribution of predation as *A. aurantii* mortality factor

According to the degree-day model the three *A. aurantii* generations were predicted to reach of their maximums at the following dates: first generation: 10/06/2015 and 12/06/2016; second generation: 29/07/2014 and 27/07/2015; and third generation: 16/09/2014 and 18/09/2015 (**SS2.1**). Exclusion trials were therefore set at those dates as indicated in **table 2.1**.

Overall 652 *A. aurantii* cohorts were used for this study; 216 in the 'exclusion' treatment, 208 in 'semi-exclusion' and 228 in the 'predation' treatment. Mortality found in the cohorts under exclusion (natural mortality) ranged around 60% (**fig. 2.1**). No differences within this treatment were found between generations ($F = 1.48$; df = 2, 210; $P = 0.231$), seasons ($F = 0.10$; df = 1, 210; $P = 0.747$) and only marginal differences were found between locations ($F = 3.79$; df = 1, 210; $P = 0.053$). Natural mortality was nevertheless significantly higher on the leaves than the fruit ($F = 15.87$; df = 1, 210; $P < 0.0001$). Mortality within cohorts was significantly influenced by treatment ($F = 122.75$; df = 2, 649; $P < 0.0001$). The highest mortality was found under 'predation', the lowest under

Sites	Season	Generation	Establishment date	Ending date	Exposure time (days)
Moncada	1	1	3-jun.-2015	15-jul.-2015	42
		2	5-ago.-2014	16-sep.-2014	42
		3	3-oct.-2014	2-dic.-2014	60
	2	1	4-jun.-2016	27-jul.-2016	53
		2	29-jul.-2015	17-sep.-2015	50
		3	2-oct.-2015	26-nov.-2015	55
Algimia	1	1	1-jun.-2015	13-jul.-2015	42
		2	8-ago.-2014	19-sep.-2014	42
		3	1-oct.-2014	2-dic.-2014	62
	2	1	7-jun.-2016	28-jul.-2016	51
		2	31-jul.-2015	16-sep.-2015	47
		3	1-oct.-2015	26-nov.-2015	56

Table 2.1 Calendar for the exclusion trials conducted during the three *A. aurantii* generations, at two locations and two different seasons. Establishment dates of the experimental units, ending dates of each trial and exposure time of cohorts in the field are displayed.

'exclusion'; the 'semi-exclusion' treatment presented *A. aurantii* mortality levels between those found in the other two treatments (**fig. 2.1**). Mortality attributed to parasitism accounted for less than 3% and 6% of all mortality in the 'semi-exclusion' and the 'predation' treatments respectively.

Corrected mortality owing to predation of *A. aurantii*, on the other hand, ranged between 51% and 97% throughout the study. No differences in corrected mortality were found between organs (F = 0.51; df = 1, 220; P = 0.4768), sites (F = 0.57; df = 1, 220; P = 0.4495) and only marginal differences were observed between seasons (F = 3.48, df = 220, P = 0.0634) (**fig. 1.2**). Corrected mortality was affected by *A. aurantii* generation (F = 4.21; df = 2, 220; P = 0.0161); being higher during the first generation (**fig. 1.3**).

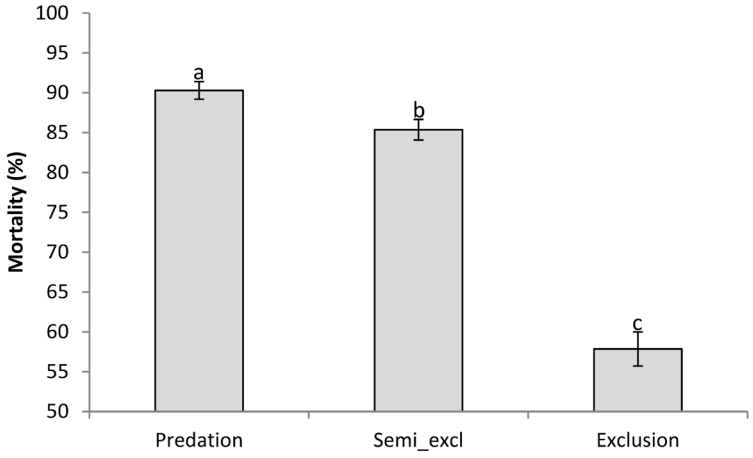


Figure 2.1 Mortality (%) of *A. aurantii* cohorts from the beginning of the experiments until the end, in the following treatments: exclusion, semi-exclusion and predation. Different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

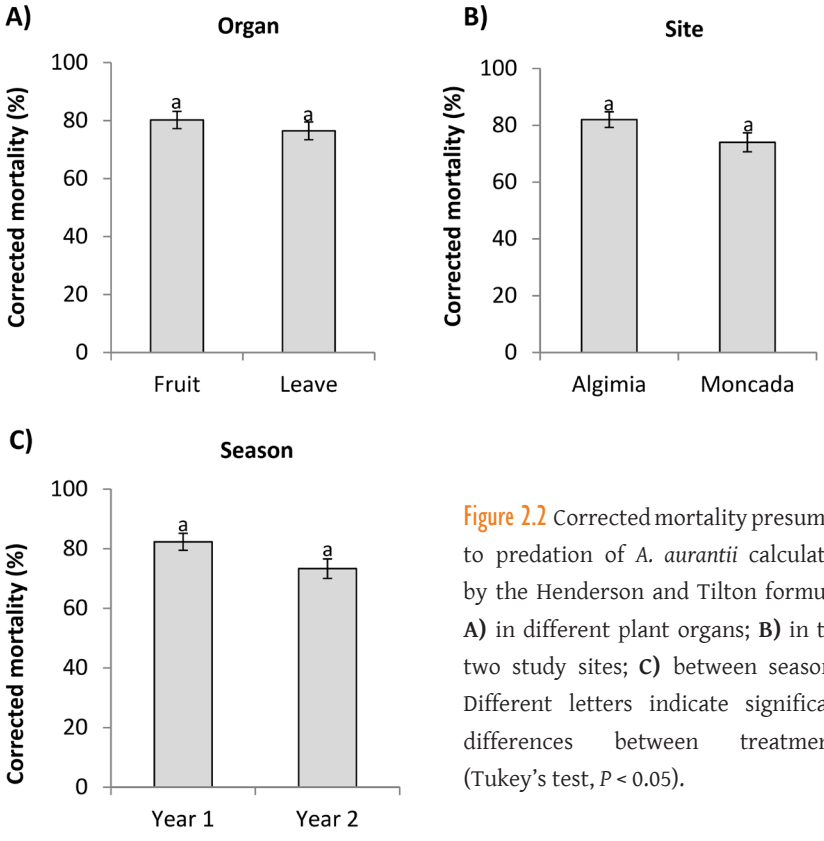


Figure 2.2 Corrected mortality presumed to predation of *A. aurantii* calculated by the Henderson and Tilton formula: **A)** in different plant organs; **B)** in the two study sites; **C)** between seasons. Different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

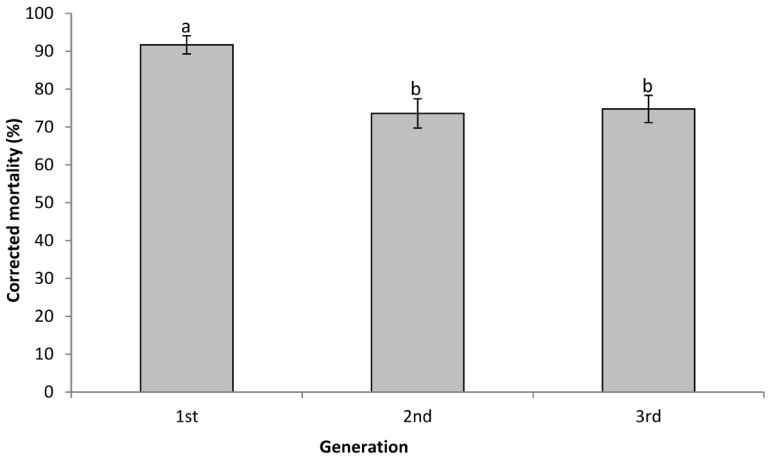


Figure 2.3 Corrected mortality presumed to be due to predation of *A. aurantii* calculated by the Henderson and Tilton formula in the three generations of *A. aurantii*. Different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

2.3.2. *Aonidiella aurantii* predatory agents and their relevance

2.3.2.1. *Aonidiella aurantii* predators

More than 10,000 specimens of potential *A. aurantii* predators from five taxonomic orders were collected by stem-tap sampling throughout the experiments (**SS2.2**). Araneae was the most diverse and frequently encountered predatory group; it accounted for 40% of all specimens collected; it presented more than 20 species. Coleoptera was the second most diverse and frequently found group with 18 species identified; all of which belong to the Coccinellidae family; it represented 34% of all specimens. Only 5 Hemipteran species were found, but they accounted for 18.6% of all the predators captured. Neuroptera and Dermaptera were the least captured and diverse groups accounting for only 7% and 0.4% of captures; 4 and 2 species, respectively.

No differences in the mean number of predators per stem-tap and tree were found for any of the taxonomic groups between seasons (**table 2.2**). In general, predators were more frequently found in Moncada than in Algimia; with differences between taxonomic groups. Araneae and Hemiptera were more frequently found in Moncada than in Algimia; whereas the contrary was observed for Coleoptera, Neuroptera and Dermaptera. Predators were most frequently found during the first *A. aurantii* generation with further differences between taxonomical groups. Coleoptera and Hemiptera were most frequently found during the first *A. aurantii* generation, whereas Araneae was mostly captured during the last generation. No differences between generations were found for Dermaptera and Neuroptera captures.

2.3.2.2. Molecular study

For the subsequent molecular analyses, the five most frequently found species of Coccinellidae (Coleoptera) and Araneae (except *Dipoena melanogaster* who is an ant predator) (Simon, 1997) were selected. In addition, the already known specific coccinellid predators of *A. aurantii*, *Rhyzobius lophantae, Coccidophilus citricola* and *Chilocorus bipustulatus* were also included in the study. The three most frequently captured Hemiptera and Neuroptera species and the only two Dermaptera species captured were also chosen for study.

		Araneae	Coleoptera	Hemiptera	Neuroptera	Dermaptera	Total predators
Season	1	5.42 ± 0.25a	5.36 ± 0.34a	2.02 ± 0.18a	0.74 ± 0.10a	0.04 ± 0.02a	15.11 ± 0.64a
	2	5.12 ± 0.16a	4.44 ± 0.20b	1.43 ± 0.09b	0.90 ± 0.05a	0.04 ± 0.01a	13.01 ± 0.33b
	F – value	1.10	6.59	10.39	2.72	0.07	9.22
	df	1, 774	1, 771	1, 771	1, 771	1, 771	1, 771
	P	0.30	0.01	0.001	0.10	0.79	0.003
Plot	Moncada	6.29 ± 0.23a	3.92 ± 0.21b	3.21 ± 0.21a	0.64 ± 0.06b	0.01 ± 0.01b	14.90 ± 0.51a
	Algimia	4.40 ± 0.17b	6.05 ± 0.29a	0.90 ± 0.08b	1.05 ± 0.08a	0.10 ± 0.02a	13.20 ± 0.44b
	F – value	49.20	37.94	147.19	20.48	11.79	6.74
	df	1, 774	1, 771	1, 771	1, 771	1, 771	1, 771
	P	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0006	0.0096
Generation	1	4.60 ± 0.19b	6.09 ± 0.30a	3.77 ± 0.23a	0.71 ± 0.06a	0.06 ± 0.02a	16.63 ± 0.55a
	2	5.20 ± 0.25b	4.65 ± 0.35b	1.06 ± 0.13b	0.87 ± 0.10a	0.03 ± 0.02a	12.84 ± 0.65b
	3	6.15 ± 0.28a	4.09 ± 0.26b	1.22 ± 0.11b	0.90 ± 0.09a	0.03 ± 0.02a	12.91 ± 0.55b
	F – value	11.75	13.14	75.24	1.86	0.76	15.09
	df	2, 774	2, 771	2, 771	2, 771	2, 771	2, 771
	P	0.001	< 0.001	< 0.001	0.16	0.47	< 0.001

Table 2.2 Mean number (±SE) of predators collected per stem-tap and tree during the exclusion trials. Captures are grouped by taxonomical orders, season, study site and *A. aurantii* generation. Different letters within taxonomical groups indicate significant differences between treatments: season, site and *A. aurantii* generation (Tukey's test, $P < 0.05$).

2.3.2.3. Primer design, sensitivity and cross-reactivity

Sequenced *Aonidiella aurantii* COI fragments were accessed in the GenBank database (accession number: MH384792). The CRSCOI primer pair (CRSCOIF1 and CRSCOIR1) designed on the sequenced COI fragment amplified a 259 bp fragment from *A. aurantii* DNA (**table 2.3**). Detection threshold on the sensitivity test was of 10^{-5} dilution of *A. aurantii* total DNA.

Primer	Sequence (5´-3´)	Ta (°C)
CRSCOIF1	AATGAAGGAAATAAAAATCAGAATC	48.4
CRSCOIR1	GGAATTTGATCAGGAATAATAGGAA	50.9

Ta, annealing temperature

Table 2.3 Primers designed from COI sequence of *Aonidiella aurantii.*

From the 61 arthropod species DNA tested (31 species were considered to be predators and 30 as potential target prey), no amplicon was found except in two *A. aurantii* closely related species of the Diaspididae family: *Aspidiotus nerii* and *Chrysomphalus aonidum* (**SS2.3**).

2.3.2.4. Relevance of *A. aurantii* predators

Aonidiella aurantii DNA was detected in the gut content of 11 of the 21 predatory species from the 1,676 specimens analyzed (**table 2.4**). *Pilophorus* cf *gallicus* had the highest proportion of positive detections followed by *R. lophantae, S. aleyrodiformis* and *S. interruptus*. Fewer positive detections were obtained from *C. mildei* and *S. subvillosus* specimens. Positive detections

were sporadic in *C. carnea*, *P. cespitum*, *B. chalybeus*, *R. cardinalis* and *I. hamatus* (less than 2% of all the specimens tested).

Species	Specimens analyzed				% Positives			
	1	2	3	Total	1	2	3	Total
Pilophorus cf gallicus	40	38	27	**105**	45	57.89	92.59	**61.90**
Rhyzobius lophantae	10	2	14	**26**	40	0	21.43	**26.92**
Semidalis aleyrodiformis	19	37	40	**96**	10.53	10.81	17.50	**13.54**
Scymnus interruptus	40	40	40	**120**	0	10	15	**8.33**
Cheiracanthium mildei	40	40	40	**120**	0	10	5	**5**
Scymnus subvillosus	40	27	24	**91**	10	0	0	**4.40**
Chrysoperla carnea	40	40	33	**113**	2.50	0	3.03	**1.77**
Philodromus cespitum	40	40	40	**120**	0	0	5	**1.67**
Ballus chalybeus	36	28	34	**98**	0	3.57	0	**1.02**
Rodolia cardinalis	40	40	38	**118**	2.50	0	0	**0.85**
Icius hamatus	40	40	40	**120**	0	2.50	0	**0.83**
Stethorus punctillum	27	35	40	**102**	0	0	0	**0**
Coccidophilus citricola	0	1	3	**4**	0	0	0	**0**
Chilocorus bipustulatus	0	1	1	**2**	0	0	0	**0**
Cryptolaemus montrouzieri	11	34	27	**72**	0	0	0	**0**
Conwentzia psociformis	7	7	21	**35**	0	0	0	**0**
Orius laevigatus	32	16	40	**88**	0	0	0	**0**
Cardiastethus fasciiventris	27	27	30	**84**	0	0	0	**0**
Forficula auricularia	12	7	6	**25**	0	0	0	**0**
Euborellia annulipes	7	4	8	**19**	0	0	0	**0**
Philodromus albidus	40	40	38	**118**	0	0	0	**0**
			TOTAL	**1,676**				

Table 2.4 Total number of predatory arthropods analyzed per *A. aurantii* generation and proportion of positive *A. aurantii* DNA detections by gut-content PCR analysis.

According to predator abundance and subsequent PCR positive detections of gut contents, *A. aurantii* predator assemblage was estimated to be most relevant to *A. aurantii* biological control during the first generation and least in the second generation (**table 2.5**). Nevertheless, differences were found between predatory groups. The two most relevant predatory groups, Hemiptera (Miridae) and Coleoptera (Coccinellidae) were most important in the first *A. aurantii* generation. Hemiptera, most relevant Order, decreased its relevance throughout the season. On the other hand Coleoptera importance reduced as an *A. aurantii* mortality factor after the first generation; there were no differences between the second and the third generations. Neuroptera significantly increased its relevance as the season went on. Spiders presented low and variable relevance between generations.

Generation	Araneae	Coleoptera	Hemiptera	Neuroptera	Total predators
1	0.04 ± 0.01b	0.31 ± 0.02a	1.87 ± 0.14a	0.02 ± 0.01b	2.20 ± 0.12a
2	0.08 ± 0.02a	0.20 ± 0.02b	0.46 ± 0.06b	0.03 ± 0.01b	0.79 ± 0.07b
3	0.05 ± 0.02ab	0.20 ± 0.02b	0.25 ± 0.04c	0.11 ± 0.02a	0.64 ± 0.06ab
F – value	2.13	9.84	96.77	10.2	89.76
dF	2, 778	2, 778	2, 778	2, 778	2, 778
P – value	0.12	< 0.001	< 0.001	< 0.001	< 0.001

Table 2.5 Mean number (±SE) of estimated relevance (proportion of positive detections x number of captures) of *A. aurantii* predators grouped by taxonomical orders for each generation. Different letters within taxonomical groups indicate significant differences between generations (Tukey's test, $P < 0.05$).

PCR based gut content analysis strongly changed the perspective of the estimated relevance of each predatory group in comparison to diversity and abundance data (**fig. 2.4**). In addition, estimated relative relevance at the species level also changed throughout *A. aurantii* generations. During the first *A. aurantii* generation, of the three most frequently captured predatory species (*P.* cf *gallicus*, *S. subvillosus* and *S. interrupts*, encountered at equal proportions) and the six species collected in the next most significant numbers, only two species, *P.* cf *gallicus* and *S. subvillosus*, seemed to be highly relevant in the control of *A. aurantii* as detected by positive PCR gut content analysis. In the second and third *A. aurantii* generations, relative abundance of *P.* cf *gallicus* reduced drastically, but nevertheless remained one of the most relevant predators. *Scymnus interruptus* importantly increased its relevance throughout the season. A similar trend was found with *S. aleyrodiformis* and to a lesser extent with *C. carnea*. The relative relevance of spiders was negligible in the first *A. aurantii* generation, yet was higher at the end of the season. The *A. aurantii* specific predator *R. lophantae* seemed to only significantly contribute to the control of its preferential prey at the end of the season (in the third *A. aurantii* generation) and its relevance in terms of positive detections and seasonal-activity was lower than the generalist predatory species.

2.3.2.5. Abundance *vs* Relevance

Changes in *A. aurantii* mortality attributed to predation throughout the season, at the two locations, and the two seasons was well explained by changes in predator abundance (F = 5.56, df = 1, 8.745; P = 0.0461) but was even better explained by their relevance (F = 6.14, df = 1, 8; P = 0.0383).

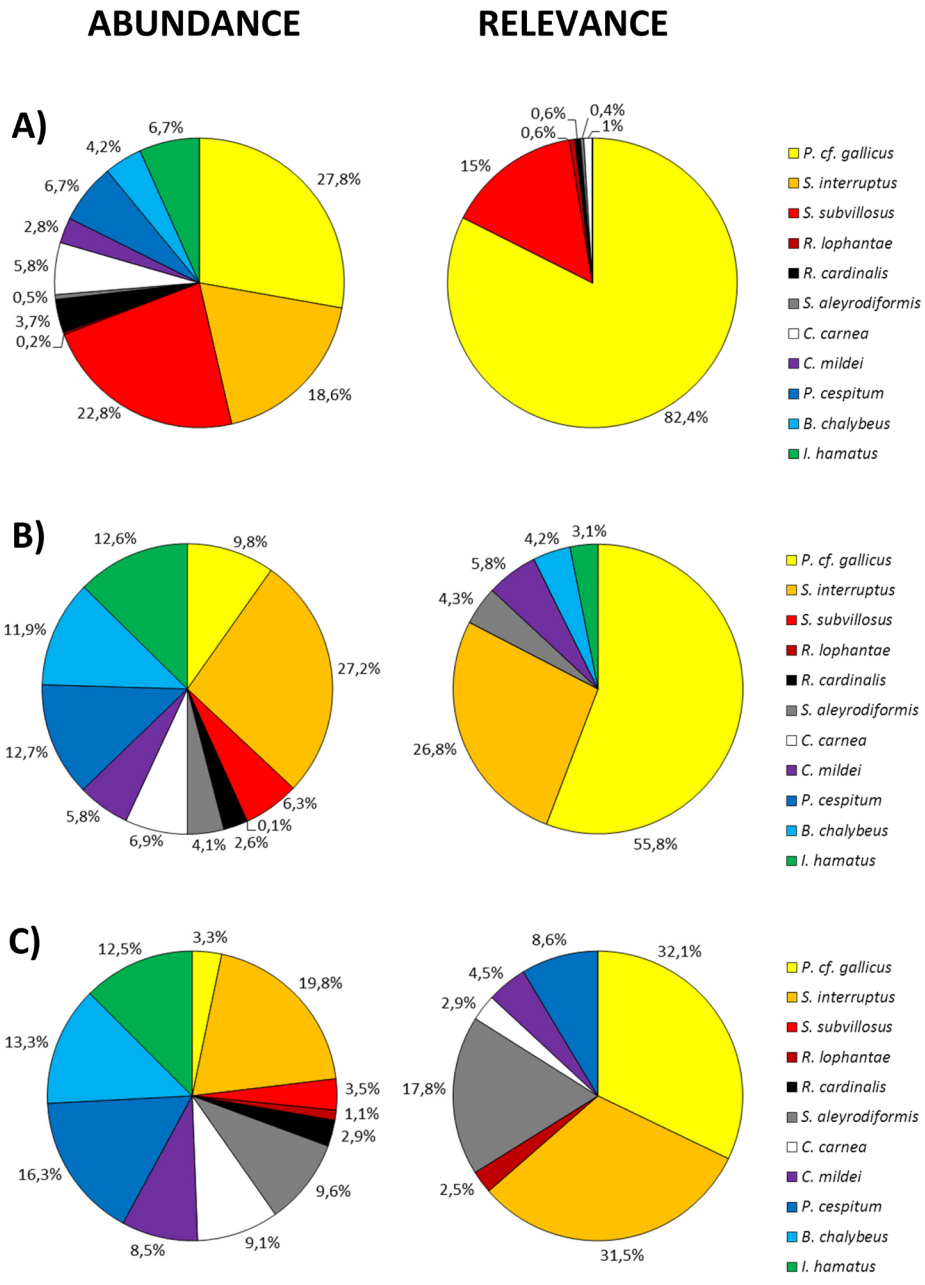


Figure 2.4 Relative abundance and estimated relevance of *A. aurantii* predators out of the most abundant predatory species found in citrus through stem-tap sampling during the three *A. aurantii* generations: A) first generation; B) second generation; C) third generation.

2.4. Discussion

The present study demonstrates the potential of predation as a determining regulating factor of a key pest in a perennial crop, and reveals how this mortality factor is mostly attributed to the combined action of non-specialist predators. Under management conditions of minimal BC disruption *A. aurantii* mortality associated to its predator complex in commercial citrus groves may rise as the main biotic mortality component; causing on average, reductions of more than 75% in recently settled cohorts. Though previous studies have named predation as a component of *A. aurantii* mortality, they were unable to accurately estimate its importance and hence it was typically underrepresented (Sorribas & García-Marí, 2010; Vanaclocha et al., 2011).

Our results present a very different view of the demographic regulation of this herbivore than Murdoch et al. (2005) modeled in citrus agroecosystems of California. In that system, *A. aurantii* populations rapidly reached temporal stability, maintaining their populations well below their economic injury levels. These authors attributed this demographic equilibrium to the single interaction between *A. aurantii* and the parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), and conclude that this interaction was purely mediated through life-history and physiological properties of only these two species. Demographic stability was consequently reached locally in each tree without the influence of metapopulations of both species. In the Western Mediterranean basin, the relationship between *A. aurantii* and *A. melinus* has been widely studied, but the stability in the

parasitoid-host system described by Murdoch et al. (2005) has never been achieved (Asplanato & García-Marí, 2001; Vanaclocha et al., 2011; Tena et al., 2011). Different factors such as specific climatic conditions or the absence of sugar food resources for *A. melinus* adult females at the end of the season have been named among the potential causes explaining this (Boyero et al., 2014; Tena et al., 2011). The high diversity of natural enemies and herbivores associated to Mediterranean citrus agroecosystems is probably the other main difference between our system and that presented by Murdoch et al. (2005) in which trophic relationships were strongly simplified to just one herbivore species and one natural enemy. As observed in the present study *A. aurantii* demographic regulation in Mediterranean citrus agroecosystems may alternatively be achieved through more complex trophic interactions between this herbivore and a guild of specific and generalist natural enemies.

Natural enemies-exclusion techniques permitted satisfactorily discrimination between biotic and abiotic *A. aurantii* mortality. The exclusion experimental units did not have a measurable micro-environmental effect on either the cohorts or the plant organs on which they were established that could significantly distort *A. aurantii* mortality values. The differences in the observed *A. aurantii* mortality rates between the 'semi-exclusion' treatment and those of either 'exclusion' or 'predation', suggest that the open experimental units partially restricted the access of predators but any altered microenvironment did not significantly affected the development of cohorts.

Aonidiella aurantii mortality attributed to predation may be overrepresented owing to the difficulty to discriminate between mortality caused by predation and that resulting from sampling and host-feeding of the *A. aurantii* specific parasitoids (Vanaclocha et al., 2011; Cebolla et al., 2018). Nonetheless, in this study, parasitism accounted for less than 5% of total CRS biotic mortality. We therefore believe that this factor would not importantly overrate our estimation of CRS mortality attributed to predators.

Mortality observed in the 'exclusion' cohorts, referred to in this study as natural mortality, could be used as an estimation of *A. aurantii* mortality due to uncontrolled environmental conditions or biotic factors such as substrate quality onto which the first instar scales adhere themselves. Although natural mortality in our study seemed to be relatively high (60% of the initially settled nymphs died), values coincide with what has been observed in other studies which surveyed *A. aurantii* mortality factors in citrus crops; thus a potential negative influence of our experimental unit could be discarded (Hare et al., 1990; Vanaclocha et al., 2011). As to be expected, natural mortality was not affected by location, season, or *A. aurantii* generation, but it nevertheless was higher on leaves than on fruit. Earlier studies demonstrated higher survival of *A. aurantii* on fruit and concluded that this parameter is mediated by the availability of nutrients in the plant organ to which individuals were adhered (Pekas, 2010; Dao et al., 2017).

Aonidiella aurantii mortality attributed to predation was not affected by plant organ or location and only partially affected by season. Preference

for food resource could be mediated by plant organ at a predator species level. In fact, parasitoid segregation between twigs and fruit has been observed in the case of *A. aurantii* control (Boyero et al., 2014). This strategy allows natural enemy coexistence when depending upon the same prey resource (Hare et al., 1990; Vanaclocha et al., 2011). With the exclusion experiments, we studied the combined effect of the whole predator complex together which may have masked the control exerted by each natural enemy species within the specific prey substrates.

The highest observed *A. aurantii* predation in its first generation, could be explained by different factors: i) climatic conditions are optimal for most predator species in the study area at the end of the spring and beginning of the summer; ii) offspring of the first generation or overwintering generation of *A. aurantii* are usually the least abundant because they are the progeny of specimens that survived the winter, thus there are less individuals to prey upon (Atkinson, 1977; Campos-Rivela et al., 2012); iii) as we observed in this study, predator seasonal-activity is highest during this generation (Lundgren, 2009; Hodek et al., 2012; Gómez-Marco et al., 2016).

Stem-tap sampling revealed an abundant complex of potential *A. aurantii* predators present in citrus agroecosystems during the pests generations. Nevertheless, some groups such as phytoseiid mites, dipterans, thrips as well as predator species with nocturnal activity that could also contribute to *A. aurantii* mortality (Vanaclocha et al., 2011; Campos-Rivela et al., 2012; Sorribas & García-Marí, 2010) cannot be accurately sampled with

this methodology and therefore, specific sampling for these groups would have been required to determine their impact on *A. aurantii* mortality.

The most frequently found natural enemy species during the *A. aurantii* first generation are mainly associated to citrus aphids (Ciancio & Mukerji, 2010). The first *A. aurantii* generation coincides with the end of aphid infestations which is during the first and major citrus flushing period of the year (Gómez-Marco et al., 2016). In fact, stenophagous Coccinellidae, Dermaptera and Miridae species associated with aphids had population peaks in this generation, then progressively reduced in number; whereas Neuroptera and Araneae generalist species that usually do not show a marked prey specific density-dependence (Riechert & Lockley, 1984; Symondson et al., 2002) progressively increased their numbers throughout the growing season as had been previously observed (Monzó et al., 2014).

In contrast to generalist and stenophagous predators, the specialist coccinellid predators of the family Diaspididae, *R. lophanthae, C. bipustulatus* and *C. citricola*, presented very low seasonal-activity and were mostly concentrated at the end of the season. Prey specific density-dependence of these natural enemies would explain the progressive increase of their activity throughout the growing season in concordance with the build-up of *A. aurantii* populations (Vanaclocha et al., 2011).

The molecular markers designed for the present study (CRSCOI primers) were able to detect *A. aurantii* DNA within the gut content of numerous

predator species associated with the citrus agroecosystem. Although CRSCOI primers were not species specific, the only two positive detections obtained with non-target organisms were found in two closely related Diaspididae species that are not found in citrus groves of the study region (Simon, 1997) and therefore, no risk of obtaining false positive detections could be expected.

Estimated relevance of *A. aurantii* predators from the gut-content studies throughout the season were more precisely explained by the seasonal changes of *A. aurantii* mortality attributed to predation, registered in the exclusion studies, than the seasonal-activity data of the citrus predatory complex obtained through stem-tap monitoring. This consequently illustrates the great utility of applying molecular tools in field studies to better understand the trophic relationships that modulate the demography of economically important pests.

Post-mortem studies with CRSCOI primers revealed, contrarily to what has traditionally been cited (Murdoch et al., 2005), a relatively rich complex of predators exploiting *A. aurantii*. In addition, most of these species are generalist or stenophagous predators never before cited as important predators of this pest. The use of the information obtained through the PCR analyses to estimate the potential relevance of each predator group and species creates a new paradigm of how the different components of the *A. aurantii* predatory complex may be contributing to its regulation. The mirid *P.* cf *gallicus* presents the most relevant *A. aurantii* predation in terms of positive detections and seasonal-activity, especially during the first generation, but also throughout

the rest of the growing season. Species of this genus have been traditionally associated with aphid control and never cited as an *A. aurantii* predator (Schuh & Schwartz, 1988). We speculate this mirid species to be exploiting *A. aurantii* as an alternative prey source to survive throughout the season when its preferential prey is not readily available (Schuh & Schwartz, 1988; Hosseini et al., 2008). A similar case would be for the two *Scymnus* species. These coccinellid species, although previously cited as potential *A. aurantii* BC agents, are also mostly associated to aphid consumption (Tawfik et al., 1973; Atlihan & Güldal, 2009; Lundgren, 2009; Hodek et al., 2012). Although these generalist predators may not be the most efficient predators of *A. aurantii*, their high relevance in terms of activity-density and positive detections revalue their importance as potential *A. aurantii* biological control agents in citrus agroecosystems. An apparent competition between the two key citrus pests mediated by their shared generalists and stenophagous predator complex (Holt & Bonsall, 2017) may be the result of the most relevant predator species population and the highest *A. aurantii* predation having occurred during the first *A. aurantii* generation, along with the fact that these predators are mostly associated to the previous aphid demographic increase in citrus clementines (Gómez-Marco et al., 2016).

Specialist *A. aurantii* predator species of the Coccinellidae family presented, nevertheless, a low relevance in terms of number of specimens found using *A. aurantii* as food source. The number of *C. bipustulatus* and *C. citricola* collected was too low to offer a significant contribution to *A. aurantii* control. *Rhyzobius lophanthae* only presented some activity and relevance at

the end of the growing season. Specialist natural enemies present a strong specific prey density-dependency; hence their population densities increase once the target pests have built up. The predatory efficiency per individual of these natural enemies is expected to be superior to that of generalists therefore their importance could be greater than what was observed in this study. On the other hand, the late presence of specialists would complement the action of generalist predator species present at the beginning of the season by helping to reduce numbers of overwintering *A. aurantii* and thus to lessening the risk of severe demographic increases during the first generation of the following year. Reduction of overwintering specimens by any means in pests that reproduce during the citrus growing season has been widely demonstrated to significantly affect the demographic potential of the first generation of the year (Qureshi & Stansly, 2009).

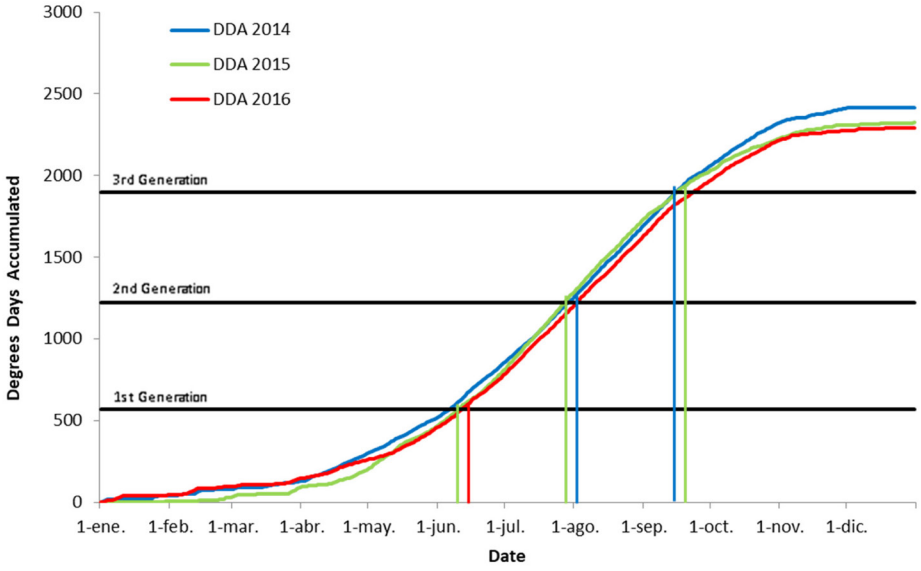
Chrysopidae and Coniopterygidae families (Neuroptera) have been previously cited as predators of *A. aurantii*. In our study the most captured and relevant species were *C. carnea* and *S. aleyrodiformis*. Both species seem to be more relevant at the end of the season, the latter being one of the most relevant *A. aurantii* predators in this study during the third *A. aurantii* generation. In this and other citrus growing regions similar seasonal-activity trends for this group of predators have been illustrated; however, no information about its predatory activity on *A. aurantii* previously existed (Alvis, 2003; Monzó et al., 2014).

The probability of obtaining positive detections in predators that fed upon the target pest is conditioned by the type of digestion of each predatory group and species. For the same molecular markers post-digestion detection can strongly vary between predator species (Monzó et al., 2011). Post-digestion time period in laboratory studies help to understand this variability. Nevertheless this kind of evaluation is not viable for studies in which a large predator range is tested. In this sense, the estimated relevance of the species associated with *A. aurantii* control could be partially conditioned by differences in digestion times. According to the extensive literature dedicated to digestion times of different predatory groups, spiders´ relevance may be overrated due to their prolonged digestions times, whereas Miridae and Coccinellidae relevancies would be underrated (Riechert & Lockley, 1984; Agustí et al., 1999; Hoogendoorn & Heimpel, 2001).

Positive detections obtained in spider specimens must also be taken into account with great caution. Secondary predation (i.e. a predator consuming a second predator, shortly after the latter consumed the target prey) has been widely described in this group of predators (King et al., 2008, Sheppard et al., 2005). Their marked generalist behavior along with their low numerical response and prolonged life cycles have traditionally driven the disregard of the BC services they may offer (Riechert & Lockley, 1984; Symondson et al., 2002). Further studies on this group would be required to understand their actual role on regulation of *A. aurantii* populations.

In conclusion, the present study reassess the importance of conservation biological control in perennial crops by highlighting the primary role of predation as the biotic mortality factor of key pests and by exposing how a rich complex of indigenous or naturalized generalist and stenophagous predators is mostly responsible for this factor. Results herein presented may therefore help to change the biological control management paradigm of one of the citrus key pests worldwide, at least in those areas where specific parasitoids of *Aphytis* genus are not able to successfully regulate their populations, and would serve as an example for other systems similar to this.

Supplemental information



SS2.1 Degrees days accumulated since January 1 in 2014, 2015 and 2016 seasons using meteorological data from the Sistema de Información Agroclimática para el Regadío (SIAR) database, using daily summaries from station Moncada IVIA (V101). First generation is predicted to reach it maximum at 570 degrees-day, second at 1220 and third at 1900.

		Moncada						Algimia						
		Season 1			Season 2			Season 1			Season 2			
Order	Predator	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	
Coleoptera	*Scymnus interruptus* (Goeze, 1777)	61	26	14	115	197	136	372	39	50	179	208	84	**1481**
	Scymnus subvillosus (Goeze, 1777)	97	38	10	255	63	35	128	1	0	409	6	5	**1047**
	Stethorus punctillum Weise, 1891	16	9	39	12	80	87	3	1	7	4	14	113	**385**
	Rodolia cardinalis (Mulsant, 1850)	34	7	5	42	14	16	30	0	9	40	24	12	**233**
	Cryptolaemus montrouzieri Mulsant, 1850	0	0	4	1	14	3	10	17	46	0	13	37	**145**
	Propylea quatuordecimpunctata (Linnaeus, 1758)	3	1	3	16	2	1	40	0	4	19	28	18	**135**
	Larvae Coccinellidae	0	0	0	70	12	0	5	1	0	19	3	0	**110**
	Scymnus mediterraneus Khnzorian, 1972	13	1	0	4	12	6	11	0	0	3	3	0	**53**
	Clitostethus arcuatus (Rossi, 1794)	0	0	0	1	5	13	0	1	0	0	2	6	**28**
	Rhyzobius lophanthae (Blaisdell, 1892)	0	0	1	0	0	0	9	0	0	0	2	15	**27**
	Staphylinidae spp. Latreille, 1802	0	0	0	4	1	5	0	0	0	0	0	6	**16**
	Delphastus pusillus (LeConte, 1852)	0	0	0	0	0	0	1	0	0	0	0	8	**9**
	Rhyzobius litura (Fabricius, 1787)	0	0	0	0	0	0	3	0	1	2	1	0	**7**
	Adalia decempunctata (Linnaeus, 1758)	0	0	0	0	0	0	0	0	0	5	0	0	**5**
	Coccidophilus citricola Brèthes, 1905	0	0	0	0	1	0	0	0	0	0	1	2	**4**
	Coccinella septempunctata (Linnaeus, 1758)	0	0	0	0	0	0	0	1	0	2	0	1	**4**
	Scymnus rufipes (Fabricius, 1798)	0	0	1	0	1	1	0	0	0	1	0	0	**4**
	Chilocorus bipustulatus (Linnaeus, 1758)	0	0	0	0	1	0	0	0	0	0	0	1	**2**
	Hippodamia variegata (Goeze, 1777)	0	0	0	0	0	0	1	0	0	0	0	0	**1**
	Total Coleoptera	**224**	**82**	**77**	**520**	**403**	**303**	**613**	**61**	**117**	**683**	**305**	**308**	**3696**

Neuroptera	*Chysoperla carnea* (Stephens, 1836)	17	3	5	31	48	15	114	19	8	64	50	103	**477**
	Semidalis aleyrodiformis (Stephens, 1836)	5	10	17	4	41	50	3	4	6	9	15	65	**229**
	Conwentzia psociformis (Curtis, 1834)	0	0	1	6	7	16	0	0	0	1	0	6	**37**
	Hemerobiidae spp. Latreille, 1803	0	0	0	0	0	0	1	0	2	0	2	3	**8**
	Total Neuroptera	**22**	**13**	**23**	**41**	**96**	**81**	**118**	**23**	**16**	**74**	**67**	**177**	**751**
Dermaptera	*Forficula auricularia* Linnaeus, 1758	1	1	0	2	0	0	3	2	2	4	4	4	**23**
	Euborellia annulipes (Lucas, 1847)	0	0	0	2	0	1	1	0	1	6	4	6	**21**
	Total Dermaptera	**1**	**1**	**0**	**4**	**0**	**1**	**4**	**2**	**3**	**10**	**8**	**10**	**44**
Hemiptera	*Pilophorus cf. Angustulus* (Reuter, 1888)	109	2	3	114	18	17	398	26	1	677	139	43	**1547**
	*Orius spp.*Wolff, 1811	11	7	15	10	2	24	2	7	26	29	2	23	**158**
	Cardiastethus fasciiventris (Garbiglietti, 1869)	4	3	6	3	4	4	2	13	42	39	9	11	**140**
	Pinalitus conspurcatus (Reuter, 1875)	1	0	0	36	0	18	0	1	0	0	0	31	**87**
	Empicoris rubromaculatus (Blackburn, 1889)	5	4	2	4	0	1	0	5	1	45	7	4	**78**
	Nymphs Anthocoridae	2	2	4	0	1	2	0	11	3	6	6	3	**40**
	Total Hemiptera	**130**	**16**	**26**	**167**	**24**	**64**	**402**	**52**	**70**	**790**	**157**	**112**	**2050**
Arachnida	*Philodromus cespitum* (Walckenaer, 1802)	28	27	33	110	83	42	100	21	44	24	88	114	**714**
	Icius hamatus (Koch, C.L., 1846)	77	18	25	72	118	78	38	9	18	75	73	58	**659**
	Ballus chalybeus (Walkenaer, 1802)	72	34	45	69	165	132	7	0	4	17	7	10	**562**
	Cheiracanthium mildei Koch, L., 1864	26	10	13	30	34	60	33	20	25	22	37	24	**334**

Philodromus albidus Kulezynski, 1911	32	18	20	83	19	16	46	3	8	20	31	38	**334**
Dipoena melanogaster (C.L. Koch, 1837)	5	28	35	43	31	64	18	9	33	41	41	146	**494**
Neoscosa subfusca (C. L. Koch, 1837)	14	4	3	28	48	43	20	6	10	14	46	56	**292**
Clubiona leucaspis Simon, 1932	13	5	8	19	22	13	5	2	10	3	11	4	**115**
Oxyopes lineatus Latreille, 1806	1	12	0	2	8	4	2	0	0	31	3	0	**63**
Olios argelasius (Walckenaer, 1805)	5	1	2	7	5	7	1	0	0	12	1	1	**42**
Kochiura aulica (C. L. Koch, 1838)	1	4	4	4	0	6	0	4	6	0	0	3	**32**
Aphantaulax trifasciata (O. Pickard-Cambridge, 1872)	3	1	1	3	3	5	4	2	3	0	1	1	**27**
Anelosimus pulchellus (Walckenaer, 1802)	1	1	13	1	1	0	1	0	2	0	1	4	**25**
Paidiscura pallens (Blackwall, 1834)	0	0	3	2	1	0	0	1	0	1	0	9	**17**
Meioneta rurestris (C. L. Koch, 1836)	1	0	1	7	0	1	4	0	0	2	0	0	**16**
Synema globosum (Fabricius, 1775)	0	0	1	3	0	0	0	0	1	3	0	2	**10**
Uloborus walckenaerius Latreille, 1806	1	1	0	1	1	1	0	0	1	2	0	0	**8**
Ero aphana (Walckenaer, 1802)	0	0	1	0	1	0	0	0	0	0	2	1	**5**
Textrix denticulata (Olivier, 1789	0	0	0	0	0	1	0	0	0	0	0	0	**1**
Araneae spp. Clerck, 1757	20	8	23	63	39	34	14	28	21	27	33	18	**328**
Total Araneae	**300**	**172**	**231**	**547**	**579**	**507**	**293**	**105**	**186**	**294**	**375**	**489**	**4078**
										TOTAL SPECIMEN			**10619**

SS2.2 Predator abundance found by stem-taps throughout the exclusion experiments, grouped by taxonomic groups, site, season and CRS generation (1st, 2nd, 3rd).

Group *	Order	Family	Species	Origin	PCR result
Preys	Hemiptera	Diaspididae	*Aonidiella aurantii* (Maskell, 1879)	IVIA Lab strain	+
			Parlatoria pergandii Comstock, 1881	Moncada, VLC	-
			Parlatoria ziziphi (Lucas, 1853)	Valencia, VLC	-
			Cornuaspis beckii (Newman, 1869)	Algimia, VLC	-
			Aspidiotus nerii (Bouche, 1833)	Valencia, VLC	+
			Chrysomphalus aonidum (Linnaeus, 1758)	Silla Lab strain	+
		Coccidae	*Coccus hesperidum* Linnaeus, 1758	Moncada, VLC	-
			Saissetia oleae (Olivier, 1791)	Moncada, VLC	-
			Ceroplastes sinensis Del Guercio, 1900	Algimia, VLC	-
		Margarodidae	*Icerya purchasi* (Maskell, 1878)	Ribesalbes, CS	-
		Pseudococcidae	*Planococcus citri* Risso, 1813	Moncada, VLC	-
			Delottococcus aberiae (De Lotto, 1961)	Algimia, VLC	-
			Pseudococcus longispinus (Targioni Tozzetti, 1867)	Moncada, VLC	-
		Aphididae	*Aphis spiraecola* Patch, 1914	Moncada, VLC	-
			Aphis gossypii Glover, 1877	Algimia, VLC	-
			Myzus persicae (Sulzer, 1776)	IVIA Lab strain	-
		Aleroydidae	*Paraleyrodes minei* Iaccarino, 1990	Moncada, VLC	-
			Alerothrixus floccosus Maskell, 1896	Moncada, VLC	-
		Triozidae	*Trioza alacris* Flor, 1861	Moncada, VLC	-
	Thysanoptera	Thripidae	*Pezothrips kellyanus* (Bagnall, 1916)	Moncada, VLC	-
			Frankliniella occidentalis Pergande, 1895	IVIA Lab strain	-
	Lepidoptera	Gracillariidae	*Phyllocnistis citrella* Stainton, 1856	Moncada, VLC	-
		Pyralidae	*Cryptoblabes gnidiella* (Millière, 1867)	IVIA Lab strain	-
		Gelechiidae	*Tuta absoluta* (Meyrick, 1917)	IVIA Lab strain	-
		Tephritidae	*Ceratitis capitata* (Wiedemann, 1824)	IVIA Lab strain	-
		Drosophilidae	*Drosophila melanogaster* Meigen, 1830	Moncada, VLC	-
	Acari	Tetranychidae	*Tetranychus urticae* C.L. Koch, 1836	IVIA Lab strain	-
			Panonychus citri (McGregor, 1916)	Algimia, VLC	-
		Tydeidae	*Tydeidae sp* Kramer, 1877	Moncada, VLC	-
	Psocoptera		*Psocoptera sp* Shipley, 1904	Moncada, VLC	-
Predators	Coleoptera	Coccinellidae	*Scymnus subvillosus* (Goeze, 1777)	Moncada, VLC	-
			Scymnus interruptus (Goeze, 1777)	Algimia, VLC	-
			Scymnus mediterraneus Khnzorian, 1972	Moncada, VLC	-
			Rhyzobius lophanthae (Blaisdell, 1892)	Moncada, VLC	-

		Chilocorus bipustulatus (Linnaeus, 1758)	Moncada, VLC	-
		Coccidophilus citricola Brèthes, 1905	Bétera, VLC	-
		Cryptolaemus montrouzieri Mulsant, 1850	Algimia, VLC	-
		Propylea quatuordecimpunctata (Linnaeus, 1758)	Moncada, VLC	-
		Delphastus pusillus (LeConte, 1852)	Bétera, VLC	-
		Rodolia cardinalis (Mulsant, 1850)	Moncada, VLC	-
		Stethorus punctillum Weise, 1891	Moncada, VLC	-
Neuroptera	Chrysopidae	*Chysoperla carnea* (Stephens, 1836)	Moncada, VLC	-
	Coniopterygidae	*Semidalis aleyrodiformis* (Stephens, 1836)	Moncada, VLC	-
Dermaptera	Forficulidae	*Forficula auricularia* Linnaeus, 1758	Moncada, VLC	-
	Anthocoridae	*Orius laevigatus* (Fieber, 1860)	Moncada, VLC	-
		Cardiastethus fasciiventris (Garbiglietti, 1869)	Moncada, VLC	-
	Reduviidae	*Empicoris rubromaculatus* (Blackburn, 1889)	Moncada, VLC	-
	Miridae	*Pilophorus cf angustulus* (Reuter, 1888)	Moncada, VLC	-
		Nesidiocoris tenuis (Reuter, 1895)	Koppert Lab strain	-
Araneae	Salticidae	*Icius hamatus* (Koch, C.L., 1846)	Moncada, VLC	-
		Ballus chalybeus (Walkenaer, 1802)	Moncada, VLC	-
	Philodromidae	*Philodromus albidus* Kulezynski, 1911	Moncada, VLC	-
		Philodromus cespitum (Walckenaer, 1802)	Moncada, VLC	-
	Clubionidae	*Cheiracanthium mildei* Koch, L., 1864	Moncada, VLC	-
		Clubiona leucaspis Simon, 1932	Moncada, VLC	-
	Theridiidae	*Dipoena melanogaster* (C.L. Koch, 1837)	Moncada, VLC	-
Hymenoptera	Encyrtidae	*Anagyrus pseudococci* (Girault, 1915)	Koppert Lab strain	-
	Aphelinidae	*Aphytis melinus* DeBach, 1959	IVIA Lab strain	-
		Encarsia formosa Gahan, 1924	Koppert Lab strain	-
	Pteromalidae	*Spalangia cameroni* Perkins, 1910	IVIA Lab strain	-
	Braconidae	*Diachasmimorpha longicaudata* (Ashmead, 1905)	IVIA Lab strain	-

SS2.3 Arthropod species used to screen CRSCOI primers specificity and result of the PCR (positive or negative amplification). Species are grouped as potential target preys and known predators. VLC = Valencia region, Spain.

capítulo 3

Limiting and regulating factors determining aphid infestation dynamics in clementine citrus

Capítulo 3

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Limiting and regulating factors determining aphid infestation dynamics in clementine citrus

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Abstract

The Spirea citrus aphid, *Aphis spiraecola* and cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) are key pests of clementine mandarines in the Mediterranean basin. Severity of aphid infestations is determined by environmental and crop variables that limit their populations as well as the regulation exerted by their associated natural enemies. However, there is no information about the role these

limiting and regulating factors. Aphid densities, citrus phenology and associated predators were weekly monitored throughout 2 shooting periods (February to July) in 4 clementine mandarin groves. Relationships between these parameters and environmental variables (temperature and precipitation) were studied. Our results show exponential increase in aphid infestation levels to coincid in with citrus phenological stages B3 and B4; shoots offer more space and nutritional resources for colony growth at these stages. Duration of these phenological stages, which was mediated by mean temperature, seems to importantly determine the severity of aphid infestations in the groves. Micro-coccinellids, mostly *Scymnus* species, were the only group of predators with the ability to efficiently regulate aphid populations. These natural enemies showed the highest temporal and spatial demographic stability. Aphid regulation success was only achieved through early presence of these natural enemies in the groves; when the first aphid colonies appear in the clementine citrus trees. Our results suggest that conservation strategies aimed to preserve and enhance *Scymnus* sp. populations may importantly contribute to future success of the biological control of these key citrus pests.

Keywords Conservation biological control, phenology, aphid, arriving early, predator, Scymnus, integrated pest management

3.1. Introduction

The Spirea citrus aphid, *Aphis spiraecola* Patch, and the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) are major pests of clementine mandarin crops (*Citrus clementina* Hort. ex Tan) in the Mediterranean basin (Marroquín et al., 2004; Franco et al., 2006; Yahiaoui et al., 2009; Tena & García-Marí, 2011; Hermoso de Mendoza et al., 2012; Vacante & Gerson, 2012; Belati & Belabed, 2014). Both aphid species use fresh, un-hardened plant tissue (shoots) for development and reproduction on citrus. Infestations consequently take place during the main sprouting periods of the season and, under the Mediterranean growing conditions, are most important in spring when citrus trees are at the height of their shoot growth (Llorens, 1990; Urbaneja et al., 2000; Hermoso de Mendoza et al., 2006; Lebbal & Laamari, 2016).

Sap suction during aphid feeding activity causes a general weakening of the plant. At high pest densities tree growth is delayed and production reduced. In the case of *A. spiraecola*, the injection of pre-digestion substances in the plant tissue causes leaf curl deformation. Colonies of this species use this strategy to protect themselves from natural enemies and adverse environmental conditions (Cole, 1925; Miller, 1928; Wang & Tsai, 2000). Development of sooty mould on leaves and fruit as a consequence of the honeydew secreted by aphids also indirectly affects tree growth, production and causes cosmetic damage to fruit. Both aphid species are known vectors of Citrus Tristeza Virus (CTV); *A. gossypii* is considered to be the most efficient one in the Mediterranean basin (Raccah et al., 1976; Hermoso de Mendoza et al., 1984; Cambra et al., 2000).

Under current crop management conditions, regulation exerted by natural enemies is generally not sufficient to keep aphid densities below their economic threshold in the Mediterranean clementine mandarin agroecosystems during the spring citrus growing period (Hermoso de Mendoza et al., 2001, 2006; Urbaneja et al., 2018). Successful pest control must therefore relay on at least one insecticide application. The extensive use of neonicotinoids either as a preventive or curative tactic for aphid control in clementine mandarins threatens the currently implemented integrated pest management strategy in this crop and highlights the need to develop less detrimental aphid management alternatives (Wang et al., 2017; Urbaneja et al., 2018).

Aphid densities are known to be strongly modulated by environmental variables such as temperature, along with biotic factors associated with their host; among them, are abundance and quality of shoots (Zhou et al., 1995; Awmack & Leather, 2002). The temperature range for *A. spiraecola* survival and proliferation is between 2.3ºC and 35ºC and between 6.2º and 35ºC for *A. gossypii*; therefore, temperatures below and above these ranges limit their infestation potential (Kersting et al., 1999; Wang & Tsai, 2000). Shoots are an ephemeral resource essential to aphid development. The presence of these in citrus occurs in specific periods of the year; thus the risk of infestation is confined to those periods of citrus shoot growth. The three major sprouting periods of spring, beginning of summer and mid-autumn have been identified under Mediterranean basin crop conditions; spring being the most important in terms of shoot abundance (Hermoso de Mendoza et al., 2012; Lebbal & Laamari, 2016). Cultivars and species of citrus strongly determine plant growing patterns. Crop

management practices also have an important influence on citrus phenology (Iglesias et al., 2007). Abiotic variables such as temperature and precipitation ultimately mediate citrus phenology throughout the season. Nevertheless, there is no information about how all these limiting factors specifically influence the severity of aphid infestations in citrus of western Mediterranean basin.

In the last two decades numerous efforts have been addressed to understand the causes restraining aphid biological control success in citrus (van Steenis, 1995; Yokomi & Tang, 1995; Bañol et al., 2012). In this search, complex trophic interactions between *A. spiraecola,* its primary parasitoid, *Binodoxys angelicae* (Haliday), and six hyperparasitoids have completely nullified the potential control of this pest by parasitism (Michelena & Sanchis, 1997; Gómez-Marco et al., 2015). Biological control services offered by the predator complex associated with citrus aphids were traditionally relegated to a secondary role. A rich predatory assemblage has been described to feed on aphid colonies (Hermoso de Mendoza et al., 2012). These authors observed a demographic synchrony between aphid and predator densities with the typical predator-prey response lag. Nevertheless, the high reproductive rate of aphids makes this lag sufficient to escape from an efficient biological control. Aphidophagous predators achieve maximum impact if they arrive early and in sufficient numbers (Michaud, 2012). Recent studies aimed at reducing the numeric lag response of predators demonstrated the use of monocotyledon based crop covers help advance the arrival of aphidophagous predators in the beginning of spring. Earlier arrival resulted in a significant reduction of aphid densities (Gómez-Marco et al., 2016a, b). Nevertheless, the components of the

predatory complex that contribute most to aphid regulation are not yet well know.

This research thus seeks to understand how environmental and host plant related biotic factors influence the infestation potential of *A. gossypii* and *A. spiraecola* in clementine mandarins and how biotic community factors regulate their populations during the spring sprouting period. The specific objectives were i) describe the dynamics of aphid populations in clementine mandarin groves in relation to temperature, precipitation and the phenology of its host; and ii) analyse the relationship between aphid infestation rates and their associated predators.

3.2. Material and methods

3.2.1. Location and experimental conditions

The experiments were conducted during the spring seasons of 2015 and 2016 in four clementine mandarins (*Citrus clementina* Hort. ex Tan) plots containing approximately 15 year-old trees at the Valencian citrus growing region. Two of them were experimental plots belonging to the Valencian Institute of Agricultural Research (IVIA) in Moncada (39°35'17.43"N / 0°23'53.28"O) [Valencia Province] and the Jaume I University (UJI) in Castellón (39°59'29.92"N / 0° 4'12.77"O) [Castellón Province]. The other two were commercial plots located in Algimia (39°42'55.11"N / 0°18'57.46"O) [Valencia Province] and Ribesalbes (40° 0'53.30"N / 0° 8'48.21"O) [Castellon Province]. All

plots were drip-irrigated and had not been treated with insecticides for at least two years before the onset of the study and during the course of it.

3.2.2. Meteorological data

Daily mean temperatures and rainfall from June 21, 2014 to June 20, 2016 were obtained from the 'Sistema de Información Agroclimática para el Regadío' (SIAR, www.siar.es) database, using daily summaries from two meteorological stations: Moncada IVIA (V101) at Valencia Province, and Castellón Benadresa (CS05) at Castellón Province. The greatest distance between meteorological stations and study plots was 15 km.

3.2.3. Temporal sampling of aphids, citrus phenology and predators

From February to July in 2015 and 2016, aphid populations were monitored weekly on 10 randomly selected trees of each plot. A 0.25 m^2 ring was randomly placed on two points of the surface of the canopy of each tree. The number of suitable aphid-infested and un-infested shoots within the ring area was counted (Hermoso de Mendoza et al., 2001, 2006).

The dominant phenological stage of clementine shoots in each ring was also recorded. A simplified BBCH phenological scale for citrus was used (Garrán et al., 1993; Agustí et al., 1995). Specifically, buds from the beginning of lengthening until shoots reach 20% of their final size were assigned to the **B1** stage (corresponding to the values of the BBCH scale: 31-32); we assigned

shoots between 20% and 40% of their final size to the **B2** stage (BBCH: 32-34); **B3** was defined as shoots with 40% - 70% of their final size (BBCH: 34-37); **B4**, shoots with 70% -90% of their final size (BBCH: 37-39); **B5**, tender shoots with final size (BBCH: 19); and **B6**, shoots with final size and leaves totally mature and hardened (BBCH: 91).

In the same trees selected for phenology and aphid surveys, potential predators of aphids were also monitored at the same time by stem-tap sampling. Two randomly selected branches per tree were stricken three times with a 40 cm-length of PVC pipe. A plastic tray (45 x 30 cm) was previously placed under each branch to capture the dislodged arthropods. Adults and larvae's predators were identified *in-situ*. In the cases in which determinations to species level was not possible specimens were collected with an entomological aspirator and determined in the laboratory under stereoscope microscope.

3.2.4. Spatial sampling of aphids and predators

Using the same methodologies previously described to measure aphid and predator populations, an extensive sampling was carried out through the main citrus growing areas of the Valencia region, representing an area of approximately 240,000 ha. From April 22 to May 31, 2016, at the end of the spring major citrus sprouting period, 60 commercial citrus plots containing aphid infestations and with trees at B3 to B5 phenological stages were randomly selected. The minimum distance between plots was 0.5 km.

3.2.5. Statistical analysis

Meteorological data were grouped per month. Differences in mean temperatures between meteorological stations, years, and months were studied using general linear model analysis (Wolfinger & O'connell, 1993). Normal distribution of the variable was tested prior to the model selection.

Differences in citrus shooting patterns between years were studied by comparing the following response variables: greatest shoot density, day when greatest shoot density was reached, day of the beginning of B2 phenological phase (upon which aphid colonies start to develop), and days of duration of B3 and B4 phenological phases (upon which aphid colonies can exhibit their highest growth potential). Student's t–test was used in all the cases and 'year' was used as predictor variable. Prior to model selection, data was tested for normality. Pearson Correlation was used to study potential relationships between duration of B3 and B4 phenological stages and mean temperatures during these phenological phases.

Differences in aphid infestation patterns between years were studied by comparing the following response variables: beginning of the first colonies and day of highest infestation rate. Student's *t*–test for two samples was used and 'year' was selected as the predictor variable. Percentage of shoots infested by *A. gossypii* and *A. spiraecola* in the spatial sampling was also compared using a Student's *t*–test for two samples.

Temporal and spatial variability of the activity-density of the most important predator groups associated to aphids in citrus was also compared. Coefficients of Variation (ratio of the standard deviation σ to the mean μ) were calculated for the data collected by stem-taps of each predatory group (micro-coccinellids, macro-coccinellids, neuropterans, hemipterans and dermapterans) in each sampling date and citrus grove. Differences in temporal variability between groups was studied through general linear model analysis. Differences between predator groups in the beginning of their seasonal activity (after the winter period) was studied by comparing the date for which 10% of all captured individuals was reached each year. General linear model analysis was used wherein 'date of 10% of all captures' was the response variable and 'predator groups' the explanatory variable. Post-hoc t-test (Tukey) comparisons were made in each case having a significant effect ($P < 0.05$) for the two analyses.

To study the potential relationship between peak aphid infestation levels and the presence of natural enemies early in the season and throughout the spring aphid infestation period, the cumulative number of individuals captured by stem-tap sampling of each predatory group, i) from the beginning of the sampling until the beginning of aphid infestations and ii) throughout the study period, was related through linear regression to the maximum aphid infestation rates measured in each grove and year.

3.3. Results

3.3.1. Meteorological data

The average temperatures in the study period varied between 10.5 °C and 22.7 °C in the months of January and June, respectively (**figure 3.1**).

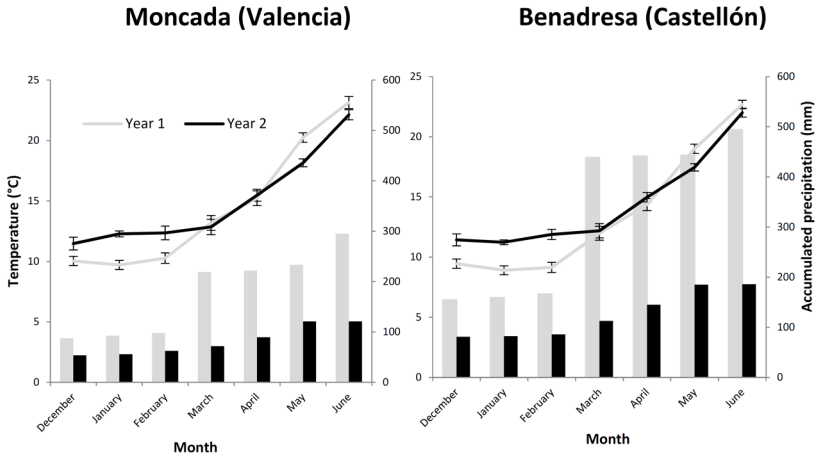

Figure 3.1 Monthly Mean temperature and accumulated precipitation in two meteorological stations of the Valencian Region: Moncada IVIA (V101) Station in the province of Valencia and Castellón Benadresa (CS05) Station in the province of Castellón.

There were no differences in mean temperatures between years (t = 1.15, df = 1, 1460, P = 0.28) for the whole study period. Nevertheless, winter months (December-January-February) were significantly colder in year 1 than year 2 (F = 65.77, df = 1, 360, P < 0.0001); early spring (March-April) had similar mean temperatures between years (F = 0.20, df = 1, 242, P = 0.66) whereas mean temperatures in late spring (May-June) were significantly higher in year 1 than year 2 at the two weather stations (F = 9.38, df = 1, 242, P = 0.002).

Accumulated rainfall throughout the months of the study was higher in both stations in year 1 than year 2, and higher in Benadresa than in the Moncada weather station. Temporal rainfall distribution throughout the study period was similar in the two weather stations with the greatest precipitation in December, March and June in year 1 and in December, April and May in year 2.

3.3.2. Citrus phenology

Clementine shooting patterns were similar between plots, but differed between years of study (**figure 3.2**). Highest shoot density occurred in year 1 (t = 66.69, df = 1, 6, P < 0.0001) but appeared earlier in year 2 (t = 20.06, df = 1, 6, P = 0.004) (**table 3.1**). Similarly, the beginning of B2 phenological phase, upon which aphid colonies start to develop, occurred approximately 25 days earlier in year 2 than year 1 (t = 67.00, df = 1, 6, P = 0.0002). Duration of B3 and B4 phenological phases, upon which aphid colonies can exhibit their highest growth potential, was nevertheless significantly longer in year 2 than year 1 (t = 23.57, df = 1, 5, P = 0.005) (**table 3.1**). Duration of these phases was strongly correlated to temperatures during that period of time (ρ = -0.94, P = 0.0017).

Table 3.1 Mean of maximum number of shoots per square meter on tree canopy surface, days from December 21th to the peak of shoots, days to the beginning of B2 and days of duration of B3 to B4. Different letters indicate significant differences between treatments (Tukey's test, P < 0.05).

	Maximum number of shoots	Days to peak	Days to B2	Days B3 to B4
Year 1	213.27 ± 9.50a	80.8 ± 1.8a	97.8 ± 3.1a	18.8 ± 1.9a
Year 2	89.07 ± 2.27b	68.5 ± 2.1b	72.0 ± 0.6b	47.3 ± 5.8b

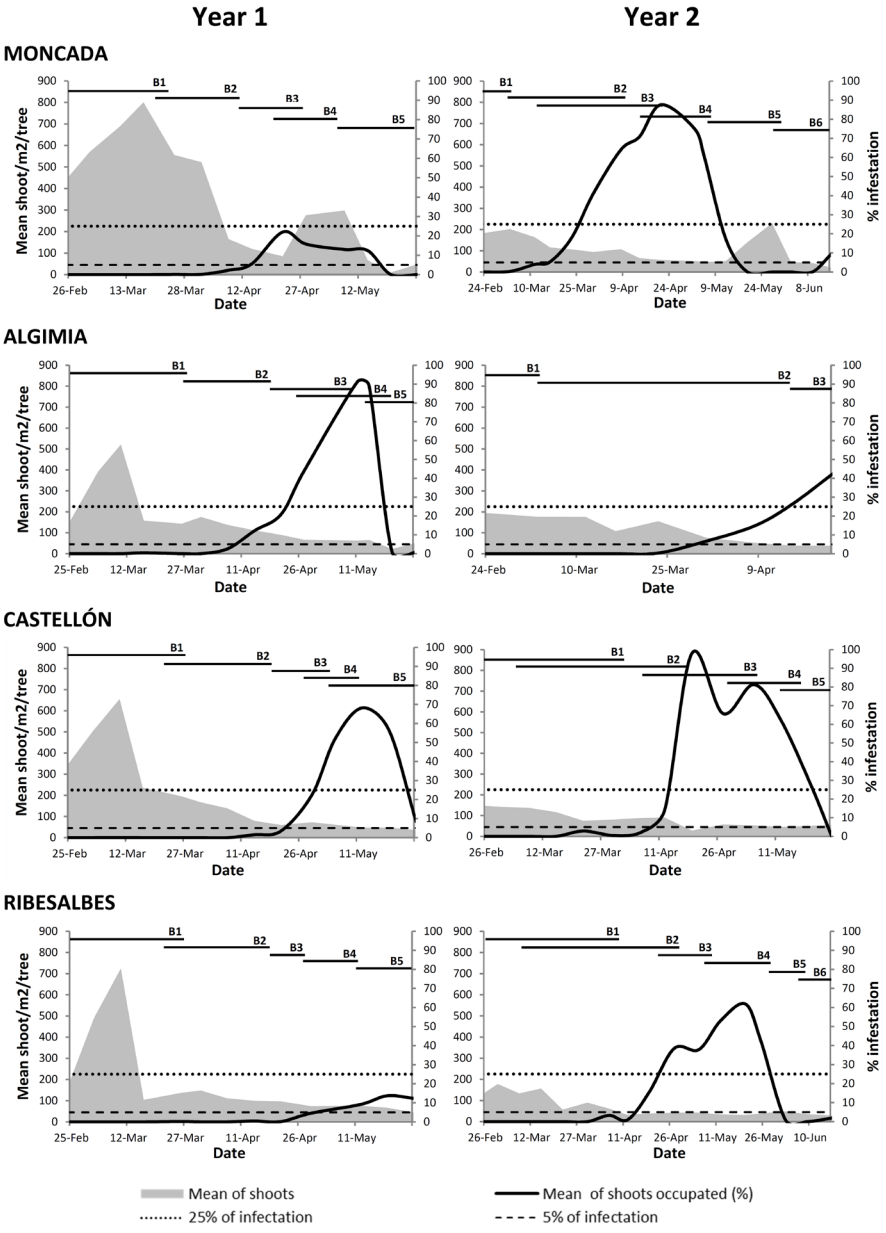

Figure 3.2 Mean number of shoots per square meter on tree canopy surface, percentage of shoots occupied by aphid colonies (infestation) and type and duration of the dominant clementine tree phenological state measured in four clementine citrus groves from February to June in 2015 and 2016 seasons. Dotted lines represent the thresholds of 5% and 25% of shoots occupied by aphid colonies.

3.3.3. Aphid phenology

Aphid infestation patterns where closely related to citrus shooting patterns. Aphid colonies were first found on average 10.78 ± 4.64 days later than the beginning of B2 phase with no differences between years (t = 1.123, df = 1, 6, P = 0.330). Exponential growth of aphid infestations in groves coincided with B3 and B4 phenological phases (**figure 3.2**). Peak infestation rates observed were 35.14 ± 3.18 days after the beginning of aphid infestations with no differences between years (t = 1.58, df = 1, 5, P = 0.175). Proportion of infested shoots exceeded the economic threshold of 25% of infested shoots, above which insecticide treatments are recommended (Hermonso de Mendoza et al., 2001, 2006), in two groves in year 1 (Algimia and Castellón) and in all groves in year 2 (**figure 3.2**). This threshold was exceeded two weeks earlier in year 2 than in year 1.

3.3.4. Citrus aphid species

In 2016, *Aphis spiraecola* and *A. gossypii* were the only two aphid species found that were associated with citrus groves in the spatial sampling across the Valencia region. *Aphis spiraecola* was present in all the groves whereas *A. gossypii* was found in only 62% of the groves. In all cases, infestations with *A. gossypii* were associated to *A. spiraecola*; whereas in 38% of all groves sampled *A. spiraecola* was the only species found. Average infestation rate was 50.0 ± 5.5% of infested shoots. Percentage of total shoots infested by *A. spiraecola* (45.5 ± 5.4%) was significantly higher than those infested by *A. gossypii* (12.3 ± 3.5%) (t = 7.92, df = 1, 118, P < 0.0001). *Aphis spiraecola* colonies were mostly pure colonies (84%

of the observed colonies) whereas most of *A. gossypii* colonies (63%) were mixed with *A. spiraecola* specimens.

3.3.5. Aphid predator's guild

Sixteen species of predators commonly associated with aphid species were found by the stem-tap sampling in the four study sites and in both of two years of data collection (**table 3.2**). Coccinellidae was the group with the greatest abundance and species richness. Within this family, micro-coccinellid species measuring less than 3 mm long were the most abundant with more than 1,600 specimens collected belonging to 4 species. *Scymnus subvillosus* (Goeze) and *Scymnus interruptus* (Goeze) were the most captured species, whereas *Rhyzobius litura* (Fabricius) and *Scymnus mediterraneus* Khnzorian were captured less frequently. Among the macro-coccinellid species measuring more than 3 mm long *Propylea quatuordecimpunctata* (L.) was the most captured. Neuroptera was the second group of predators in terms of captures being *Chrysoperla carnea* (Stephens) the dominant species within this group with 83.7% of all captures. Only one Hemipteran species, *Pilophorus* cf *gallicus* (Hemiptera: Miridae), was found with most of its captures occurring in year 2 in Moncada. *Forficula auricularia* L. (Dermaptera: Forficulidae) was found in three of the plots sampled but in low abundance.

Table 3.2 Predator abundance sampled by stem-taps, grouped by taxonomic groups (Order, Family and Specie), site and season.

Order	Family	Species	Castellon		Moncada		Ribesalbes		Algimia		TOTAL
			Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	
Coleoptera	Coccinellidae (Micrococcinellid)	*Scymnus subvillosus* (Goeze, 1777)	95	299	79	221	125	62	72	3	**956**
		Scymnus interruptus (Goeze, 1777)	17	88	64	156	3	1	200	6	**535**
		Rhyzobius litura (Fabricius, 1787)	53	44	0	0	4	1	3	0	**105**
		Scymnus mediterraneus Khnzorian, 1972	2	8	0	1	0	0	1	0	**12**
	Coccinellidae (Macrococcinellid)	*Propylea quatuordecimpunctata* (Linnaeus, 1758)	8	40	3	33	4	20	171	8	**287**
		Coccinella septempunctata (Linnaeus, 1758)	7	0	32	0	0	0	14	0	**53**
		Adalia decempunctata (Linnaeus, 1758)	12	36	0	0	1	1	1	0	**51**
		Hippodamia variegata (Goeze, 1777)	20	3	1	0	0	0	2	0	**26**
		Adalia bipunctata (Linnaeus, 1758)	1	0	1	0	0	0	2	0	**4**
		Cryptolaemus montrouzieri Mulsant, 1850	0	0	0	0	0	0	0	2	**2**
		Total Coleoptera	**215**	**518**	**180**	**411**	**137**	**85**	**466**	**19**	**2031**
Neuroptera	Chrysopidae	*Chysoperla carnea* (Stephens, 1836)	689	137	8	30	3	52	102	100	**1121**
	Coniopterygidae	*Semidalis aleyrodiformis* (Stephens, 1836)	4	20	1	10	3	32	15	28	**113**
		Conwentzia psociformis (Curtis, 1834)	0	0	10	2	2	1	3	20	**38**
	Hemerobiidae	Brown lacewings spp	1	52	0	2	1	5	2	4	**67**
		Total Neuroptera	**694**	**209**	**19**	**44**	**9**	**90**	**122**	**152**	**1339**
Hemiptera	Miridae	*Pilophorus* cf *gallicus* Remane, 1954	0	5	26	680	0	94	0	8	**813**
		Total Hemiptera	**0**	**5**	**26**	**680**	**0**	**94**	**0**	**8**	**813**
Dermaptera	Forficulidae	*Forficula auricularia* Linnaeus, 1758	17	18	0	13	0	0	20	16	**84**
		Total Dermaptera	**17**	**18**	**0**	**13**	**0**	**0**	**20**	**16**	**84**
										TOTAL SPECIMENS	**4267**

Seasonal and spatial patterns of the most important predator groups associated with aphids in the four study sites strongly varied (**figure 3.3** and **table 3.2**). Micro-coccinellids presented the lowest temporal and spatial variability being present throughout all the study period in all the study sites. Macro-coccinellids on the contrary, presented an important spatial variability, along with the highest temporal variability, with marked seasonal-activity peaks after aphid infestations. The Neuropteran group was the second less variable in terms of captures throughout the study period and the third one as for spatial variability. Hemipterans also presented a marked seasonal-activity with a well defined activity peak after aphid infestations, and the highest spatial variability with most of the captures originating from only one of the sampled groves. The micro-coccinellids group was detected earlier in the season (**table 3.3**). In fact, 10% of all their captures were obtained just two weeks after the beginning of sampling. Neuroptera was the second group with early seasonal-activity. 10% of all their captures were obtained approximately 30 days after the beginning of sampling, whereas this threshold was reached 40-45 days after the beginning of sampling for the rest of the groups.

Table 3.3 Coefficients of variation of the different groups of predators associated to aphids in citrus, throughout all the sampling period (Temporal variability) and between sampled plots (Spatial variability), and days from the first sampling date until 10% of all captures are obtained for each predator group. Different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

Predator group	Temporal variability	Spatial variability	Day with 10% to abundance
Micrococcinellid	1.08 ± 0.2c	0.75	15.8 ± 8.0b
Macrococcinellid	1.67 ± 0.2a	1.90	41.2 ± 10.3a
Neuropteran	1.21 ± 0.2bc	1.22	31.5 ± 8.0ab
Hemipteran	1.61 ± 0.2a	2.32	44.2 ± 10.9a
Dermapteran	1.42 ± 0.2ab	0.85	45.6 ± 11.8a

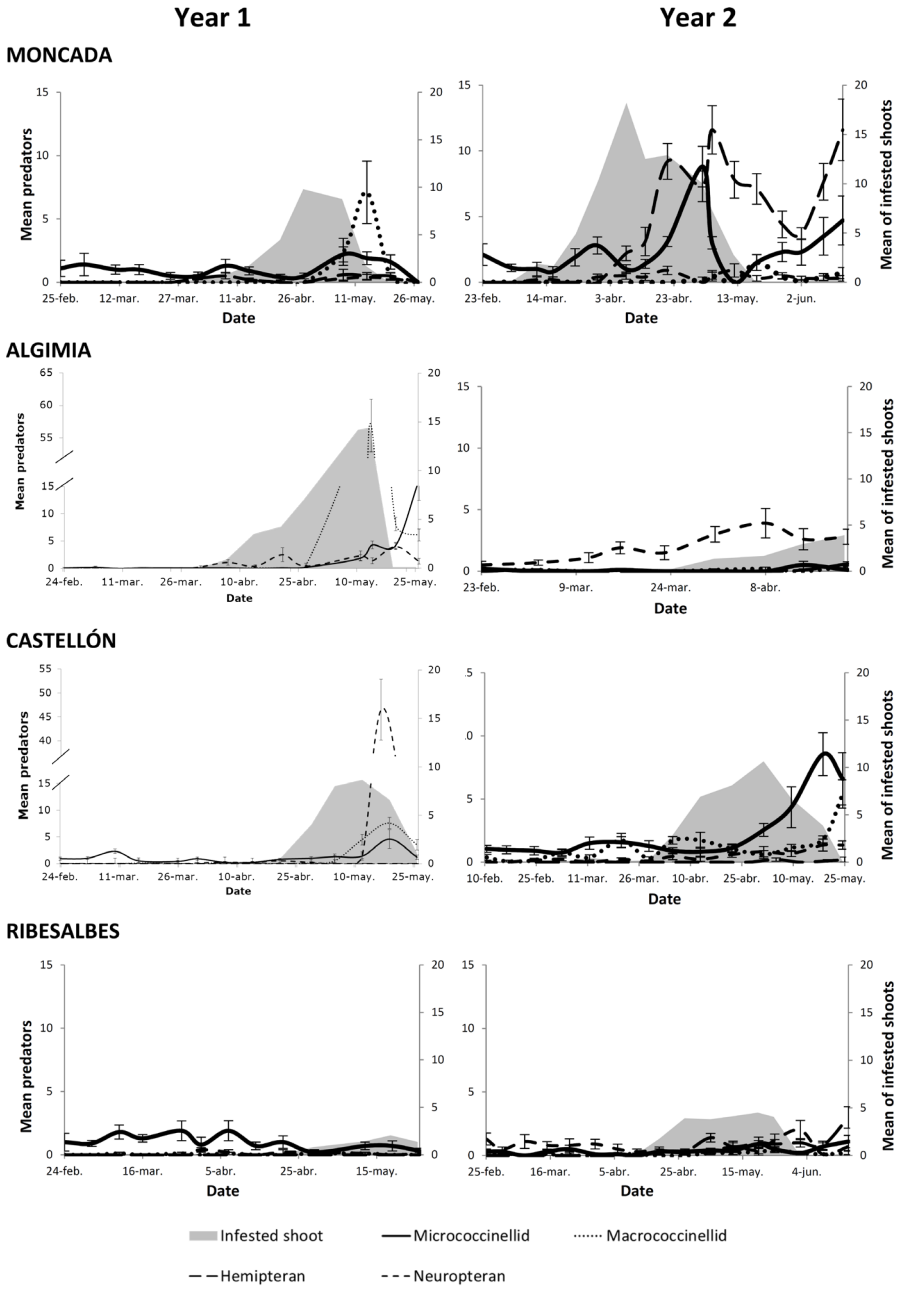

Figure 3.3 Seasonal-activity measured by stem-tap sampling of four groups of predators (Scymnus spp., macrococcinellids, neuropterans and hemipterans) associated to aphid infestations in clementine trees and percentage of shoots occupied by aphid colonies from February to June of the years 2015 and 2016.

3.3.6. Regional distribution of Coccinellidae

The micro-coccinellid *S. subvillosus* was the most captured species in the 60 sampled citrus groves through Valencia region; it was present in 80% of them (**table 3.4**). The macro-coccinellid *P. quatuordecimpunctata*, was the second most captured species and was also widely distributed in the region being present in more than 70% of the plots sampled. *Scymnus interruptus* was the third species in terms of captures and was found in more than 50% of the sampled plots. The other species of coccinellids had a marginal role in terms of captures and distribution in the Valencia region.

Table 3.4 Coccinellid abundance found by stem-taps; mean per plots, presence of predators in number and percentage of plots. Specimens grouped in micrococcinellids and macrococcinellids according to the size (less than 3 mm and more than 3 mm long).

Group	Species	Abundance	Mean	Number of plots present	% of plots present
Micrococcinellid	*Scymnus subvillosus* (Goeze, 1777)	650	0.63 ± 0.04	42	81 %
	Scymnus interruptus (Goeze, 1777)	213	0.20 ± 0.02	28	54 %
	Rhyzobius litura (Fabricius, 1787)	6	0.01 ± 0.003	1	2 %
	Scymnus rufipes (Fabricius, 1798)	2	0.002 ± 0.001	1	2 %
	Total	**871**	**0.84 ± 0.05**	**47**	**90 %**
Macococcinellid	*Propylea quatuordecimpunctata* (Linnaeus, 1758)	565	0.54 ± 0.04	38	73 %
	Hippodamia variegata (Goeze, 1777)	4	0.004 ± 0.002	2	4 %
	Coccinella septempunctata (Linnaeus, 1758)	3	0.003 ± 0.002	2	4 %
	Coccinellid larvae	48	0.05 ± 0.009	11	21 %
	Total	**620**	**0.60 ± 0.04**	**42**	**81 %**

3.2.7. Aphid-predator relationship

Maximum aphid infestation rates in the study sites were negatively correlated to the cumulative number of *Scymnus* spp. measured from the beginning of the sampling until the beginning of B2 (**Figure 3.4** and **Table 3.5**). Nevertheless, no relationship was found between the maximum aphid infestation rates and the cumulative number of *Scymnus* spp. measured throughout the study period. No relationships were found between maximum aphid infestation rates and the cumulative number of predators measured, for the rest of aphid predator groups (macro-coccinellids, neuropterans and hemipterans), neither at the beginning of aphid infestations (B2 phenological state) nor throughout the study period.

Table 3.5 Estimated parameters to the relationship between the maximum aphid infestation rates measured in the study sites and the cumulative number of predators from the beginning of the sampling until two important phenological moments of clementine trees for aphid infestations (B2 and B6).

Group	Accumulated until	df_{error}	*F*	*P*	r^2	a	b	b, SE
Scymnus spp	B2	6	39.72	7.44 E-04	0.85	41.54	-0.42	0.07
Scymnus spp	B6	6	0.64	0.45	-0.05	54.83	0.92	1.15
Macrococcinellid	B2	6	1.01	0.35	1.8 E-03	1.17	-0.01	0.01
Macrococcinellid	B6	6	0.51	0.50	-0.08	-3.88	1.59	2,23
Neuropteran	B2	6	3.46 E-02	0.99	-0.17	5.24	-8.53 E-04	0.15
Neuropteran	B6	6	0.56	0.48	-0.07	32.39	1.16	1.54
Hemipteran	B2	6	NaN	NA	NaN	0.00	0.00	0.00
Hemipteran	B6	6	0.34	0.58	-0.10	-9.62	1.15	1.98

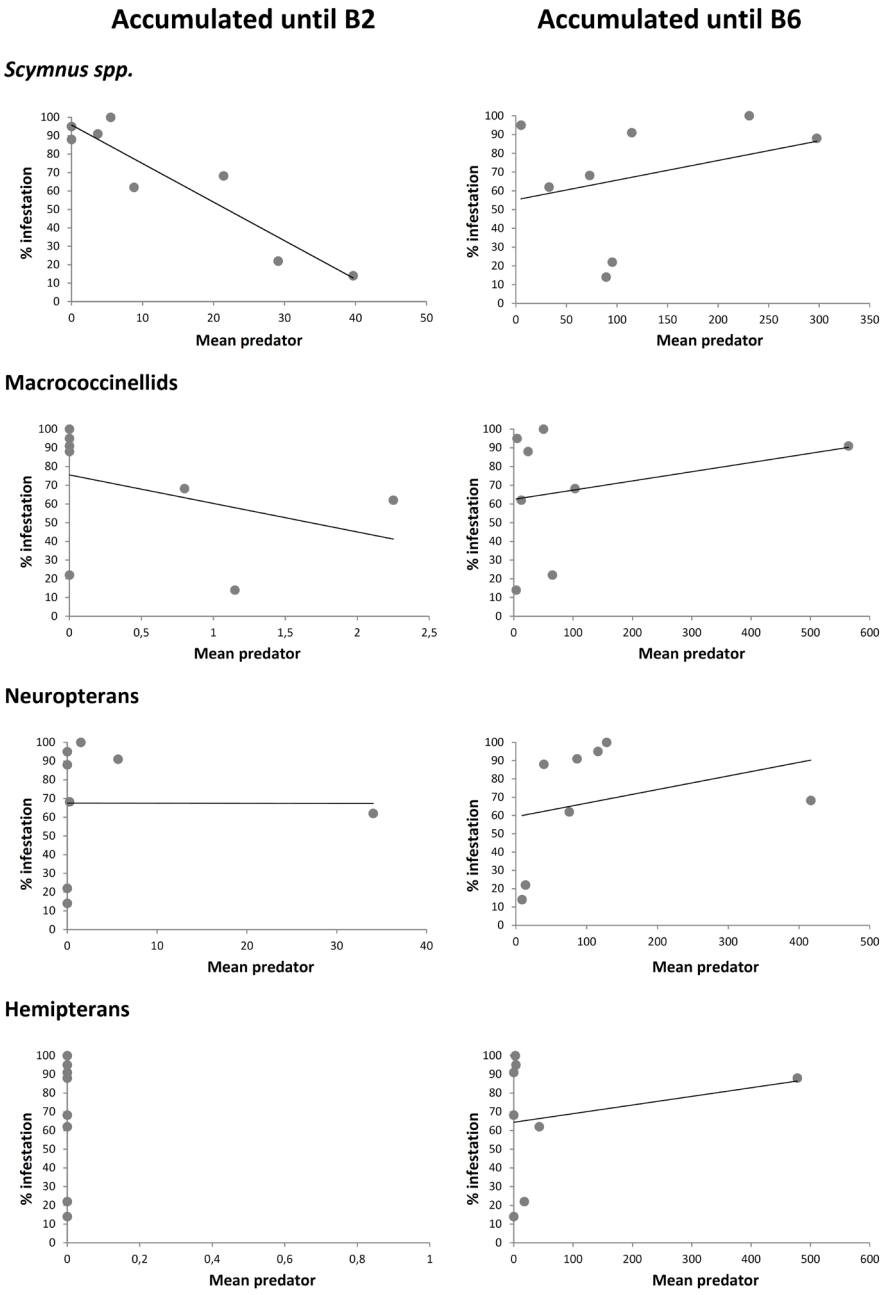

Figure 3.4 Linear regressions to the relationship between the maximum aphid infestation rates measured in the study sites and the cumulative number of predators from the beginning of the sampling until two important phenological moments of clementine trees for aphid infestations (B2 and B6).

3.4. Discussion

Aphis gossypii and *A. spiraecola* were the only two aphid species found associated with clementine citrus crops in our study; the former being the dominant one. *Aphis spiraecola* is the most abundant aphid species in citrus on the Mediterranean Basin (Algeria, Morocco, Tunisia, Italy, Spain) and in other countries such as the USA and Japan (Marroquín et al., 2004; Franco et al., 2006; Yahiaoui et al., 2009; Tena & García-Marí, 2011; Hermoso de Mendoza et al., 2012; Vacante & Gerson, 2012; Belati & Belabed, 2014). Eight species of aphids have been previously described to be associated with citrus crops in Spain; only *A. gossypii* and *A. spiraecola* are considered to be of economic importance in the crop (Hermoso de Mendoza et al., 2012). Both species are thought to be in equilibrium and although *A. spiraecola* is frequently found to be the most abundant species, their relative dominance may vary according to environmental conditions or crop management factors such as insecticide strategies (Hermoso de Mendoza et al., 1997). Several research projects have tried to explain the dominance of *A. spiraecola* when coexisting with other aphid species infesting citrus (Mostefaoui et al., 2014). One of the possible causes, is the deformation produced by the saliva of this species in the citrus shoot is thought to be a key factor since it confers protection against adverse environmental conditions as well as against natural enemies (Cole, 1925). *Aphis gossypii*, unlike *A. spiraecola*, is known to be affected by the high concentration of some plant compounds such as proline which is produced in large quantities as a result of physiological stress (Wool & Hales, 1996; Mostefaoui et al., 2014).

Environmental factors such as temperature or precipitation don´t seem to directly limit the development of the two aphid species in the western Mediterranean agroecosystems (Ro et al., 1998; Alyokhin et al., 2011; Lu et al., 2015; Holloway et al., 2018). Both the lower and higher thermal developmental thresholds for the two aphid species (*A. spiraecola*: 2.3 ºC and 35 ºC; *A. gossypii*: 6.2 ºC and 35 ºC) (Wang & Tsai, 2000; Kersting et al., 1999) were never reached throughout our study period. Our results nevertheless prove their important influence on aphid infestations which are mostly mediated by the citrus plant phenology.

Both temperature and precipitation have a determinant influence on the abundance and frequency of citrus sprouting, its timing throughout the season as well as the duration of each citrus vegetative and reproductive stage (Iglesias et al., 2007; Udell et al., 2017). Growth stages are accelerated by higher temperatures but low temperatures during the dormant season are required to release the dormancy of the buds in citrus trees and influence the intensity of spring sprouting (Iglesias et al., 2007). This effect was clearly reflected in our research, where differences in the winter temperatures between years resulted in distinct sprouting behaviours at the beginning of the growing season. Lower winter temperatures of the first year caused a greater but also later sprouting and a prolongation of the initial phenological stages of the clementine sprouts. The greater amount of rainfall accumulated during winter and early spring of the first year probably also influenced the greater sprouting activity in all the study groves.

Aphid infestations in citrus are closely related to plant phenology since these insects require tender growing tissues to develop (Zhou et al., 1995; Awmack & Leather, 2002). In the present study we were able to parameterize this association. Foundational aphid colonies were observed to be formed upon B2 phenological stage (shoots with 20% - 40% of their final size, corresponding to BBCH: 32-34). The exponential increase in aphid infestation levels coincided in most cases with phenological stage B3 in clementines (shoots with 40% - 70% of their final size, corresponding to BBCH: 34-37), when shoots offer more space and nutritional resources for colony growth. The maximum outbreak infestation rate occurred when the shoots reached stage B4 (shoots with 70% -90% of their final size, corresponding to BBCH: 37-39); probably due to the fact that at this phase shoots are sufficiently developed and big enough to accommodate large aphid colonies and offer the best quality food resources. Infestation rates rapidly declined once B5 phase was reached; which is probably associated with the progressive reduction of the resource quality before shoot hardening.

The important differences in the duration of B3 and B4 phenological stages (when aphid colonies are expected to exhibit their highest potential growth) observed between the two years may be the reason behind the distinct infestation rates observed. The period covered by these two phenological stages was almost three times shorter the first year than in the second one which was strongly correlated to the mean temperatures during that period of time. According to Wang & Tsai (2000) and Kersting et al. (1999), at the measured temperatures in that period and the duration of these two phenological phases,

both *A. spiraecola* and *A. gossypii* could complete just one generation during the first year (18.8 days at 19.27ºC) yet three generations during the second year (47.3 days at 15.05ºC). This may explain the higher peak in the infestation rate observed in the second year (83.2% on average) than the first year (48.8% on average).

During the two years of study, aphid peak infestations were registered at the end of April in Valencia province study sites and mid-May in Castellon province study sites. This is approximately one month earlier to what was previously recorded in studies conducted in the same region 20 years ago (Meliá, 1995; Hermoso de Mendoza et al., 1997). It would therefore be interesting to investigate the possible effects of global climatic change on the phenology of these citrus key pests and on the consequences of their management.

As previous research has shown, citrus aphids in the same region have been associated with a rich guild of predators belonging to several taxonomical orders (Michelena & Sanchis, 1997; Alvis, 2003; Hermoso de Mendoza et al., 2012). The most frequently captured predator species in our study coincided with what was previously observed; the micro-coccinellids *S. subvillosus* and *S. interruptus* and the macro-coccinellid *P. quatuordecimpunctata* were the dominant aphidophagous predators. Of all the predator groups found in this study, micro-coccinellids had the most homogenous populations throughout the season but also across the study sites. The macro-coccinellids showed the highest seasonality with peak populations strongly associated to the spring aphid infestation. Differences in demographic seasonal patterns between the

two groups of coccinellids could be associated with the behavior of gravid females; oviposition is influenced by the existence of sufficient resources for their offspring (Hodek et al., 2012). Models of reproductive behavior of predators, usually assume egg production to be directly regulated by the rate of food consumption (Kindlmann & Dixon, 1999, 2001). Resources required by micro-coccinellids are expected to be lower than those for macro-coccinellids. In addition, recent research demonstrates *Scymnus* species to frequently exploit alternative food resources such as nectar, pollen and other arthropods when its primary prey (aphids) is scarce (Hodek et al., 2012). The other groups of predators registered in this study (neuropterans, mirids, and dermapterans) did not present a defined pattern in their activity-density. Their more erratic activity makes their effective management for biological purposes a future challenge for integrate pest management strategies in this crop. Further studies will be required to understand the factors that modulate their populations.

The complex of aphidophagous predators is usually considered inefficient to control the populations of these phytophagous pests. Aphid colonies are characterized by their exponential growth as well as their further rapid decline (Kindlmann et al., 2010). Predators arriving to aphid colonies once they are established are therefore not able to respond quickly enough to the growth of the colonies (Brown, 2004; van Emden & Harrington, 2017). The success of aphid biological control by predators is attributed to effective control in two periods (Michaud, 2012): i) the one carried out in the colonies before winter, which will form new colonies in the spring and ii) the one carried out early in spring, which will prevent the initial development of the colonies

thus reducing further infestation rates in the growing season (Carroll & Hoyt, 1984). Winter control is not considered to be a key factor in Mediterranean citrus agroecosystems since aphid females initiating spring infestations are thought to come from non-citrus hosts (Komazaki, 1983; Brown, 2004). A previous study in the region nevertheless showed that the early arrival of aphidophagous and polyphagous predators to the colonies of *A. spiraecola* in citrus clementine in spring were able to reduce their populations to levels below their pre-established economic thresholds (Gómez-Marco et al., 2015). The authors however, did not identify the predator groups that successfully controlled *A. spiraecola*. Our study demonstrates a successful reduction in aphid infestation levels in citrus to be strongly correlated to the early presence of micro-coccinellids (mostly *Scymnus* species). On the other hand, a high density of predators once the colonies are formed was not a guarantee of successful aphid biological control. The ability of *Scymnus* species to stay at a more homogenous population level throughout the season as well as their high frequency of captures throughout the extensive citrus growing region in which this study was conducted makes these natural enemies potential key predators of aphids in Mediterranean citrus agroecosystems. Further studies will be required to better understand the ecological and biological traits of these coccinellid species that are currently limiting their role as biological control agents of aphids and to develop conservation strategies for these natural enemies.

capítulo 4

Life history traits of the coccinellids *Scymnus subvillosus* and *S. interruptus* on their prey *Aphis spiraecola* and *A. gossypii*: implications for biological control of aphids in clementine citrus

Capítulo 4

Submitted to: Biological Control

Life history traits of the coccinellids *Scymnus subvillosus* and *S. interruptus* on their prey *Aphis spiraecola* and *A. gossypii*: implications for biological control of aphids in clementine citrus

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Abstract

Predator-prey interactions are not static, but spatially and temporally dynamic. In addition to the climatic conditions and the prey density, the dynamics of predator populations may be influenced by the suitability of their diet. Therefore, to better understand aphid predator-prey relationships within food webs, it is necessary to know how their life history traits are affected by diet quality. In this research, the suitability of the two most abundant aphid species

in citrus agroecosystems of the Western Mediterranean basin, *Aphis gossypii* and *A. spiraecola* was evaluated for two of their principle natural enemies, the coccinellid predators *Scymnus subvillosus* and *S. interruptus* under laboratory conditions. The intrinsic rate of increase of *S. subvillosus* was found to be higher than that of *S. interruptus* regardless of the type of prey consumed. Some biological parameters of *S. interruptus* were lower when they were exclusively fed *A. spiraecola* rather than *A. gossypii*. These differences were not found with *S. subvillosus*. When a mixed diet of both aphids was offered, the fitness of both predators was higher than when they were fed a single aphid species. These laboratory observations were further confirmed under field conditions, wherein *S. subvillosus* abundance was greater in those colonies where *A. spiraecola* was predominant. On the other hand both, *S. subvillosus* and *S. interruptus* were found equally in *A. gossypii* colonies. Implications of these results for the biological control of aphids in this crop are discussed.

Keywords Conservation biological control, intrinsic rate of increase, predator-prey interactions, prey suitability

4.1. Introduction

Ladybird predators (Coleoptera: Coccinellidae) have been extensively studied due to their biological, ecological and behavioral characteristics such as polyphagy, high voracity, rapid numerical and aggregative responses (Obrycki & Kring, 1998; Dixon, 2000; Hodek et al., 2012). These characteristics are considered to be important for biological control agents; thus several species are widely used in biological control programs to control pest species such as whiteflies, aphids, mealybugs, scales or psyllids in different crops and cultivation systems, both outdoors and in greenhouses (Hodek & Michaud, 2008; Cabral et al., 2009; Hodek & Honěk, 2009; Obrycki et al., 2009; van Lenteren et al., 2018).

The quality and type of prey plays a key role in selection, consumption and trophic transfer efficiencies by predators (Kalushkov & Hodek, 2005; Keshavarz et al., 2015). Therefore, a good understanding of the behavior, biology and ecology of predators along with a clear view of how these insects interact with their prey are necessary to correctly develop pest management strategies (Evans, 1991; Tuan et al., 2015; Li et al., 2015). Many predatory coccinellids have a "mixed diet" composed of "essential" and "alternative" prey (Evans et al., 1999; Nielsen et al., 2002). Essential prey supports development and reproduction, whereas alternative prey enables adults to survive when essential prey is scarce or absent (Omkar et al., 2009; Lucas, 2005). One of the most important essential prey for coccinellids are aphids (Weber & Lundgren, 2009; Obrycki et al., 2009; Hodek et al., 2012).

Probably the most studied and recognized predator-prey association is the one established between Coccinellidae and Aphididae (Obrycki & Kring, 1998; Hodek et al., 2012). As early as 1874, *Coccinella undecimpunctata* (L.) (Coleoptera: Coccinellidae), a relatively polyphagous species, was imported into and established in New Zealand where it became an important predator of aphids and mealybugs in various fruit and forage crops (Dumbleton, 1936). From there, we can find many citations that elucidate predator contributions to significant reductions in populations of economically important aphid species (Weber & Lundgren, 2009; Obrycki et al., 2009). Therefore, coccinellids are considered to be the most important group of predators within the extensive aphid natural enemy complex (Volkl et al., 2007; Jacas & Urbaneja, 2010; Hodek et al., 2012). Within the aphidophagous coccinellids, most species are found within the subfamilies Coccinellinae and Scymninae. One of the ecological characteristics that make them so valuable is the ability of both larvae and adults to feed on aphids and their occurrence in the same habitats; unlike other groups of predators (Hagen, 1962; Brown, 2004; Van Emden & Harrington, 2007; Hodek et al., 2012).

Citrus crops are affected by several species of aphids. The two main species in the Mediterranean basin are *Aphis spiraecola* Patch and *A. gossypii* Glover (Hemiptera: Aphididae) (Zappalà, 2010; Tena & García-Marí, 2011; Hermoso de Mendoza et al., 2012; Vacante & Gerson, 2012; Belati & Belabed, 2013; Lebbal & Laamari, 2016). Both aphids infest young, tender, citrus shoots on which they can cause severe damage by consuming leaf contents, excreting large amounts of honeydew, and serving as a vector for *Citrus tristeza* virus

(Hermoso de Mendoza et al., 1984; Cambra et al., 2000). Clementine mandarins are the citrus cultivar most damaged by aphids; this cultivar has abundant and susceptible flushes in spring wherein aphid population levels normally exceed their economic threshold (Komazaki, 1982; Hermoso de Mendoza et al., 2001, 2006; Wang et al., 2000).

While citrus agroecosystems are recognized to harbor a high diversity of natural enemy species, biological control of citrus aphid populations is provided primarily by an assemblage of coccinellid, cecidomid and syrphid species (Michaud, 2005; Obrycki et al., 2009; Hodek et al., 2012; Gómez-Marco et al., 2016a). However, some comparative field studies indicate that coccinellids are the most efficient group among the predators (Michaud, 1999; Brown, 2004). 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; Gómez-Marco et al., 2016a).

In the Mediterranean basin, the most abundant species of aphidophagous coccinellids are *Scymnus* (*Pullus*) *subvillosus* (Goeze) and *Scymnus* (*Scymnus*) *interruptus* (Goeze), of which there are few studies examining their relevance and role within citrus agroecosystems (Longo & Benfatto, 1987; Raimundo & Alves, 1986; Franco et al., 1992; Magro & Hemptinne, 1999; Alvis, 2003; Kavallieratos et al., 2004).

Both species of *Scymnus* are mainly predators of aphids even though they can feed on other prey such as scales, mealybugs and spider mites (Tawfik

et al., 1973; Magro & Hemptinne, 1999; Hodek & Honěk, 2009; Bouvet el al., 2018). These coccinellids are cosmopolitan species found in Europe, Minor Asia, and Northern Africa (Tawfik et al., 1973; Gourreau, 1974; Raimundo & Alves, 1986). They are common in arboreal agroecosystems, including apple, citrus, peach, plum, walnut, oak and natural habitats (Raimundo & Alves, 1986; Aslan & Uygun, 2005; Atlihan & Güldal, 2009; Sebastião et al., 2015).

Compared to larger coccinellid species the *Scymnus* genus, due to its smaller size and lower voracity, is expected to be less competitive and efficient as biological control agents. However, their high abundance in agroecosystems, their capacity to persist at low prey densities and their high longevities (Tawfik et al., 1973; Borges et al., 2011, 2013), give these small ladybirds the ability to exploit aphid colonies in earlier and later plant physiological stages (Agarwala & Yasuda, 2001) and for longer periods of time than the larger ladybirds (Sebastião et al., 2015; Bouvet et al., 2018).

In the citrus agroecosystems of the Mediterranean Basin, *S. interruptus* and *S. subvillosus* have been registered to be the most abundant predators (Magro et al., 1999; Alvis, 2003; Hermoso de Mendoza et al., 2012). Both predators have been found coexisting with the two most abundant aphid species in this crop, *A. spiraecola* and *A. gossypii* (Zappalà, 2010; Tena & García-Marí, 2011; Hermoso de Mendoza et al., 2012). However, whether or not there is any feeding preference of either of the two predators for either of the two aphid species and whether this possible differentiation in terms of diet could explain their presence and abundance in the field have not been previously evaluated. Therefore, in this

research, the suitability of *A. spiraecola* and *A. gossypii* as prey for *S. interruptus* and *S. subvillosus* was studied. For this, the life history traits of both predators when raised on i) *A. spiraecola*, ii) *A. gossypii* or iii) a mixture in equal amounts of *A. spiraecola* and *A. gossypii* were studied. Furthermore, with the aim of corroborating the conclusions obtained in the susceptibility studies carried out in the laboratory, the presence and abundance of both species of coccinellids in the field on three types of colony i) *A. spiraecola*, ii) *A. gossypii* and iii) mixed colonies with presence of both *A. spiraecola* and *A. gossypii* was evaluated.

4.2. Materials and Methods

4.2.1. Stock cultures

Laboratory colonies of *A. spiraecola* and *A. gossypii* were initiated by collecting specimens on Clementine trees [Citrus clementina Hort. ex Tan. (Geraniales: Rutaceae)] located in orchards belonging to the Instituto Valenciano de Investigaciones Agrícolas (IVIA) in Moncada, Valencian Community, Spain (39°35'17.43"N / 0°23'53.28"O). Aphids were reared on two-year old, potted, clementine plants (*Citrus reticulata* Blanco [Clementina de Nules cultivar Iniasel 22] grafted onto *Citrange* Carrizo rootstock [*Poncirus trifoliata* (L.) Rafinesque-Schmaltz x *Citrus sinensis* (L.) Osbeck]) and kept in a heated chamber of a greenhouse at 24 ± 2ºC, 60 ± 5% relative humidity with the natural photoperiod.

A colony of *Myzus persicae* (Sulzer) (green phenotype) was also set up to provide a source of food for artificial coccinellid rearing. This aphid colony was

established on sweet pepper plants (*Capsicum annuum* L.; Solanales: Solanaceae); it was started from a stock colony maintained on potted broad bean plants (*Vicia faba* L.; Fabales: Fabaceae) at the IVIA since 2004. Colonies of *M. persicae* on pepper plants were selected for their practicality; the aphids are easy to rear and manipulate. This colony was kept in heated chambers of a greenhouse under the same environmental conditions described above.

To establish the *S. subvillosus* and *S. interruptus* colonies, specimens were collected from clementine trees in the IVIA orchard (as above), by stem-tap sampling. Around fifty insects were kept in Petri dishes (9.0 cm in diameter × 1.5 cm in height) with a muslin covered hole in the lid (4 × 4 cm) to avoid excess humidity. In each Petri dish pepper leaves with *M. persicae* were provided every two days, as well as pollen, honey, and water on a filter paper. Petri dishes were isolated in a growth chamber (SANYO MLR-350; Sanyo, Japan) at 25 ± 1 ° C, 60-70% RH with a photoperiod of 14:10 h (L:D).

4.2.2. Developmental parameters

Every two days *S. subvillosus* and *S. interruptus* eggs were selected and taken from the colonies. They were divided into three groups and were placed in Petri dishes (5.5 cm diameter and 1.0 cm tall) with a muslin covered hole in the lid (2 × 2 cm), to allow gas exchange, and a plaster ball (5 mm) at the base to keep the humidity constant within the experimental units.

Newly emerged coccinellid larvae (< 24 h old) were individually transferred to numbered Petri dishes (5.5 cm diameter as above) and reared with the aphid species corresponding to the three following experimental diet treatments: 1) *A. spiraecola*, 2) *A. gossypii* and 3) *A. spiraecola* and *A. gossypii* in equal proportions. Each larva was provided aphids *ad libitum* and water in the plaster every day until larvae either pupated or died. Experimental arenas were kept in climatic chambers in the same conditions as those for *Scymnus* spp. colonies.

Larval (the presence of exuviate was used as evidence of molting) and pupal development and survival were checked daily under a stereomicroscope until death or adulthood. After adult emergence, they were measured (photographed with a camera mounted stereomicroscope using the software package Leica Application Suite, LAS version 4.6.2), weighed (precision scale) and sexed. These adults were used to assess the reproductive parameters. 30 *Scymnus* adult replicates of each species and each prey diet treatment were made.

4.2.3. Reproductive parameters

Following adult emergence, males and females were kept individually with the corresponding diet during 5-7 days (pre-breeding period) (Tawfik et al., 1973; Sebastião et al., 2015). After that, all the adult coccinellids of the same species and treatment were put together in an empty Petri dish (9.0 x 1.5 cm) and visually observed. Every time a couple was formed (presumed male and female) it was isolated in a Petri dish (similar to the development experimental

units described above) for 24 h to allow mating. Males were later removed and gravid females were studied for 6 weeks. During this period, on a fresh piece of excised plant tissue females were provided a mixture of all nymphal stages of the corresponding aphid species *ad libitum* according to treatment. A small piece of corrugated cardboard (2 x 2 cm) was placed in the arena as oviposition substrate. Eggs were counted and removed daily. Water was supplied daily with a pipette and new aphids of the corresponding treatment were also added. Experimental arenas were kept in the same climatic conditions as described above.

4.2.4. Demographic growth indexes

The intrinsic rate of increase (r_m) was computed using the Euler equation,

$$\Sigma\, e^{-rm}\, l_x m_x$$

where I_x is survivorship of the original cohort over the age interval from day $x - 1$ to day x, and m_x is the mean number of female offspring produced per surviving female during the age interval x (Birch, 1948). The sex ratio of 0.5 based on other studies was used to calculate the statistics (Tawfik et al., 1973; Sebastião et al., 2015). Values of m_x for the population were calculated from the mean number of eggs laid per female per day. Other parameters, including reproductive rate (R_0) and generation time (T) were calculated as described by Birch (1948) using jackknife (Maia et al., 2000). The finite rate of increase ($\lambda_m = e^{rm}$) and doubling time ($DT = LN\ 2/r_m$) were also calculated (Mackauer, 1983).

4.2.5. Preference sampling

To further explore the relationships between the populations of the citrus aphid species, *A. spiraecola* and *A. gossypii*, and their predators, *S. subvillosus* and *S. interruptus,* sixty clementine orchards separated by a minimum distance of 0.5 km were sampled throughout the Valencian Community (**SS4.1**), covering an area of 240,000 hectares within the provinces of Castellón and Valencia (**figure 4.1**). From April 22 to May 31, 2016, in the full citrus sprouting stage, 10 random aphid infested trees per orchard were sampled for *Aphis* spp and *Scymnus* spp. populations.

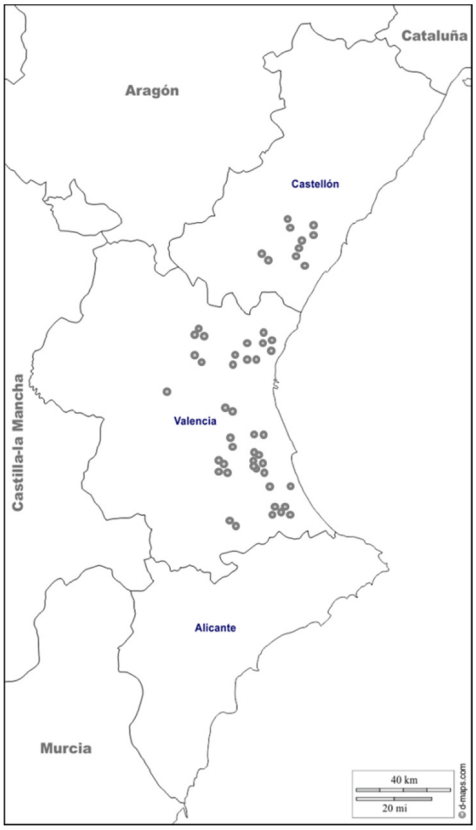

Figure 4.1 Location of sampling sites in the Valencian region (Comunidad Valenciana, Spain).

For aphid sampling, in each tree a 0.25 m^2 ring was placed twice on the canopy. Within the ring, the number of suitable aphid-infested and non-infested shoots as well as the average number of aphids per infested shoot was recorded (Hermoso de Mendoza et al., 2001, 2006). Trees were classified according to the predominant aphid species (more than 70% of shoots) as i) dominated by *A. gossypii*, ii) dominated by *A. spiraecola* or iii) presenting mixed colonies in case neither of the two aphid species was present in more than 70% of the shoots.

At the same time, by stem tap sampling, in the same trees used for the aphids surveys, *Scymnus* spp populations were also sampled. For this, we used a plastic tray (45 x 30 cm) and a PVC pipe (Polyvinyl chloride). The species of *Scymnus* (larvae and adults) dislodged from the stricken branches by the stem-tapping were identified inside the plastic tray and then released.

4.2.6. Data analysis

The effect of the prey diet on the life history traits of *S. interruptus* and *S. subvillosus* (developmental durations of the different stages, and development and reproductive parameters) were analyzed by one way ANOVA and subsequent Tukey's Honestly Significant Difference test (HSD). R Studio 1.1.383 software was used for the statistical analysis.

The Cox proportional hazards model was used to evaluate differences between the survival probabilities of the *S. interruptus* and *S. subvillosus* among

the three types of diets. Significant differences of the life table parameter means was determined using Student's *t* test. R Studio 1.1.383 software was used for the statistical analysis.

To describe the potential demographic spatial relationships between the two dominant citrus aphid species and their predators *S. interruptus* and *S. subvillosus* the number of *Scymnus* specimens captured by stem-tap sampling in each tree throughout the spatial sampling (dependent variable) was related to the *Scymnus* species (*S. interruptus* or *S. subvillosus*) and the aphid dominance (*A. spiraecola*, *A. gossypii* or mixture of the two aphid species in the colonies). The interaction between these two fixed effects was also included in the model. Generalized linear mixed model (GLMM) analysis was used for this purpose. Negative binomial distribution of the variable was assumed based on Akaike and Bayesian information criteria. Tukey's HSD test was used to investigate differences in the relative abundance of the two *Scymnus* species depending on aphid dominance.

4.3. Results

4.3.1. Developmental parameters

Duration of the different immature stages of *S. subvillosus* and *S. interruptus* when reared on *A. spiraecola* and *A. gossypii* and on the mix of both, are shown in **table 4.1**. Total developmental time for both coccinellids when

reared exclusively on *A. spiraecola* was significantly longer than when fed with *A. gossypii*, either alone or in mixture with *A. spiraecola*.

Table 4.1 Mean development times (days ±SE) of *S. subvillosus* and *S. interruptus* when reared on 1) *A. spiraecola*, 2) *A. gossypii* and 3) *A. spiraecola* + *A. gossypii*) (df = 2, 150). Within a row, means followed by the same letter are not significant different ($P < 0.05$; Tukey test).

Species	Stages	Diets			*F* value	*P* value
		1	2	3		
S. interruptus	First instar	2.52 ± 0.08a	2.20 ± 0.06b	2.23 ± 0.08b	6.02	0.003
	Second instar	1.68 ± 0.08a	1.57 ± 0.07a	1.58 ± 0.08a	0.63	0.53
	Thrird instar	1.98 ± 0.06a	1.76 ± 0.09ab	1.71 ± 0.07b	3.76	0.03
	Fourth instar	3.32 ± 0.10a	3.29 ± 0.09a	3.27 ± 0.08a	0.08	0.93
	Prepupa	2.16 ± 0.09a	2.06 ± 0.08ab	1.85 ± 0.07b	3.91	0.02
	Pupa	5.92 ± 0.08a	5.94 ± 0.07a	6.13 ± 0.06a	2.95	0.06
	Total	17.78 ± 0.18a	16.82 ± 0.16b	16.77 ± 0.12b	8.61	< 0.001
S. subvillosus	First instar	2.22 ± 0.09b	2.62 ± 0.09a	2.10 ± 0.10b	8.39	< 0.001
	Second instar	1.90 ± 0.11a	1.50 ± 0.07b	1.61 ± 0.09ab	5.28	0.01
	Thrird instar	2.00 ± 0.09a	1.48 ± 0.08b	1.59 ± 0.09b	9.62	<0.001
	Fourth instar	3.24 ± 0.13a	2.77 ± 0.12b	2.98 ± 0.12ab	3.62	0.03
	Prepupa	2.44 ± 0.11a	2.29 ± 0.09a	2.59 ± 0.10a	2.30	0.10
	Pupa	5.62 ± 0.11a	5.48 ± 0.08a	5.65 ± 0.07a	1.09	0.34
	Total	17.42 ± 0.23a	16.13 ± 0.16b	16.51 ± 0.19b	11.42	<0.001

Immature survival was higher for *S. subvillosus* than that for *S. interruptus*. Immature survival of *S. subvillosus* gradually decreased until adult emergence to values between 65 and 80% while survival of *S. interruptus* was higher than 90%. When comparing the probability of survival of each coccinellid species fed with the three diets, no statistical differences were found (*S. interruptus:* $\chi^2 = 1.9300$, $df = 2$, $P = 0.381$; *S. subvillosus*: $\chi^2 = 3.5222$, df = 2, $P = 0.17$; Cox proportional hazards model) (**figure 4.2**). The weight and size of the emerged adults (males and females) was not significantly different for all but one of the coccinellid species when fed the three diets (**table 4.2**). The only significant difference observed

was the size of the *S. interruptus* males that were fed *A. spiraecola* alone; they were smaller than those fed with the other two diets.

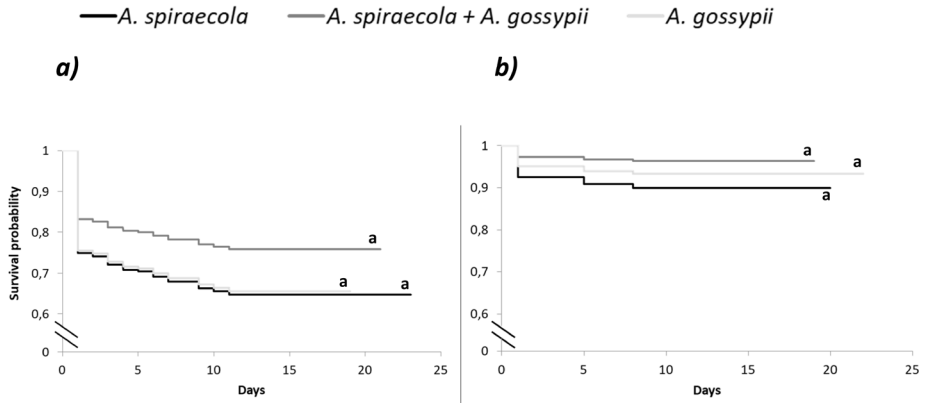

Figure 4.2 Immature survival curves of a) *S. subvillosus* and b) *S. interruptus* when reared on 1) *A. spiraecola*, 2) *A. gossypii* and 3) *A. spiraecola* + *A. gossypii* (df = 2). Survivorship curves followed by the same letter are not significantly different ($P < 0.05$; Cox regression model).

Table 4.2 Mean weight (mg ± SE) and area (mm2 ±SE) of *S. subvillosus* and *S. interruptus* when reared on 1) *A. spiraecola*, 2) *A. gossypii* and 3) *A. spiraecola* + *A. gossypii*) (df = 2, 150). Within a row, means followed by the same letter are not significant different ($P < 0.05$; Tukey test).

Species	Features	Diets			*F* value	*P* value
		1	2	3		
S. interruptus	Pupation (%)	100a	100a	100a	1.04	0.36
	Male weigth	0.97 ± 0.07a	0.88 ± 0.05a	0.95 ± 0.06a	0.46	0.63
	Female weigth	1.08 ± 0.06a	1.21 ± 0.10a	1.33 ± 0.07a	2.38	0.10
	Male area	7.30 ± 0.11b	7.74 ± 0.10ab	7.94 ± 0.14a	7.92	< 0.001
	Female area	9.61 ± 0.26a	9.65 ± 0.27a	9.83 ± 0.26a	0.17	0.85
S. subvillosus	Pupation (%)	96.49 ± 1.96a	97.01 ± 1.68a	98.46 ± 1.34a	0.25	0.78
	Male weigth	1.38 ± 0.07a	1.45 ± 0.06a	1.45 ± 0.07a	0.35	0.71
	Female weigth	1.52 ± 0.05a	1.53 ± 0.06a	1.56 ± 0.06a	0.12	0.89
	Male area	10.30 ± 0.25a	10.31 ± 0.27a	10.27 ± 0.30a	0.006	0.99
	Female area	10.76 ± 0.25a	11.11 ± 0.29a	10.62 ± 0.35a	0.64	0.53

4.3.2. Reproductive parameters

Diet had a significant effect on the reproductive parameters of both *Scymnus* species when raised on the three different diets (**table 4.3**). Fecundity of both predators was significantly higher with the mixed diet than with the *A. gossypii* diet and the lowest with the *A. spiraecola* diet. Daily fecundity (eggs/day) was higher for both coccinellids with the mixed diet which resulted in a significantly smaller egg size in *S. interruptus*, yet a larger size in *S. subvillosus* on the same diet. No significant differences in fertility of *S. interruptus* were found among the three diets; however, in the case of *S. subvillosus*, fertility was lower when fed only *A. spiraecola*. The egg incubation period was similar among diets for *S. interruptus*. However, *S. subvillosus* eggs hatched significantly sooner with the *A. gossypii* diet than with both the *A. spiraecola* and the mixed diet.

Table 4.3 Reproductive parameters (mean ± SE) of *S. subvillosus* and *S. interruptus* when reared on 1) *A. spiraecola*, 2) *A. gossypii* and 3) *A. spiraecola* + *A. gossypii*). Within a row, means followed by the same letter are not significant different ($P < 0.05$; Tukey test).

Species	Reproductive parameters	Diets			*F* value	df	*P* value
		1	2	3			
S. interruptus	Fecundity (total eggs)	115.69 ± 1.62c	158.69 ± 1.91b	174.00 ± 2.21a	326.30	2, 41	< 0.001
	Fertility (%) (hatched eggs)	81.44 ± 2.52a	86.32 ± 2.26a	84.89 ± 2.57a	1.37	2, 901	0.26
	Daily fecundity (eggs/day)	2.57 ± 0.12b	3.53 ± 0.12b	3.87 ± 0.15a	30.88	2, 1977	< 0.001
	Eggs area (mm2)	0.57 ± 0.004a	0.58 ± 0.003a	0.56 ± 0.005b	7.60	2, 901	< 0.001
	Incubation period (days)	4.63 ± 0.05a	4.54 ± 0.03a	4.51 ± 0.04a	2.28	2, 760	0.10
S. subvillosus	Fecundity (total eggs)	261.29 ± 4.02c	272.86 ± 3.37b	361.82 ± 3.22a	384.71	2, 42	< 0.001
	Fertility (%) (hatched eggs)	79.78 ± 7.89b	91.54 ± 5.45a	89.95 ± 4.07a	11.40	2, 993	< 0.001
	Daily fecundity (eggs/day)	5.81 ± 0.25b	6.06 ± 0.20b	8.04 ± 0.23a	24.38	2, 2022	< 0.001
	Eggs area (mm2)	0.38 ± 0.002b	0.39 ± 0.002a	0.39 ± 0.002a	6.55	2, 993	< 0.001
	Incubation period (days)	3.27 ± 0.04b	3.08 ± 0.03c	3.41 ± 0.03a	24.84	2, 870	< 0.001

4.3.3. Demographic parameters

Effects of diets on selected life history parameters of both coccinellids are presented in **table 4.4**. In both *Scymnus* species, when fed the mixed diet, the net reproductive rate (R_0), the intrinsic rate of increase (r_m) and the finite rate of increase (λ_m) were significantly higher. The second highest rates were found in the *A. gossypii* diet. Generation time (T) was significantly longer for *S. interruptus* when fed on *A. gossypii*, whereas for *S. subvillosus* the longest generation time occurred with the *A. spiraecola* diet. *Scymnus interruptus* doubled its population sooner when fed on *A. gossypii* than on *A. spiraecola* whereas the contrary was observed for *S. subvillosus*.

Table 4.4 Demographic parameters (mean ± SE) of *S. subvillosus* and *S. interruptus* females when reared on 1) *A. spiraecola*, 2) *A. gossypii* and 3) *A. spiraecola* + *A. gossypii*) (df = 2, 41 for *S. interruptus* and df = 2, 42 for *S. subvillosus*). Within a row, means followed by the same letter are not significant different ($P < 0.05$; Tukey test).

Species	Demographic parameters	Diets			*F* value	*P* value
		1	2	3		
S. interruptus	Net reproductive rate, R0 (♀/♀)	51.82 ± 0.79c	73.79 ± 0.91b	83.52 ± 1.06a	422.54	< 0.001
	Intrinsic rate of increase, rm (♀/♀/day)	0.11 ± 0.0006c	0.12 ± 0.0003b	0.13 ± 0.0005a	629.33	< 0.001
	Finite rate of increase, λm(♀/♀/day)	1.12 ± 0.0006c	1.12 ± 0.0004b	1.14 ± 0.0005a	630.69	< 0.001
	Generation time, T (days)	40.90 ± 0.40a	43.63 ± 0.12b	40.41 ± 0.17a	87.569	< 0.001
	Doubling time, DT (days)	7.18 ± 0.06a	7.03 ± 0.02b	6.33 ± 0.03c	207.62	< 0.001
S. subvillosus	Net reproductive rate, R0 (♀/♀)	82.31 ± 1.27c	87.31 ± 1.08b	137.86 ± 1.28a	1009.06	< 0.001
	Intrinsic rate of increase, rm (♀/♀/day)	0.14 ± 0.0004c	0.14 ± 0.0003b	0.16 ± 0.0002a	1477.10	< 0.001
	Finite rate of increase, λm(♀/♀/day)	1.15 ± 0.0005c	1.15 ± 0.0003b	1.17 ± 0.0003a	1492.00	< 0.001
	Generation time, T (days)	35.31 ± 0.14b	37.23 ± 0.11a	37.44 ± 0.18a	92.51	< 0.001
	Doubling time, DT (days)	5.55 ± 0.02b	5.77 ± 0.01a	5.27 ± 0.02c	326.07	< 0.001

4.3.4. Spatial sampling

Approximately 7,300 citrus shoots containing aphid colonies were observed. *Aphis spiraecola* was the dominant colony species in 75% of the shoots whereas only 10% of them presented dominance of *A. gossypii* colonies. The remaining 15% of the observed shoots showed mixed colonies of both aphid species.

Scymnus spp. abundance monitored through stem-taps in the regional sampling was significantly affected by the type of dominant aphid colonies in the trees (F = 4.20, df = 2, 1126, P = 0.015). Coccinellids were more frequently captured in trees presenting dominant *A. gossypii* colonies than those with *A. spiraecola* (t = 2.75, df = 1126, P = 0.006) or mixed colonies of the two species (t = 2.61, df = 1126, P = 0.009). No differences in *Scymnus* captures were found between trees mainly infested by *A. spiraecola* colonies and trees presenting mixed colonies (t = 0.64, df = 1126, P = 0.521). *Scymnus subvillosus* was the most captured coccinellid species by stem-taps throughout the regional sampling (F = 15.67, df = 1, 1126, P < 0.0001). This species was found in 80% of the plots with an average of 13.33 (±2.05) specimens per plot. *S. interruptus* was observed in 55% of the plots sampled with an abundance of less than 5.02 (±1.09) specimens per plot. Marginal differences were found in the relative abundance of the two *Scymnus* species depending on the dominant type of aphid colonies (Interaction: F = 2.23, df = 1, 1126, P = 0.108): *Scymnus subvillosus* was the most captured species in trees dominated both by *A. spiraecola* colonies (t = -7.66, df = 1126, P < 0.0001) and mixed colonies (t = -3.29, df = 1126, P = 0.001) whereas no differences

between the two *Scymnus* species were found in trees dominated by *A. gossypii* colonies (t = -0.25, df = 1126, P = 0.806) (**figure 4.3**).

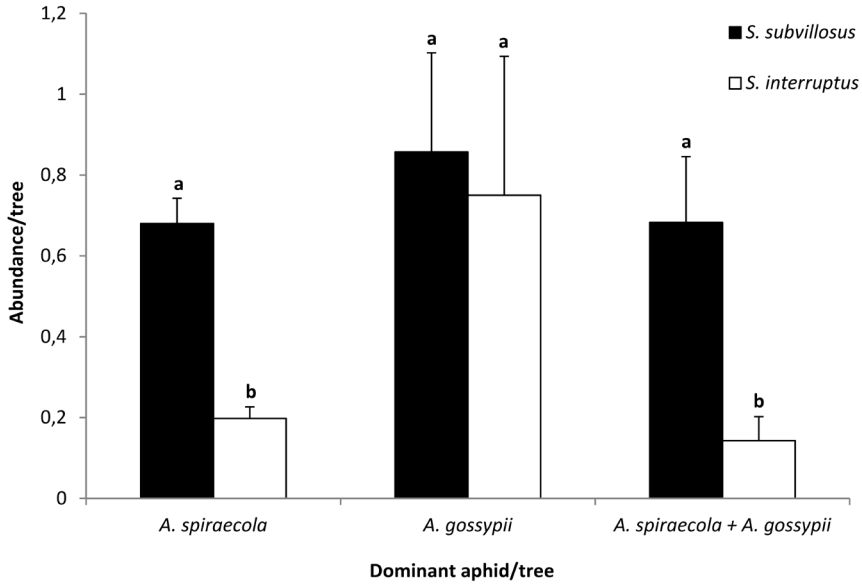

Figure 4.3 Mean number (± SE) of specimens of *S. subvillosus* and *S. interruptus* on trees with a predominant abundance of 1) *A. spiraecola*, 2) *A. gossypii* or 3) both species are found in similar proportions. Same letters between columns of the same predominance grouping indicate no significant differences (P < 0.05; Tukey test).

4.4. Discussion

For the two *Scymnus* species studied, our results presented shorter development times when offered diets that included *A. gossypii* alone and in combination with *A. spiraecola*, than those of *A. spiraecola* alone. This would indicate that *A. gossypii* is more suitable for larval development, probably because of the presence of essential nutrients for growth. Shortening of development times leads to quicker multiplication of predator populations and therefore

makes them more effective in controlling pests. Mixed diets have been shown to be the most favorable for the development of coccinellids (Evans et al., 1999; Nielsen et al., 2002; Schuldiner & Coll, 2017). Nevertheless, in our study this was not observed probably because both preys were reared on the same host plant which would not provide such a noticeable advantage as observed when coccinellids are fed on mixed aphid species diets which had been reared on different hosts (Hauge et al., 1998). Similar results were obtained by Sebastião et al. (2015) who did not find differences in *S. subvillosus* survival in any of the stages studied when fed with three different species of aphids reared in the same host plant (*Vicia faba* L.; Fabales: Fabaceae).

When we used the Cox proportional hazards model, the probability of survival during the larval developmental period was not affected by the type of diet on which they were raised, as has been observed in other research (Kalushkov, 1998; Omkar et al., 2009; Schuldiner & Coll, 2017). Again, this could be attributed to the use of the same aphid host plant species in all the treatments. On the other hand, the differences in survival rates observed among *Scymnus* species could possibly be attributed to intrinsic factors of each particular species (Tawfik et al., 1973; Sebastião et al., 2015).

The size and weight of males and females of the two *Scymnus* species were not significantly affected by any of three diets provided. Only a reduction in the male size of *S. interruptus* fed with *A. spiraecola* was observed; which supports the hypothesis that *A. spiraecola* does not meet the predator´s needs as well as *A. gossypii* as previously observed by Omkar & Bind (2004) and Kalushkov

& Hodek (2005). Low quality diets consumed during the larval period are thought to negatively influence the size of adults (Michaud, 2000).

The reproductive parameters for both predators were importantly influenced by the diet consumed. *Scymnus* females fed the aphid mixture had a significantly greater total and daily fecundity, followed by the diet composed exclusively of *A. gossypii*. Omkar & James (2004) found a relatively higher fecundity in ladybugs fed on *A. gossypii*; this was associated with the impact of early ovariole maturity, which is dependent on the prey species consumed. On the other hand, *A. spiraecola* as unique diet seemed to be deficient since it produced lower fecundity in both species of predators. In addition, *S. subvillosus* fed with *A. spiraecola* resulted in lower fertility and smaller egg size. These data clearly showed that the effect of diet on predators differentially influences the organism according to the specific requirements of each species for its development and reproduction.

Biological differences in *Scymnus* species under distinct diets can be summarized through their estimated life history parameters. The net reproductive rate (R_0) in both *Scymnus* species presented higher values when fed the mixed diet. This proves that mixed diets benefit the populations of stenophagous predators, probably because they provide a greater diversity of nutrients (Nielsen et al., 2002; Schuldiner & Coll, 2017). On the contrary, the R_0 values, when the two ladybird species were fed on *A. spiraecola* alone were the lowest; again supporting the idea that this species of aphid is of lower nutritional quality than *A. gossypii*.

Estimated R_0 values for *S. subvillosus* were similar to those obtained in other studies where the specimens were raised with only one type of prey, *Hyalopterus pruni* (Geoffroy) (Hemiptera: Aphididae) or *A. gossypii* (Atlihan & Kaydan, 2002; Atlihan & Hsin Chi, 2008; Atlihan & Güldal, 2009; Satar & Uygun, 2012). However, the R_0 values obtained with a mixed diet were higher than when only one prey was offered which is also similar to those observed by Gibson et al. (1992) for *Scymnus frontalis* (Frabricius). In contrast, estimated R_0 values for *S. interruptus* were lower than for *S. subvillosus*, independent of diet.

The high R_0 values obtained for the two *Scymnus* species may be an indication of why these predators are so abundant in the Mediterranean region and particularly *S. interruptus* and *S. subvillosus* in citrus, where both predators are thought to play a primary role in aphid control (Alvis, 2003; Soler et al., 2006; Santos et al., 2012; Bouvet el al., 2018).

As expected, the intrinsic growth rate (r_m) and the finite rate of increase (λ_m) showed the same pattern as R_0. These parameters were highest in the populations reared on the mixed diet and lowest in that fed the *A. spiraecola* diet. Similar values of these rates were observed in other studies with *S. subvillosus*, *S. levaillanti* Mulsant, *S. scyriacus* (Marseul), *S. nubilus* (Mocquard) and *S. frontalis* (Gibson et al., 1992; Uygun & Atlihan, 2000; Atlihan & Kaydan, 2002; Atlihan & Chi, 2008; Soroushmehr et al., 2008; Atlihan & Güldal, 2009; Satar & Uygun, 2012; Borges et al., 2013). The intrinsic growth rate represents the intrinsic potential of a certain species in a given situation. The low effectiveness of both predators in the control of aphids observed in some field studies (Brown, 2004; Hermoso

de Mendoza et al., 2012), could be due to the fact that *A. spiraecola* and *A. gossypii* present r_m values three times greater than those found for *S. subvillosus* and *S. interruptus* as in this research (Kocourek et al., 1994; Kersting et al., 1999; Wang & Tsai, 2000). Therefore, the potential of these predator species should be exploited through other means such as early arrival to aphid colonies through the use of banker plants or the management of cover crops (Hodek et al., 2012; Gómez-Marco et al., 2016a, b). Bouvet et al. (2018) recently found aphid biological control success in western Mediterranean citrus clementine crops to only be achieved through an early presence of *Scymnus* species in the orchards. Gómez-Marco et al. (2016a, b) demonstrated that a sown grass cover enriched with wild forbs improved the biological control of aphids in citrus since it promoted the early presence of predators, including *Scymnus* species, in citrus canopies, before aphid exponential increase.

At a regional scale, either by the samplings made in this study, or by those carried out by other researchers previously in the Mediterranean region (Hermoso de Mendoza et al., 2012; Mostefaoui et al., 2014; Lebbal & Laamari, 2016), the most abundant aphid species in citrus crops has been identified to be *A. spiraecola*. However, although both *Scymnus* species are the most abundant predators registered in the region, there is some controversy about which of the two could be the most important based on their demography. Some authors reported greater *S. interruptus* abundance in citrus crops (Magro & Hemptinne, 1999; Alvis, 2003), while others cited *S. subvillosus* as the dominant species (Hermoso de Mendoza et al., 2012), as observed in the present study. The greater abundance of *S. subvillosus* in the field could be explained by their estimated life

history parameters. On one hand, estimated R_0, r_m and λ_m for *S. subvillosus* with any of the three diets were much higher than those obtained for *S. interruptus*. Nevertheless, developmental time (T) of *S. subvillosus* was reduced when it was fed *A. spiraecola*. Developmental time of these aphid species is particularly short. A reduction of T would therefore give *S. subvillosus* a considerable selective advantage over *S. interruptus* making the former especially more effective in the control of *A. spiraecola* (Hemptinne & Dixon, 1997).

Due to the common heterogeneity in predator (Alvis, 2003) and prey distribution we initially hypothesized there to be some type of diet preference by the predators since females choose the best diet to achieve maximum fitness. The field sampling revealed the abundance of *S. interruptus* to be higher in shoots occupied by *A. gossypii*. In contrast, *S. subvillosus* did not show a preference based on the aphid species occupying the citrus shoots. Therefore, our results, from both the laboratory and field support our initial hypothesis. In addition, the preference observed by *S. interruptus* could be one of the causes of the low rates of *A. gossypii* in the field compared to *A. spiraecola*, which was dominant. On the other hand, the existence of this preference leads us to believe a competitive pressure between the *Scymnus* species could be higher in shoots with *A. gossypii*. The fact of finding greater abundance of *S. interruptus* in the shoots with greater competition indicates that this species of predator would be a better competitor than *S. subvillosus* in this situation. Studies on these aspects of competition must be carried out to clarify how these two closely related species of predators can coexist in the citrus agroecosystem without one displacing the other.

4.5. Conclusion

This research provides novel information about *Scymnus* species and their relationships with the aphid species found in citrus crops in the Mediterranean basin. Our hypothesis on the quality of the diet influencing both predators´ development and reproductive parameters, and consequently their field abundance and field distribution was confirmed. Even though the mixed diet that included *A. gossypii* and *A. spiraecola* was the most suitable for the development of both predator species, in the laboratory and field *S. interruptus*, in particular, demonstrated a preference for *A. gossypii*. This preference may be one of the main factors determining the distribution and dynamics of the citrus aphid complex and its natural enemies in the Mediterranean basin.

Supplemental information

Plot	*A. spiraecola*	*A. gossypii*	*A. spiraecola* + *A. gossypii*	*S. subvillosus*	*S. interruptus*	Longitud	Latitud
Alcira	P	P	P	P	A	39° 9'46.42"N	0°26'39.38"O
Algemesi	P	P	P	P	A	39°10'29.91"N	0°26'36.90"O
Algimia 1	A	P	P	P	P	39°42'55.11"N	0°18'57.46"O
Algimia 2	P	P	P	A	P	39°42'23.13"N	0°18'37.41"O
Algimia poble	P	A	A	P	P	39°44'57.99"N	0°22'5.65"O
Alquerias I	P	A	A	P	P	39°54'40.12"N	0° 8'9.28"O
Alquerias II	P	A	A	P	P	39°54'3.39"N	0° 6'30.18"O
Barx	P	A	A	P	P	39° 1'16.41"N	0°18'21.86"O
Belgida I	P	P	P	P	P	38°51'11.60"N	0°27'29.56"O
Belgida II	P	A	A	A	P	38°51'30.16"N	0°27'39.92"O
Belgida III	P	P	P	A	P	38°51'16.27"N	0°27'38.42"O
Belgida IV	P	A	A	P	P	38°51'26.45"N	0°27'55.75"O
Benaguacil	P	A	A	P	A	39°34'55.18"N	0°36'44.72"O
Benicull	P	A	P	P	A	39°11'23.54"N	0°22'55.31"O
Benifairó I	P	P	P	P	A	39° 3'39.77"N	0°18'48.00"O
Benifairó II	P	A	P	P	P	39° 3'42.76"N	0°19'3.52"O
Bétera	P	A	A	P	A	39°35'10.94"N	0°24'38.78"O
Betxí	P	A	A	P	P	39°56'30.80"N	0°12'45.88"O
Borriol I	P	P	P	P	P	40° 1'7.23"N	0° 5'46.26"O
Borriol II	P	P	P	P	P	40° 1'23.08"N	0° 5'56.10"O
Cabes Bort	P	A	P	P	P	39°36'54.67"N	0°22'14.83"O
Carcaixent I	P	P	P	P	A	39° 6'36.62"N	0°26'54.76"O
Carcaixent II	P	P	P	P	P	39° 6'21.01"N	0°27'8.25"O
Carcaixent III	P	P	P	P	P	39° 5'48.80"N	0°27'18.92"O
Castellon I	P	A	A	P	P	39°59'29.72"N	0° 4'12.96"O
Castellon II	P	A	A	P	P	39°59'58.43"N	0° 5'2.72"O
Castellonet	P	P	P	P	P	38°55'5.83"N	0°16'11.60"O
Faura	P	P	P	P	A	39°43'41.36"N	0°15'11.60"O
Garbí	P	A	A	P	P	39°44'25.99"N	0°23'10.03"O
Liria	P	P	P	P	P	39°36'39.92"N	0°39'25.79"O
Maxuquera	P	P	A	P	A	38°56'24.57"N	0°13'33.09"O

Monasterio St. Jeroni I	P	A	A	P	A	38°56'18.63"N	0°14'36.65"O
Monasterio St. Jeroni II	P	A	A	P	P	38°56'23.16"N	0°14'5.21"O
Moncada I	P	P	P	P	P	39°35'17.51"N	0°23'53.48"O
Moncada II	P	A	A	A	A	39°34'41.98"N	0°23'57.03"O
Onda	P	A	A	A	P	39°57'12.39"N	0°13'53.65"O
Palma de Gandía	P	P	P	P	P	38°55'14.75"N	0°13'5.99"O
Polinya de Xúquer	P	P	P	P	A	39°11'27.58"N	0°22'16.45"O
Puig	P	A	A	A	A	39°35'2.08"N	0°19'1.56"O
Puzol I	P	A	A	A	A	39°36'39.96"N	0°17'51.60"O
Puzol II	P	A	A	A	A	39°36'12.76"N	0°17'52.43"O
Puzol III	P	A	A	A	A	39°36'58.84"N	0°19'2.81"O
Ribesalbes I	P	P	P	P	A	40° 0'41.83"N	0° 8'53.76"O
Ribesalbes II	P	A	P	P	P	40° 0'21.32"N	0° 8'27.56"O
Ribesalbes III	P	P	P	P	A	40° 0'53.30"N	0° 8'48.21"O
Ribesalbes IV	P	P	P	P	A	40° 0'39.13"N	0° 7'56.65"O
San Isidro I	P	A	A	A	P	39°34'31.24"N	0°23'13.90"O
San Isidro II	P	A	A	P	P	39°34'14.53"N	0°23'14.24"O
Segorbe	P	P	P	P	A	39°49'39.45"N	0°27'46.67"O
Simat I	P	P	P	P	A	39° 4'10.88"N	0°20'1.12"O
Simat II	P	A	P	P	A	39° 4'1.14"N	0°20'19.42"O
Simat III	P	P	P	P	A	39° 3'52.75"N	0°20'31.46"O
Simat IV	P	P	P	P	P	39° 3'40.67"N	0°20'18.74"O
Simat V	P	P	P	P	A	39° 3'30.25"N	0°20'10.73"O
Simat VI	P	A	P	P	A	39° 3'6.39"N	0°19'23.72"O
Simat VII	P	A	P	P	P	39° 2'59.71"N	0°19'14.30"O
Tabernes Valldigna	P	P	P	P	A	39° 4'20.18"N	0°15'10.09"O
Villareal	P	A	A	A	P	39°55'29.73"N	0° 8'13.52"O
Villareal estación	P	A	P	P	P	39°56'37.71"N	0° 8'12.35"O
Xeraco	P	P	P	A	A	39° 2'5.95"N	0°12'29.43"O

SS4.1 Geographical location of mandarin plots sampled in the Valencian Community.

capítulo 5

Discusión general y Conclusiones

Capítulo 5

Discusión general y Conclusiones

5.1. Discusión General

Las restricciones del mercado y las preocupaciones ambientales y de salud humana son cada vez mayores debido al uso de plaguicidas en los cultivos. Nuevas reglamentaciones internacionales (Reglamento (CE) n. ° 1107/2009) han impulsado la implementación de enfoques de gestión de plagas con bajo impacto como las estrategias de control biológico. En los cultivos de cítricos españoles, la estrategia de gestión integrada de plagas se han convertido en una solución para la industria citrícola que necesita responder a estas demandas del mercado internacional (Tscharntke, 2012; Urbaneja et al., 2014).

La conservación y el uso de los recursos ofrecidos por los agroecosistemas se consideran actualmente como la estrategia más prometedora para la gestión sostenible de plagas en los cultivos perennes como los cítricos (Rusch et al., 2017). Ésta busca favorecer a los enemigos naturales y mejora sus servicios como controladores biológicos, por lo que la creación de infraestructuras ecológicas y evaluaciones sobre el papel de los enemigos naturales se han convertido en el foco principal de la investigación sobre la gestión de plagas (Woltz et al., 2012; Tscharntke, 2012; Rusch et al., 2017).

5.1.1. Revalorizando la importancia de los depredadores

Dentro de los enemigos naturales, los depredadores siempre han sido valorados como enemigos naturales de gran importancia en la agricultura. Sin embargo, su compleja biología y ecología ha obstaculizado con frecuencia la evaluación de su verdadero papel como agentes de control biológico (Riechert & Lockley, 1984; Symondson et al., 2002). En las últimas décadas, existe un creciente interés en el control ejercido por los depredadores generalistas, principalmente indígenas y naturalizados, porque se reconoce que tienen capacidad de mantener la densidad de los fitófagos en equilibrio estable en forma no dependiente de la densidad de sus presas (Symondson et al., 2002; Harwood & Obrycki, 2005).

Los métodos indirectos como la exclusión, permiten evaluar el impacto de los depredadores de forma cuantitativa en los agroecosistemas. En el presente trabajo, se utilizó esta técnica y como modelo de plaga al piojo rojo

de California, *A. aurantii*, porque es una de las plagas claves del cultivo. De esta forma, las técnicas de exclusión permitieron discriminar satisfactoriamente la mortalidad biótica y abiótica en las poblaciones del fitófago. Los resultados obtenidos en el estudio, muestran que la mortalidad asociada al complejo de depredadores del cultivo supone su principal componente de mortalidad biótica, causando en promedio, reducciones de más del 75% en las cohortes de piojo rojo de California. Aunque estudios previos habían mencionado a la depredación como un componente de la mortalidad de esta plaga clave, no se había estimado hasta la actualidad su importancia con precisión y además, ésta había sido claramente subestimada (Sorribas & García Mari, 2010; Vanaclocha et al., 2011).

El golpeo de rama (stem-tap) y las técnicas moleculares (PCR) aplicadas al análisis del ADN del contenido intestinal de los depredadores más abundantes asociados al piojo rojo de California, revelaron que existe un complejo relativamente rico de depredadores que explotan las poblaciones de este fitófago. Además, la mayoría de estas especies son depredadores generalistas o estenófagas nunca antes citados como depredadores importantes de esta plaga (Alvis, 2003; Jacas & Urbaneja, 2008). La información obtenida crea por lo tanto un nuevo paradigma de cómo los diferentes componentes del complejo de depredadores de *A. aurantii* pueden estar contribuyendo a su regulación. El mírido *Pilophorus* cf *gallicus* se presenta como el depredador más relevante en términos de detecciones positivas y actividad estacional, especialmente durante la primera generación, como así también en las otras dos generaciones. Tanto esta especie como las dos especies de *Scymnus* encontradas, que también

presentan altos valores de relevancia relativa, han sido tradicionalmente asociadas a pulgones y nunca se habían citado como depredadores importantes del piojo rojo de California (Tawfik et al., 1973; Schuh & Schwartz, 1988; Atlihan & Güldal, 2009; Lundgren, 2009; Hodek et al., 2012). En el caso de los enemigos naturales especialistas del piojo rojo de California, presentan una fuerte dependencia específica de la densidad de presas y por lo tanto, no aumentan sus poblaciones de manera significativa hasta el final de la temporada cuando la población de la plaga objetivo se ha acumulado en su tercera generación. Se espera que la eficiencia predatoria de estos enemigos naturales sea superior a la de los generalistas, pero al presentar una densidad baja, su efecto probablemente no será tan determinante como se creía. Igualmente, la mayor presencia de los depredadores especialistas antes del invierno podría ser clave para ayudar a reducir el número de *A. aurantii* invernantes y, por lo tanto, disminuir el riesgo de aumentos demográficos severos durante la primera generación de la siguiente temporada (Qureshi & Stansly, 2009).

La información obtenida a través de los ensayos de exclusión y los estudios *post-mortem* de ADN en el tracto digestivo de los depredadores evidencian por lo tanto que las relaciones tróficas asociadas al piojo rojo de California son más complejas de lo esperado, encontrándose indicios de competencia aparente entre esta plaga y los pulgones. Además, se demuestra el alto potencial de la depredación como un factor regulador determinante de una plaga clave en el cultivo de clementino y también se revela cómo este factor de mortalidad se atribuye principalmente a una combinación de depredadores especialistas y no especializados que complementan su acción a lo largo de la temporada.

5.1.2. Dinámica poblacional de los depredadores

Para entender la dinámica poblacional de las plagas, además de conocer las relaciones tróficas involucradas que estarían regulando sus poblaciones, es necesario tener conocimientos sobre los factores limitantes, como son las condiciones climáticas y el vínculo con la planta hospedera (Herms, 2004; Atlihan & Chi, 2008; Atlihan & Güldal, 2009; Barredo et al., 2015). En el caso de los pulgones, se sabe que su demografía está fuertemente modulada por variables ambientales, como es la temperatura, y factores bióticos asociados a su hospedador, como son la abundancia y calidad de los brotes. Estos últimos, a su vez también se ven condicionados por las variables ambientales ya que son las que median los estados fenológicos de las plantas (Komazaki, 1982; Kersting et al., 1999; Wang & Tsai, 2000; Valiente & Albrigo, 2000).

Bajo las condiciones actuales de manejo de cultivo en clementinos de la cuenca Mediterránea, la regulación ejercida por los enemigos naturales sobre los pulgones generalmente no es suficiente para mantener sus densidades por debajo de sus niveles de daño económico en primavera (Hermoso de Mendoza et al., 2001, 2006). Por lo tanto, el control exitoso de estas plagas está basado en la aplicación de insecticidas, al menos una vez en la estación. Dicha práctica pone en peligro las estrategias de gestión integrada de plagas actualmente implementadas en este cultivo y resalta la importancia de desarrollar alternativas de gestión de pulgones menos agresivas (Urbaneja et al., 2014; Goméz-Marco et al., 2016; Wang et al., 2017).

Entre los factores climáticos que afectan el desarrollo y crecimiento de los cítricos, la temperatura y la precipitación son consideradas las más importantes. Algunos trabajos demuestran cómo las temperaturas bajas y altas prolongan o aceleran las etapas de crecimiento o fenológicas. Este efecto se refleja en nuestro estudio, donde las diferencias entre las temperaturas invernales y primaverales de los años en los que se realizaron los muestreos, causaron una brotación diferencial entre años, como también variación en la duración de los estados fenológicos (Valiente & Albrigo, 2000; Iglesias et al., 2007).

Debido a la estrecha relación entre los pulgones y su planta hospedera, esto ha generado que la dinámica poblacional de estas plagas se vea alterada y consecuentemente también la dinámica de los depredadores asociados. Los resultados de este trabajo muestran una alta relación entre los estadios fenológicos de los clementinos con la evolución de la infestación de pulgones en parcelas de cítricos. Se observa que los estadios B2 de las plantas hospederas son los primeros susceptibles a la colonización de los pulgones en el cultivo. Cuando los clementinos presentan un estadio más avanzado de maduración de sus brotes (B3) es cuando las poblaciones presentan un crecimiento exponencial y en el estadio fenológico B4 es cuando la superficie de los brotes permite el pico poblacional de la plaga. Estadios fenológicos más avanzados, estimulan a la colonia de pulgones a producir individuos alados que emigraran a otros brotes de mejor calidad o a otros hospederos con brotes de calidad para continuar su ciclo biológico. De esta forma, queda en evidencia que bajo las condiciones ambientales de la cuenca mediterránea, solamente la fenología de la planta hospedera se comporta como factor limitante de importancia en la dinámica

poblacional de los pulgones en las plantaciones de clementinos. Por otro lado, la mediación de las temperaturas sobre el tiempo de desarrollo de las fases B3 y B4 (en las que las colonias de pulgones pueden exhibir su máximo potencial de crecimiento) parece ser un factor determinante en la virulencia de las infestaciones de pulgones en clementino. Temperaturas más bajas durante las fases B3 y B4 prolongaron el tiempo de desarrollo de estas dos fases fenológicas lo suficiente como para que tanto *A. gossypii* como *A. spiraecola* pudiesen completar más de una generación.

Además de la dinámica de los pulgones, se observa que la especie dominante actualmente en la Comunidad Valenciana es *A. spiraecola* sobre *A. gossypii*. Esto podría ser explicado por la saliva producida por esta especie dominante, que ocasiona deformación en los brotes donde se alimenta y le confiere protección contra las condiciones climáticas y los enemigos naturales. Por otro lado, Mostefaoui et al. (2014) demostraron que *A. gossypii*, a diferencia de *A. spiraecola*, se ve afectada por la alta concentración de algunos compuestos como la prolina, este compuesto se produce en grandes cantidades como resultado del estrés fisiológico y reduce la calidad nutricional del huésped (Wool & Hales, 1996). La relación entre estas especies de pulgones y sus depredadores mediadas por la calidad que suponen estas presas como recurso alimenticio de éstos, es probablemente otro factor determinante en la actual dominancia de las especies de pulgones en citricos.

La diversidad de depredadores asociada a los pulgones ha sido estudiada en numerosos trabajos en la cuenca Mediterránea (Michelena & Sanchis, 1997;

Magro et al., 1999; Alvis, 2003; Kavallieratos et al., 2004). Dentro del presente estudio, se identificaron 15 especies diferentes que ya han sido registradas en la Comunidad Valenciana y pueden agruparse en cuatro órdenes de insectos: Coleoptera, Neuroptera, Dermaptera y Hemiptera. El orden más destacado es Coleoptera, con 10 especies pertenecientes a la familia Coccinellidae que han sido agrupados en dos grupos separados de acuerdo con su tamaño y comportamiento. Los micrococcinélidos incluyen especímenes de 3 mm o menos, son los más abundantes y en general presentan mayor estabilidad en su demografía tanto espacial como temporal. En este grupo destacan dos especies por su alta abundancia: *Scymnus subvillosus* y *S. interruptus*. Dentro de los macrococcinélidos, se incluyen especímenes de más de 3 mm. Aquí se registraron 6 especies diferentes, siendo *P. quatuordecimpunctata* la más abundante en el presente trabajo.

Si bien la dinámica poblacional de los diferentes grupos de depredadores se relaciona con la de su presa (Brown, 2004; Gómez-Marco et al., 2016), en nuestros estudios se observan algunas diferencias entre los grupos que podrían ser útiles para identificar cuál de estos enemigos naturales es más eficiente en el control biológico de pulgones. Los neurópteros, dermápteros y hemípteros no presentaron un patrón definido de dinámica poblacional en los sitios estudiados, por lo que, bajo una estrategia de control biológico se los considera poco eficientes. Esto no significa que de manera puntual e impredecible, no puedan ser de importancia. A diferencia de estos, los coccinélidos fueron registrados en todas las parcelas estudiadas, presentando un patrón definido según el grupo anteriormente descripto. Los micrococcinélidos en la mayoría

de los casos registran actividad antes de la aparición de las poblaciones de pulgones. Por el contrario, los macrococcinélidos aparecen una vez ya las poblaciones de pulgones son elevadas. Esto podría estar asociado al comportamiento de oviposición de las hembras, quienes, según los modelos de comportamiento reproductivo, producirán más huevos según su tasa de consumo de alimento (Kindlmann & Dixon, 1999; Hodek et al., 2012). Por lo tanto, los recursos que requieren los micrococcinélidos son menores y en cambio, los macrococcinélidos al ser especies de mayor tamaño requieren una densidad alta de las poblaciones de pulgones para estimular a que las hembras pongan huevos (Hemptinne et al., 1992; Hemptinne & Dixon, 1997). Por otro lado, el carácter depredador más generalista de las especies más abundantes de micrococcinelidos, les proporcionaría también mayor estabilidad estacional demográfica.

De esta forma, podemos considerar a las dos especies de *Scymnus* registradas en este estudio como buenas depredadoras de pulgones, pues llegan tempranamente a las colonias de su presa, son voraces y muy abundantes. Nuestros resultados nos permiten asegurar que la clave del éxito de las especies de *Scymnus* tiene que ver con estas características en el periodo de formación de las colonias de pulgones, ya que evitan que las poblaciones de esta plaga lleguen al umbral de daño económico, por lo tanto son el factor biológico regulador más importante en el desarrollo de la dinámica poblacional de los pulgones.

5.1.3. Influencia de la presa en el desarrollo de los depredadores

La calidad y el tipo de presas que consumen los depredadores determinan cambios en los parámetros poblacionales (Kalushkov & Hodek, 2005; Keshavarz et al., 2015). Para desarrollar estrategias de gestión de plagas eficientes, es necesario tener un buen entendimiento del comportamiento, la biología y la ecología de los depredadores, junto con un amplio conocimiento de cómo estos insectos interactúan con sus presas (Evans, 1991; Tuan et al., 2015; Li et al., 2015). Dentro del grupo de depredadores, los coccinélidos han sido objeto de diversos estudios, los cuales han podido revelar que estos insectos sobreviven con una "dieta mixta", compuesta de presas "esenciales" y "alternativas" (Evans et al., 1999; Nielsen et al., 2002). La presa esencial se refiere a aquella utilizada para el desarrollo y la reproducción, mientras que la presa alternativa permite que los adultos sobrevivan cuando la presa esencial es escasa o está ausente (Lucas, 2005; Omkar et al., 2009). Una de las presas esenciales más importantes para los coccinélidos son los pulgones y por ello han sido objeto de estudio en los trabajos realizados en el laboratorio.

Al analizar cómo se ven afectados los parámetros de desarrollo, de supervivencia, reproductivos y de tabla de vida de las dos especies de coccinélidos más abundantes en la cuenca Mediterránea (*S. subvillosus* y *S. interruptus*), cuando se los alimenta con las dos especies de pulgones más abundantes en la región (*A. spiraecola*, *A. gossypii* y la mezcla de ambas), se observan diferencias que podrían estar involucradas en la distribución espacial y la abundancia de estas especies de depredadores en las plantaciones de clementinos.

No se observaron diferencias en la supervivencia de los estadios inmaduros, ni en el tamaño y peso de los adultos alimentados con las tres dietas definidas, lo que podría estar relacionado con la planta huésped que fue utilizada para criar los pulgones (clementinos) (Sebastião et al., 2015). Sí que se observaron diferencias en el tiempo de desarrollo larval, que fue menor en los ejemplares de ambas especies de *Scymnus* criados con *A. gossypii* o con la mezcla de pulgones (Evans et al., 1999; Nielsen et al., 2002; Schuldiner & Coll, 2017). Además, se encontró una alta fecundidad en ambas especies de *Scymnus* cuando se alimentaban con ambas especies de pulgones, lo que confirma los beneficios de una dieta mixta, aunque también se observaron altos valores de fecundidad cuando se alimentaban con *A. gossypii*. Esto coincide con otros estudios que encontraron una fecundidad relativamente alta cuando los coccinélidos estudiados se alimentaban de *A. gossypii*, lo cual lo asociaban con una maduración temprana de los ovariolos, que dependía de las especies presa consumidas (Omkar & James, 2004).

En concordancia con la fecundidad, cuando se analizan los parámetros poblacionales se observa un patrón similar en la tasa de reproducción neta (R_0), la tasa de intrínseca de crecimiento (r_m) y la tasa finita de incremento (λ_m), donde la dieta mixta favorece el crecimiento poblacional de estas especies de depredadores. Esto demuestra que una dieta mixta podría ser beneficiosa, ya que proporcionan una mayor diversidad de nutrientes que beneficia el desarrollo de los mismos (Nielsen et al., 2002; Schuldiner & Coll, 2017). Los valores de estas tasas fueron bajas cuando las dos especies de *Scymnus* se alimentan de *A. spiraecola*, por esta razón consideramos que esta especie de pulgón es de baja

calidad nutricional. Por otro lado, los altos valores de estas tasas en las especies de *Scymnus*, nos explicaría por qué estos depredadores son tan abundantes en la región. En varios trabajos realizados en la Comunidad Valenciana, *S. interruptus* y *S. subvillosus* son mencionados como las especies de depredadores más abundantes y que podrían jugar un papel de gran importancia en el control de presas preferenciales como los pulgones (Alvis, 2003; Soler et al., 2006; Santos et al., 2012).

El muestreo espacial realizado en 60 parcelas de mandarinos en la Comunidad Valenciana, nos permite discernir que en el caso de *S. subvillosus*, al igual que lo observado en el laboratorio, no presenta preferencia por ninguna de las dos especies de pulgones. Es decir, no se encontraron diferencias en la abundancia de esta especie en los tres tipos de árboles definidos (árboles con *A. gossypii* o con *A. spiraecola* como especie dominante o aquellos con ambas especies en similar proporción). Por el contrario, *S. interruptus* presenta una marcada preferencia por los arboles donde la especie dominante es *A. gossypii*, lo que estaría en concordancia con los resultados obtenidos de los parámetros de desarrollo y reproductivos. Además, se observa que en estos árboles la densidad de ambas especies de *Scymnus* es similar, lo que también nos estaría indicando que posiblemente *S. interruptus* sea mejor competidor que *S. subvillosus* cuando se encuentran explotando una presa preferencial.

5.2. Conclusiones

- Los resultados obtenidos en este trabajo ponen de manifiesto la importancia de los depredadores generalistas en la regulación de plagas clave del cultivo de los cítricos y demuestran que pueden ser su principal componente de mortalidad biótica.

- El control biológico realizado por este complejo de enemigos naturales se realiza a través de complejas relaciones troficas que implican a diferentes plagas del cultivo, hábitos alimenticios de sus depredadores y a otros enemigos naturales.

- Los resultados de este trabajo además, enfatizan en la importancia de algunos enemigos naturales tales como *Pilophorus* cf *gallicus*, *Scymnus subvillosus* y *Scymnus interruptus* que tradicionalmente se les atribuía un papel secundario en la gestión integrada de plagas.

- Las estrategias de conservación del complejo de depredadores generalistas asociado al cultvio de citricos se convierte por lo tanto en una herramienta fundamental dentro de los presentes y futuros programas de gestión integrada de plagas clave de este cultivo.

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