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El papel de *Anaphothrips obscurus* (Müller) en la mejora del control biológico de *Tetranychus urticae* Koch en clementino

TESIS DOCTORAL

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Resumen

ABSTRACT

Tetranychus urticae Koch is a key pest of clementine orchards of the Eastern Iberian Peninsula. The establishment of a perennial grass cover crop of *Festuca arundinacea* Schreber reduces considerably the populations of this phytophagous mite in tree canopies. The initial hypothesis is that this ground cover houses high amounts of thrips, which constitute an alternative food for natural enemies (phytoseiid mites) and favors its conservation. The thrips species present throughout the year with the greatest abundance in this cover is *Anaphothrips obscurus* (Müller). Therefore, the main objective of this doctoral thesis has been to unveil whether *A. obscurus* is involved in the natural regulation of *T. urticae* populations and whether its presence in the citrus agroecosystem favors or impairs the biological control of this herbivore. To achieve this goal, the biological and ecological characteristics of this thrips species and its potential relationships with the crop, the herbivore and the predatory mites of the family Phytoseiidae have been studied. In addition, a molecular tool to verify under field conditions to what extent *A. obscurus* is a prey for phytoseiids and if its presence could contribute to reduce the intraguild predation has been designed and optimized.

Anaphothrips obscurus exhibits wing dimorphism. This characteristic influenced its demographic and reproductive parameters with brachypterous forms exhibiting higher ecological fitness than macropterous. In both wing morphotypes, these parameters confirmed their potential to develop and reproduce in plants of *F. arundinacea* with an intrinsic rate of development of 0.17 d^{-1} for brachypterous and 0.14 d^{-1} for macropterous forms. These values are higher than those described for *T. urticae*, suggesting that both phytophagous species may be competing. Based on the results of our experiments, we can rule out both the zoophytophagy of *A. obscurus*, because it does not feed on *T. urticae* eggs, and the herbivory on citrus. Field observations highlighted changes in the composition of the arthropods studied. A progressive reduction of *T. urticae* densities and an increase of *A. obscurus* and phytoseiids since the implementation of the *F. arundinacea* cover were observed. Therefore, the combination of laboratory experiments and field observations suggest that this thrips

may be a better competitor than *T. urticae* in the *F. arundinacea* ground cover in clementine orchards.

This ground cover houses an abundant and diverse community of predatory mites. Some representative species are *Euseius stipulatus* (Athias-Henriot), *Neoseiulus barkeri* (Hughes), *Neoseiulus californicus* (McGregor) and *Phytoseiulus persimilis* Athias - Henriot. *Euseius stipulatus*, *N. barkeri* and *N. californicus* present a type II functional response when feeding on *A. obscurus* nymphs, whereas *P. persimilis* rarely feed on them. In addition, *N. barkeri* and *N. californicus* can reproduce and thus increase their populations by feeding on this thrips only, which may lead to an increase in predation pressure on *T. urticae*. In the prey preference analysis, the *Tetranychus* spp. specialist *P. persimilis* preyed preferentially on *T. urticae*, the generalists *E. stipulatus* and *N. barkeri* preferred *A. obscurus*, and the tetranychid specialist *N. californicus* showed no preference for any of the two prey species. Therefore, *A. obscurus* may be positively contributing to the biological control of *T. urticae* when a ground cover of *F. arundinacea* is established, since this thrips constitutes an alternative prey for non-specialist phytoseiids without altering the regulation exerted by *P. persimilis*, the most effective natural enemy of *T. urticae* in citrus. In addition, the availability of this alternative prey throughout the year in the ground cover could lead to a reduction of the intraguild predation between phytoseiids, which would also favor the maintenance of the most effective phytoseiid species, resulting in improved biological control of *T. urticae* by conservation of this pest mite.

Intraguild predation (IGP) is a widespread behavior in nature and occurs more frequently when prey food sources are scarce. The presence of this behavior can interfere in the biological control of a pest species, since it reduces the densities of its natural enemies. In this thesis, we have designed a molecular tool to identify prey DNA in the gut of the predator, which allows appraising IGP, under variable natural conditions including the presence of *A. obscurus* and other alternative food sources. We have successfully incorporated primers that detect phytoseiid feeding events in a single amplification reaction on: i) four species of mites [*Panonychus citri* (McGregor), *T. urticae, Tetranychus evansi* Baker and Pritchard and *Tetranychus turkestani* Ugarov

and Nikoskii]; ii) a wide range of thrips and plant species frequently found in citrus and in the ground cover, and iii) the five most representative phytoseiid species of this agricultural ecosystem. Amplification products that identify phytoseiid species have short (bp) lengths extending DNA detection time within the predator (*P. persimilis* DS_{50} in *E. stipulatus* is 18.7 h), which allows this multiplex PCR to detect intraguild predation events, occurred much earlier. The complexity of this multiplex PCR makes it applicable not only to the study of trophic webs in citrus, but to other agricultural systems.

Based on the results obtained, this doctoral thesis provides relevant information on the trophic relationships that take place in clementine orchards in which phytoseiids are involved and reveals that the role of *A. obscurus* as an alternative food source contributes to improve the conservation of the most effective natural enemies of *T. urticae*.

RESUMEN

Tetranychus urticae Koch es una plaga clave en el cultivo de los clementinos en el este de la Península Ibérica. La utilización de una cubierta vegetal constituida esencialmente por la gramínea *Festuca arundinacea* Schreber consigue reducir considerablemente las poblaciones de este fitófago en los clementinos. La hipótesis de partida es que dicha cubierta alberga cantidades elevadas de trips que actúan como alimento alternativo de los enemigos naturales (ácaros fitoseidos) y favorece así su conservación. La especie de trips que se encuentra presente durante todo el año con mayor abundancia en esta cubierta vegetal es *Anaphothrips obscurus* (Müller). Por tanto, el objetivo principal de esta tesis doctoral fue descubrir si *A. obscurus* podría estar involucrado en la regulación natural de las poblaciones de *T. urticae* y si su presencia en el agroecosistema de los cítricos con cubierta de *F. arundinacea* favorece o perjudica su control biológico. Con tal finalidad, se han estudiado las características biológicas y ecológicas de esta especie y las relaciones que podría mantener con el cultivo, el fitófago plaga *T. urticae* y los ácaros depredadores de la familia Phytoseiidae. Además se ha diseñado y optimizado una herramienta molecular que permitirá comprobar en campo en qué medida *A. obscurus* actúa como presa de los fitoseidos y si su presencia podría contribuir a reducir la depredación intragremial.

Anaphothrips obscurus presenta dimorfismo alar. Esta característica influyó en sus parámetros demográficos y reproductivos, siendo más favorables desde el punto de vista ecológico para las formas braquípteras que para las macrópteras. En los dos morfotipos alares los valores de estos parámetros confirmaron su potencial para desarrollarse y reproducirse en plantas de *F. arundinacea* presentando una tasa intrínseca de desarrollo de 0,17 d^{-1} para braquípteras y 0,14 d^{-1} para macrópteras. Estos valores son superiores al que se ha descrito para *T. urticae*, lo que sugiere que ambos fitófagos pueden estar compitiendo. Basándonos en los resultados de los ensayos de alimentación, se descarta que *A. obscurus* presente zoofitofagia y que colonice los cítricos ya que no es capaz de alimentarse de huevos de *T. urticae* ni de este sustrato vegetal. Las observaciones de campo resaltaron cambios en la composición de los artrópodos estudiados. Se ha observado que a medida que pasa el

tiempo desde la introducción de la cubierta de *F. arundinacea,* se reducen las densidades de *T. urticae* y se incrementan las de *A. obscurus* y los fitoseidos. Por tanto, la combinación de los ensayos de laboratorio y las observaciones de campo sugieren que este trips puede ser mejor competidor que *T. urticae* en la cubierta vegetal de *F. arundinacea* en los campos de clementinos.

Esta cubierta alberga una comunidad abundante y diversa de ácaros depredadores. Algunas de las especies más representativas, bien sea por su abundancia o por su eficacia en la regulación de poblaciones de ácaros plaga, son *Euseius stipulatus* (Athias-Henriot), *Neoseiulus barkeri* (Hughes), *Neoseiulus californicus* (McGregor) y *Phytoseiulus persimilis* Athias-Henriot. *Euseius stipulatus*, *N. barkeri* y *N. californicus* presentan una respuesta funcional de tipo II cuando se alimentan de ninfas de *A. obscurus*, mientras que *P. persimilis* raramente se alimenta de ellas. Además, *N. barkeri* y *N. californicus* pueden reproducirse y, por tanto, aumentar sus poblaciones alimentándose únicamente de este trips, lo que conlleva a un aumento de la presión de depredación sobre *T. urticae*. En el análisis de preferencia de presa. *P. persimilis* (especialista en *Tetranychus* spp.) depredó preferentemente *T. urticae*, los generalistas *E. stipulatus* y *N. barkeri* prefirieron *A. obscurus*, y el especialista en tetraníquidos *N. californicus* no mostró preferencia por ninguno de los dos fitófagos. Por tanto, *A. obscurus* puede estar contribuyendo positivamente en el control biológico de *T. urticae* cuando se instaura una cubierta vegetal de *F. arundinacea*, ya que constituye una presa alternativa para los fitoseidos no especialistas sin alterar la regulación que ejerce *P. persimilis*, el enemigo natural más eficaz de *T. urticae*. Además, la disponibilidad de esta presa alternativa durante todo el año en la cubierta vegetal podría conllevar una reducción de la depredación intragremial entre fitoseidos, que favorecería también la conservación de las especies de fitoseidos más eficaces, mejorando el control biológico de este ácaro plaga.

La depredación intragremial es un comportamiento muy extendido en la naturaleza y tiene lugar con mayor frecuencia cuando las fuentes de alimento de los depredadores escasean. La presencia de este comportamiento puede interferir en el control biológico de una especie plaga, ya que reduce las densidades de los enemigos

naturales. En la presente tesis, se ha diseñado una herramienta molecular que mediante la identificación de ADN de presa en el tracto digestivo del depredador, permite comprobar el nivel de depredación intragremial y la utilización de recursos alimenticios bajo condiciones naturales variables. En este trabajo se ha conseguido incorporar con éxito en una única reacción de amplificación cebadores capaces de detectar eventos de alimentación de fitoseidos sobre: i) cuatro especies de ácaros [*Panonychus citri* (McGregor), *T. urticae, Tetranychus evansi* Baker y Pritchard y *Tetranychus turkestani* Ugarov y Nikoskii]; ii) un amplio rango de especies de trips y plantas que se encuentran frecuentemente en cítricos y sus cubiertas vegetales, y iii) las cinco especies de fitoseidos más representativas de este ecosistema agrícola. Los productos de amplificación que identifican las especies de fitoseidos tienen longitudes (pb) cortas ampliando el tiempo de detección del ADN en el interior del depredador (DS_{50} de *P. persimilis* en *E. stipulatus* es 18,7 h) facilitando que esta PCR multiplex sea capaz de detectar eventos de depredación intragremial. La complejidad de esta PCR multiplex la hace aplicable no sólo en el estudio de redes tróficas en los cítricos, sino también en otros ecosistemas agrícolas.

Basándonos en los resultados obtenidos, la presente tesis doctoral aporta información relevante sobre las relaciones tróficas que tienen lugar en los campos de clementinos en las que se encuentran implicados los fitoseidos y desvela que *A. obscurus* supone una fuente de alimentación alternativa y contribuye a mejorar la conservación de los enemigos naturales más eficaces de *T. urticae*.

RESUM

Tetranychus urticae Koch és una plaga clau en el cultiu dels clementins a l'est de la península Ibèrica. La utilització d'una coberta vegetal constituïda essencialment per la gramínia *Festuca arundinacea* Schreber aconsegueix reduir considerablement les poblacions d'aquest fitòfag en els clementins. La hipòtesi de partida és que aquesta coberta hostatja quantitats elevades de trips que actuen com a aliment alternatiu dels enemics naturals (àcars fitoseids) i afavoreix així la seua conservació. L'espècie de trips que es troba present durant tot l'any amb major abundància en aquesta coberta vegetal és *Anaphothrips obscurus* (Müller). Per tant, l'objectiu principal d'aquesta tesi doctoral va ser descobrir si *A. obscurus* podria estar involucrat en la regulació natural de les poblacions de *T. urticae* i si la seua presència a l'agroecosistema dels cítrics amb coberta de *F. arundinacea* afavoreix o perjudica el seu control biològic. Amb tal finalitat, s'han estudiat les característiques biològiques i ecològiques d'aquesta espècie i les relacions que podria mantenir amb el cultiu, el fitòfag plaga *T. urticae* i els àcars depredadors de la família Phytoseiidae. A més s'ha dissenyat i optimitzat una ferramenta molecular que permetrà comprovar en camp en quina mesura *A. obscurus* actua com a presa dels fitoseids i si la seua presència podria contribuir a reduir la depredació intragremial.

Anaphothrips obscurus presenta dimorfisme alar. Aquesta característica influeix en els seus paràmetres demogràfics i reproductius, sent més favorables des del punt de vista ecològic per a les formes braquípteres que per les macròpteres. En els dos morfotips alars els valors d'aquests paràmetres van confirmar el seu potencial per desenvolupar-se i reproduir-se en plantes de *F. arundinacea* presentant una taxa intrínseca de desenvolupament de 0,17 d^{-1} per braquípteres i 0,14 d^{-1} per macròpteres. Aquests valors són superiors al que s'ha descrit per a *T. urticae*, la qual cosa suggereix que tots dos fitòfags poden estar competint. Basant-nos en els resultats dels assajos d'alimentació, es descarta que *A. obscurus* presente zoofitofàgia i que colonitze els cítrics ja que no és capaç ni d'alimentar-se d'ous de *T. urticae* ni d'aquest substrat vegetal. Les observacions de camp van ressaltar canvis en la composició dels artròpodes estudiats. S'ha observat que a mesura que passa el temps des de la

introducció de la coberta de *F. arundinacea*, es redueixen les densitats de *T. urticae* i s'incrementen les d'*A. obscurus* i els fitoseids. Per tant, la combinació dels assajos de laboratori i les observacions de camp suggereixen que aquest trips pot ser millor competidor que *T. urticae* a la coberta vegetal de *F. arundinacea* als horts de clementins.

Aquesta coberta serveix de refugi per a una comunitat abundant i diversa d'àcars depredadors. En aquesta comunitat les espècies més representatives, bé siga per la seua abundància o per la seua eficàcia en la regulació de poblacions d'àcars plaga, són *Euseius stipulatus* (Athias-Henriot), *Neoseiulus barkeri* (Hughes), *Neoseiulus californicus* (McGregor) i *Phytoseiulus persimilis* Athias-Henriot. *Euseius stipulatus*, *N. barkeri* i *N. californicus* presenten una resposta funcional de tipus II quan s'alimenten de nimfes d'*A. obscurus*, mentre que *P. persimilis* rarament se n'alimenta. A més, *N. barkeri* i *N. californicus* poden reproduir-se i, per tant, augmentar les seues poblacions alimentant-se únicament d'aquest trips, la qual cosa comporta un augment de la pressió de depredació sobre *T. urticae*. En l'anàlisi de preferència de presa, *P. persimilis* (especialista en *Tetranychus* spp.) va depredar preferentment *T. urticae*, els generalistes *E. stipulatus* i *N. barkeri* van preferir *A. obscurus*, i l'especialista en tetraníquids *N. californicus* no va mostrar cap preferència. Per tant, *A. obscurus* pot estar contribuint positivament al control biològic de *T. urticae* quan s'instaura una coberta vegetal de *F. arundinacea*, ja que constitueix una presa alternativa per als fitoseids no especialistes sense alterar la regulació que exerceix *P. persimilis*, l'enemic natural més eficaç de *T. urticae*. A més, la disponibilitat d'aquesta presa alternativa durant tot l'any a la coberta vegetal podria comportar una reducció de la depredació intragremial entre fitoseids, que afavoriria també a la conservació de les espècies de fitoseids més eficaces, millorant el control biològic d'aquest àcar plaga.

La depredació intragremial és un comportament molt estès en la natura i té lloc amb major freqüència quan les fonts d'aliment dels depredadors disminueixen. La presència d'aquest comportament pot interferir en el control biològic, ja que redueix les densitats dels enemics naturals. En aquesta tesi, s'ha dissenyat una eina molecular que mitjançant la identificació d'ADN de presa en el tracte digestiu del depredador,

permet comprovar el nivell de depredació intragremial i la utilització de recursos alimentaris sota condicions naturals variables. A més, s'ha aconseguit incorporar amb èxit en una única reacció d'amplificació encebadors capaços de detectar esdeveniments d'alimentació de fitoseids en: i) quatre espècies d'àcars [*Panonychus citri* (McGregor), *T. urticae, Tetranychus evansi* Baker i Pritchard i *Tetranychus turkestani* Ugarov i Nikoskii]; ii) un ampli ventall d'espècies de trips i plantes que es troben freqüentment en cítrics i les seues cobertes vegetals, i iii) les cinc espècies de fitoseids més representatives d'aquest ecosistema agrícola. Els productes d'amplificació que identifiquen les espècies de fitoseids tenen longituds (pb) curtes que amplien el temps de detecció de l'ADN a l'interior del depredador (DS_{50} de *P. persimilis* en *E. stipulatus* és 18,7 h) la qual cosa fa que aquesta PCR multiplex siga capaç de detectar esdeveniments de depredació intragremial que han tingut lloc molt abans. La complexitat d'aquesta PCR multiplex la fa aplicable no només en l'estudi de xarxes tròfiques dels cítrics, sinó també d'altres sistemes agrícoles.

Basant-nos en els resultats obtinguts, aquesta tesi doctoral aporta informació rellevant sobre les relacions tròfiques que tenen lloc als horts de clementins en les quals es troben implicats els fitoseids i evidència el paper d'*A. obscurus* com a font d'alimentació alternativa que contribueix a millorar la conservació dels enemics naturals més eficaços de *T. urticae*.

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Capítulo 1

Introducción

1.1 El cultivo de los cítricos

1.1.1 Importancia económica, social y cultural

El cultivo de los cítricos se extiende por numerosos países de todo el mundo donde las condiciones climáticas lo permiten (entre las latitudes 44° N y 35° S; Lovalt 2014). De hecho, es la fruta con mayor volumen de producción a nivel mundial. En la agricultura española, este cultivo es uno de los más importantes, habiéndose llegado a producir unos 7 millones de toneladas en 2014 (MAPAMA 2016). Actualmente, España es el sexto país productor de cítricos y el segundo de mandarinas en el mundo (FAO 2016). Estas últimas suponen un 33,2% del total de los cítricos en el país (MAPAMA 2016). La producción de cítricos puede destinarse tanto a su procesado (zumo, gajos, mermelada), como a la comercialización del fruto en fresco. En la citricultura española, un alto porcentaje (50,4%) se dedica a la exportación para el consumo en fresco (MAPAMA 2016) (Figura 1.1).

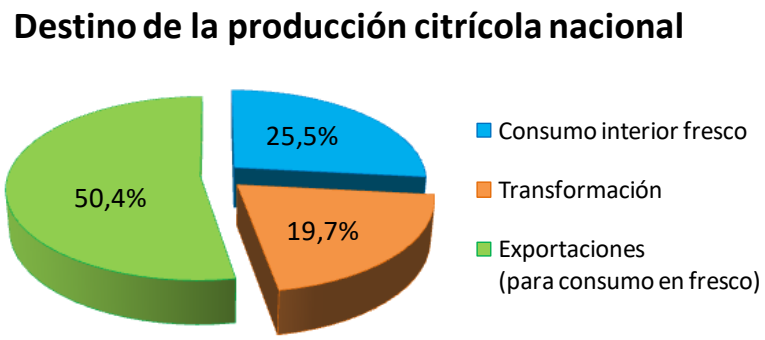

Figura 1.1 Distribución de la producción citrícola nacional del año 2014. Gráfico basado en los datos del Anuario de Estadística 2015. Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente (España).

La mayor parte de la producción citrícola nacional se concentra en la Comunidad Valenciana (3,9 millones de toneladas), constituida principalmente por naranjas y mandarinas, seguida de Andalucía y la Región de Murcia (MAPAMA 2016). Por este motivo, la Comunidad Valenciana es la que realiza gran parte de la exportación de naranjas (63,4% en la campaña de 2014/2015) y comercializa casi el total de las mandarinas exportadas (71,8% en la misma campaña) (CAMACCDR 2016). La producción citrícola de la Comunidad Valenciana en 2015 superó los 2.200 millones de

euros, lo que supone el 5% de todo el valor del sector agrario español (MAPAMA 2016). Estos datos reflejan la gran importancia de los cítricos en la agricultura valenciana y en su comercio exterior.

La relevancia de la citricultura valenciana no es reciente. Según el botánico Antonio José Cavanilles (1795), el cultivo del naranjo en plantaciones regulares se inició en tierras valencianas a finales del siglo XVIII. Sin embargo, no es hasta el siglo XIX cuando comenzó su verdadero crecimiento. En ese periodo, los países industrializados de Europa empezaron a demandar frutas frescas y en la Comunidad Valenciana coincidió con la crisis de las industrias de la seda y la vitivinícola. La confluencia de estos hechos convirtió al comercio citrícola en el motor de su economía, que pasó de una agricultura tradicional para el autoabastecimiento, a otra dirigida a la exportación, originando un cambio radical tanto en la estructura económica agrícola, como en el paisaje litoral e incluso en la sociedad valenciana (Abad y Pérez-Rojas 1996). Así pues, actualmente la citricultura no solo representa para los valencianos un medio de desarrollo económico, sino también una fuerte tradición.

1.1.2 Artrópodos plaga de los cítricos

La mayoría de los artrópodos que pueden alcanzar el nivel de plaga en cítricos suelen estar regulados por sus enemigos naturales, tanto autóctonos como naturalizados (Jacas y Urbaneja 2010). Sin embargo, como la producción se destina principalmente al consumo en fresco (75,9%; MAPAMA 2016) (Figura 1.1), los daños externos de tipo cosmético tienen gran relevancia y los umbrales económicos de daños son muy bajos. Por este motivo, el control biológico de algunos artrópodos que dañan directamente el fruto se considera insuficiente (Jacas y Urbaneja 2010). En general, las especies que pueden alcanzar el nivel de plaga se agrupan en las siguientes categorías: clave, ocasionales y secundarias. Esta agrupación considera tanto aspectos biológicos (del cultivo, de la plaga y de los factores naturales que la regulan) como económicos (valor de la producción y costes de gestión) (Funderbuk 2002). Las especies consideradas plaga clave se caracterizan por alcanzar habitualmente densidades

poblacionales superiores a las que determinan sus umbrales económicos de daño y, por tanto, si no se toman medidas de control adicionales se producen pérdidas económicas en el cultivo. Las plagas ocasionales son aquellas que generalmente se encuentran en densidades inferiores a los umbrales económicos de daños. Finalmente, las plagas secundarias se definen como aquellas especies fitófagas cuyas poblaciones sólo causan pérdidas económicas cuando alguna situación excepcional tiene lugar en el ecosistema agrícola, como por ejemplo, condiciones ambientales extremas (Urbaneja et al. 2015). Actualmente, se pueden considerar en España las siguientes plagas clave de los cítricos:

- los diaspídidos *Aonidiella aurantii* (Maskell), *Parlatoria pergandii* (Comstock) y *Lepidosaphes beckii (Newman)* (Hemiptera: Diaspididae) que afectan a todo tipo de especies de cítricos,
- los pulgones *Aphis gossypii* Glover y *Aphis spiraecola* Patch (Hemiptera: Aphididae) que afectan especialmente a clementinos y plantones,
- la mosca mediterránea de la fruta *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) en clementinos de maduración temprana y naranjos tardíos,
- y la araña roja *Tetranychus urticae* Koch (Acari: Tetranychidae) en clementinos.

El control de estas especies plaga se sigue efectuando esencialmente mediante procedimientos químicos. En el caso concreto de *T. urticae*, los tratamientos acaricidas anuales destinados a combatir los daños en el cultivo pueden ser superiores a 5 (llegando hasta 9 en algunos casos). Esto conlleva un coste elevado tanto para los agricultores como para el medio ambiente por sus efectos secundarios, incluidos aquellos sobre la fauna auxiliar (Martínez-Ferrer et al. 2006; Aguilar-Fenollosa et al. 2011c). Para conseguir reducir al máximo estas aplicaciones, y por tanto sus efectos no deseados, es necesario aunar esfuerzos hacia la mejora de otros medios de control, entre los que destaca el control biológico.

1.2 *Tetranychus urticae*: plaga clave de los clementinos

Tetranychus urticae es una especie de ácaro tetraníquido cosmopolita. Destaca por su elevada polifagia, ya que puede desarrollarse de forma adecuada en más de 150 especies vegetales de interés económico, entre las que se incluyen cultivos hortícolas, extensivos, frutales y ornamentales (Jeppson et al. 1975; Bolland et al. 1998; Zhang 2003). Asimismo, es muy común en plantas espontáneas y malas hierbas y se le considera una de las plagas de los cultivos más grave en todo el mundo (Jeppson et al. 1975; Smith et al. 1997; Jacas et al. 2010; Vacante 2010). En los cítricos españoles causa daño sobre todo en clementino y limonero (Agustí 2000; Ansaloni et al. 2008; García-Marí 2012).

1.2.1 Características biológicas y ecología

Tetranychus urticae es un ácaro de tamaño diminuto. Las hembras adultas pueden alcanzar una longitud máxima de 0,5 a 0,6 mm. Su coloración varía entre tonos amarillentos a rojizos, incluso marrones, en función de varios factores, como la planta huésped (Zhang 2003) el clima, o su estado de desarrollo (De Boer 1985). Se caracteriza por poseer dos manchas oscuras dorso-laterales sobre el idiosoma (Zhang 2003; Vacante 2010) (Figura 1.2). Los machos son de menor tamaño que las hembras, tienen el cuerpo fusiforme y las patas más largas en relación al tamaño del cuerpo. Los huevos son esféricos y translúcidos y, a medida que maduran, adquieren una tonalidad anaranjada y se oscurecen (Zhang 2003).

Figura 1.2 Individuos de *Tetranychus urticae* (longitud aproximada 0,5 mm) sobre hoja de judía.

El ciclo biológico de *T. urticae* tiene una duración aproximada de 10 días, con una puesta de entre 2 y 3 huevos diarios a su temperatura óptima, situada alrededor de los 32°C (Jeppson et al. 1975; García-Marí 2012). Esta especie tiene altas tasas de crecimiento poblacional, tiempos de generación cortos y gran capacidad para

dispersarse, por lo que desde un punto de vista ecológico se puede considerar que es una especie típica estratega de la "r" (Speight et al. 2008). *Tetranychus urticae* es capaz de producir seda creando telarañas que retienen la humedad de la transpiración de la planta y mantienen en sus colonias un microclima favorable para su crecimiento y desarrollo. Estas telarañas también protegen a las colonias ante condiciones climáticas adversas, depredadores y tratamientos acaricidas. Las colonias se localizan habitualmente en el envés de las hojas (García-Marí y Ferragut Pérez 2002; Clotuche et al. 2012). En latitudes más frías, este ácaro suele pasar el invierno en forma de hembra adulta. Sin embargo, en la zona mediterránea, se mantiene activo durante todo el año (Aucejo 2005; Martínez-Ferrer et al. 2006).

1.2.2 Daños en el cultivo

Los daños que *T. urticae* produce en el cultivo del clementino se deben básicamente a su alimentación. Para alimentarse, los individuos de esta especie introducen en la hoja su estilete a través del que ingieren los jugos celulares vaciando las células del parénquima. Este vaciado conlleva una reducción de la función fotosintética y la aparición de una mancha clorótica acompañada de un abombamiento en el haz de la hoja (Figura 1.3 A y B).

La confluencia de altos niveles de infestación de esta especie con elevadas temperaturas y/o déficit hídrico puede producir una caída prematura de las hojas, llegando a ocasionar fuertes defoliaciones que pueden mermar la producción en años sucesivos (Ansaloni et al. 2008) (Figura 1.3 D). Estas defoliaciones pueden además favorecer las migraciones de *T. urticae* desde los brotes tiernos, por los que tiene preferencia, a los frutos (Ansaloni et al. 2008), con el consiguiente daño directo sobre los mismos. La colonización del fruto, que suele coincidir con el final del verano trae como consecuencia una deficiente pigmentación al alcanzar la madurez. En ese momento, aparecen manchas herrumbrosas, más frecuentes en las zonas peduncular y estilar del fruto, que pueden extenderse por toda la corteza, lo que puede depreciar considerablemente la producción (Ansaloni et al. 2008; Pascual-Ruiz et al. 2014a) (Figura 1.3 C). Este constituye el daño más grave en clementinos.

Figura 1.3 Daños que produce *Tetranychus urticae* en el cultivo de clementino: manchas cloróticas y abombamiento de las hojas debido a la formación de colonias (A y B), manchas en el fruto (C) y defoliaciones debidas a altas infestaciones poblacionales (D).

1.2.3 Enemigos naturales

Las especies de depredadores que se encuentran alimentándose de *T. urticae* en nuestro país incluyen tanto insectos como ácaros. Entre los insectos depredadores destacan los coleópteros pertenecientes a la familia de los Coccinellidae *Scymnus mediterraneus* Lablokoff-Khnozoria, *Scymnus interruptus* (Goeze) y *Stethorus punctillum* Weise. También se ha descrito el díptero Cecidomyiidae *Feltiella acarisuga* (Vallot), los trips *Scolothrips longicornis* Priesner, *Scolothrips sexmaculatus* (Pergande) y *Aeolothrips intermedius* Bagnall (Thysanoptera: Thripidae), neurópteros pertenecientes a las familias Chrysopidae y Coniopterygidae y algunos hemípteros depredadores (Miridae y Anthocoridae) (Ripollés y Meliá 1980; García-Marí et al. 1991; Ripollés et al. 1995; Lacasa y Llorens 1998; García-Marí y González-Zamora 1999; Alvis 2003; Abad-Moyano et al. 2009). Sin embargo, los depredadores más relevantes son los ácaros de la familia de los fitoseidos (Acari: Phytoseiidae), ya que se consideran el grupo de mayor eficacia en el control biológico de *T. urticae* (Helle y Sabelis 1985). Dentro de este grupo, distribuido a nivel mundial, se incluyen más de 2.700 especies (Demite et al. 2015).

A pesar de que los fitoseidos no son especialmente voraces si los comparamos con otros artrópodos depredadores (Gotoh et al. 2004), son muy interesantes como agentes de control biológico. Esto se debe a que tienen ciclos de vida cortos, alta supervivencia, gran habilidad para sobrevivir a densidades bajas de presa y además algunos están disponibles comercialmente (Overmeer 1985; van Lenteren 2001). De hecho, muchas especies de fitoseidos se han usado satisfactoriamente en diferentes cultivos como agentes de control biológico, no sólo de ácaros fitófagos, sino también de otras especies plaga como trips, moscas blancas y otros microartrópodos (Helle y Sabelis 1985; McMurtry y Croft 1997; Nomikou et al. 2002; Gerson et al. 2003; van Baal et al. 2007; Ferragut et al. 2010; Demite et al. 2015).

Basándonos en la clasificación de McMurtry et al. (2013), los fitoseidos se agrupan en cuatro tipos en función de sus hábitos de vida. Los de tipo I se definen como depredadores especializados (entre ellos se incluyen los especializados en ácaros del género *Tetranychus*), los de tipo II como depredadores selectivos de tetraníquidos, los de tipo III como depredadores generalistas y los de tipo IV como depredadores generalistas especializados en polen. En nuestro país, las especies de fitoseidos más frecuentes asociadas a *T. urticae* en campos de clementinos son *Euseius stipulatus* (Athias-Henriot), *Neoseiulus barkeri* Hughes, *Neoseiulus californicus* (McGregor), *Phytoseiulus persimilis* Athias-Henriot y *Typhlodromus phialatus* (Abad-Moyano 2009a, Aguilar-Fenollosa et al. 2009b; Pérez-Sayas et al. 2015). A continuación, se describen las características más importantes de algunas de estas especies ordenadas alfabéticamente.

Euseius stipulatus es la especie más abundante en los campos de cítricos españoles tanto en árboles (García-Marí et al. 1991; Abad-Moyano et al. 2009a; Aguilar-Fenollosa et al. 2011b; Pérez-Sayas et al. 2015) como en la flora adventicia asociada a los mismos (Aucejo et al. 2003; Aguilar-Fenollosa et al. 2011b) (Figura 1.4 A). Es una especie omnívora clasificada como depredador generalista especializado en polen (tipo IV). Se le considera un buen agente de control biológico de *Panonychus citri* (McGregor). Sin embargo, su papel en el control de *T. urticae* es controvertido, dada su escasa capacidad para desarrollarse alimentándose exclusivamente de esta presa

(Abad-Moyano et al. 2009b; Pina et al. 2012) y la dificultad que le supone acceder a las colonias a través de las telarañas. Los resultados expuestos en el trabajo de Pérez-Sayas y colaboradores (2015) sugieren que su contribución en la regulación natural de las poblaciones de *T. urticae* se debe básicamente a una cuestión numérica más que funcional, dada su abrumadora abundancia en la copa (> 75% del total de fitoseidos) (Aguilar-Fenollosa et al. 2011b; Pérez-Sayas et al. 2015). El bajo porcentaje de *E. stipulatus* que se habían alimentado de presa tetraníquida (32,8%) en condiciones de campo apunta en esa misma dirección. En el agroecosistema de los cítricos, *E. stipulatus* está considerado como un depredador intragremial superior con respecto a otras especies de fitoseidos más eficientes en el control de *T. urticae* como *P. persimilis* o *N. californicus* (Abad-Moyano et al. 2010a, b). Este hecho, junto con la capacidad para incrementar sus poblaciones de manera explosiva cuando dispone de polen de buena calidad (Pina et al. 2012), podría explicar su predominancia y el control natural deficiente que se observa en algunos huertos comerciales de cítricos.

Figura 1.4 Fitoseidos más representativos del ecosistema agrícola de cítricos españoles: *Euseius stipulatus* (A), *Neoseiulus barkeri* (B), *Neoseiulus californicus* (C) y *Phytoseiulus persimilis* (D).

Neoseiulus barkeri es una especie ampliamente distribuida a nivel mundial (Demite et al. 2015) (Figura 1.4 B). Se considera un depredador generalista tipo III con hábitos de vida de suelo. Sin embargo, en cítricos con presencia de *T. urticae* se ha encontrado tanto en el cultivo como en la cubierta vegetal (Aguilar-Fenollosa et al. 2011b; Pérez-Sayas et al. 2015) pese a su preferencia por esta última (Ferragut et al. 2010). En Europa es uno de los fitoseidos que se utilizan mayoritariamente en el control biológico de *T. urticae* (Jafari et al. 2012) y otras plagas como *P. citri*, *Polyphagotarsonemus latus* (Banks) (Acari: Tarsonemidae), *Thrips tabaci* Lindeman o *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) y moscas blancas como

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) (Zhang 2003; Jafari et al. 2013; McMurtry et al. 2013; Niu et al. 2014; Wu et al. 2016).

Neoseiulus californicus está clasificado como un depredador selectivo de tetraníquidos (tipo II) capaz de alimentarse de polen y otros pequeños artrópodos (Figura 1.4 C). Normalmente se encuentra asociado a presas que producen estructuras de seda. Esta especie está ampliamente distribuida tanto en cítricos como en la cubierta vegetal asociada (Aucejo et al. 2003; Abad-Moyano et al. 2009; Aguilar-Fenollosa et al. 2011b) y es capaz de colonizar varios cultivos, herbáceos o leñosos. Esta flexibilidad, tanto en su alimentación como en sus hábitos de vida, hace que se consolide como una especie importante en estrategias de control biológico por conservación no sólo de tetraníquidos, sino también de otros grupos como tarsonémidos o trips (McMurtry et al. 2013).

Phytoseiulus persimilis es un depredador especializado en tetraníquidos del género *Tetranychus* (tipo I) capaces de formar estructuras de seda (McMurtry et al. 2013) (Figura 1.4 D). Su presencia se ha documentado tanto en especies cultivadas como en la vegetación asociada al cultivo (Ferragut et al. 2010; Aguilar-Fenollosa et al. 2011b). En los cítricos españoles es poco abundante (García-Marí et al. 1986; Abad-Moyano et al. 2009a). Sin embargo, es el depredador más voraz de *T. urticae* tanto en los cítricos valencianos (Pérez-Sayas et al. 2015) como australianos (Smith et al. 1997). Su especialización alimenticia y su capacidad para entrar en las colonias de *T. urticae* hacen que sea un buen candidato para regular las poblaciones de dicha plaga. No obstante, esta especie es un competidor intragremial débil y puede ser desplazado por otras especies menos selectivas como *E. stipulatus*, *N. californicus* y *N. barkeri* (Kabicek 1995; Cakmak et al. 2006; Abad-Moyano et al. 2010a, b; Momen 2010). Por este motivo el refuerzo de las poblaciones de *P. persimilis* debe ser uno de los focos de investigación en el control biológico por conservación para mantener a *T. urticae* bajo los umbrales económicos de daño en los campos de clementino.

1.3 Gestión de las poblaciones de *T. urticae* en clementino

Tetranychus urticae posee un buen complejo de enemigos naturales en el cultivo del clementino. No obstante, debido a los daños estéticos que produce en el fruto, sus umbrales económicos de daño son muy reducidos y por tanto la acción de estos enemigos naturales es a menudo insuficiente (Jacas y Urbaneja 2010; Pascual-Ruiz et al. 2014a). Como consecuencia, el control de esta especie en el cultivo de clementino en la actualidad, se basa principalmene en la aplicación de acaricidas.

El uso de acaricidas conlleva una serie de inconvenientes como la reducción de poblaciones de artrópodos beneficiosos, la inducción de proliferaciones incontroladas de otras plagas, así como el incremento de los costes del cultivo (Aguilar-Fenollosa et al. 2011c). Además *T. urticae* es conocida por su gran habilidad para desarrollar resistencias rápidamente (Van Leeuwen et al. 2010; Khajehali et al. 2011). De hecho, ocupa el octavo lugar en la lista de artrópodos con casos registrados de resistencia a plaguicidas (Michigan State University 2017). Por todo ello, los acaricidas deben ser seleccionados según su eficacia, su impacto en la fauna útil y teniendo en cuenta posibles problemas de resistencia. Asimismo, es necesario que su uso sea sostenible para reducir los perjuicios que producen en el medio ambiente y en la salud humana.

En este marco, se han establecido una serie de normativas tanto a nivel europeo (Directiva 2009/128/CE del Parlamento Europeo y del Consejo de 21 de octubre de 2009) como estatal (Real Decreto 1311/2012 de 14 de septiembre) que obligan a cumplir los principios de Gestión Integrada de Plagas (GIP) en todas las explotaciones agrícolas de la Unión Europea. El término GIP incluye el uso racional de una combinación de medidas biológicas, biotecnológicas, culturales, físicas y legales con el fin de mantener los niveles poblacionales de los fitófagos plaga por debajo de los umbrales económicos de daños. El establecimiento de estas normativas que limitan el uso de plaguicidas, junto con las restricciones legales para importar agentes de control biológico externos, hacen que en el control de *T. urticae* en cítricos adquiera especial relevancia la conservación de enemigos naturales autóctonos.

1.3.1 Control biológico por conservación: establecimiento de F. arundinacea *como cubierta vegetal*

La manipulación del medio ambiente para favorecer a los enemigos naturales se define como control biológico por conservación (DeBach 1974). Esta manipulación está dirigida tanto a mitigar factores adversos, como a proporcionar recursos necesarios que favorezcan a los enemigos naturales. Una forma de control biológico por conservación es la gestión del hábitat con la finalidad de crear una infraestructura adecuada que proporcione a la fauna beneficiosa recursos ecológicos como alimento o protección frente a condiciones adversas (Landis et al. 2000). Por tanto, las infraestructuras ecológicas, con su fauna específica de artrópodos, ofrecen a los enemigos naturales la oportunidad de desarrollar sus poblaciones en sustratos alternativos para emigrar posteriormente al cultivo y persistir e incluso mantenerse en densidades relativamente altas. Esto permite prevenir la reaparición de la plaga, evitando el desfase de tiempo que se asocia normalmente con la respuesta numérica, y contribuyendo a la regulación natural de sus poblaciones (Boller et al. 2004).

El incremento de enemigos naturales en sistemas agrícolas mediante la provisión de especies vegetales distintas al cultivo es un aspecto con un creciente interés en el control biológico por conservación (Landis et al. 2000). Las técnicas de gestión del hábitat a través de la cubierta vegetal también pueden ser importantes para regular las poblaciones de ácaros (Barbosa 1998), especialmente en sistemas perennes (Nyrop et al. 1998) como son los cítricos. En este cultivo, el establecimiento de una cubierta vegetal de *Festuca arundinacea* Schreber (Poaceae) ha demostrado ofrecer numerosos beneficios (Figura 1.5). Por un lado, tiene alta capacidad de resiembra y crecimiento reducido, lo que hace su mantenimiento más económico que el de otras especies (Aguilar-Fenollosa et al. 2011c). Además limita la presencia de flora competidora, e incluso ofrece protección contra posibles enfermedades fúngicas, como el aguado, provocado por oomicetos del género *Phytophthora* (Fibla-Queralt et al. 2003). Por otro lado, esta cubierta contribuye a regular de forma natural las poblaciones de *T. urticae* reduciendo la necesidad de aplicar tratamientos (Aguilar-Fenollosa et al. 2011c), aumentando la abundancia y diversidad de fitoseidos, tanto en la cubierta, como en la

copa (Aguilar-Fenollosa et al 2011b) y favoreciendo la formación de poblaciones de *T. urticae* adaptadas al hospedador (*F. arundinacea*) e incapaces de colonizar con éxito los clementinos (Aguilar-Fenollosa et al. 2011a; Aguilar-Fenollosa et al. 2016). Así pues, el establecimiento de una cubierta vegetal monoespecífica de la gramínea *F. arundinacea* constituye una herramienta eficaz para aumentar la sostenibilidad del control de plagas en clementino.

Figura 1.5 Campo de clementinos con cubierta vegetal de la gramínea *Festuca arundinacea.*

Un aspecto clave para que el control biológico por conservación sea efectivo es llevar a cabo prácticas que incrementen la abundancia relativa de los depredadores más eficaces de la comunidad (Straub y Snyder 2006). Aguilar-Fenollosa y colaboradores (2011b) demostraron que cuando se encuentra presente *F. arundinacea* como cubierta vegetal en los campos de clementinos, aparecen de forma consistente las especies de fitoseidos más efectivas en el control de *T. urticae* como *P. persimilis* y *N. californicus* (tipos I y II, respectivamente). Estos autores atribuyeron este resultado a la calidad y disponibilidad del alimento alternativo, ya que la cubierta vegetal de *F. arundinacea* ofrece un polen de baja calidad (Ouyang et al. 1992) para los depredadores palinófagos omnívoros (*E. stipulatus* fundamentalmente), que se encuentra disponible en una franja reducida de tiempo (primavera). Esta fuente de alimento por sí misma no es capaz de mantener poblaciones de fitoseidos a largo plazo

(Pina et al. 2012). Por ello, la cubierta vegetal de *F. arundinacea* debe estar proporcionando a los depredadores (especialmente a los de los tipos I y II) otras fuentes de alimento.

Uno de los grupos de artrópodos que raramente aparece en los campos de clementinos cuando se cultivan con suelo desnudo, pero que se encuentran en gran abundancia en la cubierta vegetal de *F. arundinacea* son los trips (orden Thysanoptera) asociados a gramíneas (Aguilar-Fenollosa y Jacas 2013). Dada la habilidad de los fitoseidos para alimentarse de varias especies de trips (Rodríguez-Reina et al. 1992; van Baal 2007; Magdy y El-Sayed 2009; McMurtry et al. 2013) y su abundancia en este agroecosistema, se ha postulado que este grupo de insectos podrían estar ejerciendo un papel esencial como alimento alternativo en la conservación de los fitoseidos implicados en la regulación natural de *T. urticae* en clementinos (Aguilar-Fenollosa y Jacas 2013), y es en este contexto en el que se enmarca la presente tesis.

1.3.2 Trips asociados a la cubierta vegetal de F. arundinacea: Anaphothrips obscurus

Los trips son insectos minúsculos (con una longitud media de 1-2 mm), ampliamente distribuidos a nivel mundial. Se dividen en dos subórdenes: los Terebrantia, caracterizados por poseer un abdomen cónico con una hendidura ventral que en las hembras aloja el ovipositor con forma de hoz (la terebra), y los Tubulifera, caracterizados por tener un abdomen acabado en tubo y sin hendidura ventral (Moritz 1994; Moritz et al. 2000). Las hembras de los terebrancios habitualmente ponen los huevos en el interior de las hojas. A pesar de que son insectos hemimetábolos, en muchas especies, como *Anaphothrips obscurus* (Müller), tiene lugar una metamorfosis extendida en la que la etapa inmadura final está en reposo y no se alimenta (análoga a una pupa de un holometábolo). Los adultos pueden presentar dimorfismo alar siendo alados o ápteros en función de la especie y el sexo, pudiendo incluso mostrar formas intermedias. Hasta el momento, se han identificado 6.147 especies de trips (ThripsWiki 2017), de las cuales algunas son beneficiosas ya que actúan como polinizadores o agentes de control biológico (Mound y Kibby 1998; Trdan et al. 2005; ThripsWiki 2017).

Otras, por el contrario, constituyen plagas agrícolas (Lewis 1997; Trdan 2007; ThripsWiki 2017). Los trips perjudiciales para los cultivos pueden causar daño no sólo debido a su alimentación, sino también por su papel como vectores de virus.

El establecimiento de la cubierta vegetal de *F. arundinacea* en los campos de clementinos determina un cambio en la composición y abundancia de las especies de trips. En este agroecosistema, las poblaciones de especies con potencial como plaga (*F. occidentalis* y *T. tabaci*, entre otras) son inferiores a las presentes en una cubierta espontánea, mientras que las poblaciones de los trips especializados en gramíneas aumentan sustancialmente. Las especies más abundantes en la cubierta vegetal de *F. arundinacea* son *A. obscurus* y *Chirothrips manicatus* (Haliday). No obstante, la primera especie se encuentra regularmente a lo largo de todo el año mientras que la segunda se detecta únicamente en primavera y otoño (Aguilar-Fenollosa y Jacas 2013). La gran abundancia de *A. obscurus* y su presencia constante en la cubierta de festuca son los motivos que han llevado a que esta tesis se centre en su estudio como modelo de los trips presentes en este ecosistema agrícola, pudiendo ser alimento alternativo de los fitoseidos depredadores de *T. urticae*.

Anaphothrips obscurus, conocido como "American grass thrips", tiene una distribución cosmopolita (Mound 1997) y se considera uno de de los trips especialistas en poáceas de mayor distribución en Europa y América del Norte (Bailey 1948). Esta especie se alimenta de varias gramíneas que en los cultivos forman parte de las cubiertas vegetales (géneros *Bromus*, *Festuca*, *Lolium*, *Phleum* y *Poa*) (Hinds 1900; Bournier 1983) pero también de algunos cereales como el trigo, la cebada, el maíz o la avena (Bailey 1957; Köppa 1967; Stannard 1968). Cuando se alimenta de las yemas produce un plateado apical característico (Hinds 1900; Kamm 1971) que da lugar a pérdidas económicas considerándose plaga en pastos como el fleo (*Phleum pratense* L.), que se cultiva para la alimentación equina (Reisig et al. 2009), y en cereales (Stannard 1968; Ananthakrishnan 1984; Brohmer et al. 1996). De hecho, en el sudeste de Polonia fue el tercer trips más frecuente en maíz dulce entre los años 2008 y 2010 (Beres et al. 2013). Esta especie se reproduce mediante partenogénesis telitoca (Hinds 1900). Los machos, cuya aparición es esporádica y local, se han descrito recientemente

y su función en la perpetuación de la especie aún se desconoce (Mirab-Balou y Chen 2010). Las hembras adultas presentan dimorfismo alar, con fenotipos macrópteros y braquípteros (Hinds 1900). Algunos autores han sugerido que el fotoperiodo, la densidad de población, y la calidad de la planta huésped, podrían ser los factores determinantes de estos fenotipos (Köppa 1970; Kamm 1972; Nakao 1996; Reisig et al. 2010). Sin embargo, estas causas no están esclarecidas ya que existe una cierta controversia al respecto. De hecho, a pesar de la gran abundancia y amplia distribución de *A. obscurus* en pastos y cultivos de cereales, esta especie aún es relativamente desconocida.

Anaphothrips obscurus tiene un ciclo de vida hemimetábolo. Los huevos son reniformes y translúcidos, y están incrustados en las hojas. Tras la eclosión se suceden dos estadios ninfales (N1 y N2) que se diferencian por su tamaño y por un proceso de muda intermedio. A continuación, siguen otras dos fases inmaduras definidas también por un proceso de muda. La primera de estas fases (prepupa) se caracteriza por presentar esbozos alares, que en el caso de las macrópteras no sobrepasan el tercer segmento abdominal, y las antenas muy cortas y sin artejos diferenciados. Por el contrario, en la última fase inmadura (pupa) las antenas ya muestran artejos diferenciados, aunque están plegadas sobre el dorso y, en el caso de las macrópteras, los esbozos alares ya sobrepasan el tercer segmento abdominal (Lacasa y Llorens 1998) (Figura 1.6).

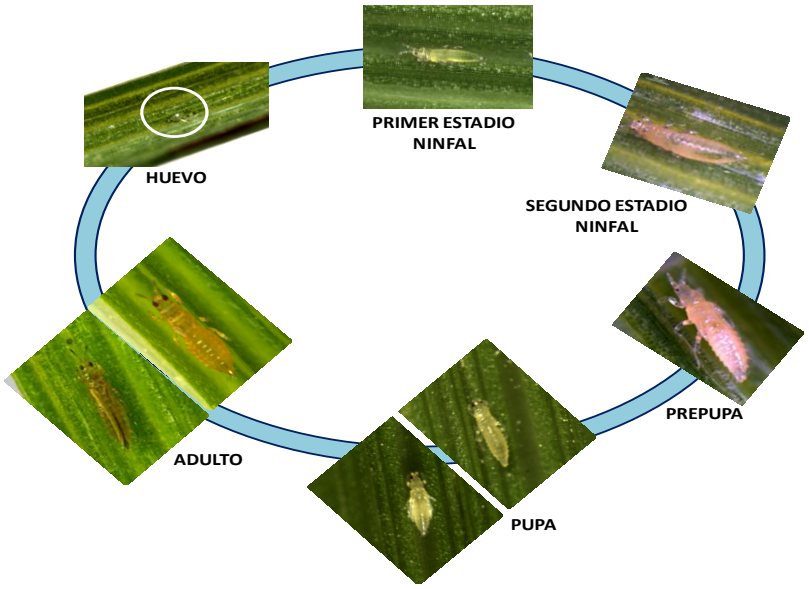

Figura 1.6 Ciclo de vida de *Anaphothrips obscurus.*

1.4 Herramientas moleculares en protección de cultivos

La incorporación de un extenso repertorio de herramientas biotecnológicas a la protección de cultivos ha abierto nuevas vías en el control de plagas permitiendo dar respuestas satisfactorias a las necesidades de sanidad y seguridad alimentaria requeridas para la producción agrícola del siglo XXI. Gran parte del éxito de un programa de control biológico se halla en la selección y conservación de enemigos naturales idóneos que contribuyan a regular de forma natural las poblaciones de la plaga (Hurtado et al. 2008b). Para ello es necesario identificar y conocer tanto la especie plaga y sus enemigos naturales, como las interacciones tróficas y ecológicas en las que se encuentran implicados. Los marcadores moleculares y la secuenciación, tanto de fragmentos de ADN como de genomas completos, nos permiten detectar la variabilidad genética entre individuos o especies (Figura 1.7) y utilizarla para conocerlos más a fondo y así mejorar el control biológico de plagas. Estas técnicas son muy versátiles haciendo posible que su utilización tenga diversas aplicaciones en la protección de cultivos.

Los marcadores moleculares se pueden emplear para identificar huevos, larvas y adultos de especies crípticas (Brunner et al. 2002; Mound et al. 2010; Matsuda et al. 2012; Hao-Sen et al. 2014; Tyagi et al. 2015), tarea que suele ser ardua y en ocasiones imposible mediante el análisis morfológico tradicional. También permiten identificar el origen geográfico de una especie invasora y sus procesos de colonización (Boubou et al. 2011, 2012) e incluso indagar sobre la emergencia de resistencias a plaguicidas en poblaciones naturales y realizar su seguimiento (Ilias et al. 2014; Kwon et al. 2014; Pascual-Ruiz et al. 2014; Ding et al. 2015; Liebman et al. 2015). Otro ejemplo de la contribución de estas técnicas en el control biológico de plagas es su uso para detectar e identificar endosimbiontes causantes de alteraciones reproductivas (Brelsfoard and Dobson 2009; Saridaki and Bourtzis 2009; Ghazy et al. 2016). Además de las utilidades citadas anteriormente, las herramientas moleculares han permitido ampliar la visión sobre las relaciones tróficas que tienen lugar en los ecosistemas. Cuando estos estudios se centran en especies tan diminutas como la mayoría de los artrópodos plaga y sus enemigos naturales, su observación directa en campo es difícil y la información

aportada por estas herramientas puede ser de gran utilidad. Así, en entomología agrícola han sido de gran ayuda para determinar las relaciones que acontecen entre las especies plaga y sus enemigos naturales. Dado que una parte de la presente tesis se enmarca en este contexto a continuación se tratará este punto con más detalle.

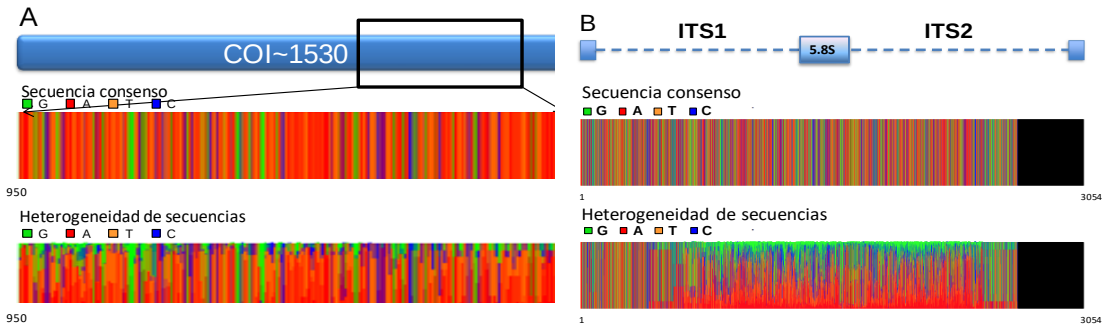

Figura 1.7 Variabilidad nucleotídica observada por Pérez-Sayas (2016) en el gen mitocondrial *Citocromo Oxidasa* I (COI) (A) y en el espaciador transcrito interno del ADN nuclear ribosomal (ITS) (B) y utilizada para llevar a cabo estudios filogenéticos en ácaros de interés agrícola.

1.4.1. Aplicación de las herramientas moleculares en la determinación de relaciones tróficas

Los sistemas de monocultivo en los que una especie vegetal se siembra en un área muy amplia se podrían percibir como una simplificación de los ecosistemas naturales. En estos sistemas se ha asumido tradicionalmente el concepto de cadena trófica en el que se tiene en cuenta la relación entre un fitófago plaga y un único agente de control biológico. Sin embargo, recientemente se ha cambiado a un nuevo paradigma en el control biológico (de cadena trófica a redes tróficas) basado en la existencia de múltiples interacciones ecológicas que forman redes complejas (González-Chang et al. 2016). Identificar, analizar y cuantificar la fuerza de estas relaciones multitróficas es muy complicado, o imposible en algunos casos, con los métodos tradicionales basados en la observación directa o la disección e identificación visual del contenido estomacal. Sin embargo, los estudios que utilizan técnicas moleculares para analizar el ADN de presas en los depredadores, así como el ADN de parasitoides en sus hospedadores, permiten arrojar luz en el entendimiento de estas relaciones multitróficas y aplicarlas

para mejorar el control biológico (Furlong 2015; González-Chang et al. 2016; Gurr y You 2016).

Las primeras técnicas moleculares aplicadas al estudio de las relaciones tróficas fueron las basadas en marcadores proteicos. La detección mediante electroforesis de isoenzimas (variantes enzimáticas con funciones similares pero diferente carga eléctrica y peso molecular) fue una de las primeras aproximaciones (Murray y Solomon 1978), aunque hoy en día está prácticamente en desuso. También forman parte de este grupo de técnicas los ensayos de inmunoadsorción ligados a enzimas (ELISA, "Enzyme-Linked ImmunoSorbent Assay"). Estos ensayos están basados en el uso de anticuerpos (en su mayoría monoclonales) que reaccionan con la presa diana de forma muy específica. Debido a lo costoso que resulta desarrollar los anticuerpos (Symondson et al. 1999; Fournier et al. 2008), el uso de los ELISA ha sido reemplazado por las técnicas basadas en la reacción en cadena de la polimerasa (PCR, "Polymerase Chain Reaction") (Symondson 2002; Gurr and You 2016; Kamenova et al. 2017) que son más versátiles y tienen un menor coste relativo respecto a su eficacia. La detección de la presa se lleva a cabo mediante el diseño de cebadores específicos que permiten amplificar fragmentos del ADN de la presa de tamaños variables en función de su identidad. Por lo general, esta detección aumenta si el tamaño de los fragmentos amplificados es relativamente corto (Agustí et al. 1999; Zaidi et al. 1999; Juen y Traugott 2005; King et al. 2008; Waldner et al. 2013). Esto se debe a que las moléculas de ADN diana de tamaños grandes se rompen en otros más pequeños durante el proceso de digestión del depredador. Otro factor que permite optimizar la detección de la presa es que los fragmentos amplificados se encuentren presentes en múltiples copias en el genoma (Zaidi et al. 1999; Chen et al. 2000; Agustí et al. 2003; King et al. 2008). Tanto la región del espaciador transcrito interno 1 y 2 (ITS1 e ITS2) del ADN nuclear ribosomal, como el gen mitocondrial de la *Citocromo Oxidasa* I y II (COI y COII) cumplen este requisito, y han demostrado ser muy útiles en los estudios de depredación en diferentes grupos de artrópodos (Hoogendoorn y Heimpel 2001; Gariepy et al. 2007; King et al. 2008; Monzó et al. 2010, 2011; Gómez-Polo et al. 2013;

González-Chang et al. 2016) y en concreto en ácaros (Fitzgerald et al. 2004; Rivera-Rivera et al. 2012; Wari et al. 2014; Pérez-Sayas et al. 2015).

El uso del COI en ácaros de interés agrícola se encuentra condicionado tanto por el número de secuencias disponibles como por la fiabilidad de las secuencias publicadas en las bases de datos (Ros y Breeuwer 2007; De Mendoça et al. 2011; Tixier et al. 2011; Auger et al. 2013; Chen et al. 2014). Por el contrario, las regiones ITS1 e ITS2 se usan con frecuencia en este taxón. En este caso, el ITS1 es más variable que el ITS2, tanto a nivel intraespecífico como interespecífico (Navajas et al. 1999; Hurtado et al. 2008a) y este último a su vez, es más variable entre especies que el COI (Navajas et al. 1994, 1998). Pérez-Sayas y colaboradores (2015) descartaron la región COI para el diseño de cebadores específicos a nivel de especie en ácaros debido a su elevada similitud. En los trips, contrariamente a lo que ocurre en ácaros, la región COI del ADN mitocondrial ha sido muy estudiada. De hecho, en las bases de datos se encuentran disponibles un gran número de secuencias fruto de su amplia utilización en estudios taxonómicos y filogenéticos (Brunner et al. 2002; Buckman et al. 2013; Nakahara y Minoura 2015).

En los estudios de relaciones tróficas con plantas, los marcadores moleculares más adecuados son aquellos que tienen regiones muy conservadas en las que diseñar cebadores muy eficaces y que resulten en productos de amplificación relativamente cortos. En este aspecto los genes cloroplásticos *trnL* y *rbcL* (Figura 1.8) y la región ITS del ADN nuclear se han presentado como buenos candidatos (Taberlet et al. 1991; Chase et al. 2005; Taberlet et al. 2007; Pumariño et al. 2010).

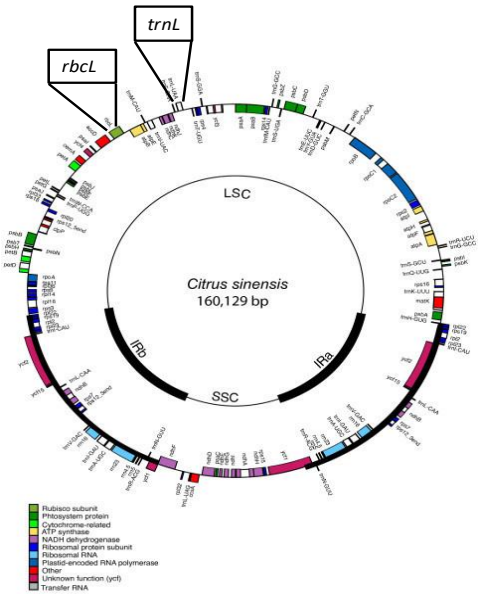

Figura 1.8 Genoma cloroplástico de *Citrus sinensis*. Imagen modificada de Bausher et al. (2006).

En el estudio de las relaciones tróficas aplicado al control biológico, es necesario analizar muchos individuos depredadores

obtenidos de campo con varios cebadores específicos capaces de detectar distintas presas para conseguir un conocimiento realista de las redes tróficas. Este requerimiento implica llevar a cabo muchas reacciones de PCR que extienden el tiempo y los recursos materiales necesarios. Además, en muchos casos, las especies de estudio son de un tamaño microscópico (como por ejemplo ácaros y trips) limitando la cantidad de ADN que se obtiene de un único individuo y el número de reacciones que podemos llevar a cabo en el estudio de un depredador concreto. Para contrarrestar este obstáculo, la técnica de la PCR multiplex permite incluir varios marcadores moleculares en una única reacción de amplificación consiguiendo la detección simultánea de un gran abanico de presas (Harper et al. 2005; King et al. 2011; Sint et al. 2012; Kamenova et al. 2017). Sin embargo, esta técnica se ve limitada a la hora de detectar relaciones tróficas insospechadas, como por ejemplo fuentes de alimentación que no estén consideradas en el diseño de la PCR multiplex, puesto que no permite identificarla si no somos conocedores de su existencia. En este caso, las novedosas técnicas basadas en la amplificación masiva de fragmentos de ADN con cebadores universales y su análisis con secuenciadores de última generación (NGS) pueden ser muy útiles dado que son capaces de aportar información mucho más detallada sobre las redes tróficas. Además, han permitido apreciar que la complejidad de las mismas es mayor de lo que se pensaba previamente, resaltando la importancia de las fuentes de alimentación alternativas o la depredación intragremial (Wirta et al. 2014, Gómez-Polo et al. 2015).

La metodología de secuenciación masiva también tiene algunas limitaciones. Aunque estos métodos permitan detectar el ADN de todas las especies de las que se alimenta un depredador, es necesario tener una base de datos potente con las secuencias de ADN de todo el espectro de especies presentes en el ecosistema para compararlas y poder identificarlas. Cuando la secuencia de ADN de una especie no está disponible en las bases de datos, ésta no se puede identificar (Gariepy et al. 2014). Por lo tanto, sería oportuno hacer un análisis previo de la diversidad de artrópodos del lugar donde se va realizar el estudio para poder contrastar los resultados. Por otro lado, estas técnicas pueden producir sesgos a la hora de cuantificar el número de

presas que realmente ha consumido el depredador (Deagle et al. 2013), por lo que la mayoría de estudios proporcionan resultados únicamente cualitativos.

Cuando se necesitan datos cuantitativos que ayuden a estimar la fuerza de las relaciones tróficas, las técnicas de PCR como la PCR-cuantitativa (qPCR) o PCR a tiempo real (real-time PCR) son preferibles a las NGS (Pompanon et al. 2012). En un estudio reciente llevado a cabo por Gómez-Polo y colaboradores (2015) se utilizó la combinación de las dos técnicas para estudiar las redes tróficas que tienen lugar en los cultivos de lechuga. El uso de secuenciación masiva les permitió identificar fuentes de alimento alternativas y relaciones de depredación intragremial aportando un conocimiento más amplio sobre la dieta de los depredadores. A su vez, el uso de la PCR a tiempo real aportó información sobre la contribución de las diferentes presas en la dieta del depredador. Este estudio resalta la importancia de combinar estas dos técnicas para evaluar los efectos de la manipulación del hábitat en las redes tróficas existentes en los ecosistemas agrícolas y su repercusión en el control biológico.

En aquellos casos donde la diversidad ecológica del agroecosistema es ya conocida, las herramientas moleculares se utilizan para evaluar en qué medida una especie considerada plaga es depredada o parasitada por un complejo de enemigos naturales. En esta situación, el número de especies diana a estudiar está acotado y por tanto, la PCR multiplex es la metodología más rápida, sencilla y económica (Pompanon et al. 2012). Uno de los trabajos más recientes que utiliza esta técnica para mejorar el conocimiento de las relaciones tróficas entre ácaros plaga y depredadores en cítricos es el llevado a cabo por Pérez-Sayas y colaboradores (2015). En este trabajo una única reacción de PCR multiplex permitió conocer la identidad de seis de los fitoseidos depredadores más relevantes del sistema agrícola y analizar satisfactoriamente su alimentación sobre dos especies plaga de cítricos, *P. citri* y *T. urticae*. Sin embargo, como el alimento alternativo y la depredación intragremial pueden tener una influencia significativa en el éxito o fracaso de la regulación de poblaciones de *T. urticae*, dada la versatilidad alimenticia de los fitoseidos, es necesario extender el estudio de las relaciones tróficas para intentar mejorar el control biológico de *T. urticae* en los campos de clementino.

1.5 Justificación y objetivos

El establecimiento de una cubierta vegetal de *F. arundinacea* en huertos de clementino puede contribuir a regular de forma natural las poblaciones de *T. urticae* en el árbol constituyendo una alternativa eficaz para el control de esta especie plaga. Dicha cubierta vegetal alberga mayor diversidad y frecuencia relativa de especies de fitoseidos más eficientes en el control de ácaros tetraníquidos. Una posible explicación de esta observación va ligada al incremento de posibles presas, como *A. obscurus*, que podrían estar jugando un papel clave al actuar como alimento alternativo y permitir a los fitoseidos más efectivos contra *T. urticae* mantener sus poblaciones en periodos de escasez de presa. Por este motivo, el objetivo principal de esta tesis doctoral es conocer las características biológicas y ecológicas de *A. obscurus* y cómo esta especie podría estar contribuyendo a la regulación natural de poblaciones de *T. urticae* en este ecosistema agrícola. Con esta finalidad se concretaron los siguientes objetivos:

1. Ampliar los conocimientos sobre la ecobiología de *A. obscurus* para determinar las relaciones ecológicas que mantiene con *T. urticae* así como con el cultivo, la cubierta vegetal y los fitoseidos.

2. Evaluar la aptitud de algunos de los fitoseidos más comunes en el sistema agrícola de los clementinos para alimentarse de *A. obscurus* mediante análisis de respuesta funcional y de preferencia de presa, y así determinar cómo puede influir esta especie en la conservación de los enemigos naturales de *T. urticae*.

3. Desarrollar una PCR multiplex que permita ampliar el conocimiento sobre las interacciones tróficas que tienen lugar *in situ* en torno a la especie plaga *T. urticae* y sus depredadores, basada en la detección de fuentes de alimentación alternativa y depredación intragremial.

Capítulo 2

Ecobiology of *Anaphothrips obscurus*, a new dweller of citrus orchards brought in by more sustainable pest management practices

Una versión de este capítulo está aceptada para su publicación:

María Antonia Gómez-Martínez, Ernestina Aguilar-Fenollosa, Josep A. Jaques and Tatiana Pina (2017) Ecobiology of *Anaphothrips obscurus*, a new dweller of citrus orchards brought in by more sustainable pest management practices. *Agricultural and Forest Entomology*. **DOI:10.1111/afe.12233**

2.1 Introduction

Conservation biological control has been defined as a "modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests" (Eilenberg et al. 2001). The implementation of ground covers as a habitat management approach increases the populations of beneficial organisms by providing them with a suitable ecological infrastructure that offers resources as food and refuge from adverse conditions (Bugg and Waddington 1994; Gurr et al. 2004; Jonsson et al. 2008; Landis et al. 2000). There is a substantial body of literature on the influence of ground covers on pest densities through the provision of either food or refuge to natural enemies. For example, in citrus orchards in California, China and Florida, ground cover plants offered pollen and alternative prey to predatory mites (Acari: Phytoseiidae), which increased their numbers and were able to control different citrus pests (Kennett et al. 1979; Liang and Huang 1994; Muma 1961).

Spanish clementine mandarins (*Citrus clementina* Hort. ex Tan.) are particularly susceptible to aphids (Hemiptera: Aphididae) and mites (Acari: Tetranychidae), which together with the Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae), are considered key pests of this citrus species (Urbaneja et al. 2015). The establishment of a perennial grass cover crop (Poaceae) in clementine orchards improved the biological control of these pests (Monzó et al. 2010; Aguilar-Fenollosa et al. 2011a, c; Gómez-Marco et al. 2016) and resulted an efficient and sustainable alternative to pesticides. More specifically, when using a *Festuca arundinacea* Schreber (Poaceae) cover in clementines, ensuing increases in the abundance and frequency of phytoseiids and a better regulation of the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), have been documented (Aguilar-Fenollosa et al. 2011a, b, c). Indeed, phytoseiids are considered as the most effective natural enemies of tetranychid mites and other microarthropods of economic importance as thrips (Helle and Sabelis 1985; McMurtry et al. 2013). Interestingly, the composition and abundance of the thrips species changed when *F. arundinacea* was established as a cover crop. On the one hand, this cover supported lower numbers of potential pest

thrips species such as *Frankliniella occidentalis* (Pergande) and *Thrips tabaci* Lindeman than a wild cover. On the other, it sustained high populations of thrips feeding preferentially on Poaceae as *Anaphothrips obscurus* (Müller) and *Chirothrips manicatus* (Haliday) (Thysanoptera: Thripidae). However, *C. manicatus* was found in spring and autumn only, whereas *A. obscurus* was consistently found throughout the year in high numbers (Aguilar-Fenollosa and Jacas 2013). *Anaphothrips obscurus* has a cosmopolitan distribution (Bailey 1948; Mound 1997). This species can feed on several Poaceae often found in orchards as weeds (Hinds 1900; Bailey 1957; Köppä 1967; Bournier 1983; Jiang et al. 2015) and can even become a pest of corn (*Zea mays* L.) (Brohmer et al. 1966; Stannard 1968) and timothy (*Phleum pratense* L.) (Reisig et al. 2009). Additionally, we cannot exclude this species from being zoophytophagous, based on Hinds' (1900) observations.

Species sharing the same niche can compete by interference (direct interaction between individuals that prevent each other from exploiting the same resource) or by resource exploitation (each individual is affected by the amount of resource that remains after being exploited by the other) (Reitz and Trumble 2002; Begon et al. 2006; Zhang et al. 2015). In the case of the zoophytophagous thrips *Frankliniella occidentalis* (Pergande) (Thripidae) and the phytophagous whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), their coexistence caused a reduction of the fecundity of the thrips by the whitefly but not vice-versa and this was tentatively attributed to interference-type competitive interactions (Wu et al. 2014). In our case, *T. urticae* can feed and reproduce on *F. arundinacea* (Aguilar-Fenollosa et al. 2012), but whether *A. obscurus* can complete development and reproduce feeding on this plant and exhibit similar competitive interactions remains unclear.

Apart from competition and direct predation, *A. obscurus* could play an important role as additional or alternative prey for phytoseiids, which might help bolster population densities of these predatory mites (van Houten et al. 1995; Grafton-Cardwell et al. 1999; Messelink et al. 2006; van Baal et al. 2007). This was hypothesized by Aguilar-Fenollosa and Jacas (2013) and could be the result of apparent competition (Holt 1977) between *A. obscurus* and *T. urticae*. This phenomenon is common in

similar systems. For instance, in apples, the predation on *T. urticae* by the phytoseiid *Euseius finlandicus* (Oudemans) was enhanced by the presence of the apparent competitor *Eotetranychus pruni* Oudemans (Tetranychidae) (Liu et al. 2006). Similar results were obtained with *Metaseiulus occidentalis* (Nesbitt) (Phytoseiidae) and *Eotetranychus villamettei* (McGregor) (Tetranychidae) on *Tetranychus pacificus* McGregor (Tetranychidae), a serious pest of grapevines in California (Hanna et al. 1997).

Hence, in the present study, with the intention of increasing our knowledge on the ecobiology of *A. obscurus* in citrus orchards grown in association with a ground cover, we aimed: i) to analyze the field dynamics of *T. urticae*, *A. obscurus* and Phytoseiidae populations; ii) to study the main biological traits of brachypterous and macropterous females of *A. obscurus* (life cycle, reproductive and demographic parameters, and feeding habits); and iii) to check whether the most abundant phytoseiid species in Spanish clementine orchards could feed on this thrips.

2.2 Materials and methods

2.2.1 Field assays. Population dynamics of A. obscurus*,* T. urticae *and phytoseiids*

We aimed to re-explore field data previously gathered (Aguilar-Fenollosa et al. 2011a, b; Aguilar-Fenollosa and Jacas 2013) to study the population dynamics of *A. obscurus*, *T. urticae* and phytoseiids. These authors studied the population dynamics of mites and thrips in a *F. arundinacea* cover associated with clementine mandarins (*Citrus clementina* Hort. ex Tan. cv. Clementina de Nules grafted on citrange Carrizo rootstock) in four commercial orchards located in eastern Spain [L'Alcúdia (30S, X: 710934.12, Y: 4339907.44, h: 25 m), Bétera (30S, X: 722427.09, Y: 43850216.97, h: 120 m), Llíria (30S, X: 706793.22, Y: 4401016.70, h: 164 m) and La Pobla de Vallbona (30S, X: 713434.80, Y: 4390362.32, h: 125 m)]. One of these orchards was organic (L'Alcúdia) and the other three followed IPM guidelines. The most relevant characteristics of these orchards (pesticide usage, ground cover management practices) as well as

weather data can be found in Aguilar-Fenollosa et al. (2011a). At all locations *F. arundinacea* was sown in autumn 2005. Orchards were sampled fortnightly from March 2006 to either March 2008 (at L'Alcúdia and Bétera) or March 2009 (at Llíria and La Pobla de Vallbona). On each sampling date, 100 g of the ground cover plants were collected, put in a plastic bag and refrigerated until reaching the laboratory. Berlese funnels were used to extract microarthropods. Extracted adult mites and thrips were characterized and quantified (Aguilar-Fenollosa et al. 2011a, b; Aguilar-Fenollosa and Jacas 2013). These direct counts (number of specimens per 100 g of ground cover plants) were transformed into seasonal cumulative counts for *A. obscurus*, *T. urticae* and phytoseiids in accordance with Aguilar-Fenollosa et al. (2011a):

$$\Sigma_{\Delta t} = \frac{(x_1 + x_2)}{2}$$

where $\sum$ is summation overall sampling dates, Δ_t is the interval between two successive sampling dates (usually 15 days in this study) and x_1 and x_2 are specimen counts on those dates. Data for each location and year were fitted to a nonlinear mixed-effect model (Pinheiro and Bates 2000) defined as:

$$N = a/(1 + \exp(b - t)/c)$$

where N is the seasonal cumulative counts for *A. obscurus*, *T. urticae* and phytoseiids and t is the time since the start of the season (days). Parameters a, b and c will be estimated, where a represents the curve's maximum value (i.e., the horizontal asymptote as $t \rightarrow \infty$), b the x-value of the sigmoid midpoint and c the steepness of the curve.

NLREG, version 6.5 (Sherrod 2008), was used to fit the nonlinear mixed-effect model, obtaining the estimates of the parameters (a, b, and c) and their 95% confidence intervals by means of a Gauss-Newton algorithm. We considered time, t, as a random effect. The a estimate was considered as an indicative of the maximum value reached by the arthropod population in that period. Subsequently, the 95% confidence

intervals of *a* were used to assess the differences between years for *A. obscurus*, *T. urticae* and phytoseiids populations at each location.

2.2.2 Laboratory assays

Stock cultures and rearing

Anaphothrips obscurus individuals were originally collected in *F. arundinacea* plants grown in an experimental citrus plot at Universitat Jaume I (UJI) (Castelló de la Plana, Spain). They were later maintained on the same kind of plants (*F. arundinacea* 'Fórmula frutales y cítricos', Semillas Fitó, S.A., Barcelona, Spain) grown in a pesticide-free greenhouse at the Institut Valencià d'Investigacions Agràries (IVIA) (Montcada, Valencia, Spain). The *A. obscurus* rearing unit consisted of detached *F. arundinacea* leaves (*A. obscurus* is a terebrantian thrips and needs a plant substrate to oviposit) set adaxially on a water saturated sponge covered by a filter paper in a closed plastic container (17 × 12.5 × 7.5 cm). Both leaf ends were fixed with wet cotton strips. Every 10 days, new leaves were added and old leaves removed to assure leaf quality for thrips oviposition. Rearing was maintained in a climatic chamber at 25 ± 1 °C, 70 ± 5% RH and a 16:8 h (light:dark; L:D) photoperiod. *Tetranychus urticae* specimens, originally collected from *F. arundinacea* (Aguilar-Fenollosa et al. 2012), were maintained in the same conditions and type of rearing units as thrips.

Laboratory colonies of *Neoseiulus californicus* (McGregor) (Acari: Phytoseiidae) were initiated with individuals from a commercial producer (SPICAL®; Koppert Biological Systems, The Netherlands). Laboratory colonies of other phytoseiid species were initiated with adults collected from different host plants: *Euseius stipulatus* (Athias-Henriot) from clementine trees at IVIA and *Neoseiulus barkeri* Hughes from *F. arundinacea* at UJI. All phytoseiid species were reared in accordance with the methodology described by Overmeer (1985). In the case of *E. stipulatus*, the plastic tile used in the rearing unit was substituted by an upside down bean leaf. A mixture of different stages of *T. urticae* obtained from a rearing maintained on lemon [*Citrus limon* (L.) Burm f. (Rutaceae)] and pollen of *Carpobrotus edulis* (L.) N. E. Br (Aizoaceae) was regularly added to the rearing as a food source. Finally, young clementine leaves

used in this study were obtained from pesticide-free plants maintained in a greenhouse.

Experimental unit

Experiments were performed in Petri dishes (diameter 9 cm) with a hole in the lid (diameter 6 cm) covered with anti-thrips mesh (14 × 95 µm) (Figure 2.1). A plastic tile (6 × 3 × 1.5 cm) used as arena was fixed in the center of the Petri dish with glue. Once the glue was dry, water was added to the setup slightly below the tile level. Tile borders were covered with wet paper to supply water to thrips and prevent them from escaping. Both parts of the Petri dishes were sealed with Parafilm® (ParaFILM®; Bemis Company, Inc. Neenah, Wisconsin) and maintained in a climatic chamber under the abovementioned conditions (Figure 2.1).

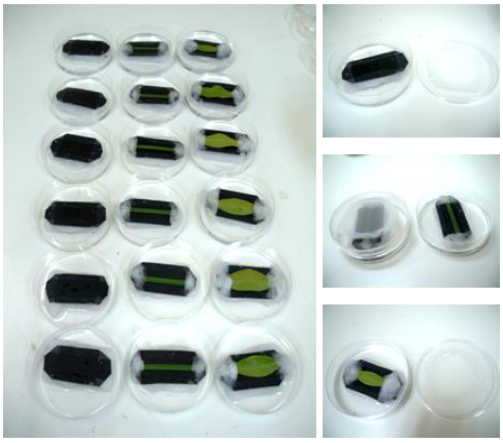

Figure 2.1 Experimental units used in the laboratory assays.

Development and survival on F. arundinacea

As this thelytokous thrips (Hinds 1900), which rarely produces males (Mirab-Balou and Chen 2010), exhibits different female wing morphs (Hinds 1900; Nakao 1996) (Figure 2.2) with different longevities and preoviposition periods on corn (Jiang et al. 2015), we considered these forms in the assays. Therefore, thirty brachypterous and thirty macropterous females of *A. obscurus* were individually introduced into experimental units including a *F. arundinacea* leaf (Figure 2.1) and left undisturbed for 24 h. Afterwards, females were removed and the arenas, which presumably contained eggs, were checked twice a day under a binocular microscope until egg hatching. The newly emerged nymphs were individually transferred to new experimental units with *F. arundinacea*. Survival and molting were recorded twice a day until adulthood or death. The presence of exuviae was taken as evidence of molting. As mother wing form does not preclude offspring wing morphology and wings can only be appraised from the prepupal stage onwards, wing morph specific development time could only

be calculated based on those individuals that actually reached at least the prepupal stage. Therefore, immature survival for the two wing morphs was differentiated only in prepupal and pupal stages.

Figure 2.2 Wing dimorphism in *A. obscurus*: macropterous (left) and brachypterous (right) females.

Data were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Levene test). Because these assumptions were not fulfilled, non-parametric tests were used. Development and survival of macropterous and brachypterous forms were compared by a Mann-Whitney *U* test and Kaplan-Meier survival analysis using the log-rank (Mantel-Cox) test, respectively. SPSS (v. 19.0.0) (IBM Corp., Armonk, New York) was used in this and subsequent statistical analyses.

Adult longevity and reproduction on F. arundinacea

Newly-emerged females (both macro- and brachyptera) reaching the adult stage were individually moved into experimental arenas containing a *F. arundinacea* leaf (Figure 2.1) and daily transferred to new ones until they died. These arenas were checked daily for oviposition and egg hatching. The progeny was maintained until they reached the adult stage. Then, they were slide-mounted to determine sex ratio. Mean pre-oviposition, oviposition and post-oviposition time, oviposition rate, egg hatching, female longevity and fecundity were calculated.

Oviposition and post-oviposition times and longevity fulfilled normality and homoscedasticity assumptions. Therefore, they were compared between wing morphs using Student's *t*-test. However, pre-oviposition time and egg hatching did not, and wing morphs were compared using Mann-Whitney *U*-test. Because fecundity and oviposition rate are discrete counts with strong dispersion around the mean, these data were analyzed with a Generalized Linear Model using a Poisson distribution.

The estimate of the intrinsic rate of increase (r_m) was calculated for both winged forms according to the formula of Birch (1948):

$$\sum (e^{-rm \cdot x} \cdot l_x \cdot m_x) = 1$$

where x means the female age (days), l_x is the age specific survival of the females at age x and m_x is the number of female progeny per female at age x. In addition, other parameters such as net reproductive rate (R_o), mean time span necessary to double the initial population (DT), mean generation time (T) and finite rate of increase (λ) were calculated by their respective equations:

$$R_0 = \sum (l_x \cdot m_x) \qquad DT = \frac{\ln (2)}{r_m}$$

$$\lambda = e^{rm} \qquad T = \frac{\ln (R_0)}{r_m}$$

The variability among individuals within a group cannot be calculated for the synthetic life table parameters. In that case variances can be calculated by the iterative Monte Carlo methods. The iterative jackknife procedure is one of them and was used to estimate standard error (Maia et al. 2000). Differences between macropterous and brachypterous forms were compared using Student's *t*-test and Mann-Whitney *U* test.

Feeding habits of A. obscurus

To characterize the feeding habits of *A. obscurus* (phytophagy, zoophagy, or zoophytophagy), one thrips specimen [second instar nymphs (N2) and macropterous adults were considered] starved for 24 h was offered either a clementine leaf or three *T. urticae* eggs using the setup already described. Clementine leaves were placed adaxially on the plastic tile and fixed by both ends with wet cotton strips (Figure 2.1). Units were checked 4, 20 and 24 h after the onset of the assay and then once a day until the thrips died. Survival, molting and oviposition were evaluated. Positive and negative controls [units containing one 5 cm long *F. arundinacea* leaf placed and fixed same as clementine leaves, or a plastic tile with water supply only, respectively (Figure

2.1)] were included in all cases. Up to 11 replicates per substrate and *A. obscurus* stage, including both control treatments, were considered. Adult longevity and development time of *A. obscurus* were analyzed with a one-factor analysis of variance with the food source as main factor. Means were separated with the conservative post-hoc test, Scheffé test.

Anaphothrips obscurus *as a prey for phytoseiids*

The potential role of *A. obscurus* as a food source for three abundant predatory mites in Spanish citrus orchards (Aguilar-Fenollosa et al. 2011b) was tested in the previously described *F. arundinacea* setup. These phytoseiids exhibit different life-styles: *N. barkeri* is a generalist predator, *N. californicus* is a tetranychid-specialist predator, and *E. stipulatus* is a pollen feeding generalist predator (McMurtry et al. 2013). Three first instar nymphs (N1) of *A. obscurus* were added to the arena and once the nymphs settled onto the substrate, one gravid phytoseiid female starved for 24 h was added. Fifteen replicates for each phytoseiid species were used. After 24 h the number of dead, preyed and live N1 *A. obscurus* was recorded. Eight replicates were used as control (arenas with no predatory mite). As data were categorical (1, 2, 3 or no prey items eaten), cross tabulation analysis was used to determine if there was a relationship between the phytoseiid species and the number of *A. obscurus* N1 consumed.

2.3 Results

2.3.1 Field assays. Population dynamics of A. obscurus*,* T. urticae *and phytoseiids*

The nonlinear mixed-effect model applied to the cumulative counts of *T. urticae*, *A. obscurus* and phytsoeiids in the *F. arundinacea* cover during the different seasons provided an accurate fit (R^2 ranging from 0.93 to 0.99: $P < 0.001$ in all cases, Table 2.1; Figure 2.3) and did not show any non-random pattern in the distribution of the residuals. The curve maximum value estimates (*a*) decreased for *T. urticae* populations along the years at all locations except L'Alcúdia, where it was not significantly different

between years. Contrarily, for *A. obscurus* and phytoseiids, *a* estimates were higher as time passed except for *A. obscurus* at La Pobla de Vallbona, where population decreased, and for phytoseiids at Bétera, where similar values of the *a* estimate were obtained (Table 2.1 and 2.2). At La Pobla de Vallbona, the parameters estimated for *A. obscurus* in 2006 were not considered (Table 2.1 D), because, in this case, the cumulative population curve did not reach a plateau (Figure 2.3).

Table 2.1 A nonlinear mixed-effect model (Pinheiro and Bates 2000) fitted to cumulative numbers of *Tetranychus urticae*, *Anaphothrips obscurus* and phytoseiids per season (2006-7 to 2008-9) at four different locations (A, L'Alcúdia; B, Bétera; C, Llíria and D, La Pobla de Vallbona) where a *Festuca arundinacea* ground cover was cultivated in association with a clementine mandarin orchard. R^2 indicates the proportion of variance explained by the model. *a* (maximum value of the curve), *b* (*x*-value of the midpoint of the sigmoid) and *c* (steepness of the curve) are the parameters estimated. Values between brackets correspond to 95% confidence intervals (CI).

A L'Alcúdia

Species	Year	R^2	*F*	*P*	*a* (95% CI)	*b* (95% CI)	*c* (95% CI)
T. urticae	2006	0.97	455.26	< 0.001	3015.58 (2862.50-3168.67)	144.15 (135.24-153.06)	31.49 (23.95-39.03)
	2007	0.97	306.47	< 0.001	3503.58 (2709.68-4297.48)	235.57 (203.37-267.77)	55.35 (40.03-70.66)
A. obscurus	2006	0.93	172.98	< 0.001	637.76 (588.23-687.29)	127.74 (112.36-143.12)	42.36 (28.93-55.79)
	2007	0.99	1019.15	< 0.001	1055.40 (1030.00-1080.80)	72.22 (68.93-75.51)	17.18 (14.33-20.04)
Phytoseiids	2006	0.98	641.99	< 0.001	165.22 (154.89-175.57)	189.60 (179.45-199.75)	40.27 (32.32-48.22)
	2007	0.99	1885.07	< 0.001	62.095 (594.11-647.80)	184.15 (178.33-189.97)	35.84 (31.79-39.89)

B Bétera

Species	Year	R^2	*F*	*P*	*a* (95% CI)	*b* (95% CI)	*c* (95% CI)
T. urticae	2006	0.99	1230.04	< 0.001	5126.45 (4927.72-5325.19)	181.87 (175.72-188.02)	33.60 (28.60-38.61)
	2007	0.95	191.58	< 0.001	1938.58 (1472.04-2405.12)	259.97 (237.80-282.14)	30.45 (17.49-43.41)
A. obscurus	2006	0.99	1037.36	< 0.001	284.32 (375.24-393.40)	99.96 (96.64-103.29)	14.94 (11.95-17.94)
	2007	0.99	27281.59	< 0.001	665.76 (662.68-668.84)	82.80 (82.23-83.37)	12.01 (11.52-12.51)
Phytoseiids	2006	0.94	204.39	< 0.001	759.05 (633.45-884.64)	197.47 (165.96-225.98)	64.14 (43.74-84.55)
	2007	0.98	504.50	< 0.001	637.18 (564.35-710.00)	198.87 (181.39-216.35)	51.05 (40.06-62.03)

C Llíria

Species	Year	R^2	*F*	*P*	*a* (95% CI)	*b* (95% CI)	*c* (95% CI)
T. urticae	2006	0.98	588.11	< 0.001	3492.80 (3259.12-3726.49)	198.65 (188.64-208.66)	37.77 (29.75-45.79)
	2007	0.96	273.54	< 0.001	1080.65 (1020.64-1140.65)	93.84 (82.83-104.84)	41.97 (31.98-51.96)
	2008	0.95	213.03	< 0.001	406.08 (342.48-469.68)	161.01 (129.70-192.32)	70.18 (49.46-90.89)
A. obscurus	2006	0.98	712.21	< 0.001	95.81 (93.03-98.59)	93.20 (88.14-98.26)	21.21 16.88-25.55)
	2007	0.98	4811.17	< 0.001	236.56 (234.15-238.96)	79.56 (78.25-80.88)	11.97 (10.81-13.14)
	2008	0.99	1198.97	< 0.001	641.63 (627.17-656.09)	87.49 (83.76-91.21)	22.92 (19.62-26.21)
Phytoseiids	2006	0.96	321.61	< 0.001	211.98 (201.26-222.69)	103.50 (92.88-114.12)	41.31 (31.93-50.69)
	2007	0.99	2043.47	< 0.001	334.93 (324.67-343.44)	147.93 (143.36-152.51)	37.13 (33.38-40.89)
	2008	0.98	611.61	< 0.001	437.51 (400.12-474.90)	192.37 (179.37-205.37)	47.31 (38.44-56.17)

D La Pobla de Vallbona

Species	Year	R^2	*F*	*P*	*a* (95% CI)	*b* (95% CI)	*c* (95% CI)
T. urticae	2006	0.97	360.12	< 0.001	2312.79 (2188.91-2436.67)	134.80 125.70-143.90)	26.76 (19.02-34.49)
	2007	0.97	326.32	< 0.001	880.82 (832.53-929.11)	144.69 (139.47-149.91)	8.41 (3.88-12.95)
	2008	0.99	922.99	< 0.001	227.76 (220.86-234.65)	113.03 (108.21-117.85)	23.91 (19.84-27.97)
A. obscurus	2006	0.97	340.84	< 0.001	-[†]	-	-
	2007	0.99	1315.98	< 0.001	7262.49 (7177.44-7347.54)	29.14 (27.45-30.84)	12.04 (10.52-13.55)
	2008	0.96	289.93	< 0.001	1634.52 (1583.10-1685.94)	43.00 (37.00-48.99)	25.08 (19.44-30.72)
Phytoseiids	2006	0.99	1156.34	< 0.001	463.65 (434.83-492.46)	229.30 (220.94-237.65)	39.01 (32.96-45.05)
	2007	0.97	357.29	< 0.001	442.79 (414.89-470.69)	103.48 (90.42-116.55)	60.01 (47.98-72.04)
	2008	0.99	2825.84	< 0.001	546.53 (537.70-555.35)	93.84 (90.85-96.82)	32.74 (30.12-35.36)

[†] Curve parameters and their confident limits were not considered, as the cumulative population curve did not reach to a plateau.

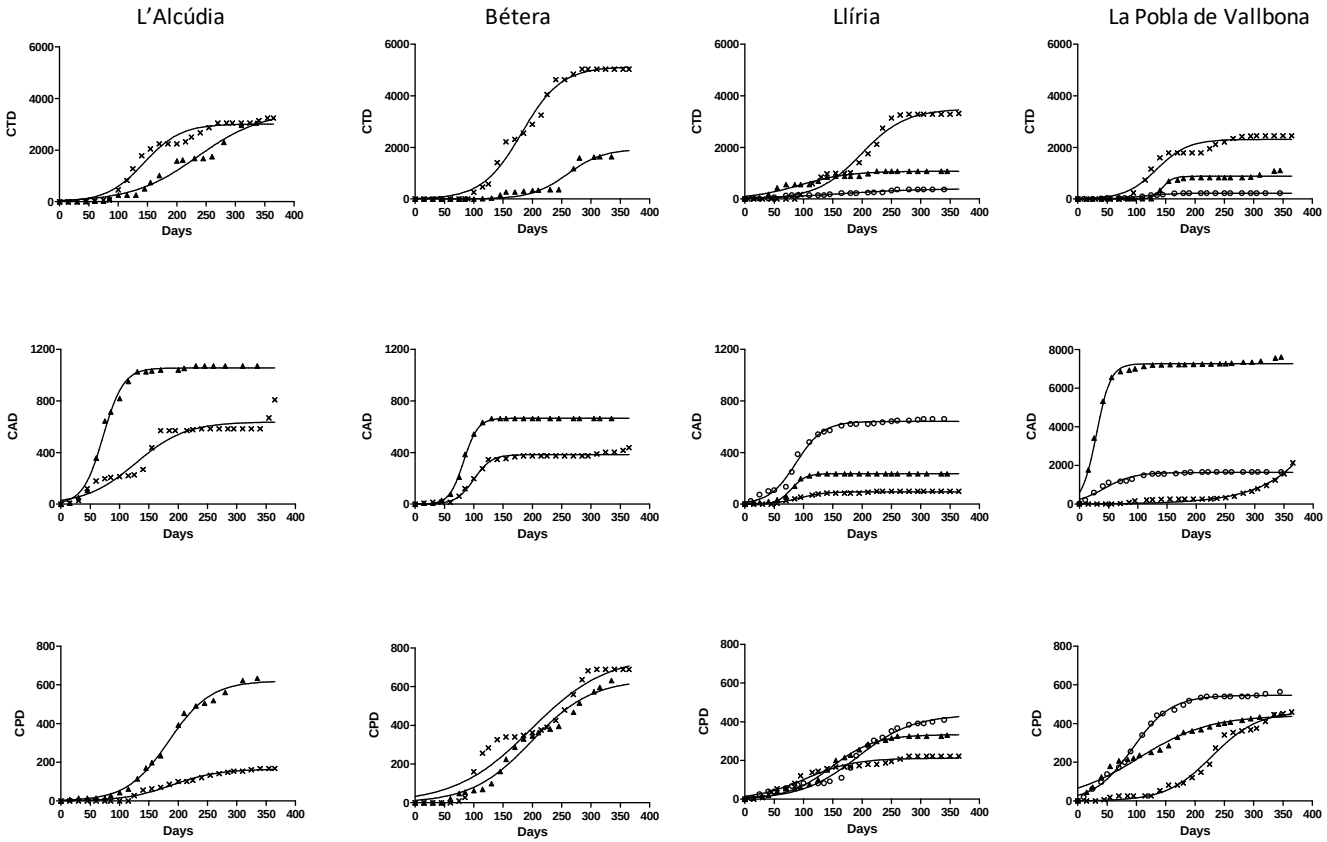

Figure 2.3 Cumulative *Tetranychus urticae* days (CTD), cumulative *Anaphothrips obscurus* days (CAD) and cumulative phytoseiid days (CPD) for *T. urticae*, *A. obscurus* and phytoseiids, respectively, and their estimated accumulation curves in *Festuca arundinacea* ground cover in 2006 (x), 2007 (▲), and 2008 (○) at four different locations (L'Alcúdia; Bétera; Llíria and La Pobla de Vallbona).

Table 2.2 Summary of significant differences between years at the curve's maximum value estimates (*a*) of *Tetranychus urticae*, *Anaphothrips obscurus* and phytoseiids at the four different locations. For all species and locations, *a* estimates were significant ($P < 0.001$).

	Location			
Species	L'Alcúdia	Bétera	Llíria	La Pobla de Vallbona
T. urticae	2006 = 2007	2006 > 2007	2006 > 2007 > 2008	2006 > 2007 > 2008
A. obscurus	2006 < 2007	2006 < 2007	2006 < 2007 < 2008	2007 > 2008
Phytoseiids	2006 < 2007	2006 = 2007	2006 < 2007 < 2008	2006 < 2007 < 2008

Table 2.3 Mean ± SE development time in days of brachypterous and macropterous forms of *Anaphothrips obscurus* reared on *Festuca arundinacea* leaves at 25 ± 1 °C, 70 ± 5% RH and a 16:8 h (L:D) photoperiod.

Wing morph	Egg	N1	N2	Prepupa	Pupa	Total immature
Brachyptera	7.50 ± 0.22 (n = 10)	2.92 ± 0.18 (n = 10)	2.89 ± 0.45 (n = 9)	1.44 ± 0.19 (n = 9)	2.20 ± 0.20 (n = 10)	16.80 ± 0.39 (n = 10)
Macroptera	7.32 ± 0.10 (n = 35)	2.82 ± 0.11 (n = 35)	2.87 ± 0.15 (n = 32)	1.19 ± 0.07 (n = 31)	2.69 ± 0.11 (n = 31)	16.76 ± 0.19 (n = 31)
Statistic†	218.50	191.00	116.00	174.50	90.50	159.00
P	0.327	0.852	0.484	0.131	0.027	0.976

Figures in brackets correspond to individuals where the molting could be actually observed and used for the calculation of development time.

Survival was 100% for all stages of brachyptera, as well as for N1 and N2 of macroptera. For the latter wing morph, one prepupa and three pupae died during their preimaginal development.

† Statistic: Mann-Whitney *U* test.

2.3.2 Laboratory assays

Development and survival on F. arundinacea

Development time for egg, N1, N2, prepupa and total development time were not significantly different between brachypterous and macropterous forms of *A. obscurus* on *F. arundinacea* leaves. Differences were found for the pupal stage only, which took longer for macropterous females (Table 2.3). Survival between successive instars/stages was high. It reached 100% in all cases except for the prepupal and pupal stages of macroptera, where it dropped to 97.1% and 91.2%, respectively. However, no significant differences between morphs were found ($P > 0.05$ in both cases).

Adult longevity and reproduction on F. arundinacea

Reproductive parameters of *A. obscurus* showed a wide dispersion that may have affected the lack of significance of the statistical analyses. Adult longevity ranged from around 1 week to more than 6 weeks, with a mean of 24.56 and 35.87 days for brachypterous and macropterous females, respectively (Table 2.4). The oviposition period was uniformly distributed along time with no clear oviposition peak in either morph (Figure 2.4). Brachypterous females had higher daily oviposition ($P < 0.001$) and fecundity ($P < 0.001$) but shorter oviposition time ($P = 0.06$) than macroptera, with most of the eggs laid during the first 30 days (Figure 2.4, Table 2.4). No differences were found between both morphs in pre- and post-oviposition times ($P > 0.05$, in both cases). Egg hatching was approximately 98% in both winged forms (Table 2.4) and no males were obtained in any case.

The intrinsic rate of increase (r_m), net reproductive rate (R_o) and finite rate of increase (λ) values for brachypterous females were significantly higher ($P < 0.001$) than for macropterous forms (Table 2.5). The opposite was true for the mean generation time (T) and the mean time span necessary to double the initial population (DT) values ($P < 0.001$).

Table 2.4 Reproductive parameters (mean ± SE) of brachypterous and macropterous forms of *Anaphothrips obscurus* on *Festuca arundinacea* leaves at 25 °C, 70% RH and 16:8 h (L:D).

Wing morph	Pre-oviposition (days)	Oviposition (days)	Post-oviposition (days)	Longevity (days)	Fecundity (eggs/female)	Oviposition rate (eggs/female/day)	Egg hatching
Brachyptera	1.21 ± 0.19	20.78 ± 4.09	2.22 ± 0.55	24.56 ± 4.60	99.89 ± 20.61	4.81 ± 0.18	0.98 ± 0.00
n; range	14[†]; 0-2	9; 4-36	9; 0-5	9; 6-41	9; 22-179	9; 0-12	15; 0.95-1.00
Macroptera	1.44 ± 0.10	32.23 ± 3.24	2.50 ± 0.32	35.87 ± 3.22	76.00 ± 9.42	2.45 ± 0.53	0.97 ± 0.01
n; range	27; 1-2.5	24; 4-53.5	24; 0-7	24; 7-56	24; 13-175	24; 0-7	27; 0.90-1.00
Statistic[‡]	U = 156.500[‡]	t = 1.950	t = 0.444	t = 1.893	GLM = 40.363	GLM = 192.455	U = 206.500
P	0.330	0.060	0.660	0.068	<0.001	<0.001	0.915

[†] One female data point was removed from the pre-oviposition analysis as it was considered as an outlier.
Differences in the number of females for different parameters were a result of escape or accidental death.
[‡] Statistic: Student's *t*-test, Mann-Whitney *U* test, generalized linear model using a Poisson distribution.

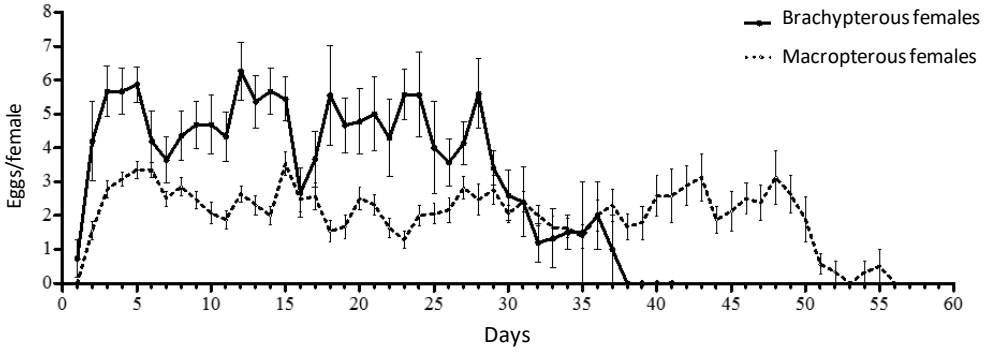

Figure 2.4 Age-specific fecundity (number of eggs produced per female and day ± SE) of *Anaphothrips obscurus* on *Festuca arundinacea* leaves at 25 ± 1 °C, 70 ± 5% RH and a 16:8 h (L:D) photoperiod.

Table 2.5 Life table parameters (mean ± SE) of brachypterous and macropterous forms of *Anaphothrips obscurus* on *Festuca arundinacea* leaves at 25 °C, 70% RH and 16:8 h (L:D).

Parameters	r_m [†] (♀/♀/day)	R_o (♀/♀)	T (days)	DT (days)	λ (♀/♀/day)
Brachyptera (n = 15)	0.171±0.001	81.215±0.784	25.741±0.054	4.058±0.006	1.186±0.001
Macroptera (n = 27)	0.144±0.001	65.585±0.293	28.967±0.256	4.800±0.004	1.155±0.001
Statistic[‡]	t = 108.273	t =18.666	t = -60.877	t = -109.462	U = 0.000
P	<0.001	<0.001	<0.001	<0.001	<0.001

Figures in brackets correspond to the number of individuals tested.

[†]The life table parameters were: the intrinsic rate of increase (r_m), net reproductive rate (R_o), mean generation time (T), mean time span necessary to double the initial population (DT), and finite rate of increase (λ).

[‡]Statistic: Student's *t*-tests; Mann-Whitney *U* test.

Feeding habits of A. obscurus

Anaphothrips obscurus females reared on *F. arundinacea* leaves survived four or five times longer than in the other treatments and the negative control (Table 2.6). *Anaphothrips obscurus* females did not feed on clementine leaves and *T. urticae* eggs, without significant differences in longevity with the negative control (Table 2.6). The color of the abdomen of *A. obscurus* exposed to those treatments was whitish and flattened contrary to those that developed on *F. arundinacea*, which were greenish and swollen (Figure 2.5). No predation symptoms were observed on *T. urticae* eggs. Furthermore, oviposition was observed in *F. arundinacea* leaves only. Similar results were found for individuals reared from N2 up to adulthood on the same substrates. Although adulthood was reached in all cases and there were no significant differences for development time between treatments, most of the individuals developing on a plastic tile without leaf substrate either escaped or died. As a result, only 30% of individuals originally introduced as N2 could reach adulthood and therefore used to calculate survival (Table 2.6).

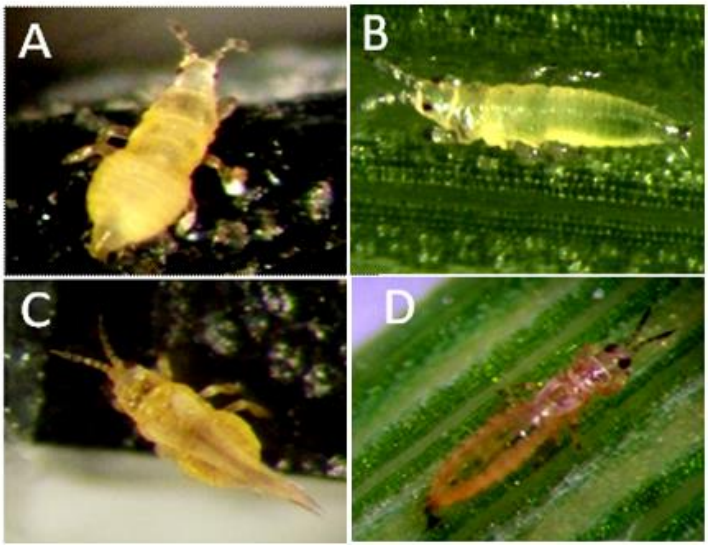

Figure 2.5 Second instar nymphs (A, B) and adult females (C, D) of *Anaphothrips obscurus* maintained either on a plastic tile (A, C) or on a *F. arundinacea* leaf unit (B, D). Those on plastic exhibit a whitish and flattened abdomen whereas those on *F. arundinacea* have a greenish, not flattened abdomen.

Table 2.6 Longevity (mean ± SE, in days) of second instar nymphs and adult females of *Anaphothrips obscurus* when feeding on different food sources.

	Adult	Second instar nymph	
Food source	Adult longevity	Developmental time	Adult longevity
Festuca arundinacea leaf	27.25± 4.52 a (4)	6.91 ± 0.67 a (11)	28.20 ± 0.58 a (5)
Tetranychus urticae eggs	7.22 ± 1.38 b (9)	6.25 ± 1.44 a (4)	4.00[†] (1)
Clementine leaf	5.30 ± 0.76 b (6)	6.00 ± 0.63 a (5)	4.80 ± 0.66 b (5)
Only water	4.91 ± 0.31 b (11)	8.17 ± 1.72 a (6)	3.00 ± 1.00 b (2)
Statistic[‡]			
F	34.24	0.59	427.66
df	3, 29	3, 25	2, 11
P	<0.001	0.660	<0.001

Figures in brackets correspond to the number of individuals tested.
[†] This single value was not included in the statistical analyses.
[‡] Statistic: ANOVA, Scheffé post-hoc comparison. Means within the same column followed by the same letter are not significantly different.

Anaphothrips obscurus *as prey for phytoseiids*

All phytoseiids tested were able to prey on *A. obscurus* N1 nymphs (Figure 2.6). Cross-correlations between predator species and number of *A. obscurus* consumed were statistically significant (Pearson's χ^2 = 18.263, P = 0.006). Prey consumption was similar for *E. stipulatus* and *N. californicus* (P = 0.612) but different from *N. barkeri* (P < 0.05). Most of *E. stipulatus* and *N. californicus* preyed on two nymphs (46 and 53%, respectively), whereas 60% of *N. barkeri* preyed on three. For *E. stipulatus*, no predation was observed in 10% of the arenas (Figure 2.6), whereas, for the other two phytoseiids, predation always occurred. Natural mortality of *A. obscurus* nymphs in the control arenas was 4%.

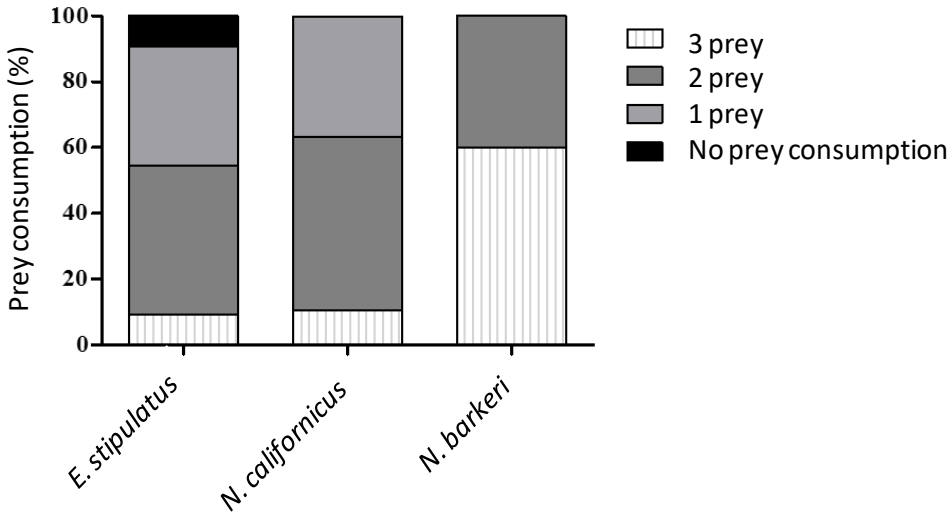

Figure 2.6 Percentage of single adult phytoseiid females eating up to three prey specimens when offered three first instar nymphs of *Anaphothrips obscurus* at 25 °C, 70% RH and 16:8 h (L:D) photoperiod for 24 h. For each phytoseiid species n = 15.

2.4 Discussion

Three main findings arose from the present study. First, during the study period, *T. urticae* populations decreased while those of *A. obscurus* and phytoseiids as a whole increased. Second, reproductive and demographic parameters for macropterous and brachypterous morphs of *A. obscurus* were different on *F. arundinacea* and higher than *T. urticae*, suggesting that competition between the two species could occur. Third, *A. obscurus* is not a predator of *T. urticae* but can be a prey for phytoseiids.

The growth rate of a population can be measured by the intrinsic rate of increase (r_m), which denotes the effects of certain climatic conditions and food provision on development, reproduction and survival. *Anaphothrips obscurus* females exhibit wing dimorphism (Hinds 1900; Nakao 1996). Based on the results of the present study, this wing dimorphism affects main biological traits, in the same way as that as observed when *A. obscurus* developed on corn (Jiang et al. 2015). Athough developmental time was affected slightly (only the pupal stage took longer for the macropterous form), total fecundity was higher for brachypterous than for macropterous females. As

expected, wing development affected the demographic parameters in the study of the classical trade-off between reproduction and dispersal (Zera and Mole 1994), with brachyptera showing higher intrinsic and finite rates of increase and net reproductive rates than macroptera.

In citrus orchards with a *F. arundinacea* ground cover, *A. obscurus* and *T. urticae* share *F. arundinacea* as a common host and hence they could be considered as potential interespecific competitors. Interspecific competition refers to the competition between two or more individuals of different species for some limiting resource as food, nutrients, or space. When one species is a better competitor, interspecific competition negatively influences the other one by reducing population sizes and/or growth rates, which in turn affects the population dynamics of the competitor (Begon et al. 2006). Even though macroptera exhibited a lower r_m than brachyptera (0.144 versus 0.171 per day, respectively) these values are slightly higher than those obtained for *T. urticae* reared on citrus leaves (0.110 - 0.150 per day) (Aucejo et al. 2004). These similar values could at least partially support our field observations (i.e., *A. obscurus* populations increased whereas *T. urticae* populations decreased), suggesting that *A. obscurus* could be actually directly and/or indirectly competing with *T. urticae*. Indeed, despite this thrips does not display any jerking behavior, when both species coexisted in our colonies *A. obscurus* prevented or reduced the settlement of *T. urticae*, suggesting that interference competition may occur. Furthermore, we cannot discard the possibility that exploitative competition is occurring concurrently when *A. obscurus* lays the eggs in the leaves and this modifies the quality and the availability of the plant substrate where they both live. Both mechanisms of competition could indeed explain the unidirectional ambulatory flux of *T. urticae* moving up from the ground cover to the canopy observed when a *F. arundinacea* cover was used in a clementine orchard (Aguilar-Fenollosa et al. 2016). Interestingly, these migrating *T. urticae* individuals were *F. arundinacea*-adapted and did not successfully establish in the citrus canopy (Aguilar-Fenollosa et al. 2016). Furthermore, because clementine mandarin plants are not a host for *A. obscurus* (extremely low adult longevity and no oviposition), the reduction of *T. urticae*

populations observed in the canopy by Aguilar-Fenollosa et al. (2011a) could be the result of a combination of interespecific competition in the cover and unsatisfactory settlement of *F. arundinacea*-adapted *T. urticae* individuals in the citrus canopy (Aguilar-Fenollosa et al. 2012, 2016). Nevertheless, this observation is compatible with apparent competition between *T. urticae* and *A. obscurus* arising from their condition of shared prey for phytoseiid mites (see below).

Hinds (1900) stated that *A. obscurus* could be not only phytophagous but zoophytophagous. Our study demonstrates that this species cannot feed on *T. urticae* eggs. Thus, in contrast to other pestiferous thrips species as *F. occidentalis* or *T. tabaci* that can feed on the eggs of this mite (Trichilo and Leigh 1986; Wilson et al. 1996; Milne and Walter 1998), the results of the present study confirm that the role of *A. obscurus* as a predator of *T. urticae* can be dismissed.

Laboratory experiments with the three phytoseiid species demonstrate that *A. obscurus* can be a potential prey for different life-style predatory mites. Therefore, the implementation of a *F. arundinacea* ground cover, as a sustainable pest management practice, may be not only providing refuge and pollen, but also supporting supplementary prey populations for key natural enemies of *T. urticae*. The presence of supplementary or alternative food sources is particularly important for generalist predators such as the phytoseiids considered in the present study and could help to maintain their populations when the main prey (in this case *T. urticae*) is scarce. Furthermore, successful dispersal of phytoseiids from the ground cover to the tree canopy has been observed in this system (Aguilar-Fenollosa et al. 2016) and this may account for the biological control exerted by these predators on *T. urticae* in the canopy. Such a phenomenon is not exclusive of this system. Phytoseiid dispersal has been documented in other woody perennial crops, including apples and peaches (Raworth et al. 1994; Wari et al. 2015). In both cases, *N. californicus* was found dispersing from either bare ground or grass and wild cover to the canopy. However, the simultaneous presence of two prey species can lead to a reduced control of the pest as a result of a dilution (larger groups dilute predation pressure among individuals in the group) or satiation (groups are larger than the maximum predator intake) effects

(Lehtonen and Jaatinen 2016). This situation deserves further research because it depends on predator preferences or maximum predator intake.

The results from this study increase our knowledge on the ecobiology of *A. obscurus*, a thrips species that can be considered as an *r*-strategist due to its early maturity, high fecundity and short generation time (Begon et al. 2006). Its presence in the ground cover can be beneficial because this thrips could be an alternative prey for relevant predators at the same time as competing with *T. urticae*. Further research focused investigating the functional and numerical response and prey preferences should address on whether phytoseiids in citrus orchards are able to maintain and enhance their populations preying on *A. obscurus* and whether this interaction could contribute to support apparent competition between *A. obscurus* and *T. urticae*.

Capítulo 3

When the ground cover brings guests: is *Anaphothrips obscurus* a friend or a foe for the biological control of *Tetranychus urticae* in clementines?

Una versión de este capítulo ha sido enviada para su publicación:

María Antonia Gómez-Martínez, Josep A. Jaques, María Victoria Ibáñez-Gual and Tatiana Pina (2017) When the ground cover brings guests: is *Anaphothrips obscurus* a friend or a foe for the biological control of *Tetranychus urticae* in clementines?

3.1 Introduction

Conservation biological control (CBC) has increased in importance as agricultural systems become more intensively managed and pesticide use becomes more restrictive (EU 2009). CBC practices usually provide shelter, refuge or alternative food to natural enemies resulting in enhanced biological control (Liang and Huang 1994; Landis et al. 2000; Boller et al. 2004; Jonsson et al. 2008). In Spanish clementine orchards, *Festuca arundinacea* Schreber (Poaceae) is used as ground cover to successfully manage some citrus key pests including the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), aphids and the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae). In the case of *C. capitata*, this cover increases the abundance of soil-dwelling predators (Monzó et al. 2011). For aphids, grassy covers promote the early arrival of aphid natural enemies (Gómez-Marco et al. 2016a, b). In the case of the clementine key pest *T. urticae*, the use of this cover improves its control by reducing the abundance of this mite in the tree canopies (Aguilar-Fenollosa et al. 2011a, 2012) and by enhancing the diversity and abundance of effective predatory species belonging to the Phytoseiidae family (Acari), both in the canopy and in the cover (Aguilar-Fenollosa et al. 2011b). *Festuca arundinacea* also provides alternative food (pollen, honeydew, different microarthropods as mites and thrips) to phytoseiids (Aguilar-Fenollosa et al. 2011a, b; Pina et al. 2012; Aguilar-Fenollosa and Jacas 2013; Gómez-Martínez et al. 2017). According to their feeding preferences, phytoseiids can be grouped from specialist selective predators of tetranychids to generalist omnivores (McMurtry and Croft 1997; McMurtry et al. 2013). The ability of some phytoseiids to exploit different food sources allows them to persist even when the prey they regulate is scarce or absent. Therefore, the maintenance of non-crop plants providing these alternative food sources may be key to enhancing biological control.

One abundant prey group brought into clementine orchards with the implementation of a *F. arundinacea* ground cover is thrips. These minute insects are scarcely present when growing clementines on bare soil but become highly abundant in the cover when growing clementines in association with *F. arundinacea* (Aguilar-

Fenollosa and Jacas 2013). Different species of Thysanoptera have been described as prey, either preferred or alternative, for the most abundant predatory mites associated with *T. urticae* (Rodríguez Reina et al. 1992; van Baal 2007; Magdy and El-Sayed 2009). *Anaphothrips obscurus* (Müller) (Thysanoptera: Thripidae) stands out among the most frequent and abundant thrips when *F. arundinacea* is used (Aguilar-Fenollosa and Jacas 2013). This thrips species feeds mainly on grasses (Poaceae) (Brohmer et al. 1966; Stannard 1968). In *F. arundinacea*, *A. obscurus* exhibits a high intrinsic rate of increase and short generation time, which could allow competition with *T. urticae* (Gómez-Martínez et al. 2017). Furthermore, Gómez-Martínez et al. (2017) demonstrated that in clementine orchards grown in association with *F. arundinacea*, *T. urticae* populations decreased while those of *A. obscurus* and phytoseiids as a whole increased in the cover.

Previous laboratory experiments demonstrated that some phytoseiids present in clementine orchards and feeding on *T. urticae* can attack *A. obscurus* as well (Gómez-Martínez et al. 2017). Two prey species sharing a natural enemy, even if they are separated in time or space, are related by indirect interactions such as apparent competition (Chailleaux et al. 2014). Holt (1977) called this indirect ecological interaction “apparent competition” because the dynamics it generates could resemble, to an observer unaware of the shared predator, that of direct competition, where a decline in one species coincides with an increase in the other. Apparent competition may have a number of consequences in biological control as prey species may affect each other. In the case of a shared predator, the presence or absence of an alternative prey species can affect the predator’s ability to control the target prey and therefore can modify trophic interactions, population dynamics and community structures (Muller and Godfray 1997; Harmon and Andow 2004; Morris et al. 2005). For instance, in California grapevines, significant reductions of the serious pest *Tetranychus pacificus* McGregor (Tetranychidae) occurred when both the apparent competitor *Eotetranychus villamettei* (McGregor) (Tetranychidae) and the predator *Metaseiulus occidentalis* (Nesbitt) (Phytoseiidae) were artificially released (Karban et al. 1994; Hanna et al. 1997). However, when the two greenhouse pests, the thrips *Frankliniella*

occidentalis (Pergande) (Thripidae) and the whitefly *Trialeurodes vaporariorum* (Westwood) (Hemiptera) shared the predators *Amblyseius swirskii* (Athias-Henriot) and *Euseius ovalis* (Evans) (Phytoseiidae), the presence of whiteflies did not affect thrips density. On the contrary, thrips presence dramatically reduced whitefly density (Messelink et al. 2008). In our context, phytoseiids in the *F. arundinacea* ground cover could be exploiting this thrips species as a new food source and, as a result, apparent competition between *T. urticae* and *A. obscurus* would appear. However, the effect of this thrips species on the regulation of *T. urticae* by phytoseiid mites remains unknown.

The suitability of a prey species for a specific predator may be unveiled by studying its functional response. This predator-prey specific response describes the relationship between individual prey consumption with food density (Solomon 1949; Holling 1959; Jeschke et al. 2002). Functional responses of predatory mites feeding on different pest species have been thoroughly studied (Fan and Petit 1994; Jalali et al. 2010; Fantinou et al. 2012; Yao et al. 2014). However, studies focusing on alternative non-pest prey species are rare. The impact of a generalist predator on a prey species will depend not only on the abundance and susceptibility of that prey species but also on those of the other species that share the predator and its prey preference (Eubanks and Denno 2000). Thus, prey preference may inform us about the success or failure of a natural enemy in a defined ecosystem.

Therefore, the objectives of this study have been to examine i) the ability of different life-style phytoseiids present in clementine orchards to feed on *A. obscurus* through functional response analysis, and ii) their feeding preferences for *T. urticae* and *A. obscurus.*

3.2 Materials and Methods

3.2.1 Mites and thrips colonies

Four species of predatory mites with different life-styles (*Phytoseiulus persimilis* Athias-Henriot, *Euseius stipulatus* (Athias-Henriot), *Neoseiulus californicus* (McGregor), and *Neoseiulus barkeri* Hughes) feeding on *T. urticae* as well as this herbivore were used in our assays. These species were initially collected in different citrus orchards near Castelló de la Plana (UTM: 30N, 753344.973 m E, 4430087.389 m N). The only exception was *N. californicus*, which was obtained from a commercial producer (Koppert Biological Systems; SPICAL®). *Euseius stipulatus* and *P. persimilis* were obtained from orange and clementine trees, respectively, whereas *N. barkeri* was collected from *F. arundi nacea* plants. The stock colony of *T. urticae* was obtained from clementine trees in the same area and maintained on lemons (Aucejo et al. 2003) (Figure 3.1).

Phytoseiids were reared following the method described by Overmeer (1985). In the case of *E. stipulatus*, the plastic tile used in the rearing unit was replaced by an upside-down set bean leaf. Pollen of *Carpobrotus edulis* (L.) N. E. Br (Aizoaceae) and a mixture of different stages of *T. urticae* obtained from a rearing maintained on lemon [*Citrus limon* (L.) Burm f. (Rutaceae)] (Figure 3.1) were regularly added to the rearing as a food source.

Figure 3.1 *Tetranychus urticae* rearing on lemon in the laboratory.

Anaphothrips obscurus individuals were originally collected from *F. arundinacea* plants grown in experimental plots at Universitat Jaume I (Castelló de la Plana, Spain). They were later maintained on the same type of plants (*F. arundinacea* 'Fórmula frutales y cítricos', Semillas Fitó S.A., Barcelona, Spain) grown in a pesticide-free greenhouse in the Institut Valencià d'Investigacions Agràries (IVIA) (Montcada, Valencia, Spain). The *A. obscurus* rearing unit consisted of detached *F. arundinacea* leaves set adaxially on a water-saturated sponge covered by filter paper in a plastic

container (17 × 12.5 × 7.5 cm). Both leaf ends were fixed with wet cotton strips, which prevented the escape of the thrips (Figure 3.2).

All stock colonies were maintained in a climatic chamber at 25 ± 1 °C, 70 ± 5% RH and a 16:8 h (L:D) photoperiod.

Figure 3.2 *Anaphothrips obscurus* rearing unit.

3.2.2 Experimental set-up

Functional response was assessed in Petri dishes (9 cm diameter) with a hole in the lid (6 cm diameter) covered with anti-thrips mesh (14 × 95 µm). Petri dishes were filled with water and provided with a plastic tile (6 × 3 × 1.5 cm) fixed to the center of the Petri dish with glue. A 6 cm long fragment of a *F. arundinacea* leaf was placed adaxially on the top of the plastic tile and both leaf ends were fixed with wet cotton strips. Tile borders were covered with wet paper to supply water to thrips and prevent them from escaping from the experimental arena (Figure 3.3 A). Dishes were sealed with Parafilm® (ParaFILM®; Bemis Company, Inc. Neenah, Wisconsin).

The experimental units used for the predation assays with *P. persimilis* immature stages consisted of a PVC plate (80 × 35 × 3 mm) containing two 15 mm in diameter chambers (Figure 3.3 B). The bottom of these chambers was covered by a fine mesh glued to the plate and closed on the upper side by a microscope slide held in place by two rubber bands (Schausberger 1997).

The choice experiment was performed using the T-shaped cages described by Schausberger and Hoffmann (2008). They consisted of a PVC plate (same dimensions as before) containing three circular chambers connected through a T-shaped excavation of 2 mm wide and 10 and 5 mm long for the horizontal and vertical bars, respectively. The two chambers located at the extremes of the horizontal bar were 15 mm in diameter and the one located at the end of the vertical bar was 5 mm. The cage was closed as before (Figure 3.3 C).

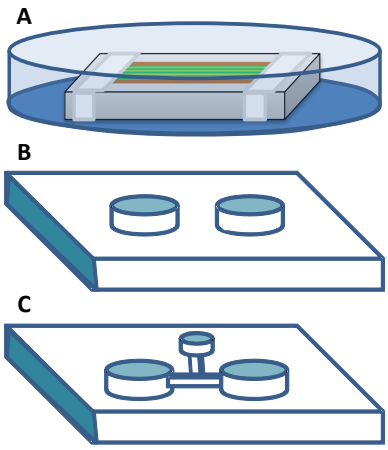

Figure 3.3 Experimental units used in the assays of: functional response (A), predation of *P. persimilis* immature stages (B) and prey preference (C).

3.2.3 Experimental design

Functional response

The functional response of each predator species when offered *A. obscurus* nymphs was investigated in different assays. First instar nymphs (N1) are the most vulnerable thrips stage (Madadi et al. 2007) and this was the prey stage chosen for these experiments. To obtain N1, cohorts of eggs less than 24 h old were established in the rearing unit described above. Newly hatched N1 were transferred to the experimental arena with a fine camel hair brush and the following densities were considered: 1, 3, 5, 10 and 20 N1. Two extra densities of 30 and 40 N1 were considered for *N. californicus* and *N. barkeri*, respectively. Once *A. obscurus* nymphs stopped moving, one gravid phytoseiid female at its maximum peak of oviposition rate (2-3 day old) was introduced into each arena. These females were obtained from less than 24 h old egg cohorts. To standardize the response, all phytoseiid females were starved for 24 h before the onset of the assay. During this period, they had access to water only. Up to 34 replicates per prey density and phytoseiid species were considered. Furthermore, up to 28 arenas without predator for each prey density were used as controls. Thrips killed during the experiment were not replaced (prey depletion method). After 24 h, the numbers of N1 alive, killed by predation and dead by other

undetermined reasons were recorded. As phytoseiids suck out the body fluids of their prey, empty N1 corpses were taken as evidence of predation. Additionally, the number of eggs laid by each predator and at each prey density offered was counted. Eventually, phytoseiids were slide mounted in Hoyer's medium (Gutierrez 1985) to confirm their identity. These assays were developed in a climatic chamber at 25 ± 1 °C, 70 ± 10% RH and 16:8 h (L: D) photoperiod.

Phytoseiulus persimilis *predation experiment*

Due to the results obtained when we tried to determine the functional response of *P. persimilis* adults (see results, section 3.3), further predation experiments were conducted with immature stages of *P. persimilis*. To determine the ability of *P. persimilis* to exploit *A. obscurus*, protonymphs and deutonymphs starved for 24 h were individually transferred to the arena where they were offered 10 N1 thrips. As in the previous experiments, the numbers of N1 alive, killed by predation and dead by other undetermined reasons were recorded the following day. Environmental conditions were the same as above.

Prey preference

One *T. urticae* deutonymph and one *A. obscurus* N1 were set at the center of each large 15 mm circular chamber and simultaneously offered to the phytoseiid, which was released in the center of the small 5 mm circular chamber (Figure 3.3 C). Both preys had been previously killed (5 min at -80 °C) to avoid any movement between chambers during the experiment. Adult females of *N. californicus*, *N. barkeri* and *E. stipulatus* and deutonymphs of *P. persimilis* were used in these experiments. Phytoseiid activity was continuously monitored using a binocular microscope. The position of the predator, the first and successive feeding events and the time spent feeding on each prey were recorded for 120 min at room conditions. The initial position of each prey was consistently exchanged among replicates to avoid any inadvertent positional effect. Each specimen was used only once and then discarded. Cages were cleaned with 70% ethanol before each use. All predators came from cohorts established on *F. arundinacea* rearing units with *C. edulis* pollen and a similar proportion of *A. obscurus*

and *T. urticae* as food supply. Because *E. stipulatus* was unable to complete its development in this rearing system, newly emerged adults from a cohort fed as the stock colonies were maintained in this new system for 5 days prior to the experiment.

3.2.4 Data analysis

Predation was corrected for control mortality using the formula proposed by Xia et al. (2003):

$$N_e = N_0 \frac{N_d - N_c}{N_0 - N_c} \quad (1)$$

where N_e represents the number of prey killed, N_0 the initial number of prey, N_d the number of prey eaten and dead in the treatment and N_c the number of prey dead in the control. Functional response of each predator species was then analyzed in two steps: i) determination of functional response type, and ii) estimation of the parameters of the fitted curve. A cubic logistic regression (2) of the relative proportion of N1 preyed was performed to evaluate the shape of the functional response curve that best fit the data for each phytoseiid species (Juliano 2001):

$$\frac{N_e}{N_0} = \frac{exp(P_0 + P_1 N_0 + P_2 N_0^2 + P_3 N_0^3)}{1 + exp(P_0 + P_1 N_0 + P_2 N_0^2 + P_3 N_0^3)} \quad (2)$$

where N_e represents the number of prey eaten; N_0 represents the initial number of prey; and P_0, P_1, P_2 and P_3 represent the estimated intercept, linear, quadratic and cubic coefficients, respectively. A linear coefficient not significantly different from 0 indicates a type I functional response, a significant negative linear coefficient indicates a type II response, while a significant positive linear term indicates a type III response.

Once the functional response type was determined, average data were further fitted by iteration to Rogers random predator equation (Rogers 1972) (3), which takes into account predator handling time and prey depletion over time:

$$N_e = N_0 \{1 - exp[a'(T_h N_e - T)]\} \quad (3)$$

where, as before, N_e represents the number of prey eaten, N_0 represents the initial density of prey, T represents total time available for attack and the estimated parameters a' and T_h represent the attack constant and handling time, respectively. The attack constant relates the predator-prey encounter rate with prey density and the handling time includes all the time the predator spends with the prey being unable to attack another prey (Juliano 2001). The 95% confidence intervals of the estimated parameters of the functional response (a' and T_h) were used to evaluate differences between phytoseiid species (Juliano 2001). The maximum predation rate was estimated from T/T_h (Hassell 1978). The value of a'/T_h indicates the effectiveness of predation. Data were analyzed using R 3.3.1 (R Core Team 2016).

The average number of eggs laid during the first 24 h by the three phytoseiids was linearly regressed against the number of nymphs offered. Analyses were performed using STATGRAPHICS Centurion XVI ver. 16.1.18.

The effect of prey position on prey choice and the effect of the first prey species attacked on the probability of a second attack and on the second prey species attacked were analyzed by Pearson's χ^2 test with Yates' continuity correction. Prey preference was analyzed by a one-sample proportion test with continuity correction, and the time feeding on each prey was analyzed by Welch's two sample *t*-test. These data were analyzed using R 3.3.1 (R Core Team 2016).

3.3 Results

Functional response

Neoseiulus californicus, *N. barkeri* and *E. stipulatus* showed a type II functional response, as determined by a negative and significant estimated linear coefficient P_1 (Table 3.1). The number of prey eaten by these predators increased with increasing prey density (Figure 3.4). Rogers random predation equation (3) fit the observed data for all phytoseiid species with determination coefficients approximately 0.98 (Table 3.2).

Estimates of the attack constant and handling time and the 95% CI for each predator are shown in table 3.2. As 95% CI did not overlap, the attack constant of *N. barkeri* was significantly higher (1.766 ± 0.339 $days^{-1}$) than those of *N. californicus* and *E. stipulatus* (0.542 ± 0.151 and 0.711± 0.175 $days^{-1}$, respectively). The attack constants of these two species were not significantly different from each other. On the contrary, handling time estimates exhibited significant differences between the three species. *Neoseiulus barkeri* spent less time handling *A. obscurus* N1 (0.080 ± 0.003 days) than *N. californicus* (0.169 ± 0.019 days), and the latter spent less time handling the prey than *E. stipulatus* (0.264 ± 0.021 days). The estimated maximum number of N1 preyed on by *N. barkeri* was 11.33, and this value is 1.9 and 2.9 times higher than *N. californicus* and *E. stipulatus*, respectively (Table 3.2). At low prey densities, which may be taken as indicative of prey searching efficiency in a worst case scenario, *N. barkeri* always consumed all prey offered at a density of 1 and an average of 2.60 prey at a density of 3. Conversely, the average of N1 preyed by *N. californicus* and *E. stipulatus* was 0.68 and 1.62 at densities of 1 and 3, respectively.

The functional response could not be described for *P. persimilis* females due to the low number of N1 preyed independently of N1 density. Predation by *P. persimilis* on *A. obscurus* N1 was positive only in 3 out of 33 replicates and only one prey was consumed. Furthermore, 36% of *P. persimilis* females died during the experiment, and the surviving females did not lay any eggs.

Table 3.1 Results of logistic regression analyses of the proportion of *A. obscurus* first instar nymphs eaten by adult females of *Neoseiulus californicus*, *N. barkeri* and *Euseius stipulatus* on initial density of prey offered (predator species are listed in decreasing order of diet specialization according to McMurtry et al. 2013).

Phytoseiid species	Parameter	Estimate	SE	*t*	df	*P*
N. californicus	Intercept	1.211	0.343	3.528	107	< 0.001
	Linear	-0.465	0.126	-3.692	107	< 0.001
	Quadratic	0.027	0.010	2.727	107	0.007
	Cubic	-0.001	0.000	-2.326	107	0.022
N. barkeri	Intercept	2.898	0.477	6.073	102	< 0.001
	Linear	-0.305	0.107	-2.843	102	0.005
	Quadratic	0.009	0.006	1.348	102	0.181
	Cubic	0.000	0.000	-0.869	102	0.387
E. stipulatus	Intercept	1.215	0.308	3.950	115	< 0.001
	Linear	-0.327	0.079	-4.116	115	< 0.001
	Quadratic	0.009	0.004	2.160	115	0.033
	Cubic					

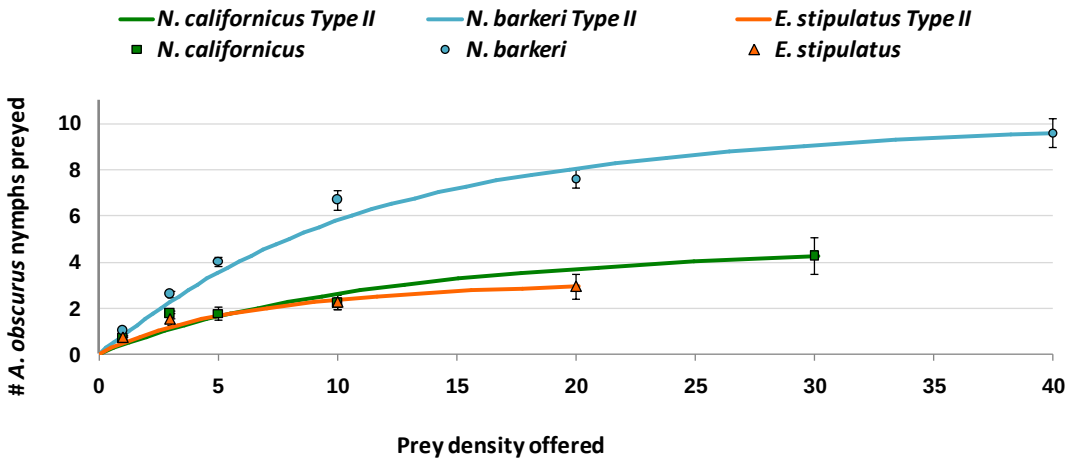

Figure 3.4 Functional response of *Neoseiulus californicus*, *N. barkeri* and *Euseius stipulatus* to different densities of *Anaphothrips obscurus* first instar nymphs during 24 h. Symbols represent the observed mean ± SE. The lines represent the functional response curves predicted from the model (random predation equation with prey depletion).

The number of eggs laid per day increased linearly as a function of the nymphs offered, with a positive and highly significant correlation in *N. californicus* (R^2 = 0.704, P = 0.023) and *N. barkeri* (R^2 = 0.647, P = 0.033) (Figure 3.5). The number of eggs laid by *E. stipulatus* was independent of the nymph density (P = 0.974).

Table 3.2 R^2 values and parameters of the functional response, attack rate constant (a') (days^{-1}) and handling time (T_h) (days), estimated by the random predator equation for *Neoseiulus californicus*, *N. barkeri* and *Euseius stipulatus* (predator species are listed in decreasing order of diet specialization according to McMurtry et al. 2013).

	FR	R^2 [a]	a' (days^{-1}) Mean ± SE [b]	T_h(days) Mean ± SE	T/T_h	a'/T_h
N. californicus	Type II	0.977	0.542 ± 0.051 (0.245 - 0.838)	0.169 ± 0.019 (0.131 - 0.207)	5.90	3.20
N. barkeri	Type II	0.985	1.766 ± 0.339 (1.102 - 2.429)	0.088 ± 0.003 (0.082 - 0.095)	11.33	20.01
E. stipulatus	Type II	0.984	0.711 ± 0.178 (0.362 - 1.061)	0.264 ± 0.021 (0.224 - 0.305)	3.78	2.69

[a] R^2 are the coefficients of determination obtained from R^2 = 1 - (sum of squares of residuals/total sum of squares).
[b] Values in parenthesis are 95% confidence intervals.

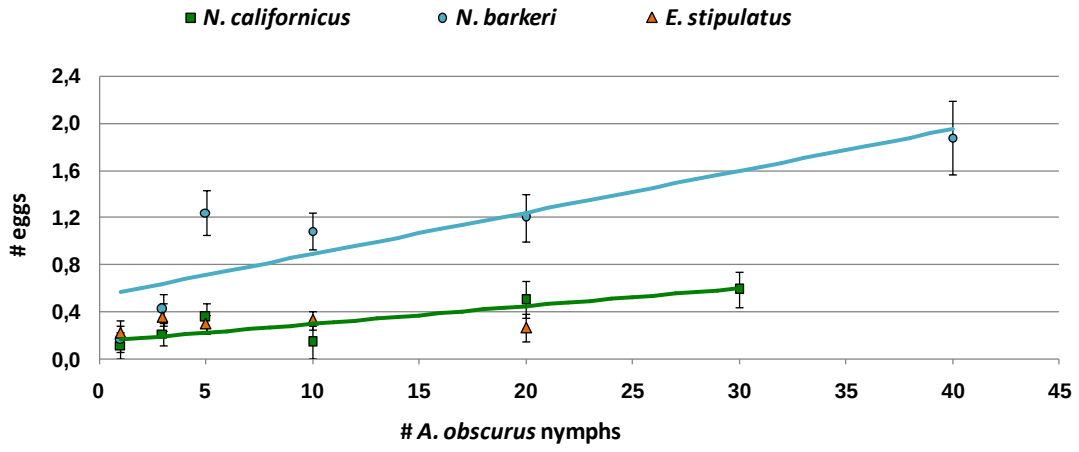

Figure 3.5 Oviposition by *Neoseiulus californicus*, *N. barkeri* and *Euseius stipulatus* when offered different densities of *Anaphothrips obscurus* N1 during 24 h. Symbols represent the observed number of eggs (mean ± SE). The blue line represents the regression line predicted for *N. barkeri* (y = 0.532 + 0.035x), and the green line represents the regression line for *N. californicus* (y = 0.143 + 0.015x).

Phytoseiulus persimilis *predation experiment*

Phytoseiulus persimilis protonymphs and deutonymphs were more aggressive than adult females, as prey attacks occurred more frequently. All protonymphs (n = 14) and deutonymphs (n = 9) preyed on *A. obscurus* N1. Mean predation rates for protonymphs (0.43 ± 0.04) and deutonymphs (0.47 ± 0.04) were not significantly different (Mann-Whitney *U* test, $P > 0.05$). Mortality during the assay was null for protonymphs and 18% for deutonymphs.

Prey preference

Feeding behavior was different for each predator tested. More than one half of the *E. stipulatus* (n = 61) and almost half of the *P. persimilis* specimens tested (n = 35) did not feed on either prey species. This percentage dropped to 10 and 14% for *N. barkeri* (n = 66) and *N. californicus* (n = 44), respectively. Prey location within the arena did not affect first prey choice (Pearson's χ^2 test with Yates' continuity correction, $P > 0.05$ in all predators).

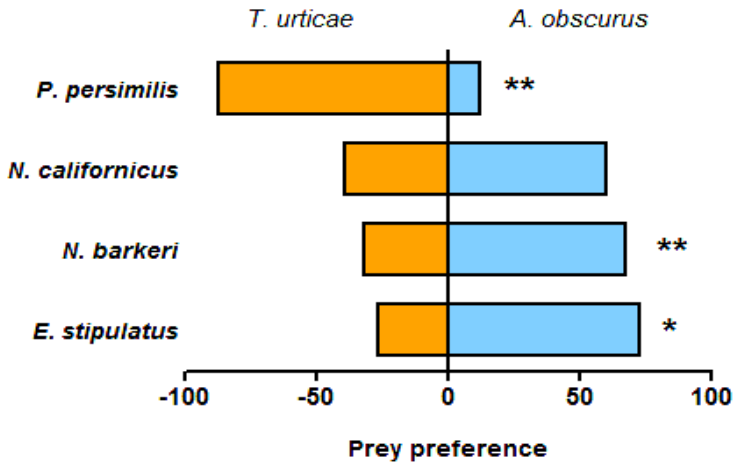

Figure 3.6 Percentages of each phytoseiid species that have chosen *Tetranychus urticae* or *Anaphothrips obscurus* as prey when offered simultaneously. Significant differences are based on a one-sample proportion test with continuity correction. One asterisk (*) represents $P < 0.05$; two asterisks (**) represent $P < 0.01$ (predator species are ordered in decreasing order of diet specialization according to McMurtry et al. 2013).

All predator species, except *N. californicus*, preferentially fed on one prey species. *Phytoseiulus persimilis* showed a strong preference for *T. urticae* (χ^2 = 7.563, *P* = 0.006), whereas *N. barkeri* and *E. stipulatus* preferred *A. obscurus* (χ^2 = 6.780, *P* = 0.009; χ^2 = 4.654, *P* = 0.031, respectively) (Figure 3.6). These preferences were also reflected in the time spent feeding on each prey species (Table 3.3). A second feeding event was observed in 68.4% of *N. californicus*, 56.3% of *P. persimilis*, 35.6% of *N. barkeri* and 30.8% of *E. stipulatus*. Only for *N. barkeri* did the identity of the first prey species chosen determine the second feeding event (χ^2 = 7.599, *P* = 0.006). The highest number of second feeding events was observed when *T. urticae* was the first prey (χ^2 = 15.429, *P* < 0.001). Furthermore, prey change during the second feeding event was observed for *N. barkeri* and *N. californicus* (χ^2 = 0.805, *P* = 0.045; χ^2 = 7.583, *P* = 0.006, respectively) whereas *P. persimilis* always fed on the same prey (*T. urticae*) even when this prey had been previously handled and partially or totally consumed. *Euseius stipulatus* usually fed on the same prey species in the second feeding event even though differences were not significant (χ^2 = 2.133, *P* = 0.144).

Table 3.3 Mean time ± SE (min) spent by each phytoseiid species feeding on a *Tetranychus urticae* deutonymph or an *Anaphothrips obscurus* N1 (predator species are listed in decreasing order of diet specialization according to McMurtry et al. 2013).

	Time feeding on *T. urticae*	Time feeding on A. *obscurus*	t^a	df	*P*
Phytoseiulus persimilis	13.66 ± 13.93	2.65 ± 3.32	2.502	8.297	0.018
Neoseiulus californicus	15.25 ± 6.13	20.17 ± 4.90	2.550	23.033	0.009
Neoseiulus barkeri	11.26 ± 7.04	14.83 ± 4.87	1.989	26.488	0.028
Euseius stipulatus	8.14 ± 5.49	19.89 ± 6.85	4.467	13.693	< 0.001

[a] Significance level set at α = 0.05; Welch two sample *t*-test.

3.4 Discussion

Understanding the interactions between pests and their natural enemies is essential for a successful pest management program. In the present work, we have demonstrated that most phytoseiid species exploiting *T. urticae* can also exploit *A. obscurus* and therefore the occurrence of apparent competition between these herbivores can be anticipated. Below, we will discuss the relationship between each phytoseiid species, *A. obscurus* and *T. urticae*, and their potential implications on the biological control of this pest mite in clementine orchards with a *F. arundinacea* ground cover.

Phytoseiulus persimilis is considered a *Tetranychus* spp. specialist predator (McMurtry et al. 2013). It can also feed on thrips as *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), a food resource allowing full immature development (Walzer et al. 2004). Although in our assays *P. persimilis* immature stages fed on *A. obscurus,* they exhibited a strong preference for *T. urticae*. Indeed, in case of *T. urticae* depletion, they still preferred to repeat feeding on *T. urticae* corpses rather than changing to *A. obscurus*. This tetranychid specialization was even stronger for adult females, which rarely fed on the thrips (only in 3 out of 33 replicates). Indeed, one third of them died without feeding on it. These results are in agreement with Walzer et al. (2004) who found that *P. persimilis* diet specialization changes with development. Therefore, the availability of *A. obscurus* as an alternative prey should not negatively affect the natural regulation of *T. urticae* by *P. persimilis*. Rather, the presence of this thrips in the cover might enhance biological control of *T. urticae* due to a reduction of *P. persimilis* immature mortality. This reduction could result from preying, even in low numbers, on the non-preferred prey. As a consequence, increased predation could be expected from a higher number of immature stages reaching the adult stage (Sabelis and van Rijn 2006). As a successful dispersal of phytoseiids from ground cover to the tree canopy has been observed in this system (Aguilar-Fenollosa et al. 2016), a better regulation of *T. urticae* both in the canopy and in the ground cover could be expected.

Neoseiulus californicus is considered a tetranychid specialist predator (McMurtry and Croft 1997; McMurtry et al. 2013). Moreover, it has been described as a biological control agent of some pestiferous thrips species (Walzer et al. 2004; van Baal et al. 2007). Herein we have demonstrated that *N. californicus* benefits from feeding on *A. obscurus* by increasing prey consumption (type II functional response) and oviposition with increasing thrips densities. Determining the suitability of a given prey for predator reproduction by starving the predators for one day and subsequently feeding them for another day on the target prey could be a priori inadequate or insufficient, as predators do not immediately convert the ingested food into eggs. However, taking into account that all individuals had the same feeding status at the onset of the assay (i.e., ad libitum feeding and standard 24 h of starvation), the increase observed in oviposition with increasing thrips density suggests that this is not an artifact. These aptitudes (prey consumption and oviposition) should allow the maintenance and augmentation of *N. californicus* populations solely feeding on *A. obscurus*. Furthermore, as *N. californicus* did not show any preference for the two herbivores, a prey switch would be expected in response to the relative availability of *A. obscurus* and *T. urticae*, as it often happens in non-specific entomophagous species (Murdoch 1969; Murdoch and Oaten 1975; Holt 1977, Holt and Lawton 1994). As *T. urticae* is present in both the *F. arundinacea* cover and the clementine canopy (Aguilar-Fenollosa et al. 2011a) whereas *A. obscurus* is mostly found in the cover (Aguilar-Fenollosa and Jacas 2013), *N. californicus* would be expected to prey randomly on both prey species in the cover and mostly on *T. urticae* in the tree canopies. Therefore, the presence of *A. obscurus* in clementine orchards could result in higher *N. californicus* densities and stronger predation pressure on both herbivores, thus benefiting *T. urticae* biological control by apparent competition. If this was the case, it would be similar to that reported by Liu et al. (2006) in apples, where *T. urticae* populations were reduced by the addition of the apparent competitor *Eotetranychus pruni* Oudemans with *Euseius finlandicus* (Oudemans) as a shared predator.

Neoseiulus barkeri is a generalist predator from soil/litter habitats (McMurtry et al. 2013) that has been reported as a biological control agent of *T. urticae* (Karg et al.

1987, Bonde 1989, Fan and Petit 1994) and used for the biological control of thrips (Ramakers and van Lieburg 1982; Hansen 1988). In our study, *N. barkeri* presented a type II functional response, which is in agreement with the results of Fan and Petit (1994) when this species fed on *T. urticae*. Among the species considered in this study, *N. barkeri* was the most effective predator of *A. obscurus* as it exhibited the highest attack constant and the lowest handling time. Furthermore, an increment in oviposition was observed with increasing prey densities. In the prey preference assays, *N. barkeri* preferred *A. obscurus* as a first prey and attacked the other species in the second attack. When a shared predator prefers the non-pest prey species, the potential of negative indirect interactions (i.e., apparent competition) to enhance the biological control of the pest are reduced (Chailleux et al. 2014). However, as *A. obscurus* and *N. barkeri* are rare in the clementine canopy, especially when the trees are grown in association with *F. arundinacea* (Aguilar-Fenollosa et al. 2011a, b), these negative interactions may not be relevant for the biological control of *T. urticae*.

Euseius stipulatus is a pollen-feeding generalist predator able to feed on microarthropods and vegetal or animal exudates (McMurtry et al. 2013). In clementine orchards, this species can feed on *T. urticae* and *P. citri* (Pérez-Sayas et al. 2015). Moreover, the populations of this omnivore can boost when pollen is available (Pina et al. 2012) and outcompete more efficient *T. urticae* specialist phytoseiids (*P. persimilis* and *N. californicus*) (Abad-Moyano et al. 2010a, b). For this reason, wild cover crops, producing an abundant pollen supply throughout the year, are not considered suitable for the management of *T. urticae* in citrus orchards (Aguilar-Fenollosa et al. 2011b). *Euseius stipulatus* has also been described feeding on thrips species as *F. occidentalis* (Rodriguez-Reina et al. 1992). In this study, this phytoseiid preferred to feed on *A. obscurus* and increased prey consumption as thrips density increased (type II functional response). Despite the fact that *E. stipulatus* laid some eggs when feeding on *A. obscurus*, oviposition could not be related to prey density, same as when *T. urticae* was the prey (Ferragut et al. 1987; Abad-Moyano et al. 2009). Therefore, both prey species alone are unsuitable for increasing *E. stipulatus* populations. This might preclude the occurrence of apparent competition and, importantly, the buildup of high

populations of this predator, which is usually accompanied by a reduction and even the disappearance of the most efficient *T. urticae* predators from clementine orchards (Aguilar-Fenollosa et al. 2011b).

Up until now, we have discussed the effects of the presence of *A. obscurus* in the cover on *T. urticae* regulation in clementine orchards at a predator species-specific level. However, we have not considered how this presence could affect interactions within the mite predatory guild. At the third trophic level, competition and intraguild predation may alter the species composition and therefore affect herbivore suppression (Polis et al. 1989, Polis and Holt 1992, Rosenheim 1998). Additional prey may change the outcome of competition and intraguild predation by promoting one species over the others (Sabelis and van Rijn 2006). Superior intraguild predators in Spanish clementine orchards are mainly *E. stipulatus* (Abad-Moyano et al. 2010a, b) and *N. barkeri* (Momen 2010). The former occurs in the canopy and the cover, whereas the latter is mostly found in the cover (Aguilar-Fenollosa et al. 2011b). In this study, both species showed a marked preference for *A. obscurus*. They have also been described to competitively displace the *Tetranychus* spp. specialist predator *P. persimilis* (Kabicek 1995), even in clementines (Abad-Moyano et al. 2010a, b). Interestingly, *N. californicus,* which could predate effectively on *A. obscurus* and probably increase its populations feeding on this thrips species, can also outcompete *P. persimilis* (Abad-Moyano et al. 2010a, b). Therefore, when using a *F. arundinacea* cover the disappearance of *P. persimilis* from the system would be anticipated. Nevertheless, Guzmán et al. (2016) pointed at the presence of a shared resource as a key factor to reduce, or even prevent, intra-guild predation in the phytoseiids, which may not be as common as previously thought within this family. Consequently, the presence of large amounts of *A. obscurus* in the *F. arundinacea* cover during the whole season could diminish intraguild predation in the system and result in better biological control of the target pest (*T. urticae*). Indeed, field results showing that *P. persimilis* is consistently present in clementine orchards grown in association with *F. arundinacea* (Aguilar-Fenollosa et al. 2011b) may be partly due to the presence of this alternative food source for *E. stipulatus*, *N. californicus* and *N. barkeri* in the cover. These results

note the importance of the type of the alternative food source for the success of the biological control of a shared pest prey. Contrary to *A. obscurus*, high quality pollen allows the boost of *E. stipulatus* populations (Pina et al. 2012). As this type of pollen is available during the whole year when clementine trees are grown in association with a resident (not sown) cover, pollen availability both in the cover and in the canopy allows the populations of *E. stipulatus* to outcompete the specialist *P. persimilis*. However, the low quality of pollen produced by *F. arundinacea* only once in spring does not allow for such an explosion of *E. stipulatus*. This fact, together with the provision of *A. obscurus* during the whole season in the cover only, is probably key for the success of the implementation of a *F. arundinacea* cover in clementine orchards as a means to control *T. urticae*. Now we can answer our initial question and state that *A. obscurus* is actually a friend, which allows better regulation of the citrus key pest *T. urticae*.

Capítulo 4

Multiplex PCR for understanding trophic webs including phytoseiid mites

4.1 Introduction

Successful biological control of agricultural pests requires a thorough knowledge of the trophic relationships between second and third trophic levels (i.e., phytophagous pests and their natural enemies). However, the study and understanding of these interactions can be highly challenging, especially when generalist predators that frequently exploit multiple prey species may interact with the target pest and other alternative preys. The only conclusive evidences of predation are direct observation of prey consumption by a predator and identification of prey remains within the predator gut or feces. Confirming trophic links under unaltered field conditions in some cases is nevertheless almost impossible, especially when working on minute species with cryptic morphology and/or lifestyle (i.e., nocturnal, hidden or elusive predation habits). Moreover, in some cases such as mites and thrips, microscopic analysis of the predator gut content should be discarded as they engage into extra-oral digestion and/or fluid feeding (Chant 1985; Sunderland et al. 1987; Breene et al. 1990; Agustí et al. 2003; Juen and Traugott 2007). A large amount of studies has demonstrated that molecular DNA techniques, based on genetic markers, can overcome these limitations. They enable the identification of the predator and the prey present in the gut content, and make possible unveiling these trophic interactions (Symondson 2002; Sheppard and Harwood 2005; Gariepy et al. 2007; King et al. 2008, Furlong 2015; González-Chang et al. 2016; Gurr and You 2016; Kamenova et al. 2017).

Since 2002, the use of molecular tools for disentangling food webs and implementing biological control has increased with the dominance of PCR-COI based markers (González-Chang et al. 2016). However, other markers as ITS have also proved useful for this purpose (Hoogendoorn and Heimpel 2001; Gariepy et al. 2007; King et al. 2008; González-Chang et al. 2016). This increase is related to the simplicity of these techniques associated with relatively low costs of development, the availability of DNA sequences, and well-developed primers for a growing number of agricultural pest species including tetranychids (Acari: Prostigmata) and thrips (Insecta: Thysanoptera). The number of studies related with the phylogeny and trophic relationships of these two groups has significantly augmented in recent years and this has resulted in an

increase of the availability of DNA sequences (Brunner et al. 2002; Rugman-Jones et al. 2006; Glover et al. 2010; Buckman et al. 2013; Pérez-Sayas et al. 2015). Moreover, the implementation of multiplex PCR techniques, in which the DNA of multiple target species is amplified simultaneously, can reveal trophic interactions between multiple pest species and their associated predator guild (King et al. 2011; Pérez-Sayas et al. 2015). This has led us to a better understanding of the effects of natural enemies on pest regulation. Multiplex PCR is especially useful in minute arthropods as mites because the amount of DNA that can be extracted from a single individual is limited and restricts the number of rounds of amplification (Navajas et al. 1998; Pérez-Sayas et al. 2015).

In Spanish citrus crops, especially in clementine mandarins (*Citrus clementina* Hort. ex. Tan.), the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is considered a key pest. Many aspects of the biology of this species, as its rapid development, high fecundity, haplo-diploid sex determination and the plasticity of its genome, facilitate rapid evolution of pesticide resistance (Van Leeuwen et al. 2010; Grbić et al. 2011; Khajehali et al. 2011; Pascual-Ruiz et al. 2014; Hada et al. 2016). Consequently, emphasis has been placed on implementing safer and more effective control measures including conservation biological control (Eilenberg et al. 2001). The establishment of a *Festuca arundinacea* Schreber (Poaceae) ground cover in Spanish clementines contributes to a better regulation of *T. urticae* populations (Jaques et al. 2015), by increasing the frequency and abundance of Phytoseiidae (Acari: Mesostigmata), the most important family of mite predators specialized on tetranychid preys (Aguilar-Fenollosa et al. 2011b), as well as by providing alternative food sources for phytoseiid species as pollen and thrips (Pina et al. 2012; Gómez-Martínez et al. 2017a, b). Although thrips are abundant microarthropods in citrus orchards (Blank and Gill 1997; Froud et al. 2001; Childers and Nakahara 2006; Costa et al. 2006; Morse and Hoddle 2006; Navarro et al. 2008; Tekşam and Tunc 2009), their specific composition can change depending on the management of the ground cover (Aguilar-Fenollosa and Jacas 2013). A wild cover consisting of many different plant species belonging to different families may promote the appearance of citrus potential

pest species as *Frankliniella occidentalis* (Pergande) and *Thrips tabaci* Lindeman (Aguilar-Fenollosa and Jacas 2013). However, a ground cover of the Poaceae *F. arundinacea* hosts high numbers of grass-specialized thrips, where *Anaphothrips obscurus* (Müller) (Thysanoptera: Thripidae) stands out because of its regular abundance along the season. The most effective phytoseiids species preying on *T. urticae* in this agricultural ecosystem (Aguilar-Fenollosa et al. 2011b) are able to exploit thrips species, at least under laboratory conditions (Ramakers 1988; Rodríguez-Reina et al. 1992; Walzer et al. 2004; Van Baal 2007; Magdy and El Sayed 2009; Jafari et al. 2013; Gómez-Martínez et al. 2017a, b). Furthermore, recent studies suggest that *A. obscurus* could compete with *T. urticae* by resource exploitation and interference and also mediate apparent competition with the mite from their condition of shared prey for phytoseiids (Gómez-Martínez et al. 2017a, b).

Additionally, *F. arundinacea* could be a food resource for some phytoseiid mites. McMurtry et al. (2013) proposed an additional group of phytoseiid species that pierce plant cells. This group would include phytoseiids that may complement their nutrition requirements by feeding on leaf cells without inducing any apparent damage to the plant (i.e., they cannot be considered pestiferous). This group would be mainly composed by of *Euseius* spp. but also by some species of the genera *Typhlodromus* (*Anthoseius*) and *Typhlodromus* (*Typhlodromus*), among others (Chant 1959; Porres et al. 1975; Congdon and Tanigoshi 1983; Nomikou et al. 2003; Adar et al. 2012).

In order to understand citrus mite trophic relationships, our research group designed species-specific primers for 8 species, and multiplexed them in a single amplification event to identify the most abundant phytoseiid predators and tetraychid prey mites in this system, (Pérez-Sayas et al. 2015). In that study, Pérez-Sayas et al. (2015) demonstrated that the designed multiplex PCR was a useful complementary tool to classic taxonomy to determine mite species composition and for the first time, empirically demonstrated the mite-mite trophic relationships in citrus crops under field conditions. However, other potential food sources including other tetranychids different from *T. urticae* and *Panonychus citri* (McGregor), thrips, or even plants were not considered in that study.

In addition to interspecific predation, intraguild predation (IGP), defined as ‘‘predator-prey interactions among consumers potentially competing for limiting resources’’ (Holt and Huxel 2007), cannot be discarded as it commonly occurs within many predatory guilds (Arim and Marquet 2004; Vance-Chalcraft et al. 2007; Gagnon et al. 2011b; Traugott et al. 2012), including generalist and specialist phytoseiid mites (e.g. Schausberger 1999a, b; Schausberger and Croft 2000; Hatherly et al. 2005; Maleknia et al. 2016). Indeed, with the previously mentioned multiplex PCR (Pérez-Sayas et al. 2015), some intraguild events were detected, although Phytoseiidae specific primers were not specifically designed for IGP detection. These primers amplified DNA fragments larger (369-605 bp) than recommended for prey detection (Agustí et al. 1999, 2003; King et al. 2008). IGP is a relevant issue in applied ecology, including biological control. It may have a negative impact on pest suppression depending on the force and frequency with which it occurs and the role of the species that interact (Polis et al. 1989; Polis and Holt 1992; Rosenheim 1998; Traugott et al. 2012). The availability of alternative prey or food sources could contribute to reduce IGP (Sabelis and van Rijn 2006; Eitzinger et al. 2017). In fact, Guzmán et al. (2016) observed that high availability of a shared food sources entails a negligible IGP in phytoseiids. Among the predatory guild described in Spanish clementines (Aguilar-Fenollosa et al. 2011b), at least the highly abundant generalist pollen feeder *Euseius stipulatus* (Athias-Henriot), and the generalist predator from soil/litter habitats *Neoseiulus barkeri* Hughes, are potential intraguild predators (Abad-Moyano et al. 2010a, b; Momen 2010). Whether they are involved in IGP or they exploit alternative food sources, and consequently reduce IGP, deserve further studies that can be tackled with molecular tools.

The aim of this study has been to develop a multiplex PCR for the identification of the phytoseiid predator species and its food sources, including tetranychids, thrips, plants and other predatory mites within the same guild (IGP) in order to understand the trophic webs involved in the natural regulation of *T. urticae* populations in field conditions.

4.2 Materials and Methods

We included for the development of the multiplex PCR the most relevant species involved in the biological control of *T. urticae* in citrus orchards. Phytoseiidae were considered as representatives of the third trophic level, Tetranychidae and Thysanoptera of the second level, and both clementine mandarine and *F. arundinacea* of the first level.

4.2.1 Arthropods for laboratory studies

Tetranychus urticae individuals were originally collected in clementine mandarin orchards in the region of La Plana (Castelló de la Plana, Spain) and subsequently reared on bean plants (*Phaseolus vulgaris* L.) at room temperature and natural photoperiod.

The initial individuals of *Neoseiulus californicus* (McGregor) were obtained from Koppert Biological Systems (Spical®). *Neoseiulus barkeri* were collected on *F. arundinacea* plants in a greenhouse at Universitat Jaume I (Castelló de la Plana, Spain). *Euseius stipulatus* and *Typhlodromus phialatus* Athias-Henriot individuals were collected in clementine orchards located in Montcada (Valencia, Spain) and *Phytoseiulus persimilis* Athias-Henriot in Les Alqueries (Castelló, Spain). *Neoseiulus barkeri*, *N. californicus*, and *P. persimilis* were reared following the procedures described by Overmeer (1985) and fed with *T. urticae* on bean leaves. *Euseius stipulatus* and *T. phialatus* were reared on upside down placed bean leaves and fed with a mixture of *T. urticae* and *Carpobrotus edulis* (L.) N. E. Br pollen. All phytoseiid species were maintained in separate climatic chambers at 25 ± 1 °C, 70 ± 10% RH, and a photoperiod of 16:8 (light:dark; L:D) h. These conditions were also used for the laboratory assays involving live mites.

Anaphothrips obscurus individuals were originally collected in *F. arundinacea* plants grown in an experimental citrus plot at Universitat Jaume I (Castelló de la Plana, Spain). They were later maintained on the same kind of plants (*F. arundinacea* 'Fórmula frutales y cítricos', Semillas Fitó, S.A., Barcelona, Spain) grown in a pesticide-free greenhouse at the Institut Valencià d'Investigacions Agràries (IVIA) (Montcada,

Valencia, Spain). *Anaphothrips obscurus* specimens were reared following the procedures described in Gómez-Martínez et al. (2017a). *Frankliniella occidentalis* individuals were obtained from a colony established at IVIA initiated in 2010 and originally collected at Campo de Cartagena (Murcia, Spain) (Guillén et al. 2014). They were later reared following the procedures described by Debreczeni et al. (2014). Both thrips species colonies were maintained in separate climatic chambers at 25 ± 1 °C, 70 ± 5 % RH and 16:8 h (L:D) photoperiod.

4.2.2 DNA extraction

DNA of Phytoseiidae, Tetranychidae, Thysanoptera and other potential food sources for Phytoseiidae mites in citrus orchards and those provided in the laboratory rearings was extracted following different protocols depending on the organism and the objective of the study. The DNA of Acari and Hemiptera (Table 4.1 and 4.2) was extracted following the modified "salting out" protocol (Monzó et al. 2010). The DNA of Thysanoptera used in the feeding trials (see *Gut content detection and feeding trials* in section 4.2.3) was also extracted using this protocol. However, those thrips specimens obtained from field samples and isolated in 99% ethanol were handled according to Rugman-Jones et al. (2006). In this case, DNA was extracted following the modified "salting out" protocol, where the grinding of the specimen was substituted by piercing one side of the abdomen using a sterilized minute pin, in 100 µl of TNES. This method allows the recovery of the remains of the original microfuge tube for species-specific identification. Adult thrips were slide mounted on Hoyer's medium for microscope observation and identified using morphological characters (Lacasa and Llorens 1998; Mound and Kibby 1998, Moritz et al. 2004). *Festuca arundinacea* leaf DNA was also extracted following the modified "salting out" protocol. Clementine mandarin (*C. clementina* Hort. ex. Tan. cvar. Clemenules) leaf DNA and *C. edulis* anther DNA were extracted using the REDExtract-N-AmpTM Plant PCR Kit (Sigma), following the manufacturer protocol. Finally, fungal DNA was extracted following the protocol described by Sánchez-Torres et al. (2008).

4.2.3 Multiplex PCR design

Alignment and primer design

Multiplex-PCR was designed considering Phytoseiidae mites (*E. stipulatus*, *N. barkeri*, *N. californicus, P. persimilis* and *T. phialatus*) as predator and intraguild prey, and tetranychid mites, thrips and plant as food sources. Therefore, different genes were selected for primer design according to the target organism. For Phytoseiidae and Tetranychidae detection, the Internal Transcribed Spacer 1 (ITS1) region (nuclear ribosomal DNA) was selected (Figure 4.1 A). Most part of ITS1 sequences were obtained in a previous work (Pérez-Sayas et al. 2015). For thrips and plant detection, the mitochondrial cytochrome oxidase I (COI) gene and the chloroplast region of the *trnL* gene were used respectively (Figure 4.1 B and C). COI sequences were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/genbank).

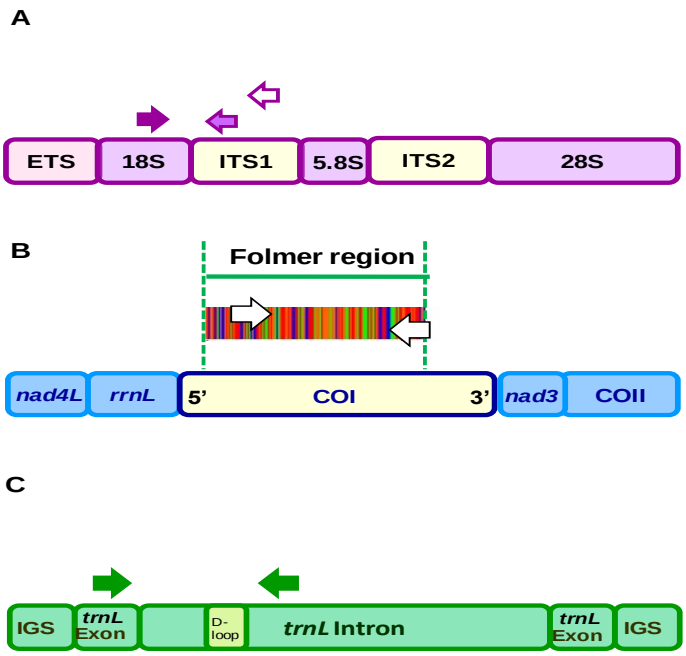

Figure 4.1 Location of the primers included in the multiplex PCR. A: Location of primers for Phytoseiidae and Tetranychidae detection (dark arrow represents the commun forward, light purple arrow represents the five reverse primers for Phytoseiidae detection and white arrow represents the Tetranychidae reverse primer). B: Location of primers for Thysanoptera detection. C: Location of primers for plant detection.

For primer design, sequence alignment was performed with the MEGA 5 program (Tamura et al. 2011). For identifying each Phytoseiidae species, different reverse primers were designed in non-conserved nucleotide sequences of the ITS1 region, using as forward primer the 18S (Navajas et al. 1999) (Table 4.3). To allow the detection of any species belonging to Thysanoptera and Tetranychidae, in both cases, forward and reverse primers, were designed in conserved regions (Table 4.3). The 18S primer (Navajas et al. 1999) was also used as forward for Tetranychidae species detection and the barcoding primer HCO2198 (Folmer et al. 1994) was used as a reverse for Thysanoptera species. The universal primers designed by Taberlet et al. (1991; 2007) were used for plant detection (Table 4.3).

Primers specificity test and multiplex PCR design

Dominant Tetranychidae, Phytoseiidae and Thripidae species found in Spanish citrus orchards (Aguilar-Fenollosa et al. 2011a, b; Aguilar-Fenollosa and Jacas 2013) reared under laboratory conditions or field collected on citrus orchards were included in our study for primer specificity test and multiplex PCR design (Table 4.1). Furthermore, some specimens of *Heliothrips haemorrhoidalis* (Bouché) and *T. tabaci* coming from persimmon [*Diospyros kaki* Thunb. (Ebenaceae)] and leeks [*Allium ampeloprasum* var. *porrum* (L.) J. Gay (Amaryllidaceae)], respectively, were also used for testing the specificity of primers designed for thrips detection (Table 4.1). The universal primers for plant detection were tested in the selected plant species, either present in citrus orchards or used in laboratory rearings (Table 4.1).

All combination of primers pairs (Table 4.3) were tested in at least three individuals of each species included at the primers specificity test. A single DNA template (5-10 ng/µL) of the target species was used as a positive control. Amplification reactions were performed on a final volume of 25 µL: 1× Taq polymerase buffer (Roche Applied Science, Mannheim, Germany), 200 µM of each dNTP (5 PRIME GmbH, D·22767 Hamburg), 2.5 mM of $MgCl_2$, 0.4 µM of each primer, 1 unit of DNA Taq polymerase (Roche), and 1 µL of DNA template. Amplifications were performed in a Bio-Rad C1000™ Thermal Cycler. PCR parameters were as follows: denaturation for 4

min at 94 °C; 27 cycles of 30 s at 92 °C, 30 s at 50 °C, and 30 s at 72 °C; and a 10-min final extension at 72 °C. PCR products were run on 1.5 % agarose D-A low EEO (Pronadisa, Sumilab S.L., Madrid, Spain) visualized on agarose gel under UV light.

Once the specificity of the primers had been tested by singleplex, the multiplex PCR conditions with all primers were adjusted on agarose following the steps defined in Henegariu et al. (1997). Reactions were performed in a final volume of 25 µL: 1.4× Taq polymerase buffer, 200 µM of each dNTP, 2.5 mM of $MgCl_2$, 1.2 µM of the *E. stipulatus* reverse primer, forward and reverse thrips primers and 18S forward primer, 0.8 µM of Tetranychidae and *N. barkeri* reverse primers, 0.4 µM of each *P. persimilis* and *T. phialatus* reverse primers, 0.2 µM of forward and reverse chloroplast *trnL* primers and 0.1 µM of *N. californicus* reverse primer, 1 unit of DNA Taq polymerase, and 1 µL of DNA template. Assessment of amplification conditions and PCR products was performed as described for the cross-reactivity test.

Multiplex PCR conditions were checked on agarose gel, modified, and adapted to analysis with marked primers in the ABI/PE 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) at the Servei Central de Suport a la Investigació Experimental (SCSIE) (Universitat de València, Spain). An equimolar mix (5 ng/µL) of the most representative DNA target species was used as a positive control (i.e., all Phytoseiidae, *T. urticae* for Tetranychidae*, A. obscurus* and *F. occidentalis* for Thysanoptera and *C. clementina* and *F. arundinacea* for plants). Final multiplex PCR conditions are described in the results section. Fragment length reads were carried out with Peak Scanner™ Software v1.0 (Applied Biosystems 2006). All samples that produced peaks of the expected size were considered positive. Sensitivity of prey DNA detection was determined by assaying multiplex PCR, at nine-fold dilutions starting with 10 ng of total independently *P. persimilis*, *A. obscurus*, *F. occidentalis* and *T. urticae* DNA till $1:10^{-10}$.

Table 4.1 Target organisms considered in the multiplex PCR.

Kingdom	Phylum	Class	Order	Family	Species	Origin
Animalia	Arthropoda	Arachnida	Acari	Phytoseiidae	*Euseius stipulatus* (Athias-Henriot)	Montcada, Spain
					Neoseiulus barkeri Hughes	Castelló de la Plana, Spain
					Neoseiulus californicus (McGregor)	Koppert Biol. Syst.
					Phytoseiulus persimilis Athias-Henriot	Les Alqueríes, Spain
					Typhlodromus phialatus Athias-Henriot	Montcada
				Tetranychidae	*Aplonobia histricina* (Berlese)	Montcada
					Eutetranychus banksi (McGregor)	Huelva, Spain
					Eutetranychus orientalis (Klein)	Málaga, Spain
					Panonychus citri (McGregor)	Montcada
					Tetranychus evansi Baker and Pritchard	Valencia, Spain
					Tetranychus turkestani Ugarov and Nikolskii	Castelló de la Plana
					Tetranychus urticae Koch	Betxí, Spain
		Insecta	Thysanoptera	Aeolothripidae	*Aeolothrips* sp.	Montcada
				(S.O. Terebrantia)	*Rhipidothrips brunneus* Williams	Montcada
				Melanthripidae (S.O. Terebrantia)	*Melanthrips fuscus* Sulzer	Montcada
				Thripidae	*Anaphothrips obscurus* (Muller)	Castelló de la Plana
				(S.O. Terebrantia)	*Anaphothrips sudanensis* Trybom	Montcada
					Aptinothrips rufus (Haliday)	Montcada
					Chirothrips manicatus Haliday	Montcada

					Frankliniella occidentalis (Pergande)	Montcada
					Frankliniella tenuicornis (Uzel)	Montcada
					Heliothrips haemorrhoidalis (Bouché)	Castelló de la Plana
					Limothrips cerealium Haliday	Montcada
					Pezothrips kellyanus (Bagnall)	Montcada
					Stenothrips graminum Uzel	Montcada
					Tenothrips frici (Uzel)	Montcada
					Thrips angusticeps Uzel	Montcada
					Thrips tabaci Lindeman	Viena (Boku lab. strain)
					Thrips vulgatissimus Haliday	Montcada
				Phlaeothripidae (S.O. Tubulifera)	*Haplothrips tritici* Kurdjumov	Montcada
Plantae	Streptophyta	Eudicotyledoneae	Caryophyllales	Aizoaceae	*Carpobrotus edulis* (L.) N. E. Br	Montcada
		Liliopsida	Poales	Poaceae	*Festuca arundinacea* Schreb.	Castelló de la Plana
		Magnoliopsida	Fabales	Fabaceae	*Phaseolus vulgaris* L.	Montcada
			Sapindales	Rutaceae	*Citrus clementina* Hort. ex. Tan.	Montcada

Table 4.2 Non-target organisms screened with multiplex PCR for cross-reactivity test.

Kingdom	Phylum	Class	Order	Family	Species	Origin
Animalia	Arthropoda	Arachnida	Acari	Acarididae	-	Montcada, Spain
				Phytoseiidae	*Amblyseius andersoni* (Chant)	Syngenta
					Amblyseius cucumeris (Oudemans, 1930)	Syngenta
					Amblyseius swirskii (Athias-Henriot)	Syngenta
					Typhlodromus (*Anthoseius*) *rhenanoides* Athias-Henriot	Montcada
				Tydeidae	-	Montcada
		Insecta	Hemiptera	Aleyrodidae	*Aleurothrixus floccosus* (Maskell)	Les Alqueries, Spain
					Aphis (*Aphis*) *gossypii* Glover	Montcada
				Aphididae	*Aphis* (*Aphis*) *spiraecola* Patch	Montcada
					Toxoptera aurantii (Boyer de Fonscolombe)	Montcada
					Aonidiella aurantii (Maskell)	Carcaixent, Spain
				Diaspididae	*Aspidiotus nerii* Bouché	Montcada
					Parlatoria pergandii Comstock	Montcada
				Coccidae	*Saissetia oleae* (Olivier)	Bétera, Spain
				Margarodidae	*Icerya purchasi* Maskell	Montcada
				Pseudococcidae	*Planococcus citri* (Risso)	Bétera
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	*Cladosporium* sp.	Montcada
			Pleosporales	Pleosporaceae	*Alternaria* sp.	Montcada
		Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium* sp.	Montcada

Table 4.3 Primer sequences and amplified fragment length of the target organism groups considered in the multiplex PCR.

Target group	Target region	Primer name	Primer sequence (5' -> 3')[1]		Length (bp)
Phytoseiidae; Tetranychidae	ITS1	18S	AGA GGA AGT AAA AGT CGT AAC AAG [2]	F	
Phytoseiidae	ITS1				
Euseius stipulatus		Esdep2	CGC GTC TGT GGA CGG TAA CG	R	247; 257
Neoseiulus barkeri		Abpr	CAT TCT TCC ATG TGAT GGA GTG	R	93
Neoseiulus californicus		Ncpr2	ACG TAC GAC GGC CAG CAG GC	R	155
Phytoseiulus persimilis		Pppr2	CTG GTT GGT ACC GAC TCG CG	R	277
Typhlodromus phialatus		Tppr2	CGA GCA GTA GGA CTG ACC TC	R	234
Tetranychidae	ITS1	TeUniITS1	CCA AGT ATG TAG CAA GAC AGG C	R	350-371[6]
Thysanoptera	COI	TripUniCOI	TCA ACA TTT TTT CAT TCT GG	F	
		HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA[3]	R	330
Plants	*trn*L	trnL_a	CGA AAT CGG TAG ACG CTA CG[4]	F	
		trnL_h	CCA TTG AGT CTC TGC ACC TAT C[5]	R	190

[1] F: forward primer; R: reverse primer.

Primer references: [2] Navajas et al. 1999; [3] Folmer et al. 1994; [4] Taberlet et al. 1991; [5] Taberlet et al. 2007.

[6] Amplified fragment length for Tetranychidae species are: *T. evansi* (350 bp), *P. citri* and *T. urticae* (367 bp) and *T. turkestani* (371 bp).

Alternative food sources and cross reactivity test

Species specificity of the multiplex PCR assay was tested on non-target food sources available in citrus orchards and not so frequently incorporated in phytoseiid diets (McMurtry et al. 2013) (Table 4.2). The aims of this test is to avoid false positives when predators can feed on alternative food sources not included in the designed multiplex PCR. We used the same positive control as in the cross-reactivity test for species-specific primers. To discriminate between unsuccessful multiplex PCR amplification (i.e., absence of target DNA) and lack of DNA in the PCR reaction (i.e., absence of both target and non-target DNA), we used the universal primer pair Univ18SrDNA and PCR conditions described in Monzó et al. (2010).

Gut content detection and feeding trials

In order to test if multiplex PCR was able to detect all the possible food sources in the gut content of the target species, the following experiments were conducted:

For tetranychid detection, we tested 11 samples of both *E. stipulatus* and *P. persimilis* that had resulted positive for this prey in a previous study at different starvation times (from 0 to 28h) where one adult *T. urticae* had been offered (Pérez-Sayas et al. 2015).

For plant detection, we extracted DNA from *E. stipulatus* and *A. obscurus* individuals directly taken from the rearings. We also included thrips and tetranychid specimens of each species from field samples. Additionally, to ascertain if *E. stipulatus* was able to pierce and take up liquids, we performed a feeding trial using a slightly modified membrane feeding system described in Ingwell et al. (2012). The membrane feeding chamber was prepared using a modified Huffaker cell (Overmeer 1985). This unit consisted of a PVC plate (80 x 40 x 10 mm) with a central circular hole (diameter 2 cm). The bottom surface of the hole was closed by a microscope slide held in place with two rubber bands. One *E. stipulatus* female randomly chosen from the colony was placed into the chamber. Immediately after, the upper opening was covered by a Parafilm® (ParaFILM®; Bemis Company, Inc. Neenah, Wisconsin) stretched tightly

across the hole and a drop of 5% sucrose diet dyed with blue colorant was pipetted onto the membrane. Finally, a second layer of Parafilm® was stretched tightly to sandwich the diet in order to obtain a uniform distribution along the surface of the membrane. The Huffaker cells were maintained in a climatic chamber at 25 ± 1 °C, 70 ± 10% RH and 16:8 h (L:D) photoperiod during the whole experiment. A color change from yellow or white into blue would imply the phytoseiid piercing the membrane and taking up liquids.

For thrips and intraguild prey detection, more specific feeding trials were conducted. In this case, we chose the most probable events in the field (i.e., *E. stipulatus* as a predator, *P. persimilis* as intraguild prey, and *F. occidentalis* and *A. obscurus* -two abundant thrips in clementine orchards- as alternative prey) to estimate prey DNA detectability success over time (DS_{50}). Experimental units (cells) used for the predation assays consisted of a PVC plate (80 × 35 × 3 mm) containing two chambers of 15 mm of diameter. The bottom of these chambers was covered by a fine mesh glued to the plate and closed on the upper side by a microscope slide hold in place by two rubber bands (Schausberger 1997). Three to 5 days-old adult females of *E. stipulatus* were individually placed in the cells and starved for 48 h in a climatic chamber at 25 ± 1 °C, 70 ± 10% RH, and a photoperiod of 16:8 h (L:D). After starvation, each adult female was transferred to a new cell containing one prey. This prey was a first instar nymph for *A. obscurus* and *F. occidentalis*, and a protonymph in the case of *P. persimilis.* Phytoseiid activity was continuously monitored under a binocular microscope. Time after feeding was set to 0 when phytoseiid released the dead prey. Then, phytoseiid specimens were maintained individually in new cells for different time periods (0 to 20 h; Table 4.5) after feeding. Next, they were transferred to 1.5 mL tubes, frozen at -80 °C, and processed for molecular assessment. Additionally, we used as negative controls phytoseiids starved for 48 h. DNA from all phytoseiids was extracted and screened with the multiplex PCR described in the results section.

Probit analysis was used to determine the prey DNA detectability success (DS_{50}) defined as “the time after which half of the predators of a cohort that fed at the same time test positive for the presence of a species of prey, considering that the rate of

prey decay is usually exponential" (Greenstone et al. 2007; Gagnon et al. 2011a). Chi-square (χ^2) tests were used to determine the fit of the probit model. To assess whether there were significant differences between lines, when applicable, we performed a χ^2 test of parallelism and a comparison of relative median potency. Analyses were performed using SPSS (v. 21).

4.3 Results

Multiplex PCR design

The primers chosen and designed amplified specific bands within the expected rank length with DNA of the target organism (Table 4.1 and 4.3).

Primers used for Tetranychidae, plant and Thysanoptera detection were successful in most species tested (Table 4.4). Tetranychidae primers gave positive detection with

Table 4.4 Species DNA detection with the target group primers included in the multiplex PCR.

Plantae	**DNA detection**
Alliumn ampeloprasum var. *porrum*	+
Carpobrotus edulis	+
Citrus clementina	+
Diospyros kaki	+
Festuca arundinacea	+
Oxalis pes-caprae	+
Phaseolus vulgaris	+
Taraxacum sp	+

Tetranychidae	**DNA detection**
Aplonobia histricina	-
Eutetranychus banksi	-
Eutetranychus orientalis	-
Panonychus citri	+
Tetranychus evansi	+
Tetranychus turkestani	+
Tetranychus urticae	+

Thysanoptera	**DNA detection**
Aeolothrips sp.	+
Anaphothrips obscurus	+
Anaphothrips sudanensis	-
Aptinothrips rufus	+
Chirothrips manicatus	+
Frankliniella occidentalis	+
Frankliniella tenuicornis	-
Haplothrips tritici	-
Heliothrips haemorrhoidalis	+
Limothrips cerealium	+
Melanthrips fuscus	+
Pezothrips kellyanus	+
Rhipidothrips brunneus	+
Stenothrips graminum	-
Tenothrips frici	-
Thrips angusticeps	+
Thrips tabaci	+
Thrips vulgatissimus	+

a different fragment length amplification in *P. citri* and *T. urticae* (367 bp), *Tetranychus evansi* Baker and Pritchard (350 bp) and *T. turkestani* Ugarov and Nikoskii (371 bp). Thysanoptera primers amplified in 13 out of 18 species tested and those for plants detection amplified in the 4 species tested with the expected amplified fragment length (Table 4.3 and 4.4).

The final multiplex PCR reaction was adjusted to a final volume of 25 µL: 1.4× Taq polymerase buffer, 200 µM of each dNTP, 2.5 mM of MgCl2, 1.2 µM of each *E. stipulatus* reverse primer, forward primer and labeled with FAM-6 and unlabeled reverse Thysanoptera primers and 18S labeled with FAM-6 and unlabeled forward primer, 0.8 µM of Tetranychidae and *N. barkeri* reverse primers, 0.3 µM of *P. persimilis* reverse primer, 0.2 µM *T. phialatus* reverse primer, 0.1 µM of forward and labeled with FAM-6 and unlabeled reverse chloroplast trnL primers and 0.05 µM of *N. californicus* reverse primer, 1 unit of DNA Taq polymerase, and 1 µL of DNA template. The multiplex PCR design was performed at the same amplification conditions as described for the cross-reactivity test. All the primers were successfully multiplexed in a single PCR discarding primers interference and allowing the identification of the target species/groups using the previously described automated sequencer. Positive control with single target DNA and equimolar mixes of DNA templates from the Phytoseiidae species and five representing species of Tetranychidae, Thysanoptera and plant amplified the expected fragment lengths (Figure 4.2). Multiplex PCR sensitivity with fluorescent markers was independently estimated at 0.1 pg for *C. clementina* total DNA, 1 pg for *P. persimilis* and *T. urticae* total DNA, 10 pg for *A. obscurus* total DNA and 1000 pg for *F. occidentalis* total DNA.

Multiplex PCR exhibited no cross-amplification of the alternative food sources tested except for *Aonidiella aurantii* (Maskell), *Aspidiotus neri* Bouché, *Aphis spiraecola* Patch and *Toxoptera aurantii* (Boyer de Fonscolombe) where a multi-peak pattern was observed and for *Cladosporium* sp. where a 290 bp peak, not coincident with any of our target organisms, was detected.

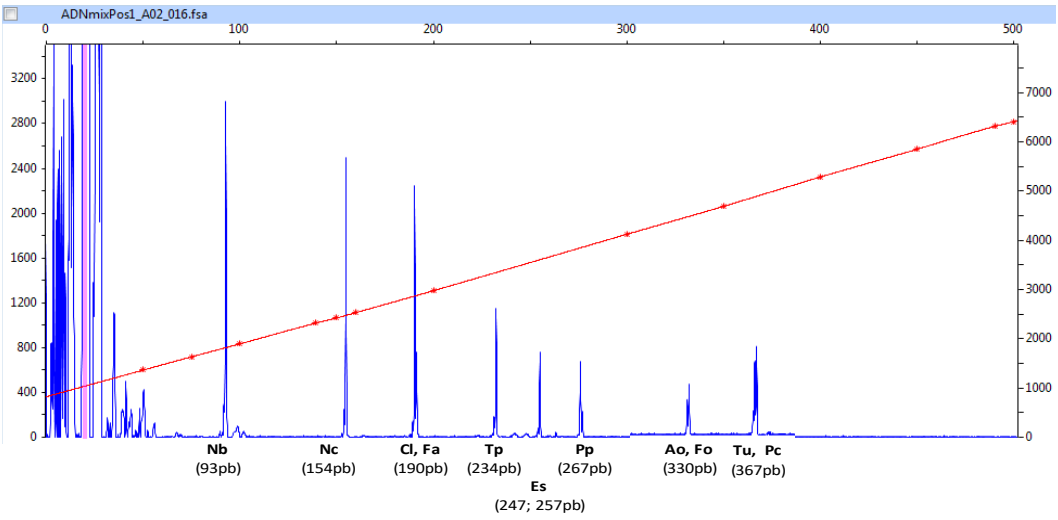

Figure 4.2 Example of amplification of multiplex PCR with all species considered together in the same electropherogram. Nb: *N. barkeri*; Nc: *N. californicus*; Tp: *T. phialatus*; Es: *E. stipulatus*; Pp: *P. persimilis* (Phytoseiidae species included in PCR multiplex); Cc: *C. clementina* and Fa: *F. arundinacea* as representative species of plant detection; Ao: *A. obscurus* and Fo: *F occidentalis* as representative species of thrips detection; Tu: *T. urticae* and Pc: *P. citri* as representative species of tetranychid detection.

Gut content detection and feeding trials

The designed multiplex PCR was able to detect degraded DNA of all food sources considered in the gut content after a digestive process. Plant DNA was detected within Thysanoptera, Tetranychidae and Phytoseiidae. In Thysanoptera, plant DNA was detected in all specimens of *A. obscurus* tested (n = 3). Furthermore, plant DNA detection within field collected thrips specimens was positive for *Chirothrips manicatus* Haliday and *Aptinothrips rufus* (Haliday) when feeding on *F. arundinacea*, for *Thrips angusticeps* Uzel feeding on *Taraxacum* sp. (Asteraceae), for *T. tabaci* directly taken from leeks and for *H. haemorrhoidalis* when feeding on persimmon. In Tetranychidae, plant DNA was detected in *Aplonobia histricina* feeding on *Oxalis pes-caprae* L. (Oxalidaceae) and in *T. urticae* on bean. Finally, in Phytoseiidae we detected plant in *Typhlodromus* (*Anthoseius*) *rhenanoides* (Athias-Henriot) (Acari: Phytoseiidae) field collected from lemon trees and later reared on bean plants. However, we did not detect plant DNA within *E. stipulatus* although it was able to take up liquid by piercing the membrane feeding system (n = 3; blue colored digestive caeca) used in the assay

(Figure 4.3). Multiplex PCR allowed detection of tetranychid DNA in the gut of *E. stipulatus* and *P. persimilis* up to 28 hours after the feeding event.

Figure 4.3 Colored digestive caeca of *Euseius stipulatus* after taking up blue colored liquid by piercing the feeding membrane.

Positive prey DNA detection within *E. stipulatus* at time = 0 h (immediately after ingestion) was 92.31%, 81.82% and 63.64% for *P. persimilis*, *A. obscurus* and *F. occidentalis* respectively (Table 4.5). Detectability of prey DNA for *P. persimilis*, *A. obscurus* and *F. occidentalis* fitted the assumptions of the probit model for *E. stipulatus* (Figure 4.4 and Table 4.6). DS_{50} values depended on the prey species considered. For *P. persimilis* DS_{50} was 18.7 h, for *A. obscurus* 2.3 h, and for *F. occidentalis* 1.3 h. Probit curves corresponding to the three prey species considered could not be successfully forced to parallelism (χ^2 = 12.853; d.f. = 2; *P-value* = 0.002). Parallelism was only significant between *A. obscurus* and *F. occidentalis* probit curves (χ^2 = 0.002; d.f. = 1; *P-value* = 0.967). Relative median potencies for *A. obscurus* suggested that detection in *E. stipulatus* lasted 1.93 times longer than in *F. occidentalis* (*P-value* < 0.05).

Table 4.5 Number of positive detections for each predator and prey combination at different time intervals since feeding.

		Time since feeding (h)					
Predator	**Prey**	**0**	**2**	**4**	**6**	**16**	**20**
Euseius stipulatus	*Anaphothrips obscurus*	9(11)[1]	5(11)	4(11)	1(12)	0(12)	-
	Frankliniella occidentalis	7(11)	5(11)	2(11)	0(12)	-	-
	Phytoseiulus persimilis	12(13)	12(13)	10(11)	10(12)	7(11)	6(14)

[1] Number in parenthesis represents the number of individuals tested.

Table 4.6 Probit curves adjusted for positive detections and prey DNA detectability success (DS_{50}) from a single prey specimen.

Predator	Prey	n	Slope	Intercept	d.f.	χ^2	*P*	DS_{50} (h)	95% f.l.[1]
Euseius stipulatus	*Anaphothrips obscurus*	57	-0.344 ± 0.101	0.802 ± 0.352	3	0.829	0.843	2.33	0.59 - 3.61
	Frankliniella occidentalis	45	-0.392 ± 0.118	0.504 ± 0.340	2	0.889	0.641	1.29	0 - 2.42
	Phytoseiulus persimilis	74	-0.082 ± 0.023	1.541 ± 0.298	4	0.302	0.990	18.72	14.11 -30.03

[1] f.l.: fiducial limits at 95%.

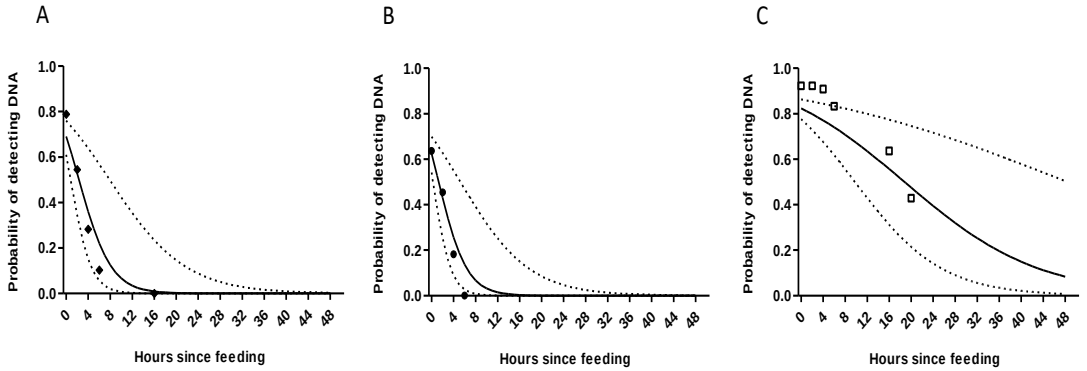

Figure 4.4 *Anaphothrips obscurus* (A), *Frankliniella occidentalis* (B) and *Phytoseiulus persimilis* (C) DNA detection probability curves in *Euseius stipulatus* samples after feeding. Lines are fitted with probit model with 95% confidence intervals (dashed lines).

4.4 Discussion

The multiplex PCR designed in this study allows us to identify in a single amplification event the phytoseiid predator and to detect three of its potential food sources commonly found in citrus orchards: Tetranychidae, Thysanoptera and plants, as well as to detect IGP events.

In the case of Tetranychidae, in addition to the citrus pests *T. urticae* and *P. citri*, already detected in a previously designed multiplex PCR (Pérez-Sayas et al 2015), we increased the spectra of tetranychid species by also detecting *T. turkestani* and *T.*

evansi, two species commonly found in this agrosystem. The later is considered a worldwide invasive pest of solanaceous crops that can appear in citrus orchards associated with the wild cover (Ferragut and Escudero 1999; Aucejo et al. 2003; Boubou et al. 2011; Migeon and Dorkeld 2006-2017) and outcompeting *T. urticae* and *T. turkestani* (Ferragut et al. 2013). Furthermore, even if it was not the initial objective, the multiplex designed allowed not only to detect but also to differentiate Tetranychidae species using the same pair of primers. The exception was for *P. citri* and *T. urticae* because they displayed a band of the same fragment length. However, if identification of both preys were needed, primers designed in Pérez-Sayas et al. 2015 could be used to differentiate them. Tetranychidae primers are designed in the ITS1 region, which is characterized by a higher level of variability than the ITS2 (Hurtado et al. 2008a, Pérez-Sayas et al. 2016). This nucleotide variability (insertions / deletions) explains the differences in length of the amplified fragments that allow species differentiation.

For thrips detection, primers were designed in the more conserved COI region. The reason for this election was that we were interested on Thysanoptera in general as a potential food source of citrus-dwelling phytoseiids. Therefore, in this case, we could detect thrips but could not differentiate species (same size of the amplification product in all species tested), contrarily to what happened with tetranychid primers. The multiplex PCR could amplify in the most abundant thrips species present in the *F. arundinacea* ground cover, including *A. obscurus* and *C. manicatus*, and the main thrips species causing fruit damage in citrus in the Mediterranean basin as *H. haemorrhoidalis* (Tekşam and Tunc¸ 2009) and *Pezothrips kellyanus* (Bagnall) (Navarro et al. 2008; Jacas et al. 2010; Vassiliou 2010; Navarro-Campos et al. 2012). Apart from these species, the multiplex PCR is able to amplify DNA of thrips species considered economically important agricultural pest worldwide as *F. occidentalis* and *T. tabaci* (Fournier et al. 1995; Kirk and Terry 2003; Morse and Hoddle 2006; Reitz 2009; Reitz et al. 2011), which could be occasional or potential pests in citrus (Jacas et al. 2010). In case that species-specific thrips identification is required, a singleplex with new or already designed species-specific primers should be used after thrips molecular

detection. This is the case of the identification of *F. occidentalis* (Gómez-Polo et al. 2015). Another possibility could be to use the PCR multiplex developed by Nakahara and Minoura (2015), which allows the identification of at least 3 of the thrips species inhabiting our citrus system: *T. tabaci*, *Frankliniella intonsa* (Trybom) and *F. occidentalis* (Navarro et al. 2008; Aguilar-Fenollosa and Jacas 2013).

Plant primers used allowed the detection of a wide range of plant species belonging to taxonomically distant families included in the Magnoliopsida, Liliopsida and Eudicotyledonea classes. These results could be attributed to the primer sequence, which is located in the highly conserved chloroplast *trnL* gene (Chase et al. 2005; Taberlet et al. 2007). In fact, the primer used as forward is greatly conserved among land plants, from Angiosperms to Bryophytes, same as the reverse primer in Angiosperms and Gymnosperms (Taberlet et al. 2007). Furthermore, the robustness of this amplification system, partly due to this region includes a conserved loop, allows the amplification of highly degraded DNA as, for example, that obtained when analyzing the diet on feces (Taberlet et al. 2007). Indeed, we detected plant DNA in the gut content of some of the phytophagous thrips and mite specimens obtained from field samples (feeding on different plant species) and, additionally, within the generalist phytoseiid *T. (A.) rhenanoides*. In this phytoseiid species, the detection of plant DNA was fortuitous when using the multiplex PCR for the cross-reactivity test. However, we did not detect plant DNA in *E. stipulatus* obtained from a laboratory colony kept on bean leaves. This species is considered a pollen feeding generalist predator, but whether it can feed or not on the plant remains unclear. In fact, Porres et al. (1975) could not detect ingestion traces of Eureka lemon leaves previously labelled with radioactive phosphoric acid or at least not detectable quantities of sap in this mite. As suggested by McMurtry et al. (2013), the leaf cell piercing may be probably related to water uptake and therefore the uptake of nutrients could be a consequence of their presence in the imbibed liquid reducing extremely the amount of plant DNA available for plant detection in the gut content. Furthermore, when food (pollen) and water is abundant, as it occurs in laboratory stock colonies, the need for piercing plant cells by phytoseiids may be less frequent or even disappear. However,

when we forced *E. stipulatus* to starve, we demonstrated that this species is able to obtain liquid by piercing a double parafilm membrane containing 5% sucrose. Further studies should be performed considering other methodologies and other plant species to test whether plant cell content uptake by *E. stipulatus* allows detection of plant DNA. Whatever the case, the multiplex PCR designed may broaden the tools available to explore the ability of phytoseiid mites to use plant as food source (allowing the detection of a wide range of plant species) and to study plant feeding habits on other zoophytophagous arthropod species.

It is possible to obtain valid quantitative data to ascertain the relevancy of each trophic link when assays of field-captured predators are complemented with laboratory studies that determine the detectability periods for prey DNA for each combination of predator and prey (Gurr and You 2016). As a first step to determine the impact of each trophic link, we studied the detectability periods of the most probable field events considering *E. stipulatus* as a predator and three different preys, *P. persimilis*, *A. obscurus* and *F. occidentalis*. Our results confirmed previous observations (Harwood et al. 2007, Gagnon et al. 2011a, Waldner et al. 2013, Greenstone et al. 2014; Pérez-Sayas et al. 2015) where the identity of the prey affected prey DNA-detection rates. In our case, DS_{50} ranged from 1.3 to 18.7 hours post-feeding (Figure 4.4 and Table 4.6), which corresponded to *F. occidentalis* and *P. persimilis* when preyed upon by *E. stipulatus*, respectively. This fact could be associated with the accessibility of the target DNA, the efficiency of the primer to target the prey DNA and the digestion factor that determines different detection efficiency (de León et al. 2006). Some authors argued that shorter fragment length resulted in longer prey DNA detectability (Agustí et al. 1999; Zaidi et al. 1999; Juen and Traugott 2005; King et al. 2008; Waldner et al. 2013). The results obtained in the present study are in agreement with this hypothesis as shorter DS_{50} corresponded to species producing longer amplification fragments (Tables 4.3 and 4.6). The only study that estimated prey DNA detection of a target organism included in this work was that conducted by Gómez-Polo et al. (2016). These authors obtained an 8.6 h half-life detection of *F. occidentalis* on *Orius majusculus* (Reuter) with a similar amplification band length (292 bp).

Differences in the feeding trial performance related with the meal size (both, prey number and size) offered could be behind these results. On the one hand, Gómez-Polo et al. (2016) offered as prey second instars nymphs (N2) of *F. occidentalis* instead of the two fold smaller first instar nymphs, as we did. On the other, Gómez-Polo et al. (2016) only analyzed those specimens that had been feeding on 2 to 4 N2 *F. occidentalis* whereas we offered only one N1 specimen. In fact, some authors pointed out the influence of meal size in prey DNA detection within predators (de León et al. 2006; King et al. 2008, 2010; Gagnon et al. 2011a; Greenstone et al. 2014). Furthermore, other non-excluding factors could also explain these differences such as the predator size (*O. majusculus* is 6 times larger than *E. stipulatus)*, the feeding habits (generalist predator *vs* pollen generalist predator) and the specific digestive processes resulting from the distance between their respective taxonomical groups (Hemiptera *vs* Acari). In the case of *P. persimilis* as a prey of *Euseius stipulatus*, we obtained the longest DS_{50} (18.7 h), similar to that observed when feeding on *T. urticae* (18.3 h) (Pérez-Sayas et al. 2015). These results could be related with the non-preference of *E. stipulatus* for these types of prey. Indeed, DS_{50} for the preferred prey (*A. obscurus*, Gómez-Martínez et al. 2017b, third chapter of this thesis) is one order of magnitude shorter (2.3 h).

Intraguild predation is considered a widespread interaction, reaching frequencies between 58.4 and 86.7% (Arim and Marquet 2004), occurring in a great diversity of animal taxa (Polis et al. 1989; Gagnon et al. 2011b). However, the study of intraguild predation occurring among predatory mites in the field is still in its infancy, due to the scarcity of suitable methodologies allowing the observation of this phenomenon without altering predator behavior. Just a few studies have quantified IGP rates in the field (Gagnon et al. 2011a; Traugott et al. 2012). The multiplex PCR designed in the present work is suitable for the study of IGP among phytoseiids. The detection of this trophic relation in the most probable context in clementine orchards, i.e., predation of *E. stipulatus* on *P. persimilis* nymphs, was highly efficient long time after the feeding event happened (detection efficiency of 42.83% 20 hours after feeding). Furthermore, it would be possible to use the present multiplex PCR in other systems where

phytoseiid species could act as prey in order to detect biological control disruption. For example, Janssen et al. (2003) observed that *F. occidentalis* preyed on *T. urticae* eggs and on eggs of predators *P. persimilis* and *Iphiseius degenerans* (Berlese) when host plants were of low quality. Detecting IGP should be easier with the newly developed multiplex.

A better knowledge of the trophic relationships established within the citrus acarofauna, including *T. urticae*, the solely mite key pest of Spanish citrus, and of the role of alternative food sources to conserve and enhance predator populations will allow us to improve the biological control of this key pest. Now, a suitable molecular tool developed with this aim is ready to use.

Capítulo 5

Discusión general

El cultivo del clementino supone una de las bases de la agricultura en la Comunidad Valenciana. En este cultivo, el ácaro fitófago *Tetranychus urticae* se considera plaga clave ya que sus poblaciones pueden superar los umbrales económicos de daño en repetidas ocasiones a lo largo del ciclo del cultivo, y en consecuencia se pueden producir pérdidas económicas todos los años. Para reducir el número de tratamientos químicos necesarios destinados a combatir esta plaga y hacer que su gestión sea más sostenible se están implementando técnicas basadas en el control biológico por conservación.

Una de las técnicas recientemente estudiada y que ofrece buenos resultados es el establecimiento de una cubierta vegetal de gramíneas compuesta esencialmente por *Festuca arundinacea* (Aguilar-Fenollosa et al. 2011c). Esta cubierta, además de proporcionar varias ventajas agronómicas, ha demostrado contribuir en la reducción de las densidades poblacionales de *T. urticae* en las copas de los clementinos (Fibla-Queralt et al. 2000; Aguilar-Fenollosa et al. 2011a). En estudios anteriores llevados a cabo por Aguilar-Fenollosa y colaboradores (2011b; 2012; 2016) se demostró que varias razones pueden explicar este efecto. En primer lugar, esta cubierta favorece la selección de una raza de *T. urticae* especializada en *F. arundinacea* y con dificultades para colonizar el clementino, impidiendo que la cubierta actúe como reservorio del fitófago plaga. En segundo lugar, el complejo de fitoseidos depredadores en los clementinos cuando se cultivan asociados a *F. arundinacea* es más diverso y alberga mayor frecuencia relativa de las especies más efectivas en la regulación de poblaciones de *T. urticae*. Esto puede deberse a la provisión de refugio y a la naturaleza de las fuentes de alimento alternativo, que puede influir en las relaciones tróficas que se establecen entre los ácaros de este ecosistema agrícola. Con una cubierta de *F. arundinacea*, el polen disponible como fuente de alimento es de menor calidad para los fitoseidos generalistas (Pina et al. 2012) y se produce durante un periodo de tiempo más corto, en comparación con el que proporciona una cubierta vegetal espontánea multifloral (Aguilar-Fenollosa et al. 2011b). Además, esta cubierta no permite el establecimiento del ácaro tetraníquido *Tetranychus evansi* y ofrece mejor regulación de las poblaciones de *Panonychus citri* (Aguilar-Fenollosa et al. 2011a).

A simple vista, podría parecer que el establecimiento de *F. arundinacea* reduce las fuentes de alimento alternativo (ácaros tetraníquidos) capaces de mantener las poblaciones de los ácaros depredadores cuando hay escasez de presa. Sin embargo, esta cubierta vegetal alberga poblaciones de trips elevadas, concretamente de trips especializados en gramíneas (Aguilar-Fenollosa y Jacas 2013), que podrían estar ocupando el nicho ecológico "vacante".

Anaphothrips obscurus es una especie perteneciente al grupo de los Thysanoptera que aparece frecuentemente en este ecosistema agrícola. De hecho, es la especie de trips más abundante y se encuentra presente durante todo el año en esta cubierta vegetal. Ante la pregunta de si *A. obscurus* podría estar implicado en la regulación natural de las poblaciones de *T. urticae* y en qué medida la favorecería o perjudicaría, se planteó esta tesis doctoral. En ella, se ha estudiado en primer lugar las características biológicas y ecológicas de esta especie para profundizar en su conocimiento, y, en segundo lugar, las relaciones que podría mantener con el cultivo, la especie plaga *T. urticae*, y los ácaros depredadores de la familia de los fitoseidos. Además, se ha diseñado una herramienta molecular que permitirá comprobar en campo en qué medida *A. obscurus* actúa como presa de los fitoseidos, y si su presencia contribuye a reducir la depredación intragremial entre fitoseidos.

El análisis de los muestreos en campo de *T. urticae*, *A. obscurus* y los fitoseidos, en cuatro parcelas de clementino con *F. arundinacea* durante varios años consecutivos, resultó en un descenso en las poblaciones de *T. urticae* acompañado con un incremento de las poblaciones de *A. obscurus* y fitoseidos, a medida que pasa el tiempo desde el establecimiento de la cubierta. Esta relación inversa con las densidades de *T. urticae* y directa con las de los fitoseidos, parece indicar que *A. obscurus* podría estar favoreciendo a los fitoseidos actuando como presa alternativa. Sin embargo, no es posible descartar que otros factores como la zoofitofagia de *A. obscurus* (Hinds 1900), y la competencia interespecífica entre *A. obscurus* y *T. urticae* pudieran estar ocurriendo también en este sistema.

Las características biológicas de *A. obscurus* resultaron ser compatibles con un estilo de vida de la "estrategia de la r" (madurez temprana, alta fecundidad y tiempos de generación cortos) (Begon et al. 2006) cuando se encuentra sobre *F. arundinacea*. Cuando dos especies compiten por un recurso limitado como el alimento o el espacio, la especie mejor competidora puede causar la reducción poblacional de la otra tal y como se ha visto que ocurre cuando la especie invasora *T. evansi* coloniza cultivos y plantas no cultivadas en la Comunidad Valenciana. En este caso, la aparición de *T. evansi* conlleva una reducción significativa de las poblaciones de las especies de tetraníquidos nativas, *T. urticae* y *T. turkestani* (Ferragut et al. 2013). La tasa intrínseca de crecimiento de *A. obscurus* sobre *F. arundinacea* es superior a la de *T. urticae* sobre clementino (Aucejo et al. 2004). Estos resultados, junto con la relación inversamente proporcional que se observa en campo entre las densidades de ambos fitófagos, apoyan la hipótesis de que existe competencia interespecífica entre *A. obscurus* y *T. urticae*, resultando el trips el mejor competidor. No obstante, *A. obscurus* resultó incapaz de alimentarse de huevos de *T. urticae*, lo que permite descartar la hipótesis de que este trips actúe como depredador de este estado de la araña, probablemente el más vulnerable de esta especie.

Anaphothrips obscurus presenta dimorfismo alar (Hinds 1900; Nakao 1996). Los resultados obtenidos en esta tesis mostraron diferencias en las características biológicas entre hembras aladas y braquípteras, teniendo las primeras un tiempo de desarrollo ligeramente superior y las últimas mayor fecundidad, cuando se desarrollan sobre *F. arundinacea*. Este mismo fenómeno se observó cuando se desarrolla sobre maíz (Jiang et al. 2015). Sin embargo, el desarrollo de alas tuvo un fuerte impacto en los parámetros demográficos, ya que las hembras macrópteras mostraron menores tasas intrínseca de crecimiento y reproductiva neta. Por tanto, en esta especie el desarrollo de alas que facilitan la dispersión tiene un coste reproductivo asociado, constituyendo un ejemplo más del clásico "trade-off" entre reproducción y dispersión que ocurre en algunas especies de artrópodos (Zera y Mole 1994).

La disponibilidad de fuentes de alimento alternativas es particularmente importante para algunos depredadores fitoseidos, ya que les ayuda a mantener sus

poblaciones cuando la presa principal escasea, evitando que reaparezcan altas infestaciones de la plaga (Boller et al. 2004). Las tres especies de fitoseidos estudiadas en el capítulo 2 de la presente tesis (*N. barkeri*, *N. californicus* y *E. stipulatus*) fueron capaces de alimentarse de *A. obscurus* confirmando su potencial para actuar como presa de los fitoseidos. Para conocer mejor la idoneidad de *A. obscurus* como fuente de alimento de los fitoseidos así como su posible influencia en la regulación natural de las poblaciones de *T. urticae* mediante su interacción en las relaciones tróficas, se estudió tanto la respuesta funcional y la puesta cuando se alimentaron de este trips, como la preferencia por *A. obscurus* o *T. urticae* como presa de *P. persimilis*, *N. californicus*, *N. barkeri*, y *E. stipulatus*.

En la presente tesis se demuestra, que los fitoseidos de la especie *P. persimilis*, considerados depredadores especialistas de *Tetranychus* spp., son capaces de alimentarse, especialmente en sus fases inmaduras, de *A. obscurus*. No obstante, tienen una preferencia marcada por *T. urticae*. Por tanto, *A. obscurus* puede actuar como presa complementaria y mejorar la supervivencia de los estadios inmaduros de *P. persimilis* cuando hay escasez de *T. urticae*, por lo que su intervención en el control biológico por conservación de esta especie plaga tendría un efecto positivo. Por otro lado, los fitoseidos de la especie *N. californicus*, considerados como depredadores especialistas de tetraníquidos, son capaces de alimentarse de *A. obscurus* e incluso aumentar sus poblaciones sólo con esta presa. Por tanto, *A. obscurus* constituye una presa alternativa adecuada para *N. californicus* permitiéndole aumentar sus poblaciones, lo que se traduce en una mayor presión de depredación sobre el trips y sobre *T. urticae*, contribuyendo a mejorar la regulación natural de las poblaciones de *T. urticae* mediante competencia aparente. La especie *N. barkeri* destacó como el depredador más efectivo de *A. obscurus* siendo capaz de aumentar sus poblaciones cuando se alimenta exclusivamente de esta presa; del mismo modo que ocurre cuando la presa es *T. urticae*. Esto aumenta la presión de depredación sobre los dos fitófagos. Sin embargo, su preferencia por *A. obscurus* conlleva que se reduzca su aportación sobre la regulación de las poblaciones de *T. urticae,* esencialmente en la cubierta vegetal. No obstante, como *A. obscurus* raramente se encuentra en la copa de los

clementinos y en caso de encontrarse sería en fase adulta y alada (más difícil de depredar) (Aguilar-Fenollosa y Jacas 2013), esta interacción negativa no parece relevante desde el punto de vista del control biológico de *T. urticae*. Finalmente, la especie *E. stipulatus* es capaz de alimentarse de *A. obscurus* y aumentar su consumo en función de la disponibilidad de presa; además muestra preferencia por este trips ante *T. urticae*. Sin embargo, no es capaz de aumentar su población alimentándose únicamente de este trips. Por tanto, la cubierta de *F. arundinacea* ofrece una fuente de alimento (polen) de baja calidad y presas alternativas para *E. stipulatus* (*T. urticae* y *A. obscurus*) que le permiten mantenerse en el cultivo pero limitando sus explosiones poblacionales. Por las razones expuestas, *A. obscurus* constituye una fuente de alimento apta para los fitoseidos más representativos del ecosistema agrícola de clementinos con cubierta vegetal de *F. arundinacea*.

En el tercer nivel trófico, la competencia y la depredación intragremial pueden alterar la composición de las especies depredadoras y, como consecuencia, afectar a la regulación de las poblaciones de la especie plaga (Polis et al. 1989, Polis y Holt 1992, Rosenheim 1998). El acceso a una fuente de alimento alternativa o complementaria puede cambiar el resultado de la depredación intragremial favoreciendo unas especies sobre otras (Sabelis y van Rijn 2006). De hecho, Guzmán y colaboradores (2016) demostraron que la presencia de una fuente de alimentación compartida es un factor determinante para reducir e incluso evitar la depredación intragremial en fitoseidos. En los clementinos, se ha descrito la existencia de depredación intragremial en fitoseidos, siendo *E. stipulatus* y *N. barkeri* competidores intragremiales superiores (Abad-Moyano et al. 2010a, b; Momen 2010). Además *N. californicus* también es capaz de desplazar a *P. persimilis* en condiciones controladas (Abad-Moyano et al. 2010a, b). Por tanto, la disponibilidad de *A. obscurus,* como fuente de alimento adecuada para los fitoseidos durante todo el año, podría reducir la competencia por el recurso alimenticio y, en consecuencia, la presión intragremial afectando positivamente en el control biológico por conservación de *T. urticae*.

Los estudios realizados hasta el momento se iniciaron con el objeto de indagar posibles explicaciones a observaciones de campo (Aguilar-Fenollosa et al. 2011a, b). Los ensayos de laboratorio se realizan con condiciones controladas y un límite de especies. Por ello, estos resultados deberían comprobarse en campo, donde las condiciones ambientales, la disponibilidad de espacios, y los artrópodos implicados en las redes tróficas son variables. Para conseguir tener este conocimiento real de lo que ocurre en los campos de clementinos es importante observar las relaciones tróficas *in situ*. Cuando se trabaja con ácaros esta observación es especialmente difícil debido a su diminuto tamaño, a que estas especies son indistinguibles a simple vista, y a sus hábitos de vida crípticos. En este aspecto, las herramientas moleculares pueden solventar estos inconvenientes.

Eitzinger y colaboradores (2017) demostraron que los estudios de respuesta funcional en laboratorio y el análisis molecular del contenido intestinal *in situ* se complementan, permitiendo demostrar y extender las predicciones teóricas en entornos naturales. En la presente tesis se ha diseñado una herramienta molecular basada en la identificación de ADN de presa en el tracto digestivo del depredador con la finalidad de validar los resultados de los estudios de respuesta funcional y comprobar el nivel de depredación intragremial bajo condiciones naturales variables y en presencia de recursos alimenticios adecuados como el que constituye *A. obscurus*.

Mediante el uso de esta herramienta es posible detectar la alimentación de fitoseidos sobre cuatro especies de ácaros que se encuentran frecuentemente en cítricos o en sus cubiertas vegetales. Los cebadores diseñados para este fin, se localizan en la región ITS1 del ADN nuclear ribosomal. Esta región se caracteriza por mostrar un nivel de variabilidad relativamente alto en ácaros (Navajas et al. 1999; Hurtado et al. 2008, Pérez-Sayas et al. 2016), y por ese motivo se ha conseguido además de detectar estos tetraníquidos, diferenciar *T. urticae* y *P. citri* de *Tetranychus turkestani* y *T. evansi.* Esta última especie está catalogada como una plaga invasora en cultivos de solanáceas, y en cítricos suele aparecer asociada al uso de cubierta vegetal espontánea (Ferragut y Escudero 1999; Aucejo et al. 2003; Boubou et al. 2011; Migeon

y Dorkeld 2006-2017) por lo que también sería interesante poderla aplicar en cultivos de solanáceas para identificar depredadores efectivos contra esta especie invasora.

En la presente tesis, el foco de atención referente a fuentes de alimento alternativas son los trips. Para conseguir la detección del mayor número de especies posible se diseñaron los cebadores en la región COI del ADN mitocondrial, que se caracteriza por estar bastante conservada y estar bien descrita en este taxón (Brunner et al. 2002; Mehle y Trdan 2012; Buckman et al. 2013; Nakahara y Minoura 2015). Por tanto, esta PCR multiplex es capaz de detectar eventos de alimentación sobre las especies de trips más abundantes de la cubierta vegetal de *F. arundinacea*, incluyendo *A. obscurus* y *Chirothrips manicatus*, las especies principales que podrían causar daño en cítricos, como *Heliothrips haemorrhoidalis* y *Pezothrips kellyanus*, y otras especies que se consideran plagas ocasionales en cítricos pero que producen daños habitualmente en otros cultivos (*Frankliniella occidentalis* y *Thrips tabaci*) (Morse and Hoddle 2006; Reitz 2009; Tekşam and Tunc¸ 2009; Jacas et al. 2010; Reitz et al. 2011; Navarro-Campos et al. 2012).

Los cebadores incluidos en la PCR multiplex para detectar ADN de planta también se localizan en una región muy conservada, el gen cloroplástico *trnL* (Chase et al. 2005; Taberlet et al. 2007). Esta característica es la responsable de que la PCR multiplex sea capaz de amplificar en todas las especies vegetales diana (entre las que se incluyen *F. arundinacea* y *Citrus clementina*) y en especies sobre las que se habían estado alimentando trips recolectados en campos de cítricos y sus cubiertas vegetales. La detección de ADN de planta en *E. stipulatus* fue negativa. Dada la capacidad de *E. stipulatus* para perforar la membrana y obtener líquidos del interior de las mismas, la no detección de planta en esta especie podría deberse a que únicamente utiliza la planta para obtener agua y no como fuente de alimento, lo que reduciría la cantidad de ADN de planta obtenido y, por tanto, su detección. Varios estudios han contemplado la posibilidad de que los fitoseidos utilicen las plantas como fuentes de alimento. Sin embargo no existe un acuerdo científico en este aspecto (Chant 1959; Porres et al. 1975; Congdon y Tanigoshi 1983; Nomikou et al. 2003; Adar et al. 2012; McMurtry et al. 2013). La PCR multiplex diseñada en la presente tesis también podría

aplicarse para explorar tanto la habilidad de los fitoseidos para alimentarse de plantas, como los hábitos de vida de otras especies de artrópodos zoofitófagos.

La depredación intragremial entre fitoseidos podría desvertebrar la regulación natural de *T. urticae*. La herramienta molecular que se ha diseñado en la presente tesis es adecuada para su estudio ya permite detectar eficientemente los eventos de depredación de *E. stipulatus* sobre *P. persimilis* incluso 20 horas después de haber tenido lugar (detección del 42.8% de los casos).

Las curvas de detección de ADN de *A. obscurus*, *F. occidentalis* y *P. persimilis* en *E. stipulatus* dieron tiempos medios de detección diferentes para las tres especies (2,3; 1,3 y 18,7 h, respectivamente) siendo mayor en *P. persimilis*, la especie con el producto de amplificación de menor longitud (pb). Estos resultados concuerdan con las observaciones de otros autores en las que tanto la identidad de la presa (Harwood et al. 2007, Gagnon et al. 2011a, Waldner et al. 2013, Greenstone et al. 2014; Pérez-Sayas et al. 2015), como la longitud del producto amplificado influyen en el tiempo de detección (Agustí et al. 1999; Zaidi et al. 1999; Juen y Traugott 2005; King et al. 2008; Waldner et al. 2013). Nuestros resultados se sitúan en el mismo rango de detección que los obtenidos en el estudio de Pérez-Sayas y colaboradores (2015), que hasta la fecha es el único trabajo en el que se han calculado tiempos de detección de ADN de presa en ácaros.

Por tanto, el desarrollo de ésta PCR multiplex supone una ampliación de las herramientas que permiten estudiar las relaciones tróficas en las que se encuentran implicados los ácaros más comunes en clementinos. La combinación de nuestra PCR multiplex junto con la desarrollada por Pérez-Sayas et al. (2015) ofrece la posibilidad de estudiar casi todo el espectro de relaciones tróficas en las que se encuentran implicados los ácaros de interés agrícola en el cultivo de clementino y obtener conocimientos que permitirán diseñar las estrategias más adecuadas para conservar las poblaciones de fitoseidos y favorecer la regulación natural de la plaga clave *T. urticae*. Además esta herramienta también se puede aplicar para buscar enemigos naturales en otros cultivos en los que *T. urticae*, *T. evansi* o los trips producen pérdidas

económicas, como por ejemplo algunos cultivos hortícolas, o para la detectar interrupción del control biológico por depredación intragremial en otros agroecosistemas donde las especies de fitoseidos incluidas pueden actuar como presas intragremiales.

Capítulo 6

Conclusiones

El objetivo principal de esta tesis doctoral es conocer las características biológicas y ecológicas de *Anaphothrips obscurus* y cómo éste podría estar contribuyendo a la regulación natural de *Tetranychus urticae* en huertos de clementino, además de desarrollar una técnica que permita determinar la contribución de diferentes fuentes de alimento en la dieta de los fitoseidos más representativos de este sistema. A continuación se presentan las principales conclusiones que responden a este objetivo:

1. *Anaphothrips obscurus* tiene un gran potencial para colonizar y desarrollarse en plantas de *Festuca arundinacea* presentes como cubierta vegetal en campos de clementinos gracias a su madurez temprana, alta fecundidad y tiempos de generación cortos que le permiten mantener un estilo de vida de la "estrategia de la r" en este hospedador vegetal. Por el contrario, es incapaz de alimentarse y reproducirse sobre hojas de clementino.
2. El desarrollo alar en las formas macrópteras de *A. obscurus* tiene un coste reproductivo asociado que se refleja en parámetros demográficos inferiores (menor tasa intrínseca de crecimiento y menor tasa neta reproductiva) a las formas braquípteras. Por tanto, esta especie constituye un nuevo ejemplo del equilibrio entre los costes de dispersión y reproducción común en artrópodos.
3. Existe una relación inversamente proporcional entre el crecimiento poblacional de *T. urticae,* y *A. obscurus* y los fitoseidos en la cubierta de *F. arundinacea*. La tasa intrínseca de desarrollo de *A. obscurus*, superior a la de *T. urticae*, sugiere que los dos fitófagos están compitiendo en la cubierta de festuca, y esta competencia favorece a *A. obscurus*.
4. Se descarta el papel de *A .obscurus* como depredador de *T. urticae*.
5. *Anaphothrips obscurus* constituye un recurso alimenticio disponible durante todo el año para *Phytoseiulus persimilis*, *Neoseiulus californicus*, *Neoseiulus barkeri* y *Euseius stipulatus* y, por tanto, puede contribuir a mantener e incluso aumentar las poblaciones de fitoseidos influyendo de forma positiva en el control biológico por conservación de *T. urticae.*

6. *Anaphothrips obscurus* actúa como presa complementaria de *P. persimilis* ya que podría permitir mejorar la supervivencia de inmaduros en periodos de escasez de su presa principal *T. urticae.*
7. *Neoseiulus californicus* aumenta la depredación conforme aumenta la densidad de la presa (respuesta funcional tipo II). Además, es capaz de incrementar sus poblaciones cuando se alimenta exclusivamente de *A. obscurus*, lo que probablemente supone en campo un incremento de la presión de depredación sobre *T. urticae* mediante competencia aparente, dado que no muestra preferencia por ninguna de las dos presas.
8. *Neoseiulus barkeri* destaca como el depredador más efectivo de *A. obscurus*, aumentando su depredación y puesta con la densidad del trips. Su preferencia marcada por *A. obscurus* podría entorpecer la regulación que ejerce sobre las poblaciones de *T. urticae* en la cubierta vegetal, pero no en los clementinos, dado que el trips rara vez aparece en la copa de los árboles.
9. *Euseius stipulatus* prefiere depredar *A. obscurus* a *T. urticae* a pesar de que el trips constituye una presa de baja calidad que le permite mantener sus poblaciones, pero no incrementarlas.
10. El uso de la cubierta de *F. arundinacea* conlleva que el alimento del que dispone *E. stipulatus* (polen y presa de baja calidad) no le permita un crecimiento poblacional explosivo. Esta condición puede ser clave para reducir la presión intragremial que ejerce sobre los depredadores más efectivos de *T. urticae* (*P. persimilis* y *N. californicus*) influyendo positivamente en el control biológico por conservación de esta especie plaga.
11. Se ha conseguido exitosamenteponer a punto una PCR multiplex para detectar en una única reacción de amplificación ADN de cinco especies de fitoseidos (*E. stipulatus*, *P. persimilis*, *N. californicus*, *N. barkeri*, *Typhlodromus* (*Typhlodromus*) *phialatus*) y sus fuentes de alimento más frecuentes en los campos de clementino y sus cubiertas vegetales (tetraníquidos y trips). Esto permite incrementar la información obtenida por reacción y por tanto reducir la cantidad de ADN requerida, el tiempo y los costes económicos.

12. Esta herramienta es capaz de detectar la alimentación de los fitoseidos sobre cuatro especies de tetraníquidos de interés agrícola (*Panonychus citri*, *T. urticae*, *Tetranychus turkestani* y *Tetranychus evansi*). Además permite diferenciar *P. citri* y *T. urticae* de *T. turkestani* y este a su vez de *T. evansi*.
13. Esta PCR multiplex detecta ADN de un amplio rango de especies de trips entre las que se encuentran los trips específicos de gramíneas más abundantes, *A. obscurus* y *Chirothrips manicatus* y los más abundantes en la copa, *Frankliniella occidentalis* y *Thrips tabaci*. Además amplifica *Heliothrips haemorrhoidalis* y *Pezothrips kellyanus*, dos especies plaga en cítricos mediterráneos.
14. La PCR multiplex diseñada en esta tesis doctoral detecta ADN de especies de plantas pertenecientes a grupos taxonómicos distantes tanto en el segundo nivel de la cadena trófica (tetraníquidos y trips) como en el tercero (fitoseidos).
15. La herramienta molecular desarrollada en esta tesis hace viable estudiar el efecto de la depredación intragremial entre ácaros fitoseidos en el campo ya que identifica ADN del depredador y de la presa intragremial haciendo posible constatar el evento de depredación sin estar presente y sin interferir en el comportamiento de las especies implicadas.
16. Esta PCR multiplex se puede aplicar en otros sistemas agrícolas en los que las especies de fitoseidos incluidas puedan ser depredadores o en caso más desfavorable para el control biológico, presa intragremial.

La presente tesis doctoral resalta la importancia de la versatilidad alimenticia de los fitoseidos para mantener sus poblaciones. Conocer las relaciones tróficas en las que se encuentran implicados es fundamental para mejorar el control biológico de una plaga clave como es *T. urticae*. A la vista de estos resultados, *A. obscurus* actúa como presa alternativa y contribuye en la conservación de los fitoseidos reafirmando que es recomendable utilizar una cubierta vegetal de *F. arundinacea* para la gestión de esta plaga. Además, la herramienta molecular desarrollada permitirá ampliar el conocimiento de estas relaciones multitróficas y, acorde a ellas, planificar estrategias que mejoren el control biológico de *T. urticae*.

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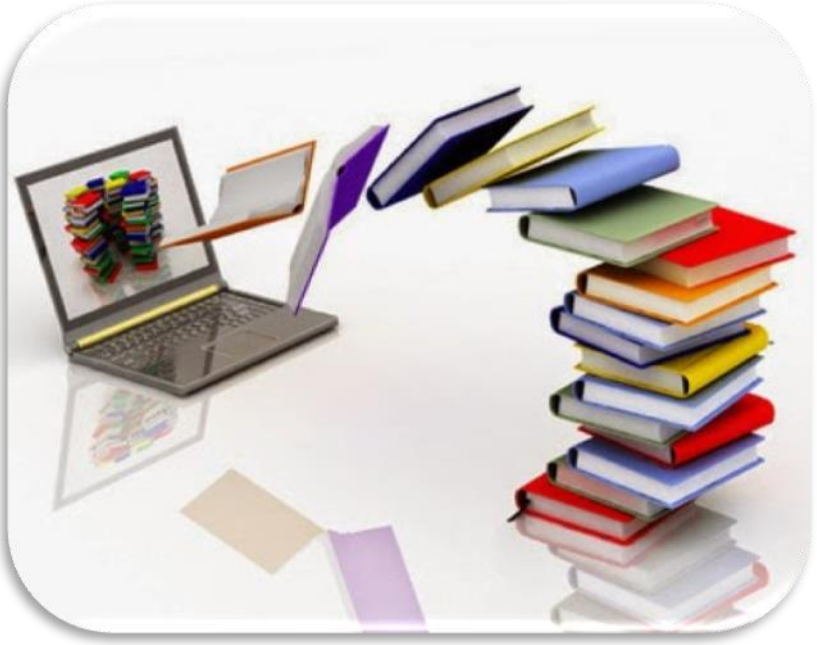

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