Departamento de Zoología
Facultad de Ciencias Biológicas

Reducing the impact of the two invasive pests, *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) and *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), by strengthening sustainability and biological control in Mediterranean citrus

International PhD Thesis
Programa de Doctorado en Biodiversidad y Biología Evolutiva

Jesica Pérez Rodríguez

Supervised by:
Dr. Alejandro Tena Barreda
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Valencia, February 2020

Universitat de València

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Institut Valencià
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“A country without research is a country without development”

Margarita Salas

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RESUMEN

1. Introducción

Actualmente, el control biológico es la base de los programas de Gestión Integrada de Plagas (GIP) de cítricos en el Mediterráneo. Uno de los mayores riesgos para estos programas de GIP en cítricos es la llegada y establecimiento de plagas exóticas que no tienen agentes de control biológico autóctonos o naturalizados que los puedan controlar. Su establecimiento obliga a los agricultores a utilizar insecticidas de amplio espectro que son incompatibles con la GIP por su elevada toxicidad sobre los agentes de control biológico. Este es el caso de dos de las últimas especies de plagas invasoras en nuestros cítricos: *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) y *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae).

Delottococcus aberiae es un pseudocóccido originario del África subsahariana que fue detectado en nuestros cítricos por primera vez en el año 2009. Este pseudocóccido, al contrario de otras especies de cochinillas algodonosas, causa graves deformaciones y reducción de tamaño en los frutos atacados, lo que conlleva importantes pérdidas económicas. Hasta su detección en nuestros cítricos, *D. aberiae* no había sido citado anteriormente como plaga de cítricos.

Desde el momento en que *D. aberiae* se estableció hasta la fecha, su control se ha basado únicamente en la utilización repetida de insecticidas de amplio espectro en primavera. En los programas de GIP, el control químico se debe limitar al momento en el que la plaga sobrepasa el Umbral Económico de Daños (UED) y Medioambiental (UEDM). Se entiende por UED la densidad poblacional mínima de plaga que origina daños económicos en el cultivo, considerando como daños económicos cuando los costes necesarios para evitar los daños en cosecha son iguales o superiores al valor económico de la pérdida de la cosecha. Los UEDM tienen además en cuenta los costes medioambientales que se producen. Para establecer estos umbrales, primero es necesario establecer un método fiable de muestreo que permita estimar la densidad poblacional de la plaga. En el caso de *D. aberiae* no se han desarrollado ni el método de muestreo ni los umbrales de tratamiento. A más largo plazo y especialmente en cítricos, el control de plagas exóticas debe priorizar el control biológico. Para ello el primer paso es identificar y determinar la eficacia de los agentes de control biológico nativos. Entre todos los agentes de control biológico, los depredadores y los parasitoides son los grupos más utilizados en el control de pseudocóccidos. Entre los parasitoides, los himenópteros pertenecientes a la familia Encyrtidae son los más importantes. Sin embargo, ninguna de las especies de encírtidos que parasitan a las especies de pseudocóccidos autóctonos han logrado parasitar eficazmente a *D. aberiae* debido a que el pseudocóccido encapsula los huevos puestos por los parasitoides. Entre todos los depredadores autóctonos o naturalizados de pseudocóccidos, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) es el depredador estenófago más voraz. Desde su introducción en España en 1928 como agente de control biológico de *Planococcus citri* Risso (Hemiptera: Pseudococcidae), se encuentra de forma natural en los campos de cítricos mediterráneos y además se utiliza en el control biológico aumentativo de otras especies de pseudocóccidos. Sin embargo, se desconoce el efecto que *C, montrouzieri* tiene sobre las poblaciones de *D. aberiae* en campo.

A diferencia de otras especies autóctonas de pseudocóccidos, *D. aberiae* migra desde la copa del árbol al suelo, un estrato en el que se pueden encontrar complejas redes tróficas de artrópodos. Entre éstos, los ácaros depredadores son unas de las especies edáficas más abundantes en cítricos donde se alimentan de otras plagas como los trips. No obstante, no existen referencias del potencial depredador de los ácaros edáficos sobre pseudocóccidos.

La reciente llegada y expansión de la psila africana de cítricos *T. erytreae* por la costa portuguesa de la Península Ibérica es probablemente el mayor desafío al que se enfrenta actualmente la gestión integrada de plagas de cítricos en el Mediterráneo. Este psílido es el vector del Huanglongbing (HLB) o "greening": la enfermedad más devastadora de los cítricos en el mundo porque no tiene cura. *Trioza erytreae* se detectó en la isla de Madeira (Portugal) en el año 1994 y en las Islas Canarias (España) en 2002. Hasta entonces, permanecía restringida a estas áreas no continentales, pero en 2014 fue detectada por primera vez en el continente europeo: en el noroeste de España y en el norte de Portugal. En estas regiones, los planes de erradicación no han tenido éxito debido a que se basan en la utilización del control químico y la mayor parte de los árboles infestados se encuentran en parcelas o jardines privados donde por cuestiones legales no siempre es posible tratar. En la actualidad *T. erytreae* sigue extendiéndose hacia el sur de Portugal, cerca del Algarve, una de las regiones de mayor producción citrícola en Portugal. Aunque el HLB no se ha detectado en Europa, el establecimiento del vector va seguido por la detección y expansión de la bacteria causante del HLB. Ante esta situación, el control biológico clásico puede ser una medida prometedora para prevenir la expansión de *T. erytreae* por las zonas citrícolas del Mediterráneo debido a que los enemigos naturales autóctonos no controlan sus poblaciones. En su lugar de origen, África subsahariana, los parasitoides son los enemigos naturales más eficaces de *T. erytreae.* Sus dos parasitoides principales son: *Tamarixia dryi* (Waterston) (Hymenoptera: Eulophidae) y *Psyllaephagus pulvinatus* Waterston (Hymenoptera: Encyrtidae). *Psyllaephagus pulvinatus* es un endoparasitoide y es la especie más común que parasita a *T. erytreae* en Camerún. En cambio, en el resto del continente africano, el ectoparasitoide *T. dryi*, es la especie más abundante y eficaz en el control de *T. erytreae.* De hecho, *T. dryi* se introdujo en los años setenta en las islas Reunión y Mauricio, donde controló las poblaciones de *T. erytreae* en pocos años. No obstante, *T. dryi* todavía no ha sido introducida en el continente europeo.

2. Objetivos

El objetivo principal de esta tesis ha sido mejorar la gestión integrada de *D. aberiae* y *T. erytreae* en cítricos fomentando la utilización del control biológico. Con este propósito, a lo largo de esta tesis doctoral se abordaron los siguientes objetivos:

i. Determinar los patrones de agregación de *D. aberiae* en cítricos, desarrollar un protocolo de muestreo eficaz para estimar la densidad poblacional y calcular los umbrales de daño económico y medioambiental. **Capítulo 2.**

ii. Evaluar el potencial depredador del ácaro del suelo *Gaeolaelaps* (*Hypoaspis*) *aculeifer* (Canestrini) (Acari: Laelapidae) sobre *D. aberiae* en condiciones de laboratorio y semicampo. **Capítulo 3.**

iii. Analizar el efecto del coccinélido *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) sobre los niveles poblacionales de *D. aberiae* en campo. **Capítulo 4.**

iv. Desentrañar el complejo de parasitoides de *T. erytreae* en Sudáfrica y analizar diferentes aspectos de la biología de sus principales parasitoides. **Capítulo 5.**

3. Material y métodos

3.1. Cálculo de los patrones de agregación de *D. aberiae*, protocolo de muestreo y umbrales de daño económico y medioambiental.

Para el desarrollo de un método de muestreo eficaz que permita estimar los niveles poblacionales de *D. aberiae* es necesario determinar con anterioridad el patrón de agregación espacial del pseudocóccido. Para ello, se tomaron muestras quincenalmente durante dos años consecutivos en hasta 19 parcelas infestadas de *D. aberiae* y en las que no se realizaron tratamientos químicos durante el periodo de muestreo. En cada parcela se muestrearon entre ocho y diez árboles, siendo en cada muestreo los mismos árboles muestreados. En cada árbol se recogieron cuatro brotes de unos 30 cm (uno en cada orientación del árbol y al azar) y una vez en el laboratorio se contabilizó la densidad poblacional de *D. aberiae* bajo la lupa, separando entre estadios.

De cada brote, se seleccionaron al azar cuatro hojas y ocho frutos o flores en los que se contabilizaron también los individuos de *D. aberiae.* Para el cálculo del umbral económico de daños, se seleccionaron al azar 40 frutos por árbol y se registraron los daños durante el periodo de cosecha. Se consideraba que un fruto estaba dañado cuando presentaba deformaciones o reducción de tamaño, suficientes para su depreciación comercial. Para el cálculo de los patrones de agregación espacial poblacional se utilizó la ley de Taylor (Taylor´s power law). Los coeficientes de agregación calculados a partir de esta ley se obtuvieron para la primera y segunda generación de *D. aberiae*. Para el desarrollo de un protocolo de muestreo, solo se consideraron los frutos ya que el patrón de agregación en este árbol no presentó diferencias entre generaciones y porque, además, el fruto se suele muestrear para determinar los umbrales económicos de daño de otras plagas. De esta manera, en un mismo muestreo podríamos calcular si diferentes plagas han sobrepasado el umbral económico de daño. Los muestreos desarrollados fueron: el muestreo binomial y el muestreo enumerativo. El muestreo binomial estima las densidades poblacionales a partir de la presencia o ausencia del insecto en el fruto. Este muestreo es más fácil en comparación con el muestreo enumerativo y permite la toma de decisiones rápidas en los programas de GIP. Para el desarrollo del muestreo binomial se utilizaron el modelo de Wilson y Room y la ecuación de Kuno, considerando diferentes niveles de precisión. Por otro lado, el muestreo enumerativo establece el tamaño de muestra mínimo para una densidad media poblacional conocida. El muestreo enumerativo se ha calculado utilizando el método de Green. Por último, el UED se ha determinado con la fórmula de Pedigo y el UEDM según el criterio de Higley y Witersteen.

3.2. Evaluación del potencial depredador del ácaro del suelo *G. aculeifer* sobre *D. aberiae* en condiciones de laboratorio y semi campo.

Para evaluar el potencial depredador de *G. aculeifer* sobre *D. aberiae* en condiciones de laboratorio, se aislaron 45 ácaros macho y 45 hembras de la misma edad y se sometieron a condiciones de ayuno. Pasadas 24 horas, se le asignó una dieta a cada ácaro: huevos de *D. aberiae*, ninfas de *D. aberiae* o ausencia de comida. Para cada dieta se realizaron 15 repeticiones por sexo. El número de presas vivas, muertas o consumidas se registró diariamente durante 14 días, reponiendo el alimento cada 24 horas. Además, se analizó la fertilidad de las hembras contabilizando la puesta de huevos cada día. A continuación, se realizó un ensayo de semicampo para determinar su eficacia. Se utilizaron 69 plántulas de *Citrus volkameriana* Tenore & Pasquale en macetas sobre las que se depositó un portaobjetos, cada uno con un ovisaco de *D. aberiae.* En 25 plantones se realizaron sueltas de unos 60 individuos de *G. aculeifer.* El número de ácaros depredadores e individuos de *D. aberiae* en el suelo se contabilizó diariamente. A los cinco días los cítricos se llevaron al laboratorio para determinar la infestación de *D. aberiae*.

3.3. Análisis del efecto del coccinélido *C. montrouzieri* sobre los niveles poblacionales de *D. aberiae* en condiciones de campo.

El estudio tuvo lugar en nueve parcelas de cítricos, infestadas con *D. aberiae.* En cada parcela se eligieron al azar entre ocho y diez árboles. Estos árboles fueron muestreados quincenalmente (entre marzo y noviembre) y mensualmente (entre diciembre y febrero) durante dos años consecutivos. En cada árbol se muestreó la copa y el tronco. Para el muestreo de la copa, en cada árbol y al azar se cortaron cuatro brotes (uno por orientación) de unos 30 cm con flores o frutos. Las muestras se observaron bajo lupa binocular en el laboratorio. En cada brote se contabilizó el número de individuos de *D. aberiae* y de *C. montrouzieri* en el brote, cuatro de sus hojas y ocho de sus flores o frutos seleccionados al azar. El muestreo del tronco consistió en el conteo visual de individuos de *D. aberiae* y *C. montrouzieri* presentes en tronco (desde la parte más baja en hasta una altura de 60 cm) durante un periodo de dos minutos. El daño en fruto también se contabilizó. Para analizar la correlación entre los picos poblacionales del depredador-presa así como los del depredador, se utilizó el test de Kendall y para determinar el efecto de *C. montrouzieri* sobre *D. aberiae* se utilizó un modelo lineal de efectos mixtos. Para analizar el efecto a largo plazo de *C. montrouzieri* sobre las tasas de crecimiento de *D. aberiae* se representaron los valores de la pendiente obtenida en la relación entre las tasas de crecimiento de *D. aberiae* y las densidades de *C. montrouzieri* en función del incremento del tiempo.

3.4. Desentrañar el complejo de parasitoides de *T. erytreae* en Sudáfrica y analizar diferentes aspectos de la biología de sus principales parasitoides.

El muestreo tuvo lugar en cuatro provincias citrícolas de Sudáfrica: Gauteng, Limpopo, Mpumalanga y el Cabo occidental. En total se muestrearon cinco parques públicos, 60 propiedades privadas y 65 parcelas citrícolas. En aquellas áreas donde se encontró *T. erytreae* se calcularon las tasas de parasitismo, se identificaron los parasitoides emergidos y la abundancia relativa de cada especie de parasitoide. Debido a la baja infestación de *T. erytreae* en algunas de las zonas muestreadas, se recolectaron entre 3 y 20 hojas por localidad y se transportaron al laboratorio. Una vez allí, se registró el número de psílidos parasitables (entre el segundo y quinto estadio ninfal) y parasitados. Cada psílido se individualizó hasta que el parasitoide emergió. Después se procedió a su identificación taxonómica. La tasa de parasitismo se calculó tomando el árbol como unidad muestral debido a que, al igual que otros psílidos, *T. erytreae* presenta un patrón de distribución espacial agregado. Una vez realizada la identificación taxonómica, ésta se verificó utilizando las secuencias de la citocromo oxidasa subunidad 1 (COI). Para ello, se amplificó el gen mitocondrial COI utilizando la técnica PCR y posteriormente se secuenció utilizando la técnica de Sanger. Las secuencias reverse y forward se ensamblaron en una secuencia consenso y se subieron al repositorio de genes GeneBank. Una vez obtenido el barcoding, utilizando el COI se analizaron las relaciones filogenéticas entre las dos especies de parasitoides: *Tamarixia dry* y *Tamarixia s*p., incluyendo a otras especies del mismo género: *Tamarixia radiata* (Waterston), *Tamarixia drukyulensis* Yefremova and Yegorenkov and *Tamarixia trioza* (Burks). Las secuencias se descargaron de GeneBank y las secuencias consenso de cada especie se alinearon utilizando el software Clustal Omega. Los posteriores análisis filogenéticos fueron realizados utilizando el software MEGA X y mediante el método Neighbor-Joining. La fiabilidad de nuestro árbol filogenético se evaluó con el test bootstrap con 10.000 réplicas y la distancia evolutiva fue calculada utilizando el Método Maximum Composite Likelihood.

La dinámica poblacional de *T. erytreae* y sus parasitoides se determinó en una parcela de limoneros infestada situada en la Unidad Experimental de la Universidad de Pretoria. La parcela no se trató con insecticidas durante el periodo de muestreo (desde octubre a diciembre). El muestreo fue quincenal y consistió en la recogida de cinco hojas infestadas de *T. erytreae* por árbol. Las muestras se procesaron según la metodología descrita para el cálculo de las tasas de parasitismo. Además, se evaluó el efecto del tamaño del hospedador en la proporción de sexos de los parasitoides primarios *T. dryi* y *Tamarixia* sp., así como el efecto sobre el hiperparasitismo. El tamaño del hospedador se calculó según la fórmula del área de una elipse. Tanto la proporción de sexos como el hiperparasitismo se analizó utilizando un modelo lineal generalizado. Para estudiar la longevidad del parasitoide primario *T. dryi* y de su principal hyperparasitoide *A. casssatus* Annecke (Hymenoptera: Encyrtidae), se seleccionaron un total de 20 hembras y 12 machos de *T. dryi* y 14 hembras y siete machos recién emergidos de *A. cassatus* muestreados en la parcela experimental de la universidad de Pretoria. Los parasitoides se introdujeron en microtubos con una gota 1M de sacarosa, que se cambió cada dos días. La supervivencia de los insectos se comprobó diariamente. Las diferencias de longevidad entre especies y sexos se analizaron utilizando un modelo de regresión de Cox.

4. Resultados

Los resultados del **capítulo 2** muestran que *D. aberiae* presentó un patrón espacial agregado en todos los órganos del árbol y que la infestación en fruto comenzó a principios de primavera. El UED obtenido fue de 7,1% de frutos ocupados y un 12,1% en el UEDM. Considerando que el UEDM es el recomendable para una puesta a punto de un programa de GIP, 12,1% de frutos ocupados corresponde a 0,24 insectos por fruto. Con esta densidad, el número de frutos requeridos para alcanzar una precisión de (D=0,25, 0,30 y 0,35) es de 210, 140 y 105 respectivamente para el método enumerativo y 390, 275 y 200 para el muestreo binomial. Aunque el muestreo enumerativo necesita un menor número de frutos muestreados y presenta una mayor precisión, requiere de más tiempo y los frutos deben ser recolectados y examinados con una lupa binocular para poder contar los individuos de *D. aberiae*. Este proceso puede durar alrededor de seis horas. En cambio, el muestreo binomial no requiere recolectar los frutos y la ocupación del fruto por *D. aberiae* puede ser contabilizada con una lupa de mano. Por ello, recomendamos la utilización de este último método de muestreo.

Los resultados del **capítulo 3** muestran que, bajo condiciones de laboratorio, el ácaro del suelo *G. aculeifer* se alimentó de ninfas de *D. aberiae*. Tanto la tasa de depredación como la fecundidad de las hembras fueron significativamente mayores cuando el ácaro se alimentó de ninfas de *D. aberiae* que de sus huevos. En los ensayos llevados a cabo en condiciones de semicampo, se observó que la infestación de las plántulas de cítricos fue menor cuando se hicieron sueltas de *G. aculeifer*. Estos resultados muestran que el ácaro puede alimentarse de *D. aberiae* en los suelos citrícolas y que, por lo tanto, se debería fomentar la presencia de ácaros depredadores en el suelo entre finales de invierno y primavera. Los resultados del **capítulo 4** evidencian que los niveles poblacionales de *D. aberiae* y de su depredador *C. montrouzieri* estuvieron sincronizados a lo largo de los dos años de estudio. Presa y depredador tuvieron dos máximos poblacionales: uno al inicio de primavera y otro en verano. A pesar de este solapamiento temporal, *C. montrouzieri* no pudo prevenir los daños provocados por *D. aberiae* en la fruta. Además, la tasa de crecimiento poblacional de *D. aberiae* no se correlacionó con la densidad poblacional de *C. montrouzieri*. No obstante, cuando se analizaron los dos años de muestreo consecutivos, el incremento poblacional de *D. aberiae* del segundo año estuvo negativamente correlacionado con la densidad poblacional de *C. montrouzieri* del verano del año anterior. Este último resultado demuestra que, aunque *C. montrouzieri* no es capaz de evitar los daños en fruto, puede ser un valioso agente de control biológico para reducir sus poblaciones a largo plazo.

El **capítulo 5** muestra que de entre todas las provincias de Sudáfrica muestreadas, solamente en la del Cabo occidental no se detectó la presencia de *T. erytreae* ni de sus síntomas. El complejo de parasitoides de *T. erytreae* estuvo formado por tres especies de parasitoides primarios: *Tamarixia dryi* (Waterston) (Hymenoptera: Encyrtidae), *Psyllaephagus pulvinatus* (Waterston) (Hymenoptera: Encyrtidae) y una nueva especie perteneciente al género *Tamarixia*. Entre ellas, *T. dryi* fue la especie más abundante pero su abundancia relativa difirió entre las zonas muestreadas. La dinámica poblacional del complejo de parasitoides muestra que la abundancia relativa de los parasitoides primarios fue mayor en el mes de octubre, pero decreció a favor de los hiperparasitoides en el mes de noviembre. El sexo de la descendencia de *T. dryi* y *Tamarixia* sp. dependió del tamaño de las ninfas de *T. erytreae* que parasitaron. Ambos parasitoides pusieron mayor proporción de hembras cuando las ninfas fueron mayores de 0,6 mm^2 en el caso de *T. dryi* y de 1,2 mm^2 para *Tamarixia* sp. Este resultado sugiere que *T. dryi* tiene mayor potencial que *Tamarixia* sp. porque pone una mayor proporción de hembras en los estadios iniciales del psílido. Las especies de parasitoides primarios fueron a su vez atacadas por tres especies de hiperparasitoides: *A. cassatus* Annecke (Hymenoptera: Encyrtidae), *Marietta javensis* (Howard) (Hymenoptera: Aphelinidae) y una especie del género *Aphanogmus*. *Aphidencyrtus cassatus* fue la especie más abundante entre los hiperparasitoides y emergió de los tamaños ninfales de *T. erytreae* de mayor tamaño. Por último, en el laboratorio se pudo determinar la longevidad del parasitoide *T. dryi* y del hiperparasitoide *A. cassatus*. Las hembras de ambas especies vivieron más de 30 días y presentaron una longevidad similar, lo que muestra el potencial problema que pueden suponer los hiperparasitoides en el programa de control biológico clásico. Como conclusión, los resultados obtenido en Sudáfrica corroboran que la introducción del parasitoide *T. dryi* en el continente europeo es una solución prometedora y, sobre todo económica, para reducir el avance de *T. erytreae*.

5. Conclusiones

5.1. Cálculo de los patrones de agregación de *D. aberiae,* protocolo de muestreo y niveles de daño económico y medioambiental.

i. *Delottococcus aberiae* presentó un patrón de distribución espacial agregado en todos los órganos del árbol (rama, hoja y fruto) sin mostrar diferencias entre la primera y la segunda generación.
ii. El daño en cosecha producido por *D. aberiae* estuvo correlacionado con la ocupación del fruto en el período en que se originan los daños (primavera).
iii. Los UED y UEDM fueron establecidos en 7,1% y 12,1% respectivamente muestreando 275 frutos con el muestreo binomial y 140 frutos con el método enumerativo.
iv. Considerando nuestros resultados, recomendamos muestrear aleatoriamente 275 frutos por parcela quincenalmente entre la caída de pétalos y el mes de julio y realizar un tratamiento químico solamente cuando el 12% de los frutos estén ocupados por *D. aberiae.*

5.2. Evaluación del potencial depredador del ácaro del suelo *G. aculeifer* sobre *D. aberiae* en condiciones de laboratorio y semi campo.

i. *Gaeolaelaps aculeifer* depredó preferiblemente ninfas de primer estadio de *D. aberiae*. Las hembras del ácaro fueron más voraces que los machos.

ii. La presencia de *G. aculeifer* en condiciones de semicampo redujo a la mitad los niveles de infestación de *D. aberiae* en plantones de cítricos.

iii. El control biológico por conservación de *G. aculeifer* y otros ácaros depredadores del suelo puede contribuir a reducir las poblaciones de *D. aberiae* en suelo en primavera.

5.3. Análisis del efecto del coccinélido *C. montrouzieri* sobre los niveles poblacionales de *D. aberiae* en condiciones de campo.

i. Las dinámicas poblacionales de *C. montrouzieri* y de su presa *D. aberiae* estuvieron sincronizadas durante los dos años consecutivos de muestreo de campo. Ambas especies presentaron dos máximos poblacionales al año: uno en primavera y otro en verano. Sin embargo, el depredador no fue capaz de evitar los daños en fruto producidos por *D. aberiae* en primavera.

ii. *Cryptolaemus montrouzieri* no redujo las poblaciones de *D. aberiae* en ese mismo año pero su abundancia en verano afectó a la densidad poblacional de *D. aberiae* del año siguiente.

iii. La utilización de *C. montrouzieri* como agente de control biológico de *D. aberiae* se debería considerar como complemento a un programa de control biológico clásico basado en la introducción de un parasitoide específico de *D. aberiae*.

5.4. Desentrañar el complejo de parasitoides de *T. erytreae* en Sudáfrica y analizar diferentes aspectos de la biología de sus principales parasitoides.

i. El complejo de parasitoides nativos de *T. erytreae* en Sudáfrica lo formaron tres especies de parasitoides primarios: *T. dryi*, *P. pulvinatus* y otro parasitoide del género *Tamarixia*. Nuestros análisis moleculares muestran que se trata de una nueva especie, cercana filogenéticamente a *T. dryi*.

ii. *Tamarixia dryi* fue la especie más abundante de parasitoide primario pero su abundancia relativa varió entre zonas muestreadas.

iii. La proporción de machos/hembras de *T. dryi* y *Tamarixia* sp. disminuyó con el aumento de tamaño de *T. erytreae* y estuvo desviada a una mayor proporción de hembras cuando el psílido tuvo un tamaño mayor a 0,6 y 1,2 mm^2, respectivamente.

iv. Los parasitoides primarios fueron atacados por tres especies de hiperparasitoides: *A. cassatus*, *M. javensis* y una especie del género *Aphanogmus*. *Aphidencyrtus cassatus* fue la especie más abundante de hyperparasitoides y emergió de tamaños más grandes de ninfas de *T. erytreae*.

v. Nuestros resultados confirman que el parasitoide *T. dryi* es el mejor candidato para ser introducido en España para el control biológico clásico de *T. erytreae*. Sin embargo, la presencia de hiperparasitoides en España podrían comprometer la eficacia de *T. dryi* como agente de control biológico de *T. erytreae*, al igual que ocurre en Sudáfrica.

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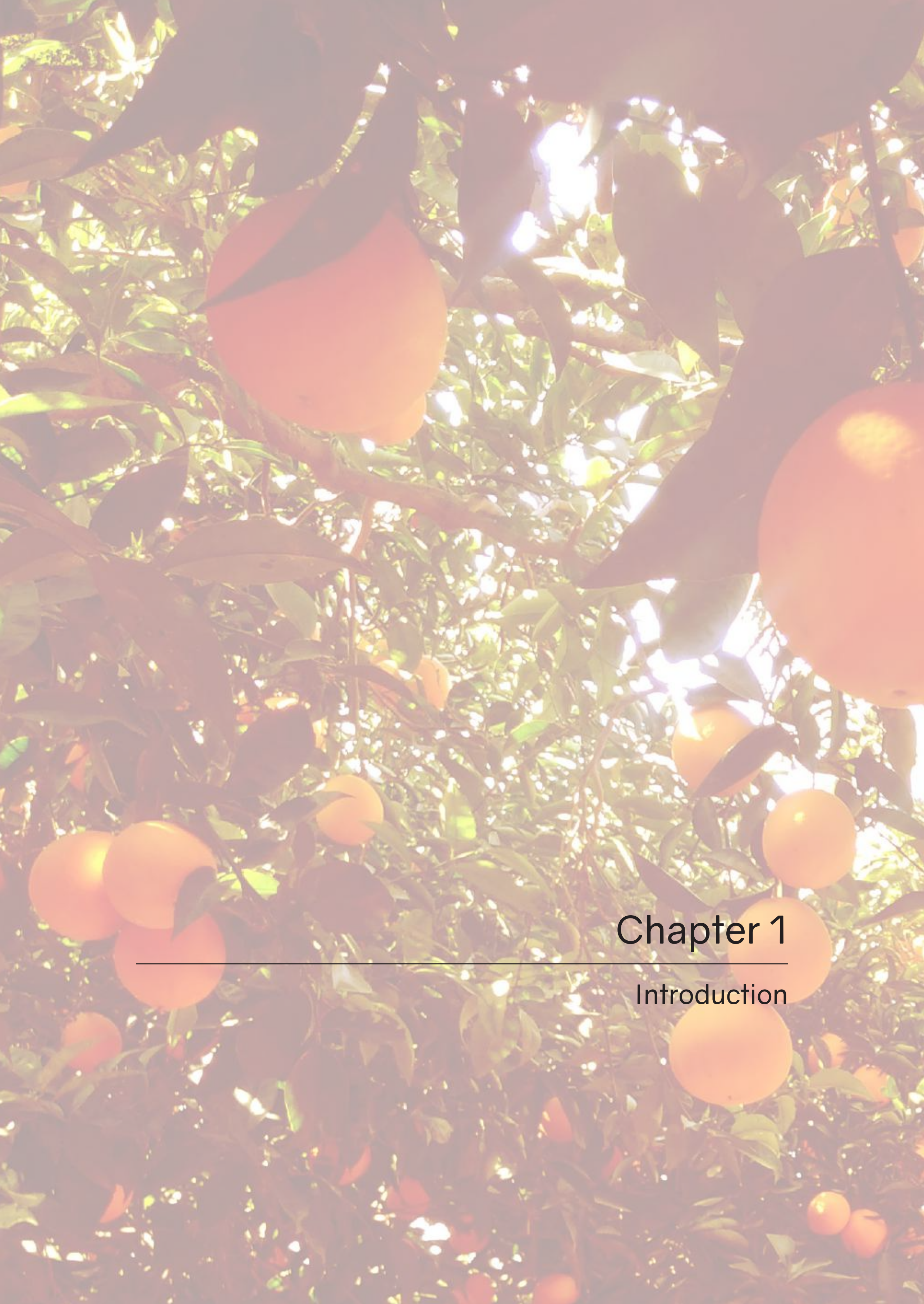
CHAPTER 2. Aggregation patterns, sampling plan, and economic injury levels for the new citrus pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae) 35



Chapter 1

Introduction

CHAPTER 1

Introduction

1.1. Integrated Pest Management in citrus crops

1.1.1. Citrus economy

Citrus is one of the most important crops in Spain. According to the Food and Agriculture Organization of the United Nations Spain produces 6,882,000 tons of citrus per year and is the largest exporter of fresh fruit worldwide; exporting 4,114,100 tons in 2017 (FAO, 2018). In Spain, the three main citrus-producing regions are located in the Mediterranean Basin: Murcia, Andalucía, and Comunidad Valenciana. Among them, the Comunidad Valenciana is the most important grower, not only because it represents the greatest citrus crop surface in Spain, but also for its long tradition of citrus farming (Peris-Moll and Juliá-Igual, 2006; MAPAMA, 2018). It produces more than half of Spanish citrus and is the largest citrus exporter in Spain; mainly for fresh consumption (MAPAMA, 2018). The Comunidad Valenciana is the major producer of mandarins (70% of the national production), the second largest producer of oranges (45%) and lemons (27%), and the third of grapefruits (30%).

Of the mandarin cultivars, clementine represents more than half total production, and more specifically the Clemenules variety (75% of clementine produced) (MAPAMA, 2018).

However, during recent years, Clemenules production has exceeded fruit demand (Tejedo et al., 2019) and what is more, Clemenules in Spain are restricted by a harvestable period between November and December. As such, commercial competitors, for example South Africa, have appeared, further saturating the European market and hence the Spanish Clemenules is becoming economically unviable to produce (Soro, 2019; Tejedo et al., 2019). As a result, other citrus varieties that could be harvested in other months are being considered (Tejedo et al., 2019).

1.1.2. IPM and current status of citrus pests in the Mediterranean Basin

Integrated Pest Management (IPM) is a wide-based pest control strategy that relies on the combination of biological, biotechnological, chemical and/or plant selection approaches (Kogan, 1998; Ehler, 2006; Chandler et al., 2011; Flint and Van den Bosh, 2012; Stenberg, 2017; FAO, 2019). The correct implementation of an IPM program restricts the use of pesticides to moments when pest population levels exceed the environmental economic injury levels (EEIL) (Naranjo et al., 2015). EEIL is defined as the lowest population density that will cause economic damage taking into consideration environmental risks (Higley and Wintersteen, 1996). Economic damage is caused when the cost (in terms of money) of suppressing the pest is equal to the potential monetary loss produced by the pest population (Stern et al., 1959; Pedigo, 19869). According to the new

European Directive 2009/128/CE, IPM has been established as mandatory for all European producers since 2014.

Similar to other crops, the correct implementation of an IPM program in citrus demands the complete knowledge of the insect´s biology and that of its natural enemies, a reliable sampling protocol which will be used to establish the economic thresholds, and the knowledge of both insecticide efficacy and the side effects on beneficial insects (Urbaneja et al., 2018).

Mediterranean citrus orchards shelter a wide range of arthropods, among them are natural enemies which exert key roles in maintaining citrus pests under the EEIL. Some well-known satisfactory biological control examples include *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) regulated by the parasitoid *Citrostichus phyllocnistoides* Narayan (Hymenoptera: Eulophidae) (García-Marí et al., 2004; Jacas et al., 2006), the citrus red mite *Panonychus citri* McGregor (Acari: Tetranychidae) controlled by *Euseius stipulatus* Athias-Henriot (Acari: Phytoseiidae) or the cottony cushion scale *Icerya purchasi* Maskell (Hemiptera: Monophlebidae) controlled by *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae) (Jacas et al., 2006; Jacas and Urbaneja, 2010) (Table 1). However, some polyphagous species still remain uncontrolled by their natural enemies; their current IPM still rely on the use of chemical control (Tena and García-Marí, 2011).

Table 1. Rank of the main Mediterranean citrus pests and the status of their biological control. (Adapted from: Jacas and Urbaneja, 2008).

Rank	Citrus pest	Biological control
1	*Aonidiella aurantii*	Unsatisfactory
2	*Ceratitis capitata*	Unsatisfactory
3	*Aphis spiraecola*	Unsatisfactory
4	*Aphis gossypii*	Unsatisfactory
5	*Tetranychus urticae*	Unsatisfactory
6	*Phyllocnistis citrella*	Satisfactory
7	*Panonychus citri*	Satisfactory
8	*Planococcus citri*	Satisfactory
9	*Prays citri*	Satisfactory
10	*Aleurothrixus floccosus*	Satisfactory
11	*Lepidosaphes becki*	Satisfactory
12	*Insulaspis gloverii*	Excellent
13	*Parlatoria pergandei*	Satisfactory
14	*Aspidiotus nerii*	Satisfactory
15	*Icerya purchasi*	Excellent

Traditionally, citrus pest management relied on chemical control and the overuse of insecticides which caused insecticide resistance, cases of hormoligosis (i.e. increased rate of reproduction due to stressor), and decreases in natural enemy populations (Grafton-Cardwell and Vehrs, 1995; Morse and Zareh, 1991; Grafton-Cardwell and Gu, 2003; Planes et al., 2015; Urbaneja et al., 2008). These natural enemies, however, play an important role not only in decreasing insect pest populations but also exerting control upon other phytophagous citrus pests (occasional or secondary pests) (Jacas and Urbaneja, 2008). The implementation of IPM programs in citrus during recent decades has reduced the use of pesticides in Spanish citrus. In fact, the number of farmers that follow an IPM program in Spanish citrus has increased fivefold (Generalitat Valenciana, 2014) and biological control has become the cornerstone for both the implementation of IPM programmes and organic agriculture (Kogan, 1988; Jacas et al., 2006; Jacas and Urbaneja, 2010; García-Marí, 2012).

However, the use of IPM in citrus is threatened by the arrival of new invasive pests (Planes, 2016). This threat is especially important when information is lacking about the pest´s life cycle, seasonal trends, natural enemies, and invasive potential.

1.1.3. Invasive pests in citrus

During the last few decades the number of invasive species has dramatically increased worldwide. Globalisation, international trade, tourism, and global warming have been described as key pathways for the unintended entry and spread of invasive species (Perrings et al., 2005; Hulme, 2009; Roques et al., 2009; Banks et al., 2014). During the last decade of the 20th century more than 1.5 established invasive species per day were recorded; this number is expected to increase (Seebens et al., 2017). In fact, throughout the 21st century more than 17% of the global land area (excluding Antarctica and glaciated Greenland) is highly vulnerable to invasion (Early et al., 2016) (Fig. 1). Among all animal taxonomic groups, Insecta is the class with the greatest number of invasive species recorded (Seebens et al., 2017) (Fig. 2). In Europe 1,306 established invasive species are insects and represent one of the major threats to global agricultural production (Dent, 2000; Pimentel et al., 2001; Keller et al., 2011; Paini et al., 2016). Of the established number of invasive insect species worldwide, Hemiptera represents the second largest Order reported (26%); following the Coleoptera (29%) (Walther et al., 2009).

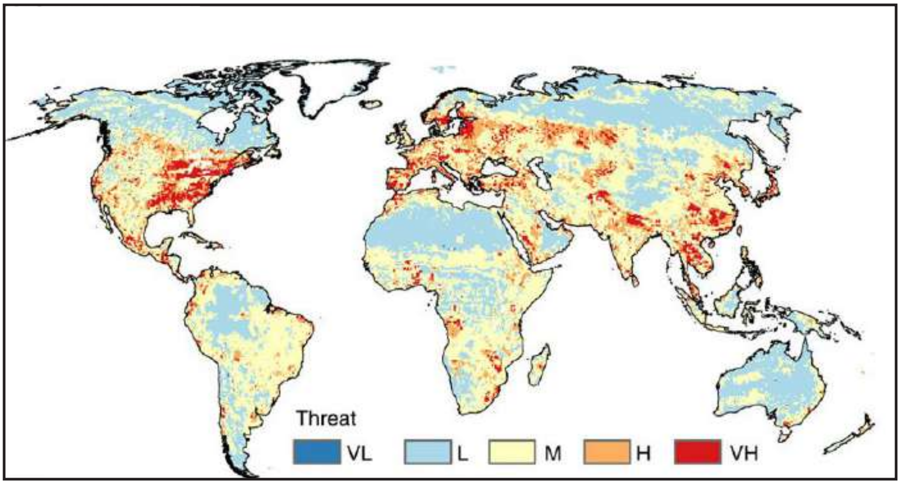


Fig. 1. Threat of emerging species invasions in the twenty first century (VL= Very low; L= Low; M= Medium; H= High; VH= Very high) (From: Early et al., 2016).

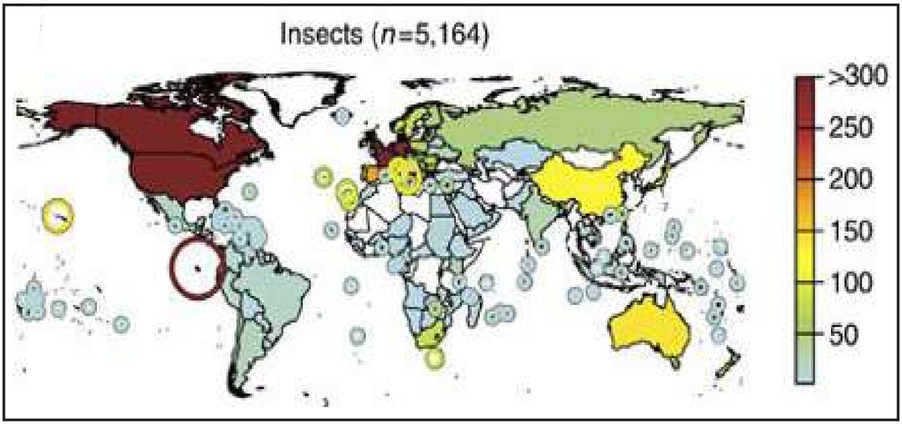


Fig. 2. Number of first records of established invasive insect species per region. Colour and size of circles indicate the number of first records of established invasive species. Circles denote first records on small islands and archipelagos (From: Seebens, 2017).

In Spain, more than 80 species of arthropods were accidentally introduced and established between 1965 and 2010 (Peña, 2013). Spanish citrus is not immune to this threat. Two of the most relevant and known invasive insect species in citrus in the nineteenth century were the woolly whitefly *Aleurothrixus floccosus* (Maskell) (Hemiptera: Aleyrodidae) in the 70´s and

Phyllocnistis citrella Stainton (Lepidoptera: Gracillariidae) in the 90´s. During the first 20 years of the new century, a new invasive pest has established or emerged every 2-3 years: the thrips *Pezothrips kellyanus* (Bagnall), *Scirtothrips dorsalis* Hood, and *Chaetanaphothrips orchidii* (Moulton) (Thysanoptera: Thripidae), the mites *Eutetranychus orientalis* (Klein), *E. banski* (McGregor) and the hemipterans *Coccus pseudomagnoliarum* (Kuwana), *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) and *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) (Beltrà et al., 2013a; Pérez-Otero, 2015; Planes, 2016). Among these new pests, the hemipterans *D. aberiae* (Fig. 3) and *T. erytreae* (Fig. 4) represent the main threat for the Spanish citrus industry as described in the following sections.

Fig. 3. *Delottococcus aberiae* adult females.

Fig. 4. *Trioza erytreae* adult.

1.2. *Delottococcus aberiae* as citrus pest

1.2.1. Origin and geographical distribution

The paraphyletic genus *Delottococcus* includes nine mealybug species native to southern areas of the Afrotropical region (Cox and Ben-Dov, 1986; Miller and Giliomee, 2011; García-Morales et al., 2016). Until the accidental introduction of *D. aberiae*, only *D. euphorbiae* (Ezzat and McConnell) and *D. proteae* (Hall) had been recorded in Europe as invasive pests of ornamental plants (Pellizzari and Germain, 2010; Franco et al., 2011). The species *D. aberiae* is native to sub-Saharan Africa and is irregularly distributed in Kenya, Mozambique, Swaziland, South Africa, Tanzania and Zimbabwe (Ben-Dov, 1994; Miller and Giliomee, 2011). In 2009 it was first recorded in the northern part of Valencia province (Spain); the centre of the main citrus producing area in the Mediterranean Basin. Since then, it has continued to spread into adjoining areas (Beltrà et al., 2013a; Soto et al., 2016). A recent study, that combined morphological and molecular characterization, has determined that the population of *D. aberiae* established in Spain has its origin in Limpopo Province (north-eastern South Africa) as they share identical COI haplotypes (Beltrà et al., 2015). Even though one specimen of *D. aberiae* was also intercepted at a U.S. ports-of-entry on citrus, at the moment this is the first record of *D. aberiae* as a citrus pest in the world (Miller and Giliomee, 2011; Beltrà et al., 2013a). Therefore, information about its biology, ecology, and management in citrus has been scarce until its detection in Spanish citrus.

1.2.2. Morphological description

Delottococcus aberiae morphological identification involves several difficulties due to its similarity with other native or naturalised citrus pseudococcids of the Mediterranean Basin. In Spanish citrus there are four different species of mealybugs: *Planococcus citri* Risso, *Pseudococcus longispinus* Targioni-Tozzetti, *Pseudococcus calceolariae* (Maskell) and *Pseudococcus viburni* (Signoret) (Franco et al., 2004; García-Marí, 2012). *Planococcus citri* is the most abundant and widespread species. It is considered to be a key citrus pest even though it has been recorded in other plant hosts (Franco et al., 2004). First instar nymphs of *P. citri* and *D. aberiae* are indistinguishable and molecular techniques are required for correct identification. However, despite their morphological similarity, older nymphs and adult females of all five species have distinctive features (Franco et al., 2000). Adult females of *D. aberiae* are characterised by an oval body, with no line along their dorsum; their pigmentation varies with environmental conditions (Cox, 1983; Charles et al., 2000). Generally they are greyish, with a white cottony cover and 18 filaments bordering the whole body. The last two anal filaments are longer than the rest and they are approximately one eighth the length of their body (Fig. 5A). Distinct to the other four native species of mealybugs, *D. aberiae* has no ventral circle (Fig. 5B) and when *D. aberiae* females lay their ovisacs they do not remain underneath their abdomen (Personal observations); they typically lay more than one.

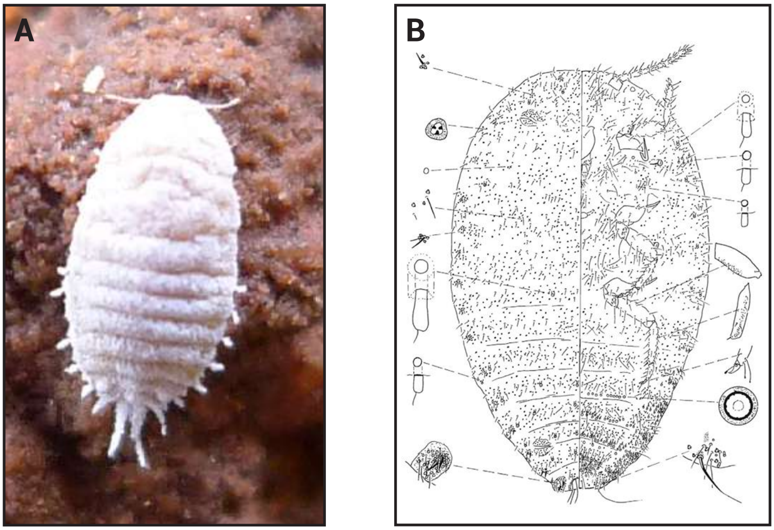


Fig. 5. *Delottococcus aberiae* adult female. **A.** Dorsal surface. **B.** Dorsal and ventral surface (From: Miller and Giliomee, 2011).

1.2.3. Biology and life history

Similar to other mealybugs, *D. aberiae* exhibits sexual dimorphism (Fig. 6) (Gullan and Kosztarab, 1997; Franco et al., 2009; Gullan and Martin, 2009). Once eggs hatch, females have three immature instars before reaching maturity. Due to neoteny, adult females resemble and keep the morphology of the immature individuals, being wingless with well-developed mouthparts. They continue feeding and growing until mating and can live for several months before laying the eggs in a cottony ovisac (Fig. 7). Every ovisac can contain more than 300 eggs (personal observations). In contrast, males show clear morphological differences between their immature and adult stages. Males have four immature instars the first two are similar to that of females. At the end of the second nymphal instar, males develop a waxy cocoon. They go throw two pupa-like stages (pre-pupa and pupa) from which a winged adult male with distinct head, thorax, and abdomen emerges (McKenzie, 1967; Cox, 1987; Gullan and Kosztarab, 1997; Franco et al., 2000; Franco et al., 2009;

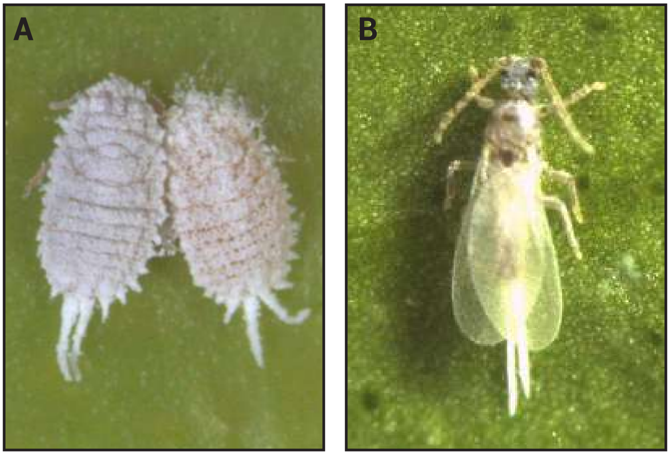


Fig. 6. *Delottococcus aberiae*. **A.** Adult females. **B.** Adult male.

Gullan and Martin, 2009; Beltrà and Soto, 2012; Mani and Shivaraju, 2016). Adult males do not feed and live only a few days, a limited time to seek out females; males can be easily overlooked in the field (Kosztarab and Kozár, 1988; Gullan and Martin, 2009; Martínez-Blay, 2018).

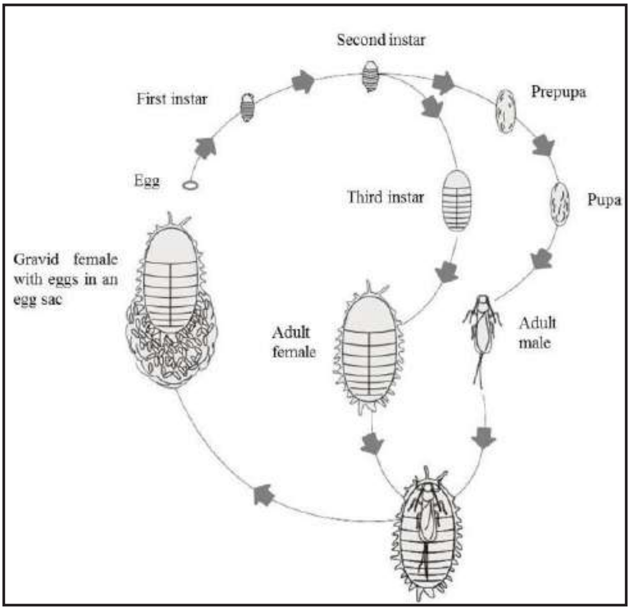


Fig. 7. Life cycle of a mealybug (From: Beltrà and Soto, 2012).

Delottococcus aberiae completes between six and seven generations per year; contrary to other Mediterranean citrus mealybug species, it remains active during winter (Martínez-Blay et al., 2017). *Delottococcus aberiae* shows two main population peaks per year in Spanish citrus: one in spring (between April and May) and another in summer (between June and July). Distinctly to other citrus mealybug species, *D. aberiae* females tend to descend to the trunk and soil to lay their ovisacs in spring (Fig. 8A) (Martínez-Blay et al., 2018b). In the canopy, all *D. aberiae* instars settle in different plant organs (Fig. 8B) but, the aggregation patterns in these organs are unknown. Mealybugs, as many hemipterans, tend to aggregate; sampling protocols can be improved with the knowledge of their particular aggregation patterns on the distinct plant organs (Binns and Nyrop, 1992; Nestel et al., 1995; Pretty and Bharucha, 2015).

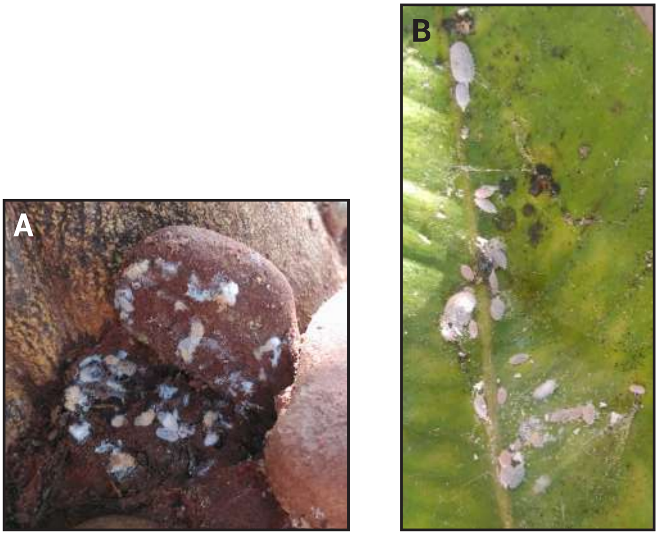


Fig. 8. Adult females of *D. aberiae* laying their ovisacs. **A.** In the soil. **B.** In leaves.

1.2.4. Host plants

Delottococcus aberiae is a polyphagous phloem feeder. It has been recorded in more than 25 different botanical families in sub-Saharan Africa (Miller and Giliomee, 2011; García-Morales et al., 2016). It feeds on various tropical and subtropical crops such as citrus, coffee, guava, persimmon, pear and olive (De Lotto 1961; Hattingh et al., 1998; Miller and Giliomee, 2011; Beltrà et al., 2013a; García-Martínez et al., 2016). It can also feed on ornamental flowering plants or trees. In some species, such as *Chrysanthemoides monilifera*

(L.) T. Norl, *D. aberiae* settles in the shrub roots (Miller and Giliomee, 2011; Martínez-Blay, 2018). In citrus, *D. aberiae* has been observed in numerous cultivars of mandarins, oranges, and lemons, however, its developmental rates and preferences have not been studied.

Delottococcus aberiae is irregularly distributed in sub-Saharan citrus where it originated. Due to this irregular distribution and the generalised use of insecticides in South African citrus groves, *D. aberiae* has been categorised as a secondary pest of citrus that can remain unnoticed for years in this region (Hattingh et al., 1998; Miller and Giliomee, 2011; Beltrà et al., 2015).

1.2.5. Plant damage

Like other mealybug species feeding on citrus, *D. aberiae* produces indirect plant damage. Its feeding behaviour reduces plant vigour and the excretion of honeydew. In turn, the honeydew fosters the growth of sooty-mould fungi which decreases photosynthesis rates and produces a niche for secondary pests such as pyralid moths (Martínez-Blay et al., 2018a). However, distinct to other citrus mealybug species, *D. aberiae* produces direct and relevant damage causing severe distortions or size reduction in fruits (Fig. 9) (Beltrà et al., 2013a). The type of damage can vary among citrus cultivars. Whilst in orange varieties it produces fruit distortions or bumps close to the fruit stem, in mandarin it produces fruit distortion and size reduction (Pérez-Rodríguez et al., 2015).

Fig. 9. Direct damage produced by *D. aberiae*. **A.** Size reduction. **B.** Fruit distortion.

1.2.6. Control methods

In citrus IPM, biological control has been widely used and is recognised as the main tool against invasive pests in the Mediterranean Basin (Kogan, 1998; Jacas et al., 2006; Jacas and Urbaneja, 2010). Long-term studies have demonstrated biological control to provide not only high economic benefits but also positive changes to the environment (De Bach, 1964; Gutierrez, 1999; Hill and Greathead, 2000; Naranjo et al., 2015). However, when a new invasive pest is established little is known about the indigenous or naturalised natural enemies. In the case of *D. aberiae*, the development of an IPM based on biological control was hindered by the fact that this species was first reported as a citrus pest.

Worldwide, parasitoids and predators are the most commonly used biological control agents against mealybugs. Among them, parasitoids play the major role in regulating mealybug populations, especially in the Encyrtidae family (Moore, 1988; Godfray, 1994; Shylesha and Mani, 2016; Hajek and Elenberg, 2018). In the Iberian Peninsula several Encyrtidae parasitoid species are reported to control mealybug populations such as *Anagyrus* sp. near *pseudococci* (Fig. 10) (Girault), *Leptomastix algirica* (Trjapitzin), *Tetracnemoidea peregrina* (Compere), *Acerophagus angustifrons* (Gahan) and *Cryptanusia comperei* (Timberlake). However, none of these species have been able to successfully parasitize *D. aberiae* because their eggs are encapsulated by the mealybug (Fig. 11) (Tena et al., 2017).

Fig. 10. *Anagyrus* sp. near *pseudococci* female.

Fig. 11. *Delottococcus aberiae* with *Anagyrus* sp. near *pseudococci* encapsulated egg (Source: Alejandro Tena).

Predators also exert an important role in controlling mealybug populations (Franco et al., 2009; Daane et al., 2012; Mani and Shivaraju, 2016). A wide range of generalist arthropod predators such as lacewings, earwigs, cecidomyid flies, mirids and spiders feed on mealybugs (Costello & Daane, 1999; Senior et al., 2001; Daane et al., 2006; Shylesha and Mani, 2016). Among these, stenophagous coccinellids are considered the most efficient mealybug predators; the mealybug destroyer *Cryptolaemus montrouzieri* Mulsant is the most widely used predator in biological control of mealybugs (Kairo et al., 2000; Hodek and Honěk, 2012; Maani et al., 2011; Maes et al., 2014; Mani and Shivaraju, 2016). *Cryptolaemus montrouzieri* (Fig. 12A and 12.B) was introduced in Spain in 1928 to control *P. citri* (Gómez-Clemente 1928). Since its introduction, it has naturally established in our citrus (Jacas et al., 2006) but it is also augmentative released annually by public and private organizations (Martínez-Ferrer, 2003). However, the effect of this stenophagous predator on *D. aberiae* is still unknown.

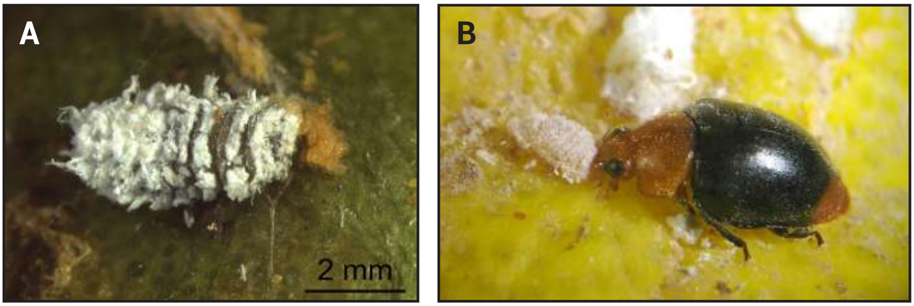


Fig. 12. *Cryptolaemus montrouzieri.* **A.** Larvae. **B.** Adult.

Unlike other native or naturalised mealybug species that feed on citrus, *D. aberiae* migrates from the tree canopy to the soil (Martínez-Blay et al., 2018). This stratum also shelters a rich complex of arthropod predators (Juen and Traugott, 2005; Ruf and Beck, 2005; Monzó, 2010). Among these predators, predatory mites are abundant in citrus and have been used in biological control of thrips, sciarids, or bulb mites that inhabit the soil (Berndt, 2004; Navarro et al., 2013). However, there are no references about its predatory potential upon mealybugs that spend only part of their life cycle in the soil.

The ineffectiveness of naturalized and indigenous parasitoids and the lack of information about the potential of naturalised or native predators to control *D. aberiae* populations have lead farmers to use insecticides to control this invasive mealybug (Tena et al., 2017). Among the recommended and authorized insecticides, chlorpyrifos methyl and chlorpyrifos can control the damaging populations of *D. aberiae*. Unfortunately, these insecticides are very toxic for most natural enemies and, what it is even worst, the sampling protocol and economic thresholds have not been developed (Newsom, 1967; Ware, 1980; Van der Werf and Hayo, 1996; Chagnon et al., 2015; James et al., 2016). In order to minimise the impact of insecticides and improve a decision-making programme when chemical control is required, *D. aberiae* aggregation patterns as well as the establishment of a sampling protocol and Economic Injury Levels are needed (Pedigo et al., 1986; Higley and Pedigo, 1993; Peterson and Hunt, 2003; Hodgson et al., 2004; Ramsden et al., 2017).

1.3. *Trioza erytreae* as citrus pest and vector of Huanglongbing

1.3.1. Origin and geographical distribution

Trioza erytreae, commonly known as the African citrus psyllid, is probably native to south-eastern Africa where it is widely distributed. Recently, it has also established in the Asian and European continents (EPPO, 2019).

In Africa, *T. erytreae* has been recorded in Angola, Cameroon, Comoro Islands, Ethiopia, Eritrea, Kenya, Madagascar, Malawi, Mauritius, Republic of Congo, Reunion Island, Rwanda, Sao Tome, Principe, South Africa, St. Helena, Sudan, Swaziland, Tanzania, Uganda, Zambia, and Zimbabwe. In the Asian continent it is distributed in the Middle East (Saudi Arabia and Yemen) (Moreira, 1967; Bové and Cassin, 1968; Ettiene and Aubert, 1980) (Fig. 13).

In Europe, *T. erytreae* was first detected at the Atlantic Islands of Madeira and Porto Santo (Portugal) in 1994 (Passos de Caevalho and Franquinho Aguiar, 1997) and in the Canary Islands (Spain) in 2002 (Pérez-Padrón and Carnero Hernández, 2002; Cocuzza et al., 2017). In 2014, *T. erytreae* was first detected in mainland Europe, in the north-western regions of the Iberian Peninsula (Pérez-Otero et al., 2015). Since then, it has also become widespread in Portugal, being recently detected in Lisbon, in their citrus producing areas (Figs. 13 and 14)

(Arenas-Arenas et al., 2018). This fast spread and establishment of *T. erytreae* is a growing threat to the European citrus industry.

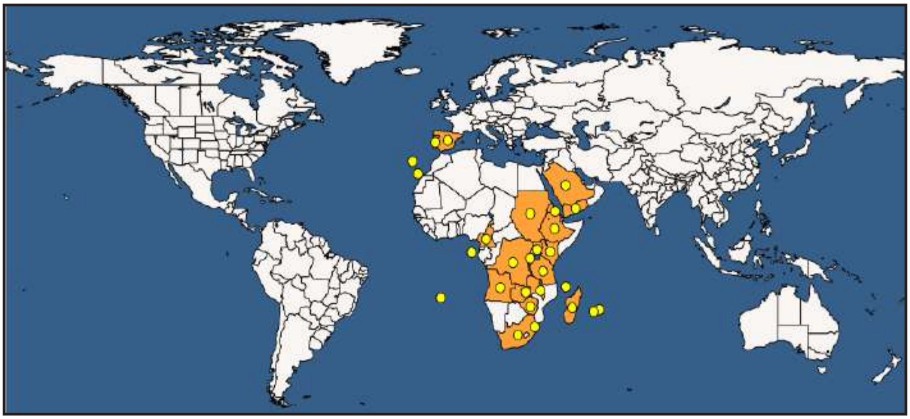


Fig. 13. Distribution of *Trioza erytreae*. Yellow spots represent the countries where *Trioza erytreae* is established (From: EPPO, 2019).

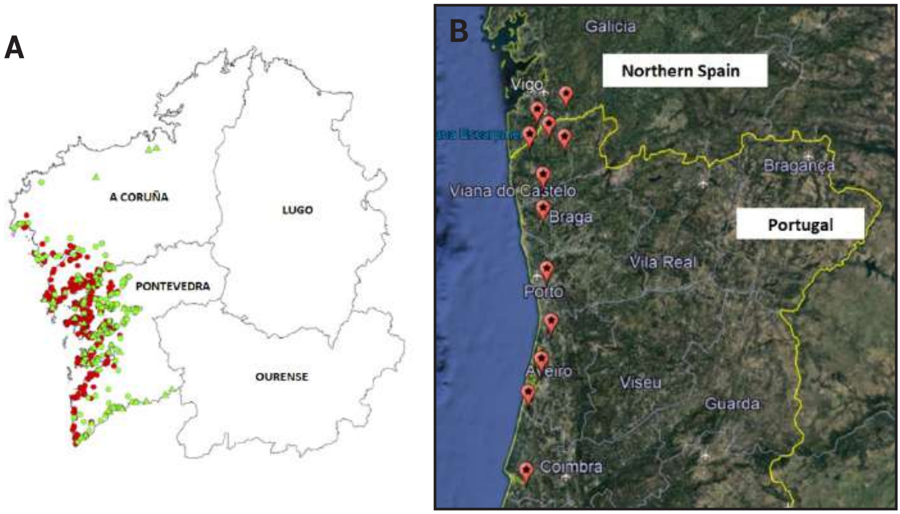


Fig. 14. Distribution of *Trioza erytreae* in Mainland Europe. **A.** North-western Spain. **B.** Portugal. Green spots represent sampled sites and red spots records of *T. erytreae* (Adapted from: Siverio et al., 2017 and Arenas-Arenas et al., 2018).

1.3.2. Morphological description

The correct identification of *T. erytreae* triggers some difficulties due to its morphological similarities with other species in the same genus or family. As other specimens belonging to the Triozidae family, *T. erytreae* fore wing venation is characterised by the split of its main vein into three others, sharing the same origin point (Fig. 15A). However, in contrast with other species of Triozidae, the first flagellomere of *T. erytreae* adults are completely white and the second one is half white and half black (Fig. 15B) (Hollis, 1984).

In the adult stage, both males and females are approximately 3-4 millimetres long, present transparent wings and are agile flying insects that skip when are disturbed. When they are newly emerged they are green coloured but progressively turn dark brownish. When feeding they lift the abdomen in a 35° angle in relation to the leaf surface where they settle (Passos de Caevalho and Franquinho Aguiar, 2007) (Fig. 16A). Females are generally longer than males and have a pointed abdomen whereas the end of the male abdomen is rounded (Fig. 16B).

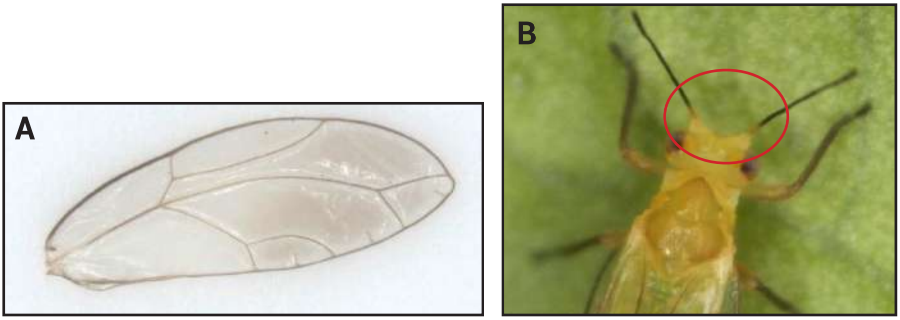


Fig. 15. *Trioza erytreae.* **A.** Wing venation. **B.** Antennae.

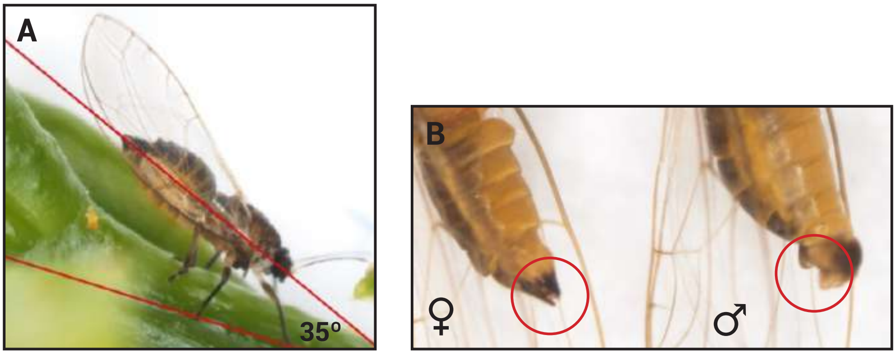


Fig. 16. *Trioza erytreae*. **A.** Position when feeding. **B.** Sexual dimorphism.

Trioza erytreae eggs are smooth, pear-shaped, and bright orange (Fig. 17A). The average length is about 0.5 mm and the plant attachment side of the egg is shortly stalked, making it appear to be inserted into the leaf tissue by a basal pedicel (White, 1968; Hokkingson, 1974). Physiological changes in the leaf tissue might affect the mechanism of water absorption and can lead to egg desiccation (Blowers and Moran, 1967; Catling, 1969c). As the embryo within the egg develops, the egg turns darker orange; before hatching the two red nymphal eye-spots become discernible under microscopic examination (Annecke and Cilliers, 1963). Once the eggs hatch, the newly emerged nymph is pale orange, flattened dorsally, lacking wing buds, and surrounded by a white, filamentous fringe. The number of filaments increases with each moult. There are approximately 50 filaments around the margin of the first instar nymph, 100 in the second, 200 in the third, 300 in the fourth and 450 in the fifth instar nymph (Catling, 1973). Nymphal colour becomes darker and greener as they go throw the latter instars. In the moult to the fourth instar two pale brown spots appear on the abdomen of the nymph (Fig. 17B) (Catling, 1973).

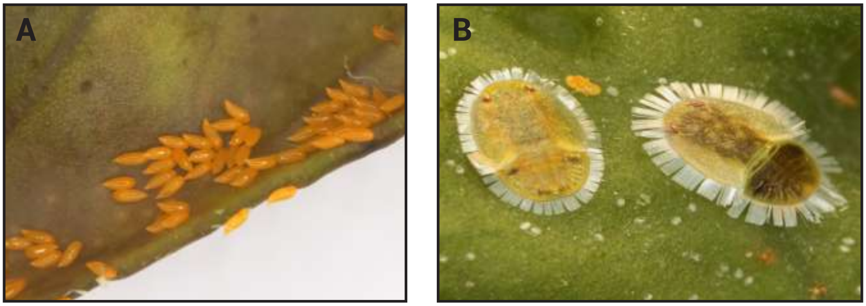


Fig. 17. *Trioza erytreae.* **A.** Eggs. **B.** Fourth and fifth nymphal instars.

1.3.3. Biology and life history

The biology and life history of *T. erytreae* is typical of a subtropical psyllid species; there is no diapause (Catling, 1973). Similar to other psyllids, *T. erytreae* goes through an egg stage and five nymphal instars before becoming adult; the final moult usually takes place during the early morning (Catling, 1973; Hodkinson, 1974) (Fig. 18).

The pre-oviposition period is approximately 3-5 days in summer (with mean temperatures around 24-26ºC) and 6-7 days in winter (14-16ºC). However, oviposition depends on the availability of young growth and tree flushes. In their absence, pre-oviposition period might be extended indefinitely (Burts and Fischer, 1967; Catling, 1969a). As soon as the cuticle of the newly emerged adult hardens, mating occurs. It is initiated, sometimes aggressively, by the male and can occur approximately three times per day during a six-day period. A mated female can lay more than 2,000 eggs (EPPO/CABI, 1997). Egg-laying ceases a few days before female death; their lifespan is around 25 days (Catling, 1973). The life cycle and longevity is similar for both sexes (Catling, 1973).

After egg-hatching, the first instar nymphs settle down preferably on the underside of the youngest available leaves. They are sedentary and highly susceptible to desiccation. Once settled they remain in one place unless disturbed or crowded (Van der Merwe, 1941). The whole nymphal period lasts about 17-43 days and the whole life cycle between 43 to 115 days, depending on climatic conditions (Catling, 1973).

Trioza erytreae can have up to eight generations per year under subtropical conditions; the span of generations extends throughout all citrus flushing periods. It is highly sensitive to temperature conditions and can only survive between 10 to 30ºC. Its optimum climatic conditions are between 20º-24ºC, around 70% RH and 500-600 m above sea level (Moran and Blowers 1967).

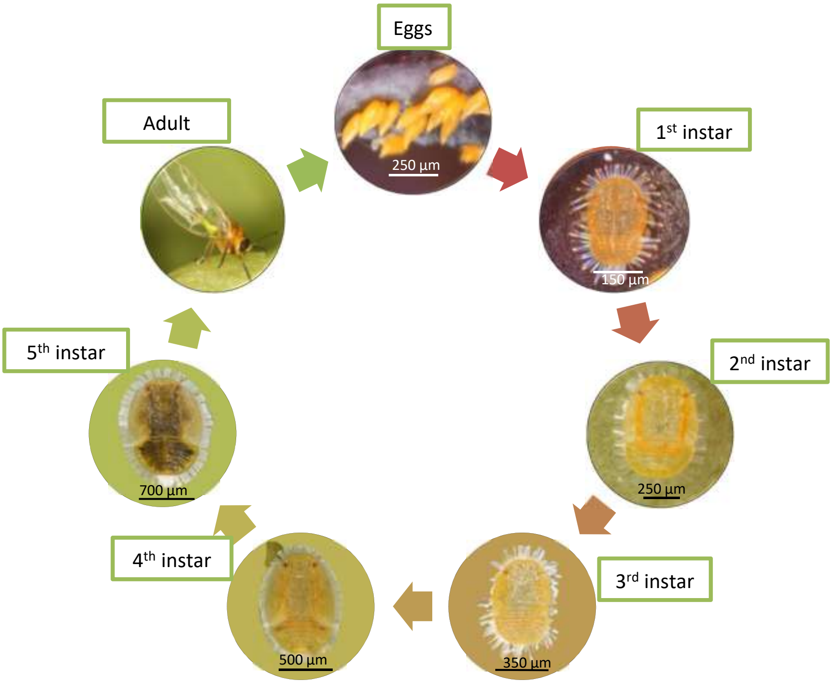


Fig. 18. Life cycle of *Trioza erytreae*.

1.3.4. Host plants

Trioza erytreae is an oligophagous phloem sap feeder of Rutaceae plants. It has been documented feeding on at least 20 species of Rutaceae, but egg laying and nymphal development are restricted to 15 and 13 species, respectively (Table 1) (Van der Merwe, 1923, Moran and Buchan, 1975; Aubert, 1987).

Even though *Clausena anisata* (Willd.) Hook.f. ex Benth. and *Vepris undulata* (Thunb.) Verdoorn & C.A. Sm. are probably the original indigenous host plants (Moran, 1968), *T. erytreae* has a marked preference for the exotic *Citrus limon* (L.) Burm due to it has larger soft flush leaves (Moran and Buchan, 1975).

Table 2. *Trioza erytreae* host plants. (+++ Very common, ++ usual + occasional, - not observed in neither natural nor experimental conditions). (Adapted from: Aubert, 1987).

		Leaf sucking	**Egg laying**	**Nymphal development**
Preferred host plants	*Citrus limon*	+++	+++	+++
	Citrus medica	+++	+++	+++
	Claussena anisata	+++	+++	+++
	Vepris lanceolata (=undulata)	+++	+++	+++
	Citrus aurantifolia	++	++	++
Common host plants	*Citrus deliciosa*	++	++	++
	Citrus nobilis	++	++	++
	Citrus paradisi	++	++	++
	Citrus reticulata	++	++	++
	Citrus sinensis	++	++	++
	Citrus grandis	+	+	+
	Murraya paniculata	+	+	+
	Fagara capense	+	+	+
Occasional host plants	*Fortunella sp.*	+	+	-
	Toddalia asiatica	+	+	-
	Calodendrom capense	+	-	-
	Microcitrus australisiaca	+	-	-
	Poncirus trifoliata	+	-	-
	Choisa ternata	+	unknown	unknown
	Murraya koenigii	+	unknown	unknown

1.3.5. Plant damage

Similar to other psyllids, the feeding activity of *T. erytreae* is detrimental to the host plant causing direct and indirect damage. Direct damage is mainly produced when *T. erytreae* newly emerged nymphs colonise the young shoots for feeding. The nesting of nymphs results in a pit-like depression (galls) beneath their body (Fig. 19A) and the excretion of honeydew, which promotes the appearance of sooty mould (Fig. 19B) (van den Berg et al., 1990). Depending on the leaf psyllid density, nymphs can also produce severe leaf distortions, chlorosis, and sap depletion (Fig. 20) (Catling, 1973; van den Berg and Deacon, 1988).

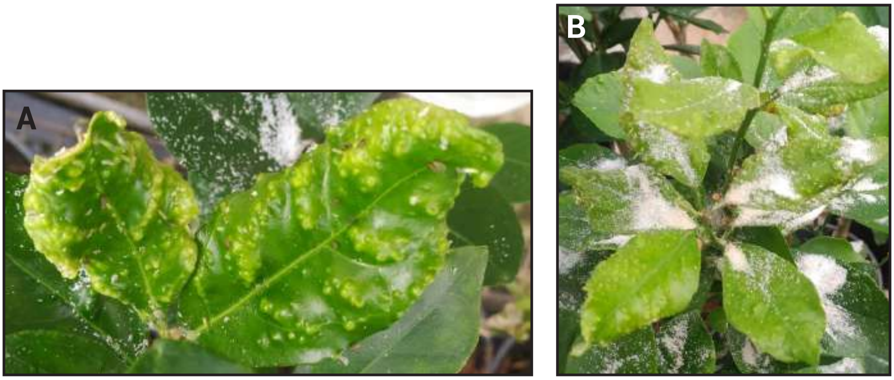


Fig. 19. *Trioza erytreae*. **A.** Leaf damage. **B.** Honeydew excretion.

Fig. 20. Severe leaves distortion and chlorosis produced by *Trioza erytreae.*

However, *T. erytreae* indirect damage is the major threat for citrus. It is a potential vector of Huanglongbing (HLB) or greening, the most devastating citrus disease (Halbert and Manjunath, 2004; Bové, 2006; Cocuzza et al., 2017). During the first stages of the disease, HLB causes asymmetrical leaf chlorosis, vein yellowing, and fruit pulp asymmetry, but in its last stages (from 5 to 10 years) tree defoliation and dieback occurs (Fig. 21). In Florida, one of the main citrus producers in the world, HLB has caused losses of 4.554 million US dollars and more than 8,000 jobs linked to the citrus industry within only 6 years (2005–2011) (Hodges and Spreen, 2012; Cocuzza et al., 2017; Singerman and Useche, 2016). Between 2014 and 2015, ten years after the first cases of HLB were identified in Florida, citrus production had been reduced by one third (Alvarez and Solis, 2018).

The disease is associated with three-gram negative, phloem-limited α-proteobacterias: *Candidatus* (*Liberibacter*) *asiaticus*, *C.* (*Liberibacter*) *americanus*, and *C.* (*Liberibacter*) *africanus* (Laf). *Trioza erytreae* has only been recorded as a *Ca*. Liberibacter africanus vector but it has been demonstrated to transmit the other bacteria strains (Garnier et al., 2000; Pietersen et al., 2010, Massonié et al., 1976; Saponari et al., 2010). Although the African form of HLB is less aggressive than the Asian HLB, it is the main cause of losses in citrus in Cameroon and numerous treatments with insecticides are necessary in South Africa to control the vector (Cocuzza et al., 2017).

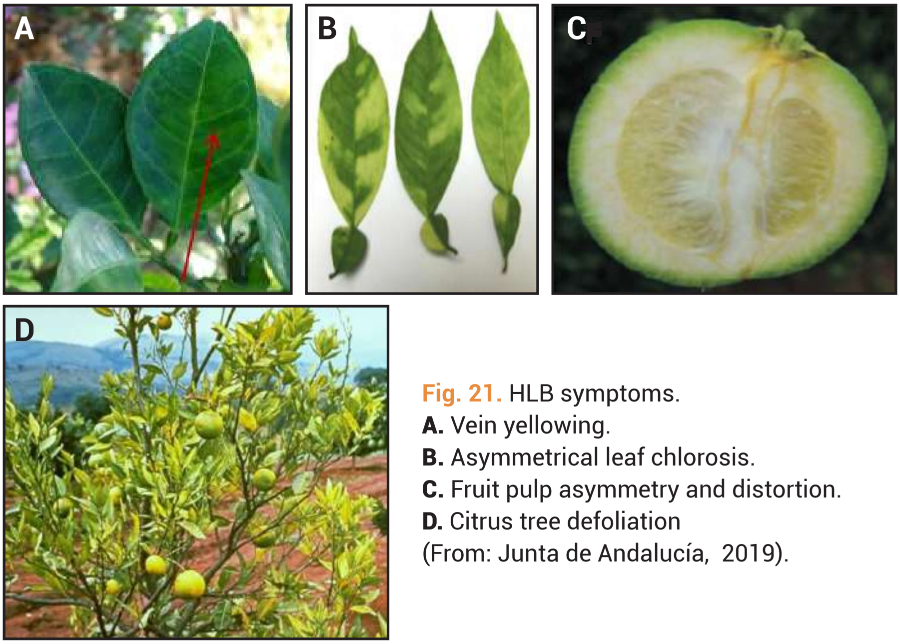


Fig. 21. HLB symptoms.
A. Vein yellowing.
B. Asymmetrical leaf chlorosis.
C. Fruit pulp asymmetry and distortion.
D. Citrus tree defoliation
(From: Junta de Andalucía, 2019).

Both *T. erytreae* nymphs and adults can acquire the bacteria when feeding and transmit the disease to healthy plants. Moreover, *T. erytreae* females can transmit Laf by transovarial transmission, yet, in low percentages (less than 10%) (Halbert and Manjunath, 2004). As reported for *D. citri,* for an effective transmission of Laf bacterial multiplication must take place inside the psyllid; once multiplied, it can remain viable for the entire life of the insect (Hung et al., 2004; Pelz-Stelinski et al., 2010). To effectively transmit the disease to the plant, only a few infected psyllids are needed, although other factors such as tree health, environmental conditions (temperature and relative humidity) or presence of new shoots can affect transmission (Schwarz and Green, 1972; van Vuuren and van der Merwe, 1992; Lee et al., 2015).

The establishment of *T. erytreae* does not necessarily imply the presence of HLB in newly invaded regions. At the moment, mainland Europe and the rest of the Mediterranean basin are free of HLB (EPPO, 2019).

1.3.6. Control methods

Due to the economic impact of HLB and the fact that there is no strategy to cure this disease, its management relies on the repeated use of broad spectrum insecticides (between 6 and 50 applications per year) against the psyllid vectors (Gottwald, 2010, Grafton-Cardwel et al., 2013; Qureshi et al., 2014). Consequently, in countries where HLB has been reported, IPM in citrus has had a significant setback (Belasque et al., 2010; Hall et al., 2013).

Although HLB is yet to be detected in Europe, *T. erytreae* has already established and spread in north-western Spain and Portugal where it is close to the main citrus producing area, Algarve (Cocuzza et al., 2017; Arenas-Arenas et al., 2018). In these regions, similar to other countries, eradication of the psyllid vectors has been unsuccessful because it is based on chemical control that has not been correctly achieved in private gardens. These gardens act as shelters for the psyllid and as sources of resurgent outbreaks (Fernandes and Aguiar, 2001; Tamesse et al., 2002a; González-Hernández, 2003; Gottwald, 2010; Cocuzza et al., 2017). Under this scenario, prompt *T. erytreae* detection, eradication of infested trees and quarantine measures in those free-psyllid contiguous areas are the most suitable measures to avoid entry and spread of both psyllid and the pathogen (Cocuzza et al., 2017). Together with these measures of contingency, biological control could be a tool for lowering population levels and spread of *T. erytreae* in mainland Europe.

Among *T. erytreae* natural enemies, parasitoids are the most abundant and successful biological agents. The two main primary parasitoids of *T. erytreae* are *Tamarixia dryi* (Waterston) (Hymenoptera: Eulophidae) and *Psyllaephagus pulvinatus* Waterston (Hymenoptera: Encyrtidae) (Catling, 1969b; McDaniel and Moran, 1972; Aubert, 1987; van den Berg and Greenland, 2000; Tamesse, 2009). *Psyllaephagus pulvinatus* is an endoparasitoid which is the most common parasitoid of *T. erytreae* in Cameroon (Fig. 22) (Tamesse et al., 2002a). By contrast, in the rest of the African continent the ectoparasitoid, *T. dryi*, plays the major role in controlling psyllid populations (Fig.23) (van den Berg and Greenland, 2000). In fact, *T. dryi* was introduced in Reunion Island and Mauritius where it controlled *T. erytreae* within a few years (Etienne, 1978; Etienne and Aubert, 1980; Aubert and Quilici, 1986; Aubert, 1987). *Tamarixia dryi*, however, has not been introduced in the Atlantic islands and mainland Europe.

Fig. 22. *Psyllaephagus pulvinatus* pupae.

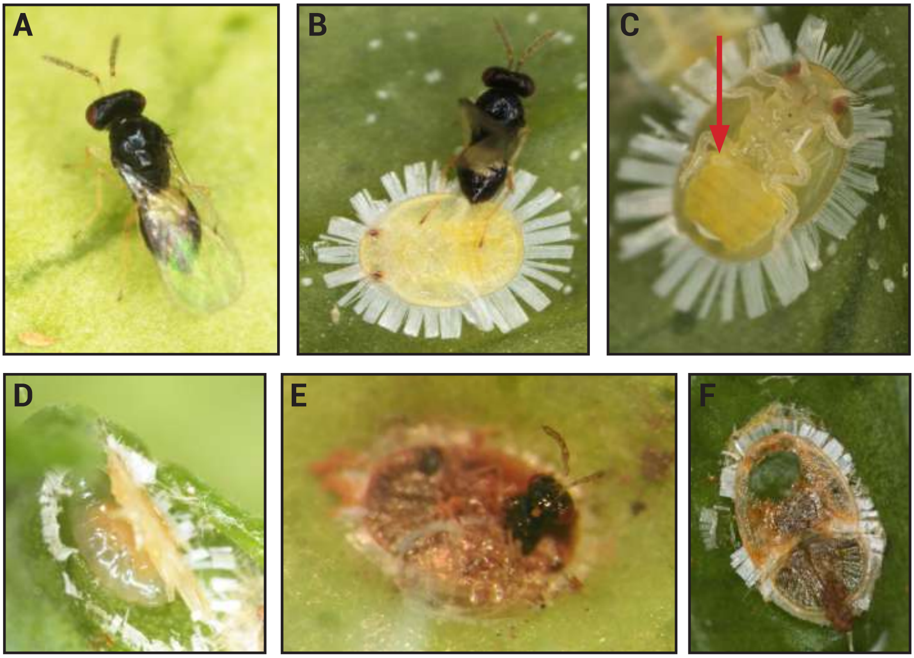


Fig. 23. *Tamarixia dryi*. **A.** Adult female. **B.** Adult female parasitizing. **C.** Egg. **D.** Larvae. **E.** Emerging. **F.** Emergence hole.

Studies of the parasitoid complex of *T. erytreae* in southern Africa and Cameroon also show that environmental conditions and the presence of a rich complex of hyperparasitoids limit the success of *T. dryi* as a biological control agent of the psyllid (Catling, 1969b; Aubert, 1987; Tamesse, 2009). Most of the studies, however, date from the 60s or 70s and are based on the relative abundance of parasitoids. The biology, parasitism, and hyperparasitism rates of these parasitoids are poorly known. All this information has to be considered prior to implementing a successful biological control for *T. erytreae*.

1.4. Research objectives

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) and *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae) are two of the latest citrus invasive pests in the Mediterranean Basin. Since both species were established, IPM of citrus has been disrupted and its management solely relied on chemical control due to the lack of indigenous natural enemies.

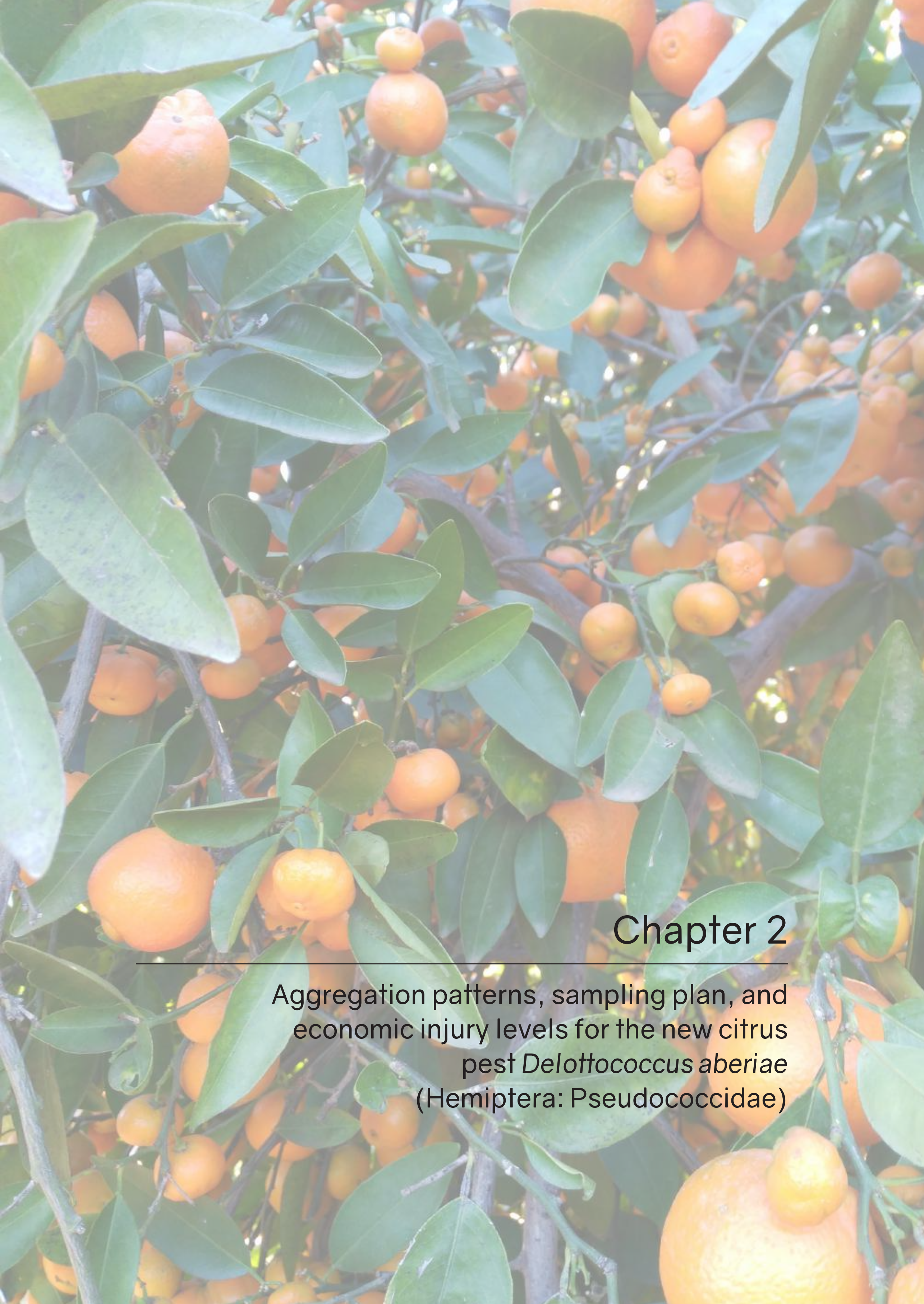
In this context, the main objectives of this thesis are:

i) To determine the aggregation patterns of *D. aberiae* in citrus, develop an efficient sampling protocol to assess its population density and calculate its Economic and Economic Environmental Injury Levels (EIL and EEIL, respectively). Chapter 2.

ii) To evaluate the potential of the soil-dwelling mite *Gaeolaelaps* (*Hypoaspis*) *aculeifer* as a predator of *D. aberiae* at laboratory and semi-field conditions. Chapter 3.

iii) To analyze the effect of the coccinellid *Cryptolaemus montrouzieri* on the population levels of *D. aberiae* in the field. Chapter 4.

iv) To disentangle the parasitoid complex of *T. erytreae* in South Africa and to determine several biological traits of its main parasitoids. Chapter 5.



Chapter 2

Aggregation patterns, sampling plan, and economic injury levels for the new citrus pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae)

CHAPTER 2

Aggregation patterns, sampling plan, and economic injury levels for the new citrus pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae)

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Abstract

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) is the latest exotic mealybug species introduced in citrus in the Mediterranean basin. It causes severe distortion and size reduction on developing fruits. Due to it is its first interaction with citrus, *D. aberiae* economic thresholds are still unknown for this crop and the current Integrated Pest Management programs have been disrupted. The objectives of this study were to determine the aggregation patterns of *D. aberiae* in citrus, develop an efficient sampling plan to assess its population density and calculate its Economic and Economic Environmental Injury Levels (EIL and EEIL, respectively). Twelve and 19 orchards were sampled in 2014 and 2015, respectively. At each orchard, population densities were monitored fortnightly in leaves, twigs and fruit and fruit damage was determined at harvest. Our results showed a clumped aggregation of *D. aberiae* in all organs with no significant differences between generations on fruit. Fruit damage at harvest was strongly correlated with fruit occupation in spring. Based on these results and using chlorpyrifos as the insecticide of reference, the EIL and EEIL were calculated as 7.1% and 12.1% of occupied fruit in spring, respectively. With all this, we recommend sampling 275 fruits using a binomial sampling method or alternatively, 140 fruits with an enumerative method bimonthly between petal fall and July.

Key words

Agricultural entomology • Citrus • Sampling • IPM • Invasive species

2.1. Introduction

The introduction of alien species in Europe has increased over the last decades (Roques et al., 2009; Bellard et al., 2016). One of the main causes is the establishment of the international trade across continents as well as the globalization process (Meyerson and Mooney, 2007; Hulme, 2009; MacDonald et al., 2015). In this regard, the number of biological invasive species is expected to rise in the near future (Pimentel et al., 2005; Mainka and Howard, 2010; Sutherland et al., 2011).The impact of non-native species may trigger not just an environmental damage upon indigenous species, but also important economic losses, especially in agriculture (Pimentel et al., 2000; Gaertner et al., 2009; Sujay et al., 2010). Within this context, mealybugs (Hemiptera: Pseudococcidae) are considered one of the main primary pests all over the world (Williams and Miller, 2002) and represent one of the groups with a major number of alien insects in Europe (Pellizzari and Germain, 2010).

Delottococcus aberiae (Hemiptera: Pseudococcidae) is the latest invasive mealybug pest introduced in Mediterranean citrus. The genus *Delottococcus* is common in South Africa and recent studies have demonstrated that invasive populations of *D. aberiae* are native to Limpopo province (NE within South Africa) where citrus is irregularly distributed (Paul, 2006; Beltrà et al., 2015). There, *D. aberiae* is also found in wild olive trees (homogeneously distributed at low densities) and on the roots of the flowering shrub *Chrysanthemoides monilifera* (L.) T. Norl (Miller and Giliomee, 2011). This mealybug is not considered a pest in South Africa, remaining unnoticeable over decades (Hatting et al., 1998; Miller and Giliomee, 2011). Contrarily, since the first individuals of *D. aberiae* were discovered in the core center of the main citrus producing area in the Mediterranean basin (northern part of Valencia province) in 2009 (Beltrà et al., 2013a), damage became noticeable on citrus fruit. Distinctively to other species of citrus mealybugs, *D. aberiae* causes severe direct effects such as distortions and size reduction in fruit which fosters a high depreciation of its commercial value (Beltrà et al., 2013a). This mealybug also triggers indirect effects arisen from the excretion of honeydew, mainly due to the growth of sooty mould fungi *Capnodium citri* Berk (Capnodiales: Capnodiaceae). In addition, it can give shelter to other secondary pests such as pyralid moths, mites or scale insects. Due to all these negative effects, in those orchards with the presence of *D. aberiae*, the integrated pest management has been partially disrupted by the urgency of the farmers to apply chemical treatments against this new pest, which in most cases are not compatible with a system based on conservation biological control (Franco et al., 2004; Wajnberg et al., 2004; Jacas and Urbaneja, 2010).

Delottococcus aberiae has many generations throughout the year but contrary to other citrus species in the Mediterranean growing conditions, it remains active during the winter. Populations tend to reach the maximum peak between June and July and decrease during the summer period (Martínez-Blay et al., 2018a). Fruit distortion and size reduction occurs from the flowering period to July and all citrus varieties are sensitive to *D. aberiae* attack (herein and Martínez-Blay et al., 2018b).

At the present, due to the lack of effective natural enemies against *D. aberiae* in the Mediterranean basin, its management relies on the application of broad-spectrum insecticides such as chlorpyrifos (Tena, 2017). For optimal control, insecticides must be applied once fruit set begins. However, there is no criterion based on pest abundance or damage to determine whether the application may be necessary or not. The estimation of Economic Injury Levels (EIL) defined as the lowest population density that will cause economic damage for this pest would therefore ease the integration of its management in the current Integrated Pest Management (IPM) strategies of citrus crops. Economic damage begins to occur at the pest density in which the monetary cost of suppressing pest-caused injury equals the potential loss resulting from this pest density (Stern et al., 1959; Pedigo et al., 1986; Pedigo and Rice, 2009). However, in IPM, environmental costs must be taken into account, producing sustainable solutions to pest problems. For this reason, another intervention threshold that incorporates both economic criteria and environmental risks is preferred: The Economic Environmental Injury Level (EEIL) (Higley and Wintersteen, 1996; Groffman et al., 2006).

In order to implement both EIL and EEIL, a methodology of sampling able to accurately estimate pest populations at the minimum cost is required. The selection of a sampling method mainly depends on the spatial distribution of the sampled population (Kuno, 1991).

EILs have been established for other species of pseudococcids in citrus such as *Planococcus citri* (Risso) (Martinez-Ferrer, 2006), as well as for *Pseudococcus viburni* (Signoret) in pome fuits (Mudavanhu et al., 2011). However, due to the recent arrival of *D. aberiae*, EILs have not yet been developed.

Herein, we sampled between 12 and 19 citrus orchards during two consecutive years to determine the distribution pattern of the new citrus pest

D. aberiae on canopies. Based on these results, we developed an accurate sampling plan and established the EIL and EEIL. These results will be used to integrate this pest within the current IPM program.

2.2. Materials and methods

2.2.1. Sampled orchards

The study was conducted in the region of Les Valls (Valencian Community, eastern Spain). A total of 12 orchards were sampled in 2014 and 19 in 2015, including eight from the previous year. Seven orchards belonged to four different varieties of sweet orange, *Citrus sinesis* (L.) Osbeck: Sanguinelli (three orchards) and Powell Navel (four orchards). Eleven orchards consisted of *Citrus reticulata* Blanco, including the varieties: Oroval (four orchards), Marisol (one orchard) and Clemenules (six orchards). The remaining five orchards consisted of the hybrid varieties Ortanique (three orchards), Moncada and Orri (one orchard each one). Sampled orchards ranged from 0.4 to 3 ha and all of them were drip-irrigated. The selected orchards were under several ground cover management strategies (Table 1). Within each orchard, the area where the evaluations were done was not sprayed with pesticides.

2.2.2. Sampling procedure

To determine the dispersion pattern of *D. aberiae* and the sampling protocol, 12 orchards were sampled in 2014 and 19 in 2015 (Table 1). In each orchard, between six and ten trees were sampled bimonthly during the 2 yr of the study. Four 30-cm long twigs with flowers and/or fruits were collected

Table 1. Sampled sites and years, number of trees sampled per orchard, citrus variety and cover crop.

Sampling year	Locality	Number of trees	Citrus variety	Ground cover	Used for
2014-2015	Algimia	8	Clemenules clementine	wild weed	EIL(2014), aggregation pattern
2015	Almenara	8	Ortanique	poaceae grass	EIL, aggregation pattern
2014-2015	Benifairó de les Valls	8	Clemenules clementine	wild weed	EIL(2014), aggregation pattern
2014	Benifairó de les Valls	6	Oroval clementine	wild weed	EIL, aggregation pattern
2015	Benifairó de les Valls	8	Marisol clementine	poaceae grass	EIL, aggregation pattern
2015	Benifairó de les Valls	10	Sanguinelli	bare soil	EIL, aggregation pattern
2015	Benifairó de les Valls	10	Oroval clementine	wild weed	EIL, aggregation pattern
2015	Faura	8	Clemenules clementine	poaceae grass	EIL, aggregation pattern
2014-2015	Quart de les Valls	8	Clemenules clementine	bare soil	EIL, aggregation pattern
2014	Quart de les Valls	8	Powell Navel	poaceae grass	EIL, aggregation pattern
2014-2015	Quart de les Valls	10	Oroval clementine	bare soil	EIL, aggregation pattern
2014-2015	Quart de les Valls	10	Oroval clementine	bare soil	EIL, aggregation pattern
2015	Quart de les Valls	8	Orri	poaceae grass	EIL, aggregation pattern
2015	Quart de les Valls	8	Ortanique	bare soil	EIL, aggregation pattern
2015	Quart de les Valls	8	Powell Navel	poaceae grass	EIL, aggregation pattern
2015	Quart de les Valls	8	Clemenules clementine	poaceae grass	EIL, aggregation pattern
2014-2015	Quartell	10	Powell Navel	wild weed	EIL, aggregation pattern
2014-2015	Quartell	10	Sanguinelli	wild weed	EIL(2015), aggregation pattern
2014-2015	Quartell	10	Powell Navel	wild weed	EIL, aggregation pattern
2015	Quartell	8	Sanguinello	wild weed	EIL, aggregation pattern
2015	Quartell	8	Ortanique	poaceae grass	EIL, aggregation pattern
2014	Vall d´Uixó	8	Moncada	bare soil	EIL
2014	Quart de les Valls	8	Clemenules clementine	wild weed	EIL, aggregation pattern

randomly from the canopy of each tree (each twig belonged to a different cardinal orientation). It means one twig randomly selected in each orientation. All samples of a tree were placed in individual plastic bags, enclosed, and transported to the laboratory. Within the next 24 hours, twigs and four leaves and a maximum of eight flowers or fruits per twig were examined under a stereomicroscope. Leaves and flowers or fruits were selected at random within the twig. All post-embryonal development stages of *D. aberiae* were counted: from first nymph instar to the third (N1, N2, N3), adult males (M) and adult females (F1) including females with ovisac (F2).

To determine the EIL, the percentage of damaged fruit was assessed at harvest in the same sampled trees. Orchards where fruit thinning operations were conducted in the summer were excluded from these analyses. We sampled 40 fruits per tree, 10 per orientation (N, S, E, and W) of the tree canopy. We considered that a fruit was damaged when distortion and size reduction could trigger commercial depreciation.

2.2.3. Aggregation indices

In order to calculate the spatial distribution of *D. aberiae*, Taylor´s power law (Taylor, 1961) index was determined. This index establishes a correlation between a population's density and variance by the power function: $s^2 = a\ m^b$ where s^2 is the sample variance, m is the sample mean density and "*a*" and "*b*" are Taylor's coefficients. In order to estimate both coefficients, the model was fit as a linear regression in logarithms ($\log s^2 = \log a + b \log m$). Coefficient "*a*" is a sampling factor that depends on sample's size and it lacks ecological meaning and "*b*" is the Taylor's aggregation index. Coefficient "*b*" is regarded as a species characteristic, which provides a basis for a sampling program and describes the aggregation pattern. When $b = 0$, the population is distributed uniformly, $b = 1$ indicates a random distribution, and $b > 1$ is an indication of a clustered distribution (Taylor, 1984).

Taylor´s coefficient was calculated for each sampled tree organ: twig, leaf and fruit. Flowers were not included because of the low number of *D. aberiae* specimens. Aggregation coefficients were calculated separately for the first (April-May) and second generations (June-July) of *D. aberiae*. To simplify the analysis and because of the difficulty differentiating some instars, N1 and N2 were combined and N3 and adult immature females (F1) were also combined. The gravid females (F2) were analyzed as a separate group.

MANCOVAs (multivariate analysis of covariance) were used to determine whether aggregation patterns differed between generations, taking the mean as the quantitative factor and variance as the dependent variable. These analyses were conducted for each citrus organ (twig, fruit and leaf) and developmental group. For the following analyses (sampling protocols and EILs), we examined all possible regressions and selected the sample unit (among leaf, twig and fruit) where all the instar groups aggregated similarly in both generations.

2.2.4. Sampling protocol

To develop the sampling protocol, we only considered the fruits because: 1) aggregation in this organ did not differ between generations and 2) fruit is already sampled during the spring to determine the population levels of another important citrus pest, *Pezothrips kellyanus* Bagnall (Thysanoptera: Thripidae) (Navarro-Campos et al., 2012; Planes et al., 2015). Therefore, farmers can use the same organ to sample both pests.

2.2.5. Binomial sampling

This sampling method estimates densities from occupied and unoccupied organs by the insect. It is used when insect populations show a high aggregation pattern and when there is a correlation between the proportion of sample units infested with the insect (p) and their mean number per sample unit (m). It also enables one to make decisions at less cost than with the enumerative sampling in IPM (Wilson and Room, 1983).

Wilson and Room´s (1983) model relates m and p according to Taylor's indices a and b:

$$p = 1 - \exp\left[-m \ln\left(a\, m^{b-1}\right) / a\, m^{b-1} - 1)\right]$$

The sample size (N) required to estimate *D. aberiae* mean density (m) for a fixed precision (D) in the binomial sampling was calculated using the expression of variance proposed by Kuno (1986):

$$N = D^{-2} (1-p_0)\, p_0^{-(2/k)-1} \left[k \left(p_0^{-1/k} - 1\right)\right]^{-2}$$

Where p_0 is the proportion of non-occupied sample units and k was calculated from the mean and the Taylor's indices by the equation:

$$k = m^2 / (am^{b} - m)$$

Although $D = 0.25$ is the value commonly used in research studies of insects' populations (Southwood and Henderson, 2000) $D = 0.30$ and $D = 0.35$ also were considered as our sampling protocol is designed to be implemented by farmers. For each level of precision, sample size was calculated at different population means.

2.2.6. Enumerative sampling

In order to calculate the minimum sample size (n) required for a known mean density (m) to achieve prefixed levels of precision ($D = 0.25$, $D = 0.30$ and $D = 0.35$), Green´s method (1970) was used. It establishes that the standard error ($s/\sqrt{n}$) is a fixed proportion (D) of the sample mean. The variance was substituted by its expression according to Taylor's indices:

$$n = a\, m^{(b\text{-}2)} / D^2$$

2.2.7. Economic injury levels

The economic injury level (EIL) for *D. aberiae* was calculated using the formula of Pedigo et al. (1986):

$$\mathrm{EIL} = C / VIDK$$

Where C is the *D. aberiae* management costs per production unit (€ ha^{-1}), V is the price of the fruit in origin (€ ha^{-1}), I is the injury unit per insect per production unit [proportion damaged fruits / (insect ha^{-1})], *D* is the damage per injury unit [kg reduction ha^{-1})/proportion fruits damaged], and K is the proportional reduction in injury with treatment (i.e. the efficacy of the treatment). *I* x *D* is the yield loss per pest and it is obtained from the slope *b* of the damage function: $y = a + bx$, where y is the percentage of damaged fruits at harvest, and x is the percentage of sample units (fruits) occupied by *D. aberiae*. Consequently:

$$\text{EIL} = C \,/\, VIDK = C \,/\, VbK$$

In the damage function, percentage of damaged fruit was obtained by dividing the number of damaged fruits by the total number of fruits sampled per tree at harvest. Percentage of occupation was obtained by dividing the highest number of occupied fruits by the total number of sampled fruits in each sampling date and then calculating the maximum percentage of occupied fruit during the season (first and second generation). We considered damaged fruit as those with a size reduction or deformation that completely depreciated them from a commercial view.

The EEIL, which takes into account environmental costs, was calculated by multiplying the EIL by 1.7 (Higley and Wintersteen, 1996).

2.3. Results

During the sampling period, a total of 6,801 specimens were collected on twigs, 13,714 on leaves and 87,895 on fruits.

2.3.1. Aggregation index

The aggregation pattern of the first and second instar (analyzed together) on twigs was similar in both generations (*F* = 3.6; df = 1, 133; *P* = 0.06; R^2 = 95.12) (Table 2). However, the aggregation pattern of the rest of the developmental groups on twigs differed between generations (third instar and adult females: *F* = 4.41; df = 1, 135; *P* = 0.04; gravid females and ovisacs: *F* = 7.58; df = 1, 98; *P* = 0.0071). When we pooled all the developmental groups, the aggregation pattern of *D. aberiae* on twigs was similar in both generations (*F* = 0.71; df = 1, 168; *P* = 0.4).

The aggregation pattern of the first and second instars (analyzed together) on leaves was similar in both generations (*F* = 1.44; df = 1, 137; *P* = 0.23) (Table 2). However, the aggregation pattern of the rest of the developmental groups on leaves differed between generations (third instar and adult females: *F* = 7.39; df = 1, 114; *P* = 0.01; gravid females and ovisacs: *F* = 12.13; df = 1, 123; *P* = 0.0007). When we pooled all the developmental groups, the aggregation pattern of *D. aberiae* on leaves was similar in both generations (*F* = 0.98; df = 1, 175; *P* = 0.32).

The aggregation pattern of all the developmental groups on fruits was similar in both generations (first and second instar: *F* = 0.02; df = 1, 146; *P* = 0.9; third instar and adult females: *F* = 0.71; df = 1, 130; *P* = 0.4; gravid females and ovisacs: *F* = 0.03; df = 1, 103; *P* = 0.87). When we pooled all the developmental groups, the aggregation pattern of *D. aberiae* on fruit was similar in both generations (*F* = 0.07; df = 1, 157; *P* = 0.8) (Table 2).

Table 2. Taylor's indices for each sample unit, generation and life instar of *D. aberiae*. (N1= first nymphal instar; N2= second nymphal instar; N3= third nymphal instar; H1= adult female; H2= gravid adult female).

Sample unit	Life stage	Generation	Samples (n)	a	b	SE(b)	R^2	t-Value for slope=1
Twigs	N1–N2	1	73	5.714	1.504	0.03	96.61	-18.665
		2	61	8.091	1.612	0.05	95.14	-16.573
		Total	134	6.653	1.548	0.03	95.64	-21.512
	N3-F1	1	76	2.63	1.31	0.03	95.92	-12.167
		2	60	3.873	1.406	0.03	97.03	-16.22
	F2-ovisac	1	55	1.517	1.138	0.05	90.82	-3.671
		2	44	3.055	1.321	0.04	96.39	-11.284
	All instars	1	89	4.508	1.512	0.03	96.05	-18.668
		2	80	6.561	1.552	0.03	96.45	-19.957
		Total	169	5.37	1.525	0.02	95.92	-23.705
Leaves	N1–N2	1	72	14.997	1.569	0.04	95.18	-16.655
		2	66	19.953	1.656	0.06	91.93	-13.455
		Total	133	16.982	1.6	0.04	93.61	-18.491
	N3-F1	1	62	3.733	1.26	0.03	95.71	-9.772
		2	53	6.823	1.425	0.05	94.63	-11.787
	F2-ovisac	1	69	1.607	1.091	0.03	94.98	-3.708
		2	55	3.266	1.248	0.03	96.32	-13.279
	All instars	1	90	11.83	1.567	0.04	94.64	-16.981
		2	86	15.241	1.627	0.04	93.56	-16.145
		Total	162	13.459	1.597	0.03	93.9	-20.423
Fruits	N1–N2	1	56	7.551	1.411	0.05	94.19	-11.213
		2	91	5.546	1.418	0.03	95.51	-15.249
		Total	147	6.252	1.398	0.03	94.87	-16.419
	N3-F1	1	44	2.761	1.21	0.03	97.53	-9.711
		2	87	2.506	1.245	0.03	96.34	-11.168
		Total	131	2.547	1.214	0.02	97.13	-13.135
	F2-ovisac	1	33	0.925	0.98	0.005	99.93	4.835
		2	71	1.096	0.99	0.03	93.59	0.4541[a]
		Total	104	1.109	1.01	0.02	96.42	-0.7221[a]
	All instars	1	61	7.079	1.41	0.04	94.75	-12.104
		2	97	4.677	1.42	0.03	95.78	-16.223
		Total	158	5.495	1.39	0.03	95.12	-17.094

[a] Indicates t-value for slope = 1 ($P > 0.05$).

2.3.2. Enumerative sampling plan

The sample size was calculated for all the instars and generations together as there were not significant differences between generations. For a mean population level of 0.1 insects per fruit, which represents a mean population value during the sampling process, 250, 210 and 150 fruits are required with a D = 0.25, 0.30 and 0.35 respectively (Fig. 1).

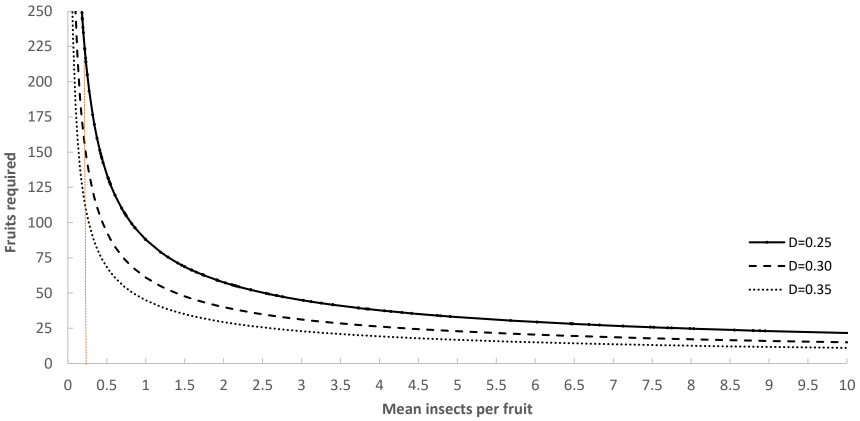

Fig. 1. Enumerative sampling for *Delottococcus aberiae* in citrus. Number of fruits required based on the mean number of mealybugs pre fruit based on Green's method (1970) to achieve a precision level of D = 0.25, 0.30 and 0.35. The vertical line represents the obtained EEIL (0.24 insects per fruit).

2.3.3. Binomial sampling plan

The model by Wilson and Room (1983) adequately fits the correlation between the number of *D. aberiae* (insect density) per fruit (sample unit) and the percentage of occupied fruits (Fig. 2). Using these data and Kuno's method (1986), for a mean density of 0.1 insects per fruit in a binomial sampling, 470, 330 and 260 fruits are needed for a D = 0.25, 0.30 and 0.35 respectively (Fig. 3). Compared to the enumerative sampling method, more samples are required.

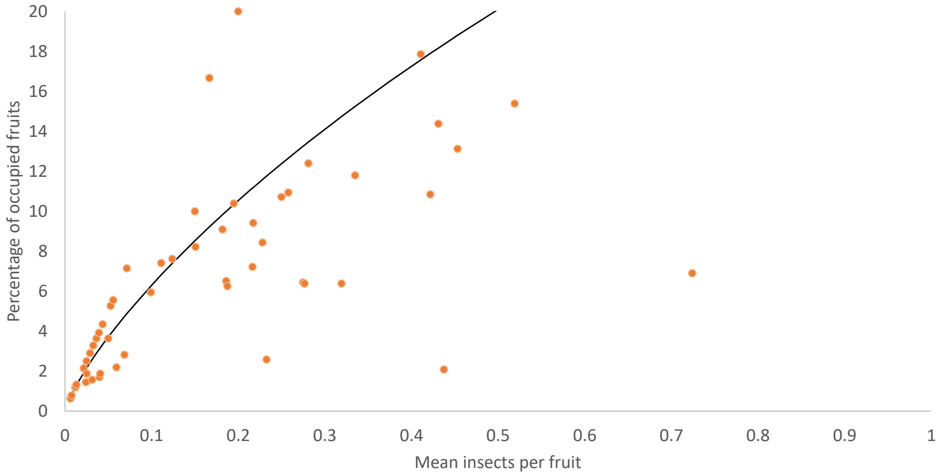

Fig. 2. Relationship between the percentage of occupied fruits and the mean population density of *Delottococcus aberiae* in citrus. Solid line represents Wilson and Room´s theoretical model.

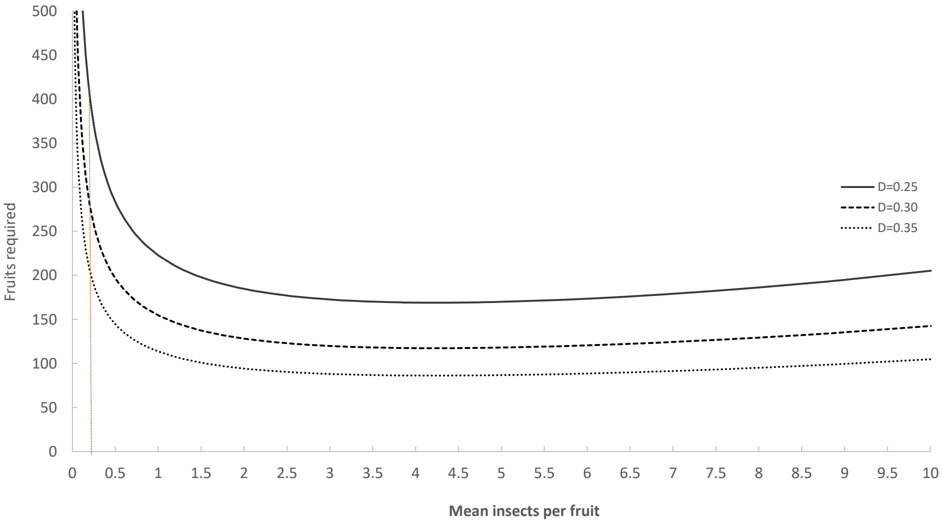

Fig. 3. Binomial sampling for *Delottococcus aberiae* in citrus. Number of fruits required based on the percentage of occupied fruit based on Kuno's method (1986) to achieve a precision level of D = 0.25, 0.30 and 0.35. The vertical line represents the obtained EEIL (0.24 insects per fruit).

2.3.4. Economic injury levels

Delotococcus aberiae management costs (C = 285 € ha^{-1}) were estimated as the sum of the product (135 € ha^{-1}) and application (150 € ha^{-1}) costs. Most of the applications against this pest are done with chlorpyrifos and, therefore, it was selected for this model. The treatment price was established from published assays with chlorpyrifos (96 g l^{-1} of water) (Tena et al., 2009).

Fruit price (V) was fixed according to official national statistics about prices on origin for navel oranges (MARM, 2016; Navarro-Campos et al., 2012) as: V = 0.22 € kg^{-1} x 30 000 kg ha^{-1} = 6 600 € ha^{-1}. The efficacy (K) of chlorpyrifos was taken as K= 0.70 (Tena et al., personal communication). The estimated value of b was 0.87 (Fig. 4). Consequently, EIL= 285 € ha^{-1}/(6 600 € ha^{-1} x 0.87 x 0.70) = 7.1 % fruits infested by *D. aberiae*. All varieties of mandarins and oranges are included as they showed a similar trend and good fit along the regression line (R^2 = 0.85).

Higley and Wintersteen (1996) proposed to estimate the EEIL for chlorpyrifos multiplying EIL x 1.7. As a result, EEIL= 12.1% of fruits occupied by *D. aberiae*. This percentage corresponds to a 0.24 insects per fruit (Fig. 3).

2.3.5. Sample size

The number of samples required to achieve the prefixed precision levels (D = 0.25, 0.30 and 0.35) at the estimated *D. aberiae* density of 0.24 insects per fruit for the obtained EEIL were 210, 140 and 105 fruits respectively for the enumerative method and 390, 275 and 200 fruits for the binomial plan.

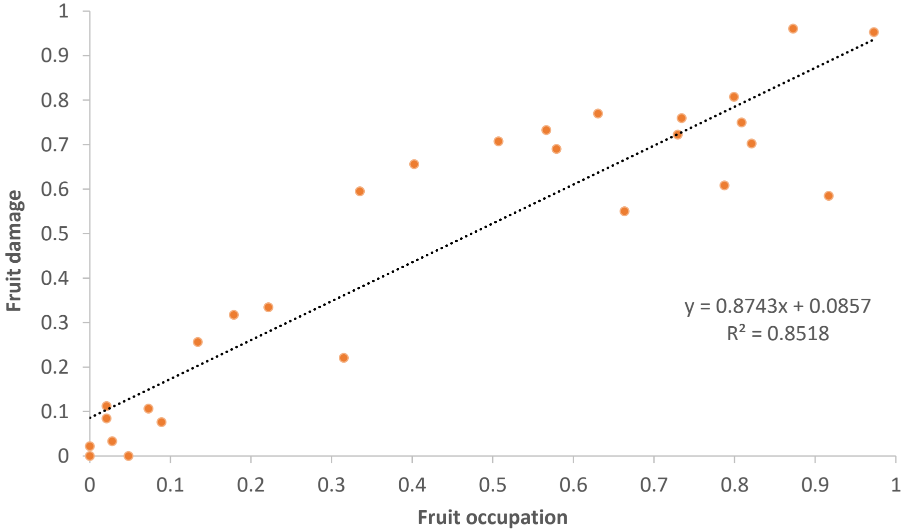

Fig. 4. Relationship between the maximum fruit occupation throughout the damaging period (petal fall until July) and the percentage of damaged fruit by *Delottococcus aberiae* at harvest (R^2 = 0.85; $P < 0.001$; n = 28). Each point represents an orchard during one year (the maximum percentage of occupied fruits throughout the damaging period was considered only if more than 12 fruits were counted per tree).

2.4. Discussion

All *Delottococcus aberiae* instars tended to aggregate in fruits, leaves and twigs of citrus trees. Other mealybugs, like *P. citri* also aggregate on citrus (Nestel et al., 1995; Martínez-Ferrer et al., 2006). In addition, other mealybugs are known to aggregate on their hosts, including *Rastrococcus invadens* Williams on mango leaves (Boavida et al., 1992), *Saccharicoccus sacchari* (Cockerell) on sugarcane stalks (Allsopp, 1991), *Pseudococcus maritimus* (Ehrhorn) on vines (Geiger and Daane, 2001) and *Phenacoccus peruvianus* Granara de Willink on ornamental plants (Beltrà et al., 2013b). Among the different instars of *D. aberiae*, the aggregation index decreased with mealybug age. *D. aberiae* crawlers (first instar), as occurs in other species when conditions are favorable (Greathead, 1997), settled close to the ovisac after hatching. As they grew and space became limited on fruits, nymphs tended to disperse during the first and second generation.

Mealybugs are multivoltine under mild Mediterranean conditions. *D. aberiae* has between five and six generations per year on citrus (Martínez-Blay et al., 2018a). In spring, *D. aberiae* has the two first and homogeneous generations (Martínez-Blay et al., 2018a). The aggregation pattern of the young instars was similar in both generations. This result is in accordance with the observations of other mealybug species such as *P. citri* also on citrus or *P. peruvianus* on ornamental plants (Martínez-Ferrer et al., 2006; Beltrà et al., 2013b). Third instar nymphs and adult females behaved similar in the first and second generation when settled on fruits. However, this pattern changed when these instars were settled on leaves and twigs, as individuals of the second generation had higher Taylor's indices that those of the first. Generally, these differences within the same species are explained by environmental variations such as temperature or the presence of natural enemies (Taylor et al., 1988). In the case of *D. aberiae* in citrus, no effective predator or parasitoid attacks these two generations as parasitoids do not develop on *D. aberiae* (Tena et al., 2017) and the density of its main predator *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinelidae) is very low until June (Pérez-Rodríguez et al., submitted).

According to our data, the EEIL for *D. aberiae* in citrus is 12.1% of infested fruits after petal fall. Economic thresholds of *D. aberiae* could be obtained because the aggregative pattern on fruit was similar for both generations. It also is worth mentioning that these values have been calculated considering only direct damage: fruit reduction and distortion. Indirect damage produced by honeydew excretion was not considered because they were much lower. For example, in some of our orchards, 90 per cent of the fruit was damaged by *D. aberiae* whereas sooty mould was scarce. In the case of *P. citri*, the main mealybug pest in citrus worldwide, economic thresholds were calculated considering indirect damage due to the lack of the direct ones. Although Cavalloro and Prota (1983) proposed thresholds for *P. citri* between 5 and 15% of infested fruit in summer, Martínez-Ferrer et al. (2006) established the EEIL in 20% of infested fruit. Following this study, the IPM of citrus in Spain recommends spraying when 20-30% of fruits are infested. As expected, these thresholds are much higher than the ones obtained for *D. aberiae*. Finally, it is noticeable that our thresholds are similar to those obtained for *P. kellyanus* and *Scirtothrips citri* Moulton (Thysanoptera: Thripidae), other citrus pests which cause serious direct damage on young fruits after petal fall (Navarro-Campos et al., 2012; Planes et al., 2015). These species produce a scar ring between petal fall and 4-6 wk later (Planes et al., 2015), the same period of *D. aberiae*. Therefore, the same sampling plan can be used to sample both citrus pests and decide whether spraying is necessary.

Here we propose a binomial sampling of 275 fruits randomly selected per orchard with a precision of $D = 0.30$. According to our results, the enumerative sampling needs a lower number of fruits and provides more accurate results but it is more time-consuming. Fruits have to be collected and examined with a stereomicroscope to count the number of *D. aberiae* nymphs under the sepals. All this process could last around six h considering that the citrus producer has a stereomicroscope in the sampled orchard. By contrast, binomial sampling does not require fruit harvest and *D. aberiae* presence can be determined with a magnifying glass. Moreover, the reduced visibility of first instars is balanced by their high aggregation patterns. Taking all into consideration, the binomial sampling process could last around 15 min and 2 h (considering that 30 s are necessary to sample a fruit). Monitoring techniques based on direct observations of fruit and counting individuals have been widely used in IPM of other mealybug species (Cavalloro, and Prota, 1983; Ripollés, 1990; Barbagallo et al., 1993; Roltsch et al., 2006; Mgocheki and Addison, 2009). However, the use of plant material is a laborious and time consuming task compared to alternative monitoring techniques based on passive sampling (Geiger and Daane, 2001; Waterworth et al., 2011). In this sense, we have recently shown that *D. aberiae* density on plant is highly related with pest level in corrugated cardboard bands in trunks (Martínez-Blay et al., 2018a). Further research might determine whether this technique can be used as a sampling method making it simpler and less time-consuming. In fact, these techniques have already been used in several biological control programs in order to monitor population densities of mealybugs and also to evaluate the impact of their natural enemies, mainly predators (Browning, 1959; Furness, 1976; Goolsby et al., 2002).

Monitoring processes should be carried out fortnightly after petal fall according to our results and the seasonal trend of *D. aberiae* presented in a companion manuscript (Martínez-Blay et al., 2018a). Sampling should start just after petal fall because spraying is forbidden during the flowering period. *D. aberiae* density increases exponentially between April and July and fruit damage is caused mainly during this period. When populations reach the EEIL, four insecticides are currently recommended against mealybugs in citrus in Spain: chlorpyrifos, chlorpyrifos-methyl, mineral oils and spirotetramat (Tena, 2017). More information is needed to evaluate the efficacy of these insecticides but it is worth mentioning that some *D. aberiae* adult females descend to the trunk and soil where they lay their ovisacs in spring (Martínez-Blay et al., 2018b). Therefore, the application should reach at least the base of the trunk. After insecticide application, the monitoring process should continue because *D. aberiae* can reach the EEIL again as occurs with *P. kellyanus* (Planes et al., 2015). Finally, it should not be overlooked that fruit of the previous year might have not been harvested during the damaging period, in some late varieties like Valencia oranges. Therefore, farmers should be cautious with insecticide residuals in the fruits of the previous year.

2.5. Acknowledgements

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Chapter 3

The soil mite *Gaeolaelaps* (*Hypoaspis*) *aculeifer* (Canestrini) (Acari: Laelapidae) as a predator of the invasive citrus mealybug *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae): implications for biological control

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CHAPTER 3

The soil mite *Gaeolaelaps* (*Hypoaspis*) *aculeifer* (Canestrini) (Acari: Laelapidae) as a predator of the invasive citrus mealybug *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae): implications for biological control

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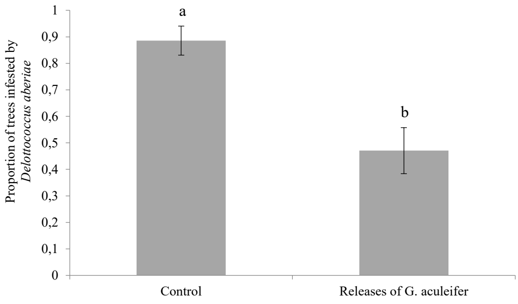
Abstract

Soil-dwelling predatory mites are important biocontrol agents of several pests. Despite their wide prey range, they have never been evaluated as predators of mealybugs that inhabit the soil. Here, we tested the potential of the soil mite *Gaeolaelaps (Hypoaspis) aculeifer* (Canestrini) (Acari: Laelapidae) as a predator of the invasive citrus mealybug *Delottococcus aberiae* (De Lotto)

(Hemiptera: Pseudococcidae). The predation and fecundity of mites over a 14-day period were analysed when fed on three different diets under laboratory conditions: *D. aberiae* eggs, first instar nymphs, and without food. *Gaeolaelaps aculeifer* preyed on *D. aberiae* and both predation rates and the proportion of females that laid eggs were significantly higher when females preyed on mealybug nymphs than on eggs. These data prompted the evaluation of *G. aculeifer* as a biological control agent of *D. aberiae* under semi-field conditions. Infestation levels of *D. aberiae* on citrus potted plants were reduced in response to *G. aculeifer* releases. The implications of these results on conservation biological control of mealybugs are discussed.

Graphical Abstract



Key words

Prey range • Citrus • *Delottococcus aberiae* • Invasive pest • Integrated pest management

3.1. Introduction

Microarthropods are well-represented in most agricultural soils (Crossley et al., 1992), playing a key role in nutrient cycling, organic matter decomposition, and establishment of multitrophic interactions between above and belowground communities (Loreau et al., 2001; Van Der Putten et al., 2001; Coleman et al., 2004). Out of all microarthropods, soil mites (Acari) form one of the most diverse and abundant group (Hendrix et al., 1986; St John et al., 2002; Kardol et al., 2011). This group includes predatory mites, which exert significant predation from the top of the food chains (Postma-Blaauw et al., 2010). Most predatory mites are mobile predators that feed predominantly on collembolan, nematodes, insect eggs, and the larvae of other microarthropods (Koehler, 1997; Baatrup et al., 2006; Walter and Proctor, 2013). Their efficiency in regulating soil ecosystems has contributed to their being used in both open field and greenhouses (Karg, 1998). In fact, some mesostigmatid mites of the family Laelapidae, namely *Gaeolaelaps aculeifer* (Canestrini) and *Stratiolaelaps miles* (Berlese), have been successfully reared and released for the augmentative biological control of nematodes, shore flies (Diptera: Sciaridae), bulb mites *Rhizoglyphus* spp. (Enkegaard et al., 1997; Koehler, 1997), and thrips pupae (Gillespied and Quiring, 1990; Wiethoff et al., 2004; Navarro-Campos et al., 2012). However, these species have not been documented as predators of mealybugs, which also inhabit soil ecosystems.

Mealybugs (Hemiptera: Pseudococcidae) are small sap feeding insects that are covered with a powdery wax (Franco et al., 2000; Tabata et al., 2012; Mani and Shivaraju, 2016). They are globally distributed, and many species are considered primary agricultural pests (Williams and Miller, 2002; Franco et al., 2004; Daane et al., 2012; Wetten et al., 2016). Mealybugs tend to aggregate and remain immobile for large periods of their lives. Some species, however, move from the aerial part of the plant to the subterranean crown or plant roots (Franco et al., 2000; Daane et al., 2012; Mani and Shivaraju, 2016), especially when they need protection against extreme climate conditions (Geiger and Daane, 2001; Gutierrez et al., 2008). In these habitats, generalist soil predators, such as predatory mites, might be potential biological control agents.

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) is the latest invasive mealybug pest that has been introduced to citrus orchards in Europe (Beltrà et al., 2012; Pérez-Rodríguez et al., 2017). It is likely native to Limpopo Province (NE part of South Africa) (Beltra et al., 2015). Unlike other species of citrus mealybugs, it causes severe damage to developing fruit, leading to major economic losses (Pérez-Rodríguez et al., 2017). *Delottococcus aberiae* is often located in the tree canopy (leaves, twigs, flowers, and fruit); however, gravid females migrate to the subterranean crown in search of shelter during spring (Martínez-Blay et al., 2018a). Once there, they lay the ovisacs, and emerging nymphs (crawlers) climb to the upper part. Today, the biological control of *D. aberiae* is limited due to the ineffectiveness of native parasitoids (Tena et al., 2017) and the late arrival of predators (Pérez-Rodríguez et al., submitted). We, thus, questioned whether *G. aculeifer* would feed on *D. aberiae* instars found in the soil.

This study is the first to evaluate the potential of a soil mite as a predator of mealybugs. We tested whether: i) *G. aculeifer* is able to feed on the eggs and first instar nymphs of the recently introduced citrus pest *D. aberiae* and ii) the fecundity of *G. aculeifer* increases when feeding on these prey items. Based on these results, we subsequently: iii) tested the efficacy of *G. aculeifer* as predator of *D. aberiae* in a semi-field assay. Our results are expected to provide new insights on the utility of *G. aculeifer* for the biological control of mealybugs.

3.2. Materials and methods

3.2.1. Arthropods

3.2.1.1. *Gaeolaelaps aculeifer* culture

Gaeolaelaps aculeifer mites were obtained from the commercial product Entomite^{TM}, in a cardboard cylinder containing ca. 50,000 mites of all growth stages mixed with vermiculite as the carrier material and storage mites *Tyrophagus putrescentiae* (Acari: Acaridae) (Koppert Biological Systems, The Netherlands). For the laboratory assay, ~350 nymphs were removed from the cylinder and kept in five ventilated Petri dishes (5.3 cm in diameter; ~70 nymphs per dish). Petri dishes were kept under controlled conditions (14:10 h light:dark [L:D], 25 ± 2°C, 60–70% relative humidity [RH]), and were checked daily until the nymphs reached the adult stage to obtain an adult cohort of the same age. The artificial diet (provided *ad libitum*) and substrate were taken from the original bottle and provided to the nymphs. Newly emerged adults were sexed based on their size and abdominal shape (Beaulieu, 2009; Ramroodi et al., 2014).

The semi-field assay was carried out using 5 ml of the commercial product content, which contained 30.2 ± 3.78 nymphs and 29.8 ± 3.99 adults of *G. aculeifer* (n = 25 replicates), as well as the substrate and artificial diet.

3.2.1.2. *Delottococcus aberiae* culture

A laboratory colony of *D. aberiae* was established at the Instituto Valenciano de Investigaciones Agrarias (IVIA), Spain, using specimens collected from a citrus orchard located in Quartell (733,915.65X 4,401,857.39Y, Les Valls, Valencia, Spain), in 2015. Mealybugs were reared on organic lemons inside cardboard boxes (30 cm × 22 cm × 25 cm), in which egg cartons were placed on the bottom. Lemons used to rear *D. aberiae* were covered with red paraffin around the mid-section to slow desiccation, as described by Tena et al. (2013b). Between four and five lemons were introduced weekly, and dried lemons were removed. The mealybug colony was maintained in darkness in a climatic chamber at 20 ± 1°C and 70 ± 5% RH.

3.2.2. Laboratory experiments: Predation experiment

Each adult mite was isolated in the cells (arenas) of plastic bioassay trays (Bio-BA-128©, Bio-Serv, Frenchtown, NJ, USA). An E995 cover (CV-16) was placed onto the tray to prevent mites from escaping. Cells were 15.9 mm in diameter and 15.9 mm deep. The bottom of each cell was covered with moist plaster of Paris (DAP$^{®}$) as the substrate, and 70–100 µl distilled water was supplied every 2 days to each cell to maintain humidity, following a similar methodology to Bernd et al. (2004b) and Prischmann et al. (2011). Mites were then starved for 24 h to homogenize their nutritional status. Then, one of three diets was assigned: no food, three alive *D. aberiae* first-instar nymphs, and three *D. aberiae* eggs. All arenas were checked daily for 14 days, and the number of live and preyed on *D. aberiae* eggs and nymphs, as well as mite fecundity (measured as the proportion of females that laid eggs) were recorded. Trays were maintained at 14:10 h L:D, 25 ± 2°C and 60–70% RH during the experiment. Initially, there were 30 replicates per treatment, and escaped mites were not considered in the analysis.

3.2.3. Semi-field predation experiment

The semi-field experiment was conducted in a glasshouse located in the IVIA. The conditions in the glasshouse were 22 ± 3°C, 65 ± 10% RH, and a natural photoperiod (June–July).

A total of 69 pesticide-free *Citrus volkameriana* Tenore & Pasquale seedling plants (~30 cm high) were planted in 8 × 8 × 8 cm pots, with sterilized peat moss and cocopeat (70:30) being assigned to one treatment with predatory mites (n = 34) and one treatment without predatory mites (n = 35), as the controls. Pots were kept on plates with a layer of water to avoid cross contaminations and mealybug scape. Plants were randomly designated for each treatment in a complete randomized design.

Mealybug ovisacs were transferred from the colony to the center of a glass cover slip, with one ovisac being used per cover slip. Ovisacs on the cover slips were kept in separate meshed-lid 5.3 cm diameter Petri dishes. The Petri dishes were checked daily until the eggs started to hatch. Then, a glass cover slip with the ovisac was transferred to the corner of a pot and *G. aculeifer* were released on the soil of plants designated for *G. aculeifer* release. Ovisacs were assigned to alternate treatments. The number of predatory mites and mealybug nymphs in the soil was counted daily by directly observing the soil for 2 min periods. Five days later, the plants were transferred to the laboratory and the number of mealybugs per plant was counted.

3.2.4. Statistical analysis

The number of eggs and nymphs of *D. aberiae* preyed on by the males and females of *G. aculeifer* was compared using a two-way ANOVA ($\alpha = 0.05$), with the number of preyed mealybugs representing the dependent variable and mealybug stage (egg and first instar) and mite sex representing the explanatory variables. The number of *D. aberiae* nymphs observed per plot over the 5-day semi-field assay was analysed using an ANOVA with repeated measures ($\alpha = 0.05$). The normality assumption was assessed using the Shapiro-Wilk test and homoscedasticity by the Levene test ($\alpha = 0.05$). These analyses were run using the package Statgraphics Centurion XVI.I. Proportional data (proportion of females that laid eggs and tree infestation) were analysed with generalized linear models. We assumed a binomial error variance, and assessed the error structures using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If we detected over- or under-dispersion, we re-evaluated the significance of the explanatory variables using an *F* test after rescaling the statistical model by a Pearson chi-square divided by the residual degrees of freedom (Crawley, 2007). Bonferroni post hoc test was used when differences between treatments were found. The means of the untransformed proportion are presented. These analyses were performed with R studio (Version 1.0.143, Rstudio, Inc., https://www.rstudio.com); (Ihaka and Gentleman, 1996).

3.3. Results

3.3.1. *Gaeolaelaps aculeifer* predation on mealybugs

Gaeolaelaps aculeifer males and females preyed on significantly more *D. aberiae* nymphs than eggs ($F = 62.01$; df = 1, 71; $P < 0.001$) (Fig. 1), even though females were more voracious than males ($F = 102.82$; df = 1, 71; $P < 0.001$). The interaction between diet and sex was significant ($F = 57.61$; df = 1, 71; $P < 0.001$), thus revealing that females preyed more than males when they had access to *D. aberiae* nymphs (Fig. 1).

3.3.2. Effect of diet on *G. aculeifer* fecundity

The diet provided to *G. aculeifer* individuals affected their fecundity. The proportion of female mites that laid eggs was significantly higher when they had access to the first instar nymphs of *D. aberiae* than for starved females ($\chi^2 = 59.63$; df = 1, 60; $P < 0.0037$) (Fig. 2).

3.3.3. Semi-field conditions

During the 2-minute observations, a mean of 0.3 ± 0.11 *G. aculeifer* individuals were observed in pots where the mite was released. In comparison, no mites were observed in pots without predator releases (Fig. 3A). The mean number of *D. aberiae* per pot was similar in pots with and without releases of *G. aculeifer* throughout the assay (ANOVA with repeated measures, treatment: $F = 2.85$; df = 1, 320; $P = 0.093$; time: $F = 6.56$; df = 4, 320; $P < 0.0001$; treatment * time: $F = 0.5$; df = 4, 320; $P = 0.74$) (Fig. 3B).

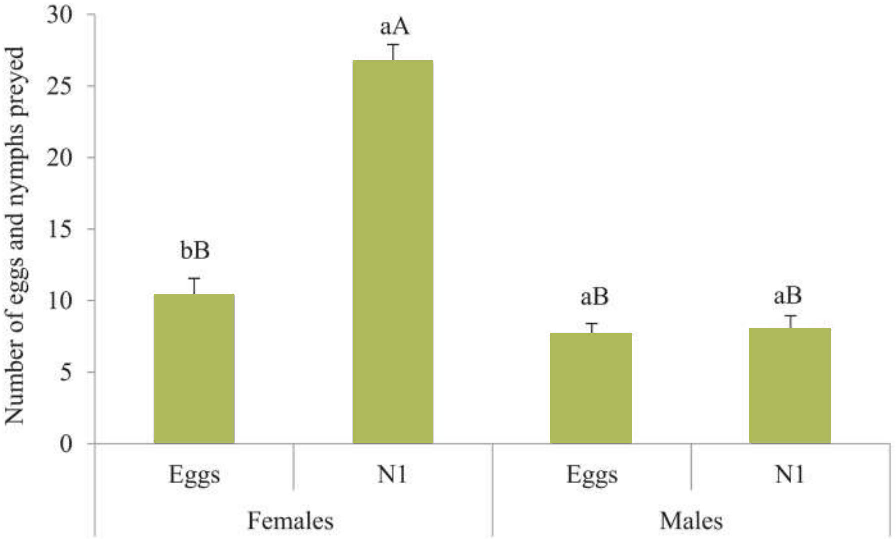


Fig. 1. Mean number (± SE) of eggs and nymphs of the mealybug *Delottococcus aberiae* preyed on by females and males of the predatory mite *Gaeolaelaps aculeifer*. Different uppercase letters above columns denote significant differences between sexes for each diet ($P < 0.05$). Different lowercase letters above columns denote significant differences between diets for each sex at $P < 0.05$.

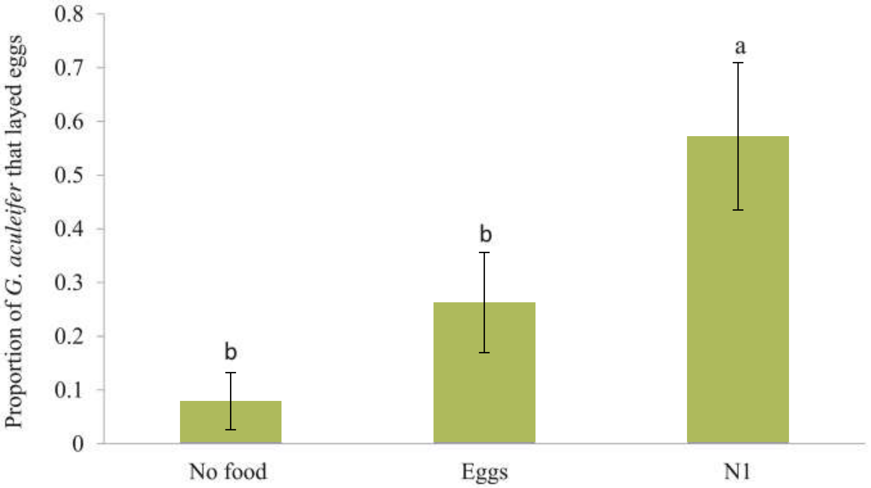


Fig. 2. Proportion of females of the predatory mite *Gaeolaelaps aculeifer* that laid eggs when they had access to three diets: without food, with *Delottococcus aberiae* eggs, with *Delottococcus aberiae* first instar nymphs (N1). Different letters above the columns show significant differences between treatments ($P < 0.05$)

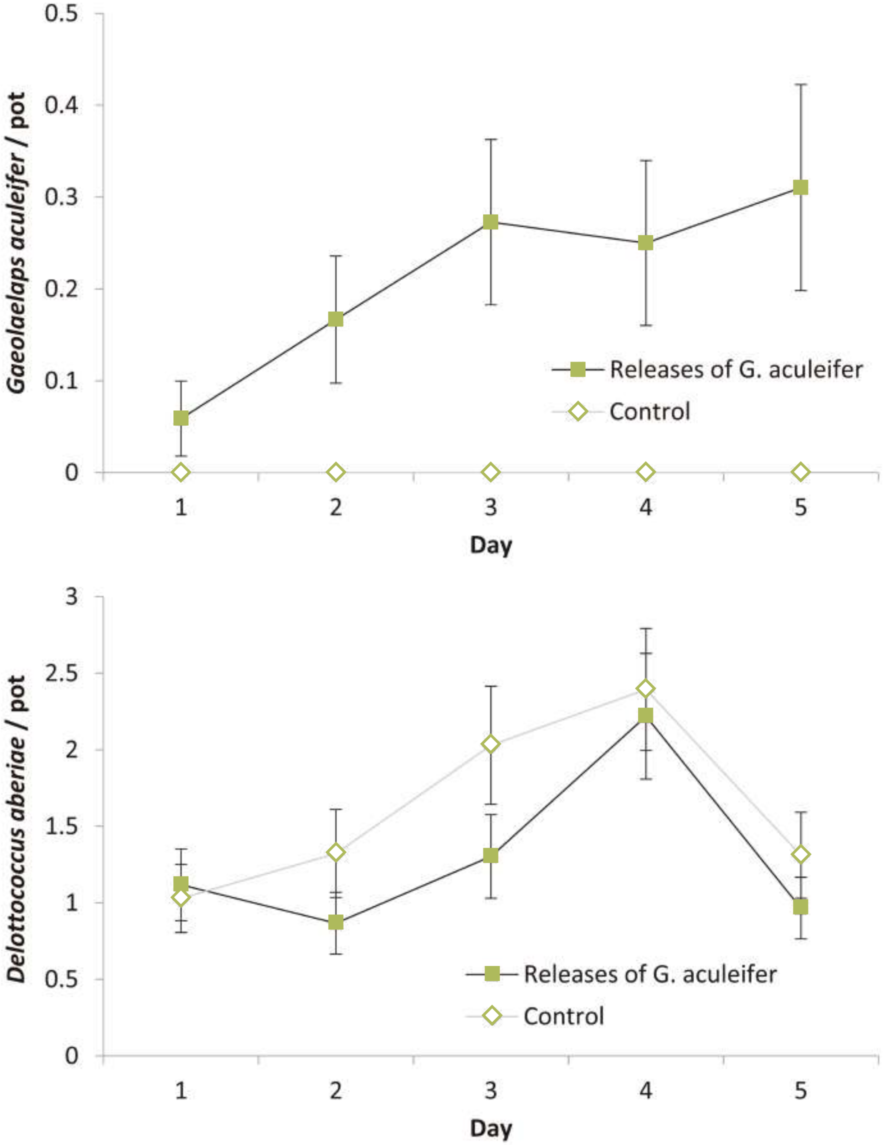


Fig. 3. Mean number (±SE) of (A) predatory mites, *Gaeolaelaps aculeifer*, and (B) *Delottococcus aberiae* nymphs counted during 2 min direct observations of pots with and without the release of the mite over the 5-day experiment.

At the end of the assay, the ratio of trees infested by *D. aberiae* (those that had at least one mealybug) was significantly higher in the control pots than in pots where mites were released ($F = 71.89$; df = 1, 67; $P < 0.001$) (Fig. 4).

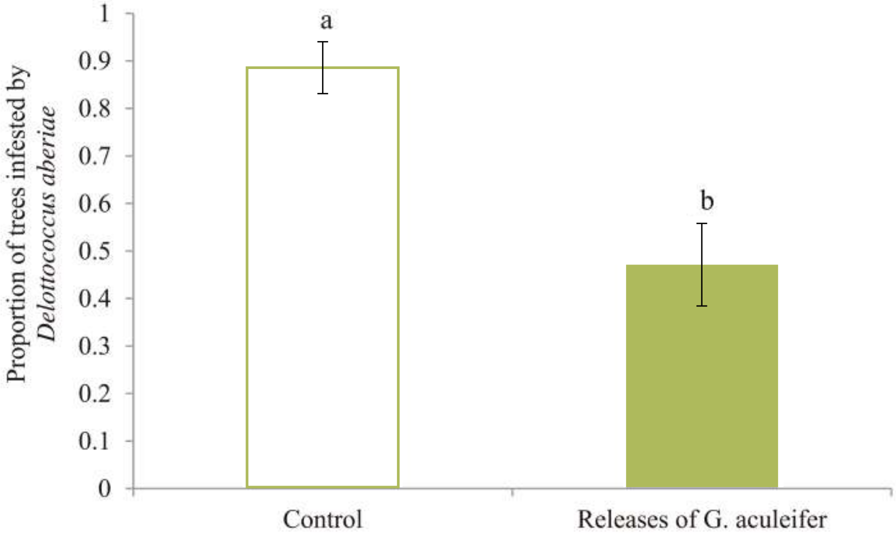


Fig. 4. Proportion of citrus trees infested by the mealybug *Delottococcus aberiae* in pots with and without the release the mite *Gaeolaelaps aculeifer* after 5 days. Different letters above the columns show significant differences between treatments ($P < 0.05$).

3.4. Discussion

Males and females of a soil dwelling mite *G. aculeifer* preyed on the first instar nymphs of *D. aberiae* mealybugs. The adult females of this mealybug lay eggs in the soil and on the trunks of citrus trees during spring, after which newly emerged nymphs return to the tree canopies (Martínez-Blay et al., 2018b). Our results demonstrate that soil predatory mites are potential biological control agents of *D. aberiae* and, possibly, other mealybug species that spend part of their life cycle in the soil, such as *Dysmicoccus brevipes*

Cockerell, *Geococcus coffeae* Green, *Rhizoecus hibisci* Kawai & Takagi, and *Rhizoecus kondonis* (Broza et al., 1995; Godfrey and Pickel, 1998; Huang et al., 2002; Kabi et al., 2016).

Gaeolaelaps aculeifer preyed two times more frequently on the nymphs than eggs of *D. aberiae* in our no-choice test. This result supports the hypothesis of Wright and Chambers (1994), which indicates that predatory mites might have difficulty recognizing immobile prey items. Other *Gaeolaelaps* species also prefer mobile stages rather than eggs (Abou-Awad et al.1989; Nawar et al., 1993). In the case of mealybugs, eggs are protected by a cottony ovisac that might also hinder the predatory behavior of *G. aculeifer*. Of note, our study supplied eggs individually (i.e., without their natural cottony protection), demonstrating that mealybug nymphs are much more vulnerable to *G. aculeifer* than eggs.

Gaeolaelaps aculeifer females were more voracious than males, as they consumed two times more *D. aberiae* nymphs than males. Similarly, Ragusa and Zedan (1988) found that *G. aculeifer* females consumed three times more immature individuals of *Rhizoglyphus echinopus* (Fumouze and Robin) (Acari: Acaridae) than males. The higher voracity of females might be explained by several biological traits. First, mite females tend to feed to repletion, unlike males (Wright and Chambers, 1994). Second, females have higher energetic needs than males, because they are bigger (Oliver Berndt et al., 2004) and repeatedly produce and mature eggs throughout their lifespan (Write and Chambers, 1994; Berndt et al., 2004a). In fact, more *G. aculeifer* females laid eggs when they fed on *D. aberiae* nymphs than when the fed on eggs or did not have access to food.

Our semi-field study demonstrated that *G. aculeifer* could be considered in future biological control programs of *D. aberiae* mealybugs inhabiting the soil. During the 5 days of the assay, we observed the presence of newly emerged nymphs of *D. aberiae* crawling on the soil of both treatments, with and without the presence of *G. aculeifer*. However, at the end of the assay, the infestation level and the number of nymphs per plant were double in the control pots compared to pots containing *G. aculeifer*. Future studies should test and compare the potential of other soil predatory mites commonly used in biological control, such as *H. miles*, or those that are common on citrus, such as *Parasitus americanus* (Parasitidae), *Gaeolaelapspraesternalis* and *Gaeolaelaps* sp. (Laelapidae), *Neomolgus* sp. (Bdellidae), *Pachylaelaps islandicus* (Pachylaelapidae), and *Macrocheles scutatiformis* (Macrochelidae) (Navarro-Campos et al., 2012).

Here, we chose *G. aculeifer* because it is commercially available and also has higher consumption rates and fecundity than other Laelapidae mites (Berndt et al., 2003). In addition, this species is able to survive under prey scarcity, as demonstrated in this study and in the study by Berndt et al. (2004b). Obviously, in natural soil environments, there is a more complex food chain structure, with the prey preference of *G. aculeifer* requiring elucidation. *Gaeolaelaps* aculeifer has a broad range of food sources, including sciarid larvae, nematodes, collembolan, other mites, and thrips (Kevan and Sharma, 1964; Ragusa and Zedan, 1988; Gillespie and Quiring, 1990; Lesna et al., 1996; Premachandra et al., 2003; Berndt et al., 2004b). To resolve this issue, future studies should determine the prey preferences of predatory mites. In this context, DNA-based approaches have shown great potential in identifying prey that remain in the gut or faeces of predators (Hoogendoorn and Heimpel, 2001; Symondson, 2002; Sheppard and Harwood, 2005; Juen and Traugott, 2006; Waldner et al., 2013; Athey et al., 2017). In fact, the development of DNA barcoding offers the opportunity to identify the complete diet of predators by simultaneously amplifying and sequencing DNA from all organisms present in a sample (Pompanon et al., 2012; Pérez-Sayas et al., 2015). Our study indicates that the optimal time to detect prey would be during spring season, when *D. aberiae* spend part of their life cycle in the soil, and might be predated on by *G. aculeifer*. Consequently, the artificial provisioning of a suitable substrate (mulching) around the trunks of trees, which could serve as a reservoir of suitable prey items for these mites, could support and increase the populations of soil-dwelling predatory mites, ultimately resulting in better pest control. Nevertheless, the presence of several suitable prey items might reduce the effectiveness in controlling the target pest (*D. aberiae*) by a generalist predator (*G. aculeifer*).

From the perspective of biological control, the presence of soil predatory mites in citrus could be enhanced in conservation biological control by adding compost (Navarro-Campos et al., 2013) or through the application of mulch (Hurlbutt et al., 1958; Parr et al., 2011). The proliferation of soil predatory mites, due to application of mulch, might be caused by an increase in prey microarthropods associated with the mulch, changes in microclimatic factors, or by providing shelter (Thomson and Hoffmann, 2007; Kawashima and Jung, 2010; Samaritani et al., 2011; Navarro-Campos et al., 2013). The positive effect of ground cover should not be disregarded, as it might also benefit the presence of these mites. Thus, ground cover based on Poaceae, such as the grass *Festuca arundinacea* L. (Poales: Poaceae), improves the biological control of mites (Aguilar-Fenollosa et al., 2011; Aguilar-Fenollosa and Jacas, 2013) and aphids (Gómez-Marco et al., 2016), and increases the number of predators of the Mediterranean fruit fly, *Ceratitis capitata* Wied (Diptera: Tephritidae) (Monzó et al., 2011).

Overall, our results demonstrate that *G. aculeifer* could contribute to the mortality of mealybugs that spend part of their life cycle in the soil. Therefore, their presence in agricultural soils should be promoted to improve biological control. To our knowledge, this study presents the first step towards understanding the importance of this group of natural enemies as biological control agents of mealybugs. Future studies should investigate whether other species are able to break the cottony defence of the mealybug eggs to feed on them, as eggs remain for longer periods in the soil than first instar nymphs.

3.5. Acknowledgements

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Chapter 4

Field evaluation of *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae) as biological control agent of the citrus invasive pest *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae)

CHAPTER 4

Field evaluation of *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae) as biological control agent of the mealybug *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae)

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Abstract

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) is one of the latest invasive mealybugs in Europe. It causes severe fruit distortion and reduced fruit size leading into important economic losses in citrus. *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae) is probably one of the most-used predators of mealybugs in biological control programs worldwide. However, its impact on *D. aberiae* is still unknown. Here, we conducted a two-year field study in nine citrus orchards located in eastern Spain in order to describe the population dynamics of *D. aberiae* and *C. montrouzieri* and evaluate the effect of the predator on *D. aberiae* using different approaches. Our results showed that *C. montrouzieri* and *D. aberiae* had two main synchronised population peaks per year: early spring and summer. Although *D. aberiae* and *C. montrouzieri* seasonal trends were synchronised, *C. montrouzieri* neither reduced mealybug density within the same year nor prevented fruit damage, which occurs early in the season. Moreover, *D. aberiae* population growth rates were not correlated with *C. montrouzieri* density. When two consecutive years were analysed, the increase of *D. aberiae* in the second year was negatively correlated with the density of *C. montrouzieri* in summer of the previous year. Based on our two years of data, *C. montrouzieri* was not able to regulate *D. aberiae* on its own or prevent the damages produced by the mealybug but might become a valuable addition to the natural enemy guild when combined with other natural enemies and rational control measures.

Key words

Conservation Biological Control • Predator–Prey Interaction • Coccinellid • Exotic Pests

Key message

- *Delottococcus aberiae* is an invasive citrus pest in Europe and its predators are still unknown.
- This study evaluated the potential use of *Cryptolaemus montrouzieri* as a biological control agent of *D. aberiae*.
- *D. aberiae* and *C. montrouzieri* seasonal trends were synchronized but the predator alone did not control the mealybug or prevent fruit damage.
- The abundance of *C. montrouzieri* in summer affected the increase of *D. aberiae* density the following year.
- The utilization of *C. montrouzieri* might be considered complementary to classical biological control.

4.1. Introduction

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) is one of the latest exotic mealybug species introduced in citrus in the Mediterranean basin (Beltrà et al., 2012; Pérez-Rodríguez et al., 2017). Likely native to Southern Africa, it was first recorded in 2009 in eastern Spain, in the region of Les Valls, Valencia (Beltrà et al., 2012). Since then, *D. aberiae* has been spreading to the surrounding areas, becoming a significant citrus pest (Martínez-Blay et al., 2018a; Pérez-Rodríguez et al., 2017; Tena et al., 2017). Unlike other species of citrus-inhabiting mealybug, *D. aberiae* causes severe fruit distortion and size reduction leading to major economic losses (Pérez-Rodríguez et al., 2017). The damaging period ranges from April to July, during the fruit set, when *D. aberiae* alters fruit cell division while feeding (Martínez-Blay et al., 2018b). Generally, *D. aberiae* is distributed throughout the entire tree canopy, but aggregate on fruits when they become available, resulting in direct damage (Pérez-Rodríguez et al., 2017). In the early spring, gravid *D. aberiae* females descend down the trunk into the soil searching for shelter in the subterranean crown (Franco et al., 2009; Martínez-Blay et al., 2018b). Once in the soil, the females deposit ovisacs, and, upon emergence, nymphs climb again to the upper part of the tree (Martínez-Blay et al., 2018a).

Integrated pest management (IPM) of *D. aberiae* mainly relies on the application of broad-spectrum insecticides such as chlorpyrifos or chlorpyrifos-methyl. Recently, the environmental economic injury level for *D. aberiae* has been established as 12% of fruit occupation between petal fall and July. When more than 12% of fruits are occupied by *D. aberiae*, a chemical treatment is recommended (Pérez-Rodríguez et al., 2017). Biological control is currently not an option because native parasitoids, the most important group of biological control agents of mealybugs, cannot develop in *D. aberiae*, and the role of predators is poorly known (Tena et al., 2017). Recently, the predatory mite *Gaeolaelaps aculeifer* (Canestrini 1883) (Acari: Laelapidae) has been recorded as a potential enemy of young instars of *D. aberiae* in the soil because it feeds on the first instar under semi-field conditions (Pérez-Rodríguez et al., 2018), but the impact of other generalist predators is still unknown.

Among mealybug predators, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) has been used worldwide in many biological control programs (Kairo et al., 2000; Stiling, 2004; Maes et al., 2014). It is native to the Australasian zoogeographical region and has been introduced to more than 64 countries to suppress important mealybug pests (Kairo et al., 2013). In the citrus-growing areas of the Mediterranean Basin such as France, Greece, Italy, and Turkey, *C. montrouzieri* has been introduced and/or inoculatively released to control *Planococcus citri* Risso (Hemiptera: Pseudococcidae) (Ripollés et al., 1995; Katsoyannos, 1996; Franco et al., 2004; Jacas et al., 2006; Villalba et al., 2006; Mani, 2018). In Spanish citrus, *C. montrouzieri* was first introduced in 1927 (Gómez-Clemente, 1928) from California. Since then, it has been reared and inoculatively released because winter temperatures, prey scarcity, and the overuse of chemical control decrease *C. montrouzieri* densities during winter (Boyero et al.,2005; Villalba et al., 2006; Planes et al., 2013). However, it has been shown that *C. montrouzieri* is able to overwinter in pesticide-free citrus orchards (Martínez-Ferrer, 2003; Franco et al., 2004).

The larvae and adults of *C. montrouzieri* are voracious feeders and prey on all stages of *P. citri* (Rosas-García et al., 2009; Attia et al., 2011). However, the earlier larvae of *C. montrouzieri* prefer to feed on eggs and young nymphs while older larvae, which have a higher predation rate, feed on mealybugs of any size (Heidari and Copland, 1992; Attia et al., 2011; Mani, 2018). For instance, fourth instar larvae have been found to consume up to 3,330 eggs or 250 young instars of *P. citri* at 28º C (Gosalwad et al., 2009; Kairo et al., 2013). Adults of *C. montrouzieri* become smaller or even infertile when prey is scarce (Torres and Marcano, 2015) and engage in cannibalism when prey is absent (Mani, 2018).

In order to locate their prey, *C. montrouzieri* larvae require physical contact whereas adults use visual and chemical stimuli (Heidari and Copland, 1992; Kairo et al., 2013). The wax secretions and honeydew produced by mealybugs act as both attractants and oviposition stimulants for *C. montrouzieri* females (Merlin et al., 1996), which are able to lay viable eggs as long as they can prey on mealybugs (Finlay-Doney and Walter, 2012). A single adult female lays about 400 eggs in 40 days of oviposition (Babu and Azam, 1987; Siddhapara et al., 2013) and this quantity can increase if multiple mating occurs (Xie et al., 2014).

In the Mediterranean, *C. montrouzieri* has four generations per year and it can overwinter depending on the climatic conditions (Kaneko, 2017; Toorani et al., 2017). Apart from *P. citri*, it has been observed feeding on other mealybug species as well as soft scales (Hemiptera: Coccidae), aphids (Hemiptera: Aphididae), and whiteflies (Hemiptera: Aleyrodidae) (Saikia and Balasubramanian, 2000; Attia et al., 2011; Maes et al., 2014; Francis et al., 2016). In the main citrus producing areas of Spain, *C. montrouzieri* follows a predator–prey population dynamic with its main prey *P. citri* (Llorens, 1990; Martínez-Ferrer, 2003; Villalba et al., 2006) and it is considered a key predator in the system (Mani and Krishnamoorthy, 2008; Kairo et al., 2013) .

During the past few years, *C. montrouzieri* has been observed feeding on *D. aberiae* in the field, but its impact as a biological control agent of this citrus invasive pest is unknown. In this study, we surveyed nine citrus orchards during two consecutive years in order to: i) describe the population dynamics of *D. aberiae* and *C. montrouzieri*; ii) evaluate the effect of *C. montrouzieri* density on the population levels of *D. aberiae* within the same year and among years; iii) evaluate the influence of *C. montrouzieri* as well as the mean temperature per day and citrus variety on *D. aberiae* population growth rates; and vi) describe damages produced by *D. aberiae* throughout the year. We used these data to discuss the potential of *C. montrouzieri* as biological control agent of *D. aberiae.*

4.2. Material and methods

4.2.1. Survey sites and procedure

The study was carried out in the core of the citrus producing area of eastern Spain (Valencian Community). A total of nine orchards were monitored during 2014 and 2015. Five orchards consisted of *Citrus reticulata* Blanco (mandarins): three were Clemenules and two were Oroval cultivars. The remaining four consisted of *Citrus sinesis* (L.) Osbeck: Navelate cultivar. Orchards monitored ranged from 0.4 to 3 ha and all were drip-irrigated. The surveyed area was pesticide-free during the study.

Eight to ten trees were observed twice a month from March to November and monthly from December to February. Sampling occurred with less frequency from December to February due to the reduced insect activity during the winter. Trees were selected at the beginning of the surveys if they were infested only with *D. aberiae* during previous years. In each tree, two different strata were surveyed: canopy and trunk. In the canopy, four 30-cm long twigs with flowers or fruits or both were collected and each twig belonged to a different cardinal orientation (i.e., one twig randomly selected in each orientation). All samples of a tree were placed in individual plastic bags, sealed, and immediately transported in a cooler to the laboratory. Within the next 24 h, twigs, four leaves, and a maximum of eight flowers or fruits per twig were examined under a stereomicroscope. Leaves, flowers, and fruits were selected at random within the twig. All nymphs and adult females of *D. aberiae* as well as all the larvae and adults of *C. montrouzieri* were counted. In the trunk stratum, sampling consisted of visual counts lasting 2 min. All *D. aberiae* and *C. montrouzieri* individuals present on the trunk (from the ground up to 60 cm in height) were recorded. During fruit formation, we also recorded whether the fruit was damaged by *D. aberiae*. We considered damaged fruit as those with a size reduction or deformation that completely depreciated them from a commercial view (Pérez-Rodríguez et al., 2017).

4.2.2. Data analyses

4.2.2.1. Prey-Predator dynamics and impact of *C. montrouzieri* on *D. aberiae*

In order to represent the population dynamics of *D. aberiae* and *C. montrouzieri*, sampling data of the different strata were averaged per tree and afterward per orchard. The ratio of fruit damaged by *D. aberiae* was also averaged per tree and afterward per orchard. The maximums of *C. montrouzieri* density throughout the two years of the study were correlated using Kendall tests

(Kendall, 1975). The same analysis was used to determine correlations between the maximums of *C. montrouzieri* density and the fluctuations of *D. aberiae* density among generations (or maximum peaks). These and all subsequent analyses were conducted using the software R (version 3.4.4, The R Foundation).

4.2.2.2. Population growth rates

Population growth was expressed as the intrinsic rate of growth, r, calculated as $\ln(N_{(t+1)}/N_t)$, in which N represents the total number of all developmental *D. aberiae* stages per tree at time t and $t + 1$. The r-value was expressed normally using the units d^{-1} by dividing $\ln(N_{(t+1)}/N_t)$ by the period of time in days between the survey date t and the subsequent survey date $t + 1$. The relative density of *C. montrouzieri* was calculated as *C. montrouzieri* / (*D. aberiae* + *C. montrouzieri*), which is the fraction of *C. montrouzieri* in an isolated predator–prey community (per tree).

A linear mixed-effects model was used to quantify the effect of *C. montrouzieri* on *D. aberiae* population growth. The model incorporated predator density, mean temperature per day, and citrus species as main effects, and orchard and tree as nested random effects to account for repeated measures. The data were also analysed for any delayed effects by plotting the slope of *D. aberiae* population growth and *C. montrouzieri* density as a function of increasing the magnitude of time (incremental increase in sampling period) between measuring *C. montrouzieri* density and the associated *D. aberiae* population growth rate. Thus, as the time delay becomes more extreme (considered as increasing values along an x-axis), this represents a larger delay in the effect of *C. montrouzieri* on *D. aberiae*. For example, a 365-day delay correlates *D. aberiae* population growth with *C. montrouzieri* densities surveyed one year prior. Citrus cultivars were analysed separately in this variation of the model because sampling periods differed across cultivars.

4.3. Results

4.3.1. Prey-predator population dynamics and impact of *C. montrouzieri* on *D. aberiae*

The population densities of *D. aberiae* and *C. montrouzieri* exhibited two major peaks in both years, and their populations were synchronized except in the summer of 2014, when the mealybug population peaked earlier than the predator (Figure 1). The first major infestation was at the end of May and the second occurred between July and August.

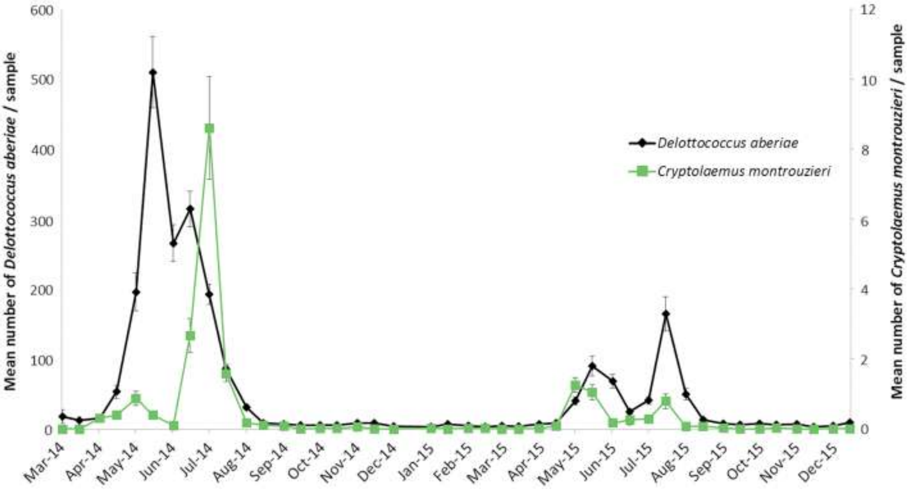

Fig. 1. Population density (mean ± SE) of the invasive mealybug *Delottococcus aberiae* and its predatory coccinellid *Cryptolaemus montrouzieri* in nine citrus orchards from Spain in 2014 and 2015.

The maximum number of *C. montrouzieri* in the first and second peaks had a positive correlation both years (2014: Z = 2.04, df = 1, 70, *P* = 0.042, Ʈ = 0.19; 2015: Z = 2.69, df = 1, 70, *P* = 0.007, Ʈ = 0.27) (Table 1). Other correlations between different peaks of *C. montrouzieri* were not significant (Table 1).

Table 1. Correlation between the densities of the predatory coccinellid *Cryptolaemus montrouzieri* in different maximum peaks of 2014 and 2015. Significant *P*-values are presented in bold (N = 71 trees).

C. montrouzieri **density**		**Z**	**P**	**Ʈ**	**slope**
(x-Axis)	**(y-Axis)**				
1st peak 2014	2nd peak 2014	2.04	**0.04**	0.19	1.29
	1st peak 2015	1.62	0.11	0.16	0.16
	2nd peak 2015	0.05	0.96	0.01	-0.12
2nd peak 2014	1st peak 2015	-0.43	0.67	-0.04	-0.001
	2nd peak 2015	-1.24	0.21	-0.12	-0.04
1st peak 2015	2nd peak 2015	2.69	**0.01**	0.27	0.46

When we analysed the effect of *C. montrouzieri* density on changes in peak *D. aberiae* abundance, the number of predators in the second peak of 2014 had a negative relationship with the increase of *D. aberiae* in 2015 [increase between the first (May) and second peak (July-August); Z = -3.75, df = 1, 70, *P* < 0.001, Ʈ = -0.31] (Table 2). Other correlations between *C. montrouzieri* density and changes in *D. aberiae* abundance were not significant (Table 2).

Table 2. Effect of *Cryptolaemus montrouzieri* density on *Delottococcus aberiae* increase among different maximum peaks of 2014 and 2015. Significant *P*-values are presented in bold. The significant interactions are graphically represented in Fig. 2.

C. montrouzieri **density**	**Population fluctuations of *D. aberiae***	**Z**	**P**	**τ**	**slope**
1st peak 2014	2nd peak 2014 – 1st peak 2014	-1.43	0.15	-0.13	-13.52
	1st peak 2015 – 2ndt peak 2014	0.35	0.72	0.032	9.83
	2nd peak 2015 – 1st peak 2015	-1.62	0.11	-0.14	-14.30
2nd peak 2014	1st peak 2015 – 2nd peak 2014	-1.13	0.26	-0.10	-1.68
	2nd peak 2015 – 1st peak 2015	-3.75	**<0.001**	-0.31	-3.50
1st peak 2015	2nd peak 2015 – 1st peak 2015	-0.79	0.43	-0.07	0.09

4.3.2. Fruit damaged by *D. aberiae*

During both years, the ratio of fruit that could be categorized as damaged by *D. aberiae* increased exponentially from the end of May until the end of June (Figure 2), and by the last week of June, approximately 70% of the fruit was damaged.

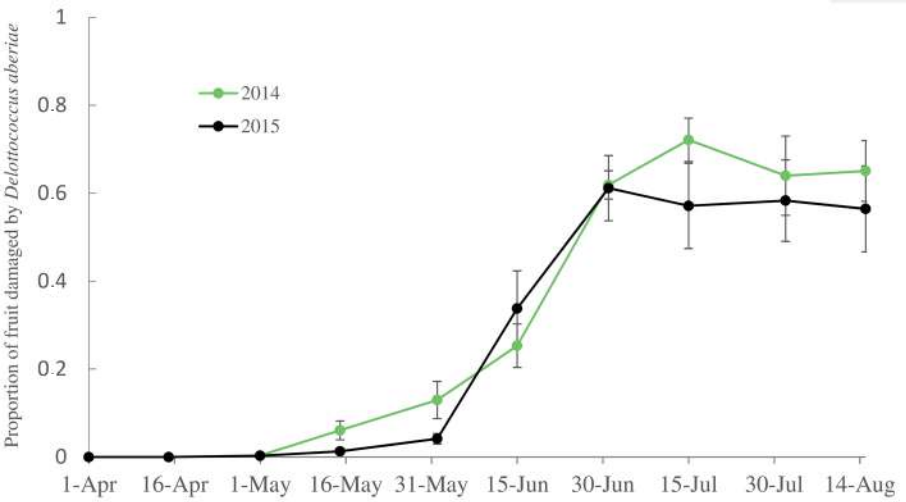

Fig. 2. Fruit damaged (mean ratio ± SE) by *Delottococcus aberiae* in nine citrus orchards from Spain throughout 2014 and 2015.

4.3.3. Analyses of population growth rates

Changes in *D. aberiae* population growth rate were associated with changes in temperature and *C. montrouzieri* density, but not citrus variety (Figure 3; Table 3). *Delottococcus aberiae* population growth rate tended to decrease with increasing temperature but to increase with declining *C. montrouzieri* population density. Similarly, analyses of a subset of the data spanning 1 May to 30 September (outbreak and decline of *D. aberiae* densities) exhibited similar trends, except that *D. aberiae* population growth rate was not associated with changes in *C. montrouzieri* density in 2014 (Table 3).

Table 3. Effect of the density of *C. montrouzieri*, mean temperature per day and citrus variety on *Delottococcus aberiae* population growth rates during 2014–2015. Significant *P* values are presented in bold and represented graphically in Fig. 3.

Time period	Factor	df	*F*-value	*P*-value
2014–2015	*C. montrouzieri*	1. 2502	10.32	**0.001**
	temperature	1. 2502	35.67	**<0.0001**
	citrus variety	1. 6	1.880	0.219
May–Sep 2014	*C. montrouzieri*	1. 749	1.500	0.221
	temperature	1. 749	53.00	< **0.0001**
	citrus variety	1. 6	1.134	0.328
May–Sep 2015	*C. montrouzieri*	1. 647	17.05	**<0.0001**
	temperature	1. 647	9.939	**0.002**
	citrus variety	1. 6	3.356	0.117

Figure 4 summarizes the time lag of predator impact on *D. aberiae* population growth rates. While the slope was positive when *C. montrouzieri* density was delayed 15 days, it tended to be zero later on.

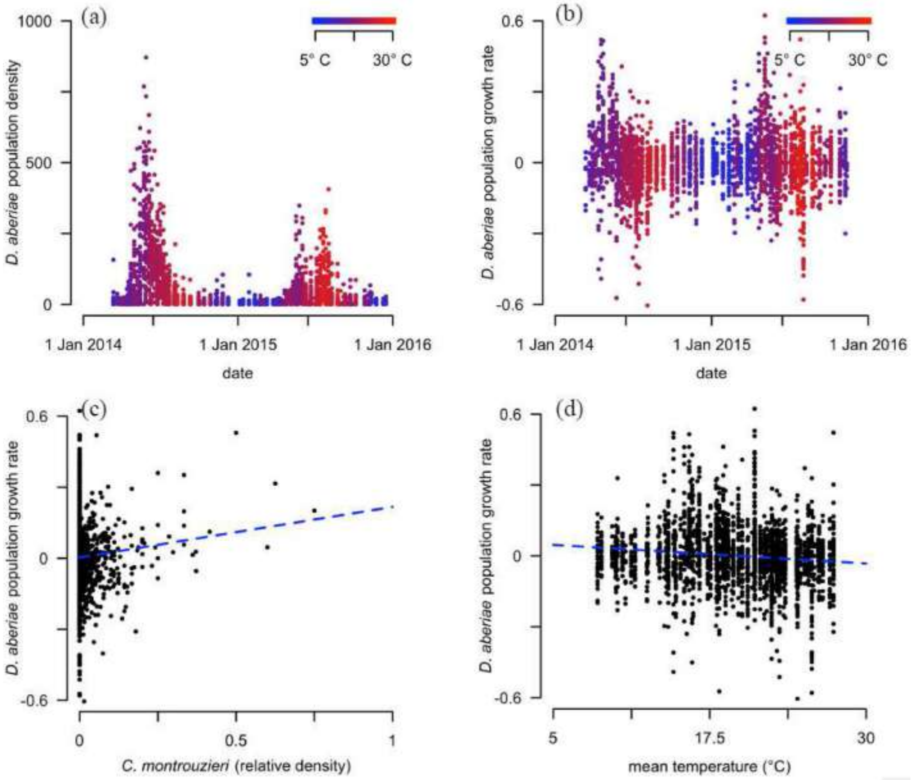

Fig. 3. a) Population density and b) population growth rate of *Delottococcus aberiae* represented per day and tree. Different colours represent mean daily temperature. c) Population growth rate of *D. aberiae* as a function of relative density of *Cryptolaemus montrouzieri* and d) mean temperature during 2014 and 2015. Dashed line plots the slope β1 from the linear mixed models presented in Table 3.

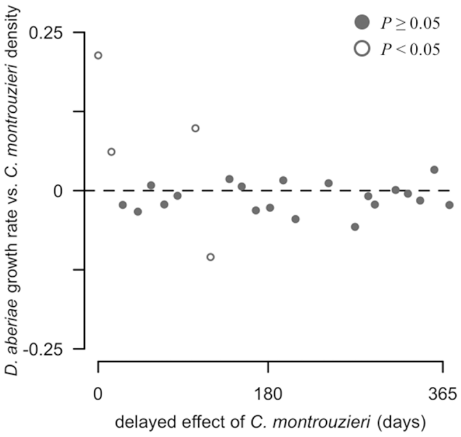

Fig. 4. Slope (β_1) of *Delottococcus aberiae* population growth rate (*r*) vs. the relative *Cryptolaemus montrouzieri* density as a function of offsetting the temporal relationship between *D. aberiae* and *C. montrouzieri* density from 2014–2015. The x-axis maps incremental delays in surveyed periods. Slopes significantly different than zero are indicated by open circles. The dashed line represents no correlation between *C. montrouzieri* and *D. aberiae* population density.

4.4. Discussion

The two-year field study showed that the seasonal abundance of *C. montrouzieri* (the lady beetle mealybug destroyer) was synchronized with that of the invasive mealybug *D. aberiae*. The mealybug and the predator had two major peaks in population density per year; the first one was in the spring and the second in the summer. The populations of *D. aberiae* and *C. montrouzieri* were synchronised for both peaks during the two years of the study. This prey-predator synchrony, as well as the absence of alternative mealybug species, suggests that *C. montrouzieri* is able to locate and survive by preying upon *D. aberiae* in the field just a few years after the mealybug established in Europe. It is worth mentioning that, although *P. citri* is abundant and widely distributed in Mediterranean citrus (Tena and García-Mari, 2011), *D. aberiae* was the predominant mealybug species in the monitored orchards; only one other mealybug, *Pseudococcus longispinus* (Targioni Tozzetti) (Hemiptera: Pseudococcidae), was recovered occasionally in one of the orchards.

The peaks in the densities of *D. aberiae* and *C. montrouzieri* tended to overlap, contrary to other prey–predator dynamics. Generally, predators peak a few weeks after their prey, showing a temporal mismatch between population cycles (Holling, 1965; Tansky, 1978; Martin and Ruan, 2001). The reason for this overlap might be due to the preference of *C. montrouzieri* adults of laying their eggs near mealybug ovisacs, just before the new generation of the prey emerge and peak (Whitcomb, 1940; Merlin et al., 1996). In fact, *C. montrouzieri* adults withhold their eggs in the oviducts, delaying oviposition when mealybug ovisacs are not present (Maes et al., 2014).

Although *D. aberiae* and *C. montrouzieri* seasonal trends were synchronised, our results indicated that *C. montrouzieri* was not able to prevent fruit damage by the mealybug. Damages produced by *D. aberiae* were observed by May and increased exponentially in June, at which point nearly 70% of fruits were damaged, so it does not appear that *C. montrouzieri* would have been able to prevent the damages produced by *D. aberiae*. *Delottococcus aberiae* damages the fruit when it feeds on flower ovaries or on very small, tender fruits (Martínez-Blay, 2017). The most plausible explanation for this lack of damage control is the high susceptibility of the fruit to be damaged even at low mealybug densities. As with other sap feeders, *D. aberiae* might interfere with fruit cell division by injecting toxic enzymes (Sharma et al., 2014; Martínez-Blay et al., 2017). During this period, which occurs during May and June, the mealybug is poorly aggregated and relatively low in abundance, which may not be favourable to *C. montrouzieri* population growth (Pérez-Rodríguez et al., 2017).

Cryptolaemus montrouzieri was not able to regulate the populations of *D. aberiae* within the same year. Density fluctuations of the mealybug between spring and summer, as well as its population growth rates were independent of the density of the coccinellid. One possible explanation could be that *D. aberiae* is an unsuitable prey for *C. montrouzieri*. The nutritional value of some prey can be insufficient to allow the development of their predators (Hodek et al., 2012), and the nutritional value of *D. aberiae* for *C. montrouzieri* still needs to be evaluated. Coccinellids are not able to distinguish between adequate food and a less suitable prey (gustatory discrimination) (Nielsen et al., 2002; Synder and Clevenger, 2004; Nedvêd and Salvucci, 2008). Therefore, *D. aberiae* could be a less suitable prey for *C. montrouzieri* than other citrus mealybugs such as *P. citri*, which density levels within the same year depend on the coccinellid (Martínez-Ferrer, 2003). Another possible factor that might explain this lack of regulation is temperature. The coccinellid is adapted to tropical temperatures and requires a minimum temperature of 21° C to feed or lay eggs and is unable to complete development between 0 and 17° C (Kairo et al., 2013). Although the minimum temperature threshold of *D. aberiae* is unknown, the mealybug might have a lower threshold than *C. montrouzieri* because it is active during our Mediterranean winter (mean maximum temperature per month around 17° C (MAPAMA, 2018) and a male flight occurs in February (Martínez-Blay et al., 2018a,b). These different temperature requirements might contribute to the lack of top-down regulation within the same year. Finally, bottom-up regulation may be more important than top-down regulation and mask the effect of *C. montrouzieri*. As with all mealybugs, *D. aberiae* is likely a phloem feeder. In citrus, the two highest concentrations of amino acids in the phloem occur in early spring (March) and during the fruit setting period (June-July) (Moreno and García-Martínez, 1983; Yang and Sadof, 1995; Sétamou et al., 2016), both of which correspond to the two main peaks of *D. aberiae*. The increase of amino acids might accelerate *D. aberiae* development and increase its reproduction in spring compared to *P. citri,* which likely has a higher minimum of temperature threshold.

Another detrimental factor that could disrupt the prey-predator dynamic between *C. montrouzieri* and *D. aberiae* is the presence of ants in citrus. In a mutualistic association, ants obtain carbohydrates and other nutrients from the mealybug honeydew in exchange for providing protection against their natural enemies (Tollerup et al., 2004; Majerus et al., 2007; Styrsky and Eubanks, 2007; Nelson and Daane, 2008; Tena et al., 2013a; Zhou et al., 2015; Calabuig et al., 2015; Beltrà et al., 2017). In Spanish citrus orchards, the two dominant ant species are *Lasius grandis* (Forel) (Hymenotera: Formicidae) and *Pheidole pallidula* (Nyl) (Hymenoptera: Formicidae). They attend the main citrus mealybug species *P. citri* (Pekas et al., 2011; Calabuig et al., 2015) and have been reported attacking *C. montrouzieri* larvae (Mansour et al., 2012; Kairo et al., 2013). However, it is unclear whether *L. grandis* and *P. pallidula* disrupt the biological control of *D. aberiae* because we observed them attending *D. aberiae* only occasionally (Pérez-Rodríguez, personal observations). Further studies should assess the effect of these ant species on *D. aberiae* population dynamics. Despite the lack of correlation between *C. montrouzieri* and *D. aberiae* populations within the same year, the increase in the density of *D. aberiae* in 2015 was negatively related to the density of *C. montrouzieri* in the summer of 2014. *Cryptolaemus montrouzieri* has been largely considered in conservation biological control programmes of other mealybug species because it complements the action of parasitoids (Prokopenko, 1982; Longo and Benfatto, 1987; Moore, 1988; Martínez-Ferrer, 2003; Kairo et al., 2013), which suggests that *C. montrouzieri* could contribute to regulate *D. aberiae* once a successful parasitoid is introduced in Spain. At this time, biological control of *D. aberiae* is limited because the mealybug encapsulates the eggs of the native or naturalized parasitoids in Spanish citrus (Tena et al., 2017). Recently, two new parasitoid species of *D. aberiae* have been described in its native area of South Africa: *Anagyrus aberiae* Guerrieri sp. nov. and *Anagyrus antoniae* Guerrieri sp. nov. (Hymenoptera: Encyrtidae) (Beltrà et al., 2015; Guerrieri and Cascone, 2018), and the introduction of these parasitoid species within a classical biological control program might contribute to the management of *D. aberiae*. Another promising control measure could be the augmentative or inoculative releases of *C. montrouzieri* larvae during winter, or before the first main population peak of *D. aberiae*. Because late nymphal and adult female *D. aberiae* aggregate to and disperse down the trunk during February and March (Martínez-Blay et al., 2018b), *C. montrouzieri* could be inoculatively released at the base of the trunk during this time. Further research in the field is needed to test this potential strategy and whether *C. montrouzieri* survive and search for *D. aberiae* under the winter conditions of the Mediterranean basin (Maes et al., 2014).

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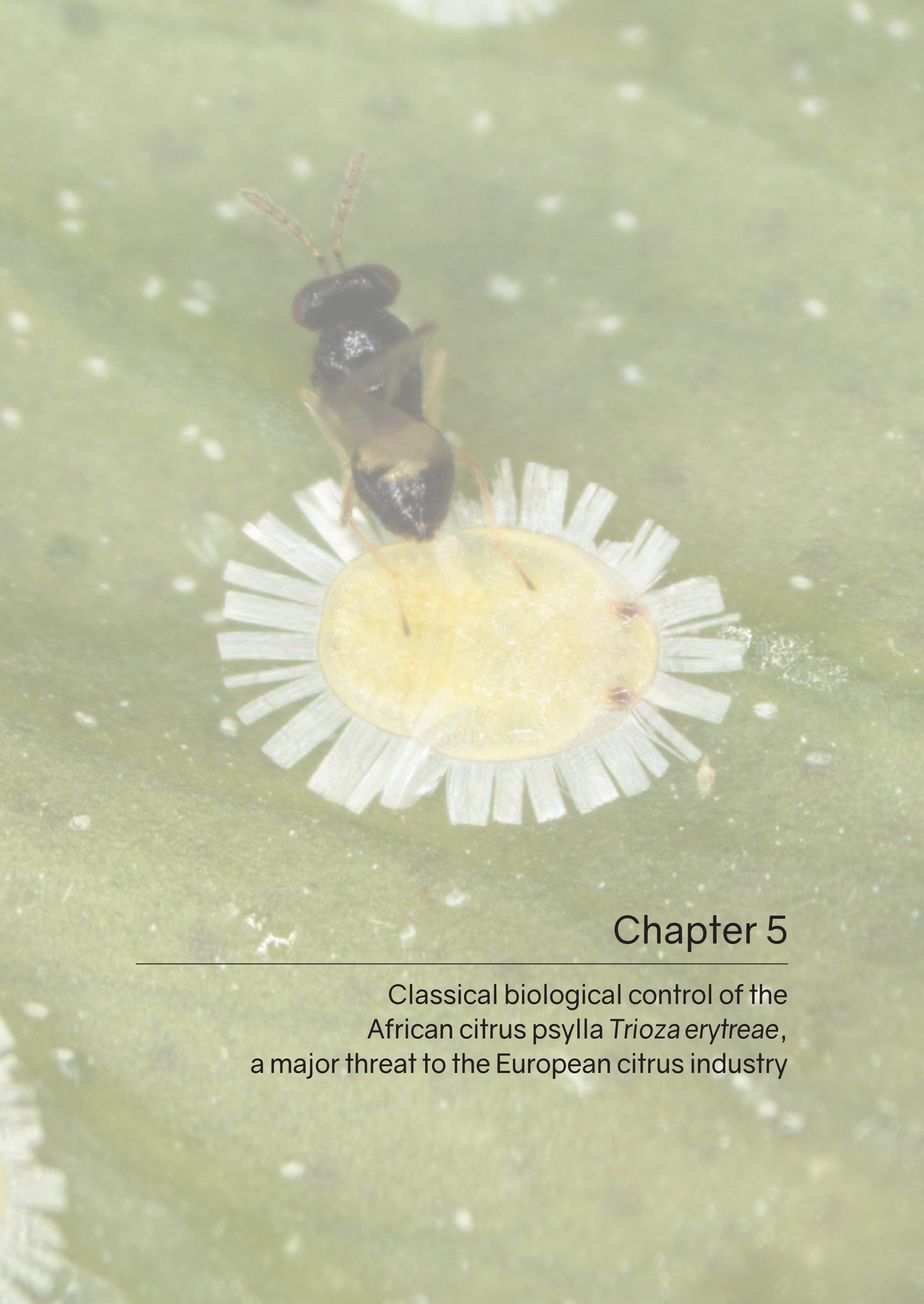
Author contribution statement

AS, JM and AT conceived and designed the research. JPR and VM collected field data. JP, JRM and AT analyzed the data. JPR, JRM and AT wrote the first version of the manuscript. All authors read and contributed to the last version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.



Chapter 5

Classical biological control of the African citrus psylla *Trioza erytreae*, a major threat to the European citrus industry

CHAPTER 5

Classical biological control of the African citrus psylla *Trioza erytreae*, a major threat to the European citrus industry

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Abstract

Citrus greening or Huanglongbing (HLB) is the main threat to the European citrus industry since one of its vectors, the African citrus psyllid, *Trioza erytreae*, has recently become established in mainland Europe. In this context, classical biological control programmes should be implemented to reduce the spread of the psyllid. The aims of this study were to: i) disentangle the parasitoid complex of *T. erytreae* combining morphological and molecular characterization; and ii) study the biology of its main parasitoids in its area of origin in South Africa for their future importation into Europe. The main citrus producing areas of South Africa were sampled during 2017. In contrast to previous studies, the parasitoid complex of *T. erytreae* included three species of primary parasitoids: *Tamarixia dryi*, *Psyllaephagus pulvinatus* and another parasitoid of the genus *Tamarixia*. Molecular analysis showed that it is a new species closely related to *T. dryi*. *Tamarixia dryi* was the most abundant parasitoid but its relative abundance varied among sampling sites. The sex ratio (males/females) of *T. dryi* and *Tamarixia* sp. decreased with *T. erytreae* size and became female biased when psyllid nymphs were larger than 0.6 and 1.2 mm^2, respectively. These parasitoids were attacked by three species of hyperparasitoids, *Aphidencyrtus cassatus*, *Marietta javensis* and a species of the genus *Aphanogmus*. *Aphidencyrtus cassatus* was the most abundant hyperparasitoid, tended to emerge from large nymphs, and adult females lived as long as those of *T. dryi*. The implications of these results are discussed within the framework to introduce *T. dryi* into Europe.

Key words

Citrus • Huanglongbing • Citrus Greening • *Tamarixia dryi* • *Aphidencyrtus cassatus* • Sex Ratio • Hyperparasitism • Barcoding

5.1. Introduction

Citrus greening or Huanglongbing (HLB) is one of the most devastating citrus diseases in the world (Carmo Teixeira et al., 2005; Bové, 2006; Gottwald, 2007; Lee et al., 2015). It is associated with the three phloem α-proteobacterias "*Candidatus* (*Liberibacter*) *asiaticus*" (CLas), "*C.* (*Liberibacter*) *americanus*" (CLam) and "*C.* (*Liberibacter*) *africanus*" (CLaf). The three bacteria are transmitted by two insect vectors: The Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) and the African citrus psyllid *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae) (Bové, 2006; Grafton-Cardwell et al., 2013). Since its first record in Taiwan in 1908 (Kuwayama, 1908), *D. citri* has been spread and has been reported transmitting Las in Asia, parts of North and South America, Africa and numerous islands in the Atlantic and Pacific oceans (Knapp et al., 1998; French et al., 2001; Bové, 2006; Lopes et al., 2009; Shimwela, 2016). In contrast, *T. erytreae* is associated with CLaf and, since its first record in 1929 in South Africa (Jagoueix et al., 1994; Li et al., 2006), has been recorded along all the African continent, Yemen and a few Atlantic Ocean islands (Bové, 2006). It has recently been reported from Portugal and Spain (Otero et al., 2015) even though HLB has not been detected yet in European countries (Otero et al., 2016; Cocuzza et al., 2017).

HLB disease manifests as asymmetrical yellow mottles or severe chlorosis of the foliage, fruit drop and dieback (Bové, 2006; Zhang et al., 2011), leading to significant economic losses (Manjunath et al., 2008; Paula et al., 2018). As an example of the destructive potential of HLB, citrus production in Florida has dropped by more than 70% since HLB was detected in 2005 (Hodges and Spreen, 2015). In the Mediterranean Basin, HLB detection could be a destructive turning point not only because it is the main producing area of citrus for the fresh market in the World (Jacas et al., 2010), but also because Mediterranean citriculture is based on small farming, where managing HLB and its vectors is more complex than in larger commercial orchards (Reig-Martínez and Picazo-Tadeo, 2004).

In Europe, *T. erytreae* was first detected in 1994 in Madeira (Portugal) (de Carvalho and Aguiar, 1997) and later on in the Canary Islands (Spain) in 2002 (Pérez Padrón and Carnero Hernández, 2002; Hernández, 2003). Until then, it seemed to be restricted to non-continental areas, but in 2014 it first appeared in the north-western Iberian Peninsula (Otero et al., 2015). Despite the initial insecticide treatments to eradicate it, *T. erytreae* is now spreading from the north-west to the south-west of the Iberian Peninsula (Cocuzza et al., 2017). Parasitoids of the genus *Tamarixia* are amongst the most effective natural enemies of HLB vectors (Etienne and Aubert, 1980; van den Berg and Greenland, 2000; Grafton-Cardwell et al., 2013; Hoddle and Pandey, 2014). However, no native parasitoids have been recorded on *T. erytreae* neither in the Atlantic islands nor in the Iberian Peninsula (Fernandes and Aguiar, 2001; Cocuzza et al., 2017). In this context, classical biological control seems to be the most feasible measure for preventing *T. erytreae* to spread further in the Mediterranean citrus growing areas.

The complex of parasitoids in South Africa and Swaziland was analysed in detail during the 1960's and 70s (Catling, 1969a; Mc Daniel and Moran, 1972) and in Cameroon twenty years ago (Tamesse and Messi, 2000). According to these studies, the two main primary parasitoids of *T. erytreae* in Southern Africa are *Tamarixia dryi* (synonym *Tetrastichus dryi*) (Waterston)

(Hymenoptera: Eulophidae) and *Psyllaephagus pulvinatus* (Waterston) (Hymenoptera: Encyrtidae). Both are solitary koinobiont parasitoids. The former is an ectoparasitoid, whereas the encyrtid is an endoparasitoid. These primary parasitoids frequently are attacked by a complex of hyperparasitoids (van den Berg and Greenland, 2000; Tamesse and Messi, 2002) that, accordingly to van den Berg and Greenland (van den Berg and Greenland, 2000), severely decrease the impact of the primary parasitoids. *Tamarixia dryi* was used in a classical biological control programme in Reunion Island when *T. erytreae* was detected in 1974 (Aubert et al., 1980; Etienne and Aubert, 1980). In less than five years, *T. dryi* became established and controlled *T. erytreae* (Etienne and Aubert, 1980; van den Berg and Greenland, 2000). Similarly, in 1982, *T. dryi* was imported into Mauritius (Aubert and Quilici, 1988). In these islands both *T. erytreae* and *D. citri* coexisted as well as the African and Asian forms of HLB. However, only the classical biological control of *T. erytreae* with *T. dryi* was successful (Aubert and Quilici, 1988; Bové, 2014). The lack of alternative hosts for *T. erytreae,* the presence of alternative hosts for *T. dryi* and the absence of hyperparasitoids were considered key aspects for the establishment of *T. dryi* and the successful control of *T. erytreae* populations (Toorawa, 1998; Chen and Stansley, 2014; Husain et al., 2016). Whether *T. dryi* would find these conditions in mainland Europe it is unknown. However, *T. dryi* is highly specific. The parasitoid did not parasitize and develop in any of the eleven alternative host species that were offered in host-specificity tests (Urbaneja-Bernat et al., 2019).

In this study, we propose a classical biological control programme to introduce the main parasitoids of *T. erytreae* from its area of origin into Europe. We first identified the parasitoid complex of *T. erytreae* in several areas of South Africa using morphological and molecular characterisation. We then determined several aspects of the biology of the main parasitoids: sex ratio, longevity and hyperparasitism. Implications of using *T. dryi* in classical biological control programmes of *T. erytreae* also are discussed.

5.2. Results

5.2.1. Insect survey

The Western Cape was the only province in South Africa where neither *T. erytreae* nor its symptoms were recorded (see supplementary material Table S1). Leaves of trees from 17 out of the 65 sampled sites in the other provinces had the characteristic open gall-like structures, indicating the presence of *T. erytreae*. Live nymphs were collected from five out of the 17 sites and parasitoids were recorded from four sites.

5.2.2. Parasitoid emergence and species abundance

A total of 580 parasitized *T. erytreae* individuals were collected during the survey. From these samples 334 parasitoids belonging to five species emerged. The parasitoids in the remaining 246 psyllids failed to develop. The parasitoid complex was composed of at least five species whose relative abundance varied with sampling site (Fig. 1). *Tamarixia dryi* was the most abundant primary parasitoid in Pretoria and Nelspruit (> 95% of the emerged parasitoids). This parasitoid species was also present in Tzaneen. On the other hand, the primary parasitoids *P. pulvinatus* (79%) and *Tamarixia* sp. (65%) were the most abundant species in Nelspruit and Tzaneen, respectively. The most abundant hyperparasitoid was *Aphidencyrtus* *cassatus* Annecke (Hymenoptera: Encyrtidae), which was recovered in Nelspruit, Tzaneen and Pretoria. One specimen of an *Aphanogmus* sp. (Hymenoptera: Ceraphronidae) was recorded in Tzaneen.

Parasitism rates ranged between 0.72 ± 0.12 in Pretoria and 0.41 ± 0.21 in Nelspruit (Table 1). Parasitoid emergence ranged between 0.57 ± 0.04 and 0.66 ± 0.19. Hyperparasitism rates ranged between 0 and 0.09.

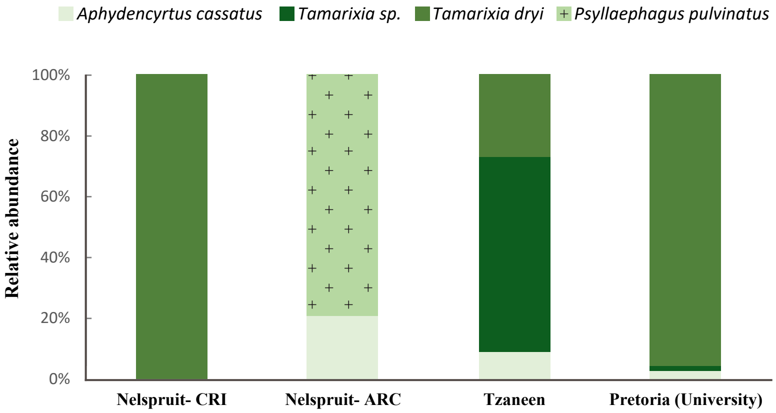


Fig. 1. Relative abundance of *Trioza erytreae* parasitoids collected from individual parasitized nymphs at four sites in South Africa in 2017.

Table 1. Parasitism and hyperparasitism rates of *Trioza erytreae* in four locations from South Africa.

		Parasitism assessment			
Date	Location	No. of nymphs examined	Parasitism rate [Mean ± EE (*n*)]	Parasitoid emergence [Mean ± EE (*n*)]	Hyperparasitism rate [Mean ± EE (*n*)]
9/28/2017	Nelspruit- CRI*	72	0.45 ± 0.19 (5)	0.64 ± 0.09 (5)	0 (5)
09/29/2017	Nelspruit- ARC*	266	0.41 ± 0.21 (4)	0.66 ± 0.19 (4)	0.09 ± 0.09 (4)
10/05/2017	Tzaneen	742	0.42 ± 0.07 (3)	0.57 ± 0.004 (3)	0.08 ± 0.04 (3)
10/05/2017	Pretoria (University)	142	0.72 ± 0.12 (5)	0.65 ± 0.12 (5)	0.02 ± 0.003(5)

*CRI: Citrus Research International (Nelspruit)

*ARC: Agricultural Research Council (Nelspruit)

5.2.3. DNA barcoding of *Tamarixia* and *Trioza* specimens

The sequences were submitted to the GenBank public sequence repository, with the following accession numbers for *T. dryi* (MK293946-MK293954), *Tamarixia* sp. (MK302489-MK302491) and *T. erytreae* (MK285548-MK285560). In a BLAST search, the COI barcode sequence obtained using the specific primers for *T. erytreae* showed 100% homology with the South African accessions KY754590 (TeSA7) and KY754594 (TeSA1) identified by Khamis *et al* (Khamis et al., 2017) as *T. erytreae*, confirming that all specimens collected at Pretoria, Nelspruit and Tzaneen corresponded indeed to these species (see Supplementary Material Fig. S1). The COI barcode fragment sequenced from *T. dryi* and a new species of *Tamarixia* collected at Pretoria, Nelspruit and Tzaneen, shared 86-91% of identity to COI barcode fragment accessions from other *Tamarixia* species available in GenBank [*Tamarixia radiata* (Waterston) (GQ912272), *Tamarixia drukyulensis* (Yefremova and Yegorenkova) (KY986293) and *Tamarixia triozae* (Burks) (GQ912288)] (Fig. 2). The new species of *Tamarixia* collected at Tzaneen showed a higher identity (90%) to *T. dryi* sequences than to *T. radiata*, *T. drukyulensis* and *T. triozae* (87, 88 and 86%, respectively).

The phylogenetic tree of these *Tamarixia* species was paraphyletic with two distinct branches (Fig. 3). The first branch separated into two clusters. The first cluster grouped together the COI barcode sequences obtained in this work from the *T. dryi* specimens, while the second cluster hosted the *T. radiata*, *T. drukyulensis* and *T. triozae* GenBank accessions included in the analysis. The COI barcode sequence from the new *Tamarixia* sp. branched separately from the rest of the *Tamarixia* species included in this analysis.

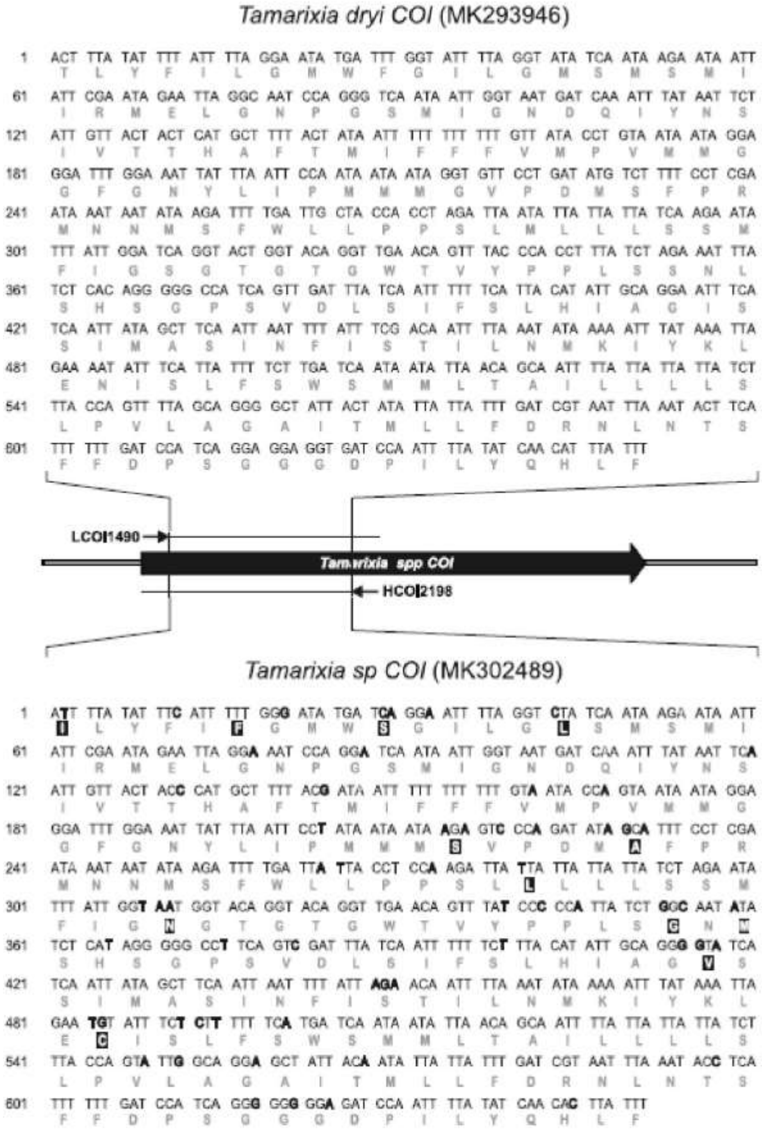


Fig. 2. Nucleotide sequence of COI barcode fragment for *Tamarixia dryi* and *T.* sp generated in the present work. Deduced amino acid (aa) sequence of the corresponding polypeptide is shown under each triplet. Nucleotide changes and non-conserved aa in the sequence of *T.* sp COI fragment are represented in boldface and in black boxes, respectively. The coding region of *Tamarixia* spp COI gene and standard primers position used for the amplification of the barcode fragment –652 bp without including the sequence of the standard primers– are shown for schematic purposes.

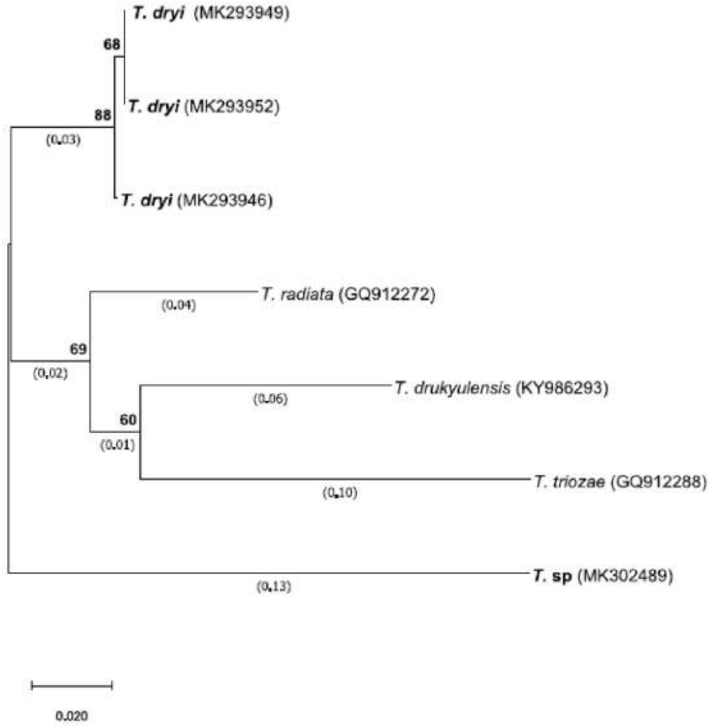


Fig. 3. Rooted phylogenetic analysis showing the evolutionary relationships between *Tamarixia* species, based on DNA sequences of COI barcode fragment. The analysis involved seven nucleotide sequences including those of *Tamarixia dryi* and one *T.* sp. generated in this study, and three closest sequences retrieved from GenBank [*T. radiata* (GQ912272), *T. drukyulensis* (KY986293) and *T. triozae* (GQ812288)].

5.2.4. Seasonal trend of the parasitoid complex of *T. erytreae*

The most abundant parasitoid was *T. dryi,* followed by its hyperparasitoid *A. cassatus*, but their relative abundance showed different trends (Fig. 4). While the relative abundance of *T. dryi* decreased over the five weeks, the number of hyperparasitoids increased during the same period. The primary parasitoid *Tamarixia* sp. was recorded during the first and third sampling periods, and the hyperparasitoid *Marietta javensis* (Howard) (Hymenoptera: Aphelinidae) was found only at the second sampling date.

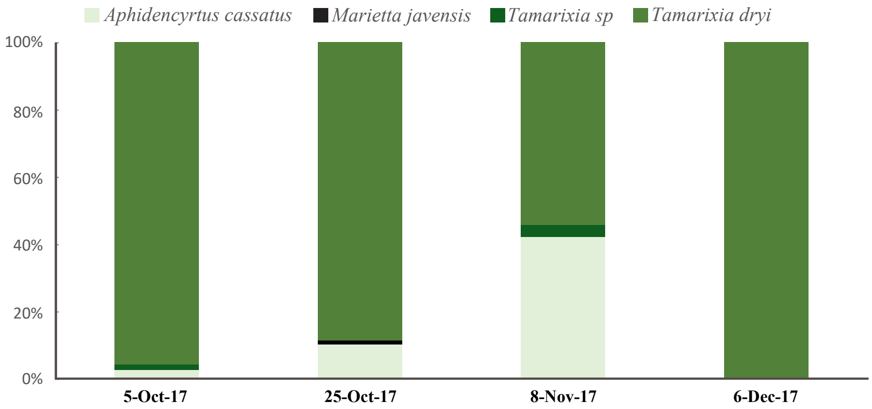


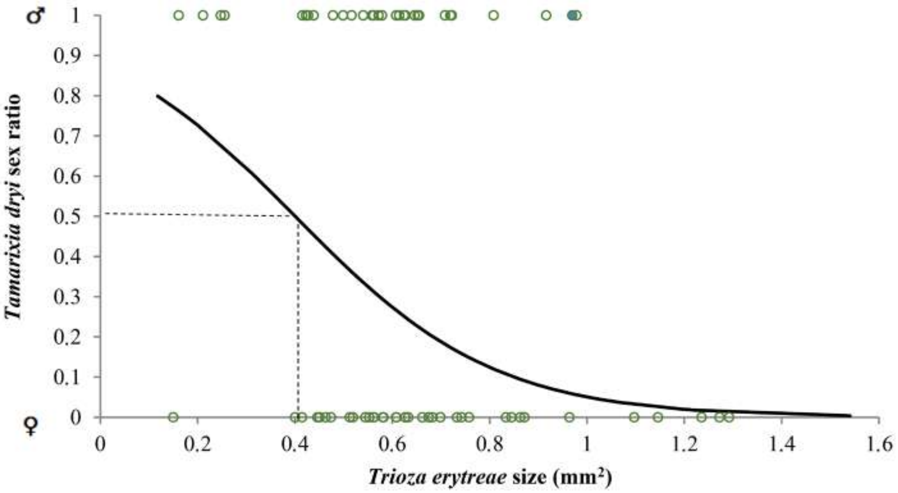
Fig. 4. Relative abundance of *Trioza erytreae* parasitoids in a citrus orchard from the University of Pretoria (Pretoria, South Africa) throughout the spring of 2017.

5.2.5. Effect of host size on the secondary sex ratio of *T. dryi* and *Tamarixia* sp.

The secondary sex ratio of the primary parasitoids *T. dryi* and *Tamarixia* sp. depended on *T. erytreae* size. In both species, females emerged from larger-sized hosts than males (*T. dryi*: $F = -3.34$; df = 1, 86; $P < 0.001$; *Tamarixia* sp.: $F = -3.99$; df = 1, 78; $P < 0.001$) (Figs. 5A and B). Sex ratio in *T. dryi* turned female-biased around 0.40 mm^2 and in *Tamarixia* sp. at around 0.90 mm^2.

5.2.6. Effect of host size on *A. cassatus* emergence

Hyperparasitism also depended on the size of *T. erytreae* individuals. The hyperparasitoid *A. cassatus* tended to emerge from large hosts ($F = 3.144$; df = 1, 80; $P = 0.002$) (Fig. 6). Hyperparasitism rates became higher than 50% when hosts were larger than 1.65 mm^2.



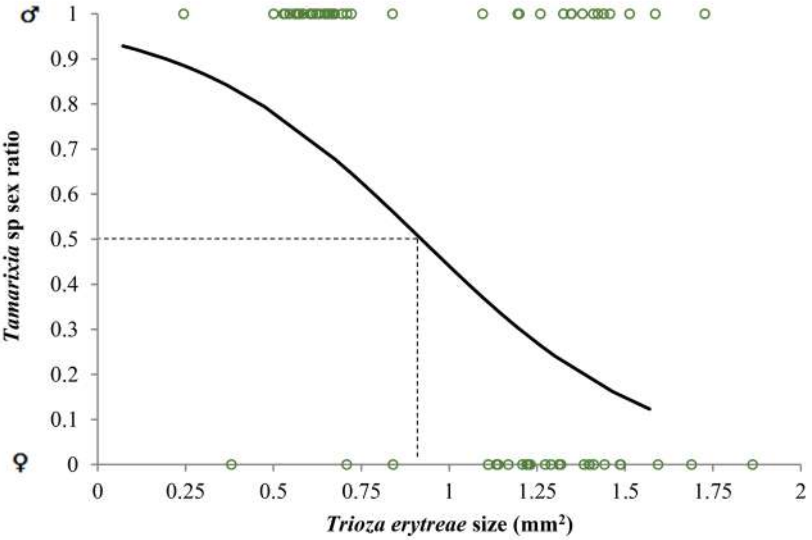


Fig. 5. Effect of *Trioza erytreae* size on the secondary sex ratio of *Tamarixia dryi* (A) and *Tamarixia* sp. (B). Sex ratio turns female-biased around 0.4 mm^2 in *T. dryi* and 0.9 mm^2 in *Tamarixia* sp.

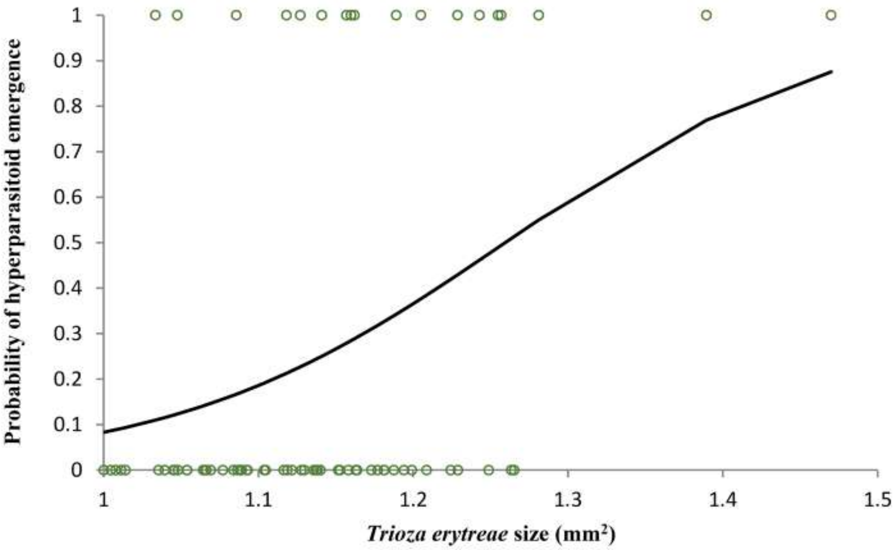


Fig. 6. Effect of *Trioza erytreae* size on the probability that an individual of *Aphidencyrtus cassatus* emerged from the nymph.

5.2.7. Longevity of *T. dryi* and its hyperparasitoid *A. cassatus*

Tamarixia dryi survivorship differed between sexes ($\chi^2_1 = 4.29$; $P = 0.038$) (Fig. 7). Females lived 19.6 ± 1.86 days on average and males 14.75 ± 1.47 days. *Aphidencyrtus cassatus* survivorship was also higher in females than in males (25.5 ± 1.14 and 17.71 ± 2.89 days, respectively) ($\chi^2_1 = 5.16$; $P = 0.023$). When females of both species were compared, no significant differences were found with respect to their longevity ($\chi^2_1 = 4.10$; $P = 0.21$).

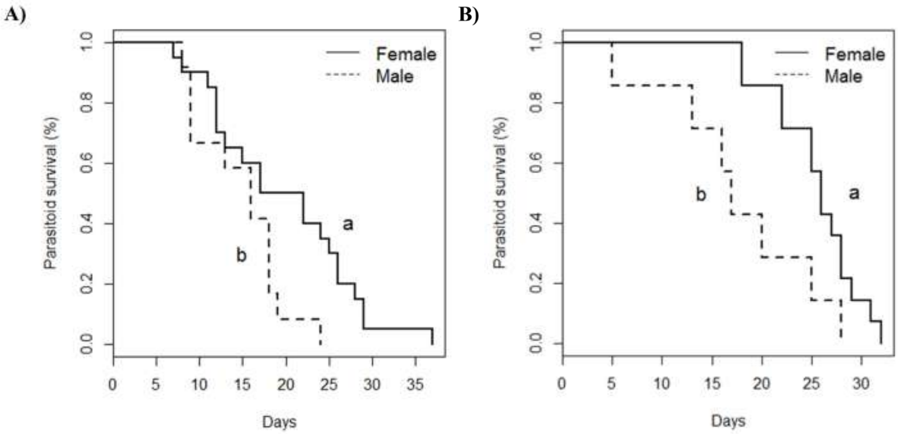


Fig. 7. Cumulative survival function of *Tamarixia dryi* (A) and its hyperparasitoid *Aphidencyrtus cassatus* (B) for both sexes.

5.3. Discussion

Trioza erytreae was highly parasitized by several species of hymenopteran parasitoids. Parasitism rates were high in all the sampled areas in spring and ranged between 0.40 and 0.70. These rates are similar to those reported by van der Merwe (1923) and Catling (1969b) in South Africa and Swaziland, respectively, and Tamesse et al. (2002) in Cameroon. Therefore, as demonstrated by Catling in the 60s, parasitoids are important biotic regulators of *T. erytreae* in those areas where insecticides are not sprayed in South Africa, e.g. abandoned and experimental orchards and public gardens (Catling, 1969a, 1970). This result reinforces the suggestion of introducing exotic parasitoids in those areas where *T. erytreae* has arrived and where effective native parasitoids are absent. This is the case of Maderia (Portugal), the Canary Islands (Spain) and, more recently, mainland Europe (Cocuzza et al., 2017).

Among the three species of primary parasitoids of *T. erytreae*, *T. dryi* was the most effective and abundant, as has been previously demonstrated by other studies in South Africa and Swaziland (Catling, 1969b; Mc Daniel and Moran, 1972). Its relative abundance was higher than 90% in two sites. Similar values were obtained by Catling (Catling, 1969b) in South Africa. Another primary parasitoid of *T. erytreae*, *P. pulvinatus,* was only present in Nelspruit-ARC, where it was the most abundant species (80%). The high abundance of *P. pulvinatus* at this site may be due to the absence of *T. dryi.* Although *P. pulvinatus* parasitizes younger nymphs than *T. dryi*, its developmental time is longer than that of *T. dryi* (Mc Daniel and Moran, 1972). This reason might partially explain why *T. dryi* tends to be more abundant where *T. erytreae* is present at low population densities.

A new parasitoid species from the genus *Tamarixia* was recorded in Tzaneen and Pretoria. It was the most abundant species in Tzaneen (70%), coexisting with *T. dryi* and the hyperparasitoid *A. cassatus*. In Pretoria, it was recorded sporadically. This new species could be the same species named as "*Tetrastichus* sp. n." in Western and Eastern Africa and classified as a primary parasitoid (Aubert, 1986). In other studies, in Cameroon, Zimbabwe and Malawi, an unknown "*Tetrastichus* sp." was also found, but it was recorded as a hyperparasitoid (Mc Daniel and Moran, 1972; Aubert and Quilici, 1988; Tamesse et al., 2002). The high abundance of *Tamarixia* sp. and the fact that we never observed the pupae or larvae of any *Tamarixia* species attacked by other larvae suggest that it is a primary parasitoid. The molecular analysis confirmed that this new species has not been reported yet in the database Genbank and it is closely related to *T. dryi.*

The sex ratio of *T. dryi* and *Tamarixia* sp. turned female biased when the size of *T. erytreae* was greater than 0.40 and 0.90 mm^2, respectively. Many species of solitary parasitoids lay male eggs on small hosts and female eggs on large hosts (Charnov et al., 1981; Beltrà et al., 2014). This host-size-dependent sex ratio is presumed to be advantageous because females gain a greater benefit from the resulting increase in body size than do males (Charnov et al., 1981; Godfray, 1994). According to Waage (1982), this occurs mostly in idiobionts, which paralyze the host, because in koinobiont parasitoids, hosts continue growing and its size at oviposition is a less reliable predictor of the resources that offspring will have available for development (Askew and Shaw, 1986; West and Sheldon, 2002). In our case, although *T. dryi* is a koinobiont, host size increases only slightly after parasitism (personal observations). Therefore, host size could be a reliable predictor of the resources when *T. dryi* recognizes and assesses the size of *T. erytreae* individuals. These results are important to mass-rear the parasitoids and maximize the production of females (Bernal et al., 1999).

Hyperparasitism rates were low and ranged between zero and 0.1 at the four sites. In Pretoria, where the seasonal trend of the parasitoids was determined in spring, hyperparasitism also reached a maximum of 0.1. These values were higher than the ones obtained by Catling and Anneke in the Letaba District [Limpopo (then Transvaal province) South Africa] from 1965 to 1967, although hyperparasitism was thought to be of little apparent significance, later on Mc Daniel and Moran (Mc Daniel and Moran, 1972) and Anneke and Moran (Annecke and Moran, 1982) determined that it was the main factor in the increase of *T. erytreae* population levels in citrus. Among the hyperparasitoids, *A. cassatus* was the most abundant and widely distributed. *Aphidencyrtus* *cassatus* is considered the most abundant hyperparasitoid of *T. erytreae* and it attacks, at least, the two primary parasitoids *T. dryi* and *P. pulvinatus* (Catling, 1969b; Aubert, 1987; Tamesse et al., 2002). Moreover, *A. cassatus* has been observed host feeding in the thoracic region of the primary parasitoid host, which sometimes killed the host (Mc Daniel and Moran, 1972). This mortality and the high temperatures reached at the end of spring could have caused the high mortality rates of the primary parasitoids observed in Pretoria.

Two traits of the biology of *A. cassatus* could explain the negative impact that this hyperparasitoid has on *T. dryi*. First, the probability that the hyperparasitoid *A. cassatus* emerged from *T. erytreae* nymphs increased with the size of the nymph. Since *T. dryi* females develop in larger nymphs than males, *A. cassatus* may affect the secondary sex ratio of *T. dryi* and more males will emerge. Second, females of the hyperparasitoid *A. cassatus* lived for more than 30 days when they had access to carbohydrates, and their longevity was similar to that of *T. dryi*. Therefore, the hyperparasitoid is capable of surviving long periods of host scarcity or, at least, as long as the primary parasitoid. Although hyperparasitoids can regulate herbivore populations by stabilising host-parasitoid interactions (Hassell and Waage, 1984; Briggs, 1993; Sullivan and Völkl, 1999), from the point of view of classical biological control, the accidental introduction of *A. cassatus* could impair the establishment of *T. dryi* (Tougeron and Tena, 2018). Therefore, great care should be taken to exclude this parasitoid when importing *T. dryi* to Europe, for example introducing only adult parasitoids, establishing isolines and following quarantine procedures before the release (van Lenteren et al., 2003; Heimpel and Mills, 2010).

5.4. Materials and methods

5.4.1. Insect survey

Sampling took place in citrus producing areas in four provinces, Gauteng, Limpopo, Mpumalanga and Western Cape, of South Africa. A total of 65 citrus orchards, 5 public parks and 60 private properties were examined from September 21st to December 9th of 2017. At all sites, the sampling date, the number of trees sampled, the variety of the trees and the presence of *T. erytreae* or its visual symptoms and parasitoids were recorded (see supplementary material Table S1). Visual symptoms of *T. erytreae* on citrus, in contrast to those of *Diaphorina citri*, consist of pit galls protruding from the upper face of the leaves, chlorosis and leaves twisting (Catling, 1973; Cocuzza et al., 2017).

5.4.2. Parasitoid identification, relative abundance and parasitism rates

From those areas and trees where *T. erytreae* was collected, the psyllids were transported to the laboratory to identify potential parasitoids, determine their relative abundance and parasitism rates in each location. From each tree, 3 to 20 leaves infested by *T. erytreae* were collected and transported in enclosed individualised plastic bags to the laboratory. Due to the scarcity of *T. erytreae* in some of the orchards, the number of sampled trees and leaves was variable (see supplementary material Table S1). Once in the laboratory, the number of live psyllids and psyllids suitable for parasitism (2nd to the 5th instar nymph) and parasitized psyllids was recorded using a stereomicroscope. In order to identify all the parasitoids and calculate the rate of emerging parasitoids, psyllid nymphs were placed individually in 1ml microtubes closed with cotton wool. Afterwards, the microtubes tubes were kept in an incubator (Labcon™ 2 LTGC 20, Laboratory Marketing Services cc, South Africa) under controlled conditions (12L: 12 Dh, 25 ºC, 60-70% RH) and checked daily until parasitoids emerged. Once emerged, their sex was determined and the identification at species level was carried out using the key of Tamesse (Tamesse, 2009). The morphological identifications of *T. erytreae* and *Tamarixia* parasitoids were confirmed by David Ouvrard (Natural History Museum, London) and Roger Burks (University of California, Riverside), respectively. Moreover, vouchers of all the parasitoid species collected during this study were deposited at the University of California, Riverside and labelled with the database number UCRC_ENT from 00517324 to 00517336.

In order to calculate the parasitism rate, each tree was used as a sampling unit because *T. erytreae*, as other psyllids, has an aggregative distribution pattern (Samways and Manicom, 1983; Chi-kun and Fasheng, 1984; Butler and Trumble, 2012).

5.4.3. DNA extraction, PCR and sequencing of barcode fragment

The morphological identifications were verified with molecular identifications using cytochrome c oxidase subunit 1 (COI) sequences of *T. erytreae* and of the parasitoids *T. dryi* and *Tamarixia* sp. The insects were collected at three different locations, Nelspruit, Pretoria, and Tzaneen, in South Africa. DNA was extracted from individual insects using a salting out method (Sunnucks and Hales, 1996) adapted from Monzó et al. (2011). Polymerase chain reaction (PCR) was performed to amplify the mitochondrial COI gene.

Standard primers LCO1490 (5’-GGTCAACAAATCATAAAGATATTGG-3’) and HCO2198 (5’-TAAACTTCA GGGTGACCAAAAAATCA-3’) (Vrijenhoek, 1994) were used for the amplification of a 708-bp fragment of the COI gene of *Tamarixia* species, while Te-6U30 (5’-ATTTTTAAGCACTAATCATAAAATTATTGG-3’) and Te-720L26 (5’-TATACTTCAGGATGTCCAAAAAATCA-3’) specific primers were used to amplify a COI barcode fragment of a 714-bp fragment of the COI of *T. erytreae*. PCR was carried out in a total reaction volume of 20 µl containing 1X Reaction Buffer (Mg free), 1625 µM MgCl2, 250 µM of all four dNTPs, 0.25 µM of each primer, 1 U of DNA polymerase (1 U/µl, Biotools), and 1 µl of DNA in a thermal cycler (Eppendorf Mastercycler). Reactions were cycled as follow: initial denaturation at 95°C for 120 seconds (s); 40 cycles of 94°C for 60 s, 45°C for *Tamarixia* or 54ºC for *T. erytreae* for 60 s and 72°C for 90 s; and a final extension at 72°C for 600 s. Amplified PCR products were resolved in a 1.2% agarose gel and successfully amplified barcode fragments were cleaned up using the UltraClean PCR Clean-up DNA Purification Kit (MO BIO Laboratories Inc., Carlsbad - California, USA). Amplified barcode fragments were bidirectional sequenced using both the forward and reverse PCR primers. Sanger-sequencing was performed by capillary electrophoresis using a 3130XL Genetic Analyser (Applied Biosystems, Carlsbad - California, USA), at the Sequencing Service of the IBMCP (Valencia, Spain). Sequences were analysed and trimmed to remove primer sequences, using Sequencer DNA Sequence Analysis Software (Gene Codes Corporation, Ann Arbor – Michigan, USA). Forward and reverse high-quality reads obtained for each individual were assembled into consensus sequences and submitted to the GeneBank public sequence repository.

5.4.4. Sequence data and phylogenetic analysis

The phylogenetic analysis of the two species of *Tamarixia* was carried out using the COI barcode sequences obtained in this work together with those already included in the GenBank: *T. radiata* (Waterston), *T. drukyulensis* Yefremova and Yegorenkov and *T. trioza* (Burks). Consensus sequences corresponding to the amplified COI barcode fragment of the *Tamarixia* parasitoids were first used as query to BLASTN against the non-redundant nucleotide collection of the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), optimising for highly similar sequences. The barcode based taxonomic assignment at the genus level was set at 94% identity over a 90% sequence overlap. Multiple alignments of consensus sequences and closest sequence identities were done using Clustal Omega software (Sievers et al., 2011). The phylogenetic analysis was carried out in MEGA X (Kumar et al., 2018), using the Neighbor-Joining method (Saitou and Nei, 1987). The reliability of the tree pattern was evaluated using a bootstrap test with 10000 replicates (Felsenstein, 1985), and the evolution distances, given as units of the number of base substitution per sites, were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). The rate variation among sites was modelled with a gamma distribution (shape parameter = 1).

5.4.5. Seasonal trend of the parasitoid complex of *T. erytreae*

The seasonal trend of *T. erytreae* and its parasitoids was studied in an infested lemon orchard located at the University of Pretoria Experimental Farm (25°44’51.1”S 28°15’31.2”E). The orchard was ~10 years old, and the trees sampled were not treated with pesticides during the sampling period.

From October to December, five infested leaves from five trees were sampled every two weeks. Samples were processed following the same methodology described above until parasitoids emerged and were identified.

5.4.6. Effect of host size on secondary sex ratio and hyperparasitism of parasitoids

The effects of host size on the secondary sex ratio of *T. dryi* and *Tamarixia* sp. and on hyperparasitism were analysed. After parasitoids emerged they were identified and their sex was determined. The psyllid nymphs have an oval shape and host size was determined by calculating the area of an ellipse by multiplying r_1 x r_2 x π (r_1: major radius, r_2: minor radius). Both sex and hyperparasitism ratios were analysed using generalized linear models assuming binomial errors. The assumed error structure was assessed by a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If an over or an under dispersion was detected, we re-evaluated the significance of the explanatory variables using an *F*-test after rescaling the statistical model by a Pearson's χ^2 divided by the residual degrees of freedom. All data analyses were performed with the R freeware statistical package (Version 1.0.143) (Team, 2015).

5.4.7. Longevity of *Tamarixia dryi* and its hyperparasitoid *Aphidencyrtus cassatus*

Tamarixia dryi and *A. cassatus* longevity was recorded. From the individual nymph sampled at the University of Pretoria Experimental Farm, a total of 20 females and 12 males of *T. dryi* as well as 14 females and 7 males of *A. cassatus* were selected. Individual parasitoids were placed singly 1ml microtubes tubes closed with a cotton wool and 1M sucrose drop renewed every two days. The microtubes were kept under controlled conditions in an incubator (Labcon 2 LTGC 20, Laboratory Marketing Services CC, Roodepoort, South Africa) at 12L: 12D, 25 ± 2 ºC and 60-70% RH, and the survival of the insects was checked daily. The Cox regression model was used to determine differences between sexes within the same species and between species. Analyses were carried out using the R freeware statistical package (Version 1.0.143) (Team, 2015).

5.5. Acknowledgements

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5.6. Author contributions

Field and laboratory assays were designed and performed by JP, KK and AT. Data were subsequently analysed by JP and AT. Molecular analyses were performed by OR and MP. All authors wrote, read and approved the manuscript.

5.7. Additional Information

Conflict of interest: The authors declare that they have no conflicts of interest. Ethical approval: This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

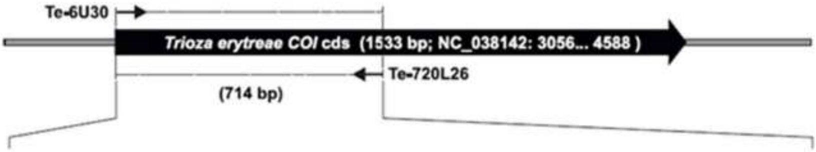
5.8. Supplementary Information

Supplementary Table S1. Sampling dates and sites, number and variety of sampled trees per site and symptoms and presence of *T. erytreae*. Asterisks are used when *T. erytreae* was parasitized.

Sampling date	Locality	Province	Coordinates	Type of orchard	Rutacea Host	No. trees sampled	*Trioza* symptons	Presence of *Trioza*
21/09/2017	Citrusdal	Western Cape	32°36'27.3"S 18°56'37.8"E	Commercial	*Citrus sinensis*	50	No	No
21/09/2017	Citrusdal	Western Cape	32°36'31.6"S 18°56'39.9"E	Commercial	*Citrus reticulata*	50	No	No
21/09/2017	Citrusdal	Western Cape	32°36'23.3"S 18°56'51.7"E	Commercial	*Citrus sinensis*	50	No	No
21/09/2017	Citrusdal	Western Cape	32°36'37.2"S 18°56'32.1"E	Commercial	*Citrus limon*	50	No	No
21/09/2017	Citrusdal	Western Cape	32°35'43.2"S 19°00'50.2"E	Private garden	*Citrus sinensis*	2	No	No
21/09/2017	Citrusdal	Western Cape	32°51'18.0"S 19°05'55.8"E	Commercial	*Citrus sinensis*	50	No	No
21/09/2017	Citrusdal	Western Cape	32°51'19.0"S 19°05'40.4"E	Commercial	*Citrus sinensis*	50	No	No
21/09/2017	Citrusdal	Western Cape	32°36'37.3"S 19°00'01.2"E	Organic commercial	*Citrus limon*	50	No	No
21/09/2017	Citrusdal	Western Cape	32°36'27.2"S 18°59'55.5"E	Organic commercial	*Citrus limon*	50	No	No
21/09/2017	Citrusdal	Western Cape	32°36'42.4"S 19°00'05.5"E	Organic commercial	*Citrus reticulata*	50	No	No
22/09/2017	Citrusdal	Western Cape	32°37'03.5"S 18°57'20.6"E	Organic commercial	*Citrus reticulata*	10	No	No
22/09/2017	Citrusdal	Western Cape	32°37'03.5"S 18°57'20.6"E	Private garden	*Citrus sinensis*	4	No	No
22/09/2017	Citrusdal	Western Cape	32°37'03.5"S 18°57'20.6"E	Private garden	*Citrus limon*	2	No	No
22/09/2017	Citrusdal	Western Cape	32°21'23.4"S 18°55'52.5"E	Organic commercial	*Citrus sinensis*	50	No	No
22/09/2017	Citrusdal	Western Cape	32°21'28.7"S 18°55'56.2"E	Organic commercial	*Citrus sinensis*	50	No	No
22/09/2017	Stellenbosch	Western Cape	33°49'21.8"S 18°55'48.1"E	Private garden	*Citrus sinensis*	100	No	No
22/09/2017	Stellenbosch	Western Cape	33°56'11.0"S 18°51'56.3"E	Private garden	*Citrus sinensis*	5	No	No
26/09/2017	Nelspruit	Mpumalanga	25°27'09.0"S 30°58'06.4"E	Organic commercial	*Citrus sinensis*	50	No	No
26/09/2017	Nelspruit	Mpumalanga	25°27'07.9"S 30°58'16.1"E	Organic commercial	*Citrus limon*	50	No	No
27/09/2017	Nelspruit	Mpumalanga	25°23'04.3"S 30°32'33.8"E	Nursery	*Citrus sinensis*	20	Yes	No
27/09/2017	Nelspruit	Mpumalanga	25°23'06.7"S 30°32'34.1"E	Commercial	*Citrus sinensis*	20	No	No
27/09/2017	Nelspruit	Mpumalanga	25°22'44.2"S 30°31'50.7"E	Organic commercial	*Citrus limon*	50	No	No

27/09/2017	Nelspruit	Mpumalanga	25°22'44.2"S 30°31'50.7"E	Abandoned orchard	*Citrus reticulata*	10	No	No
27/09/2017	Nelspruit	Mpumalanga	25°22'35.5"S 30°32'30.8"E	Private garden	*Citrus limon*	1	No	No
27/09/2017	Nelspruit	Mpumalanga	25°22'49.1"S 30°34'03.0"E	Private garden	*Citrus limon*	1	No	No
27/09/2017	Nelspruit	Mpumalanga	25°22'35.7"S 30°32'33.0"E	Commercial	*Citrus limon*	1	No	No
27/09/2017	Nelspruit	Mpumalanga	25°26'42.8"S 30°57'57.4"E	Public garden	*Citrus aurantium*	0	No	No
28/09/2017	Nelspruit	Mpumalanga	25°27'31.5"S 31°02'46.5"E	Commercial	*Citrus sinensis*	0	Yes	No
28/09/2017	Nelspruit	Mpumalanga	25°27'12.2"S 31°01'58.2"E	Abandoned orchard	*Citrus sinensis*	20	No	No
28/09/2017	Nelspruit	Mpumalanga	25°27'30.2"S 31°02'35.0"E	Abandoned orchard	*Citrus limon*	20	No	No
28/09/2017	Nelspruit	Mpumalanga	25°27'58.2"S 31°02'32.4"E	Commercial	*Citrus limon*	50	No	No
28/09/2017	Nelspruit	Mpumalanga	25°28'48.4"S 30°59'38.2"E	Experimental farm	*Citrus limon*	30	Yes	Yes*
29/09/2017	Nelspruit	Mpumalanga	25°27'07.7"S 30°58'09.5"E	Nursery	*Citrus sinensis*	50	No	No
29/09/2017	Nelspruit	Mpumalanga	25°27'07.4"S 30°58'15.9"E	Assay	*Citrus limon*	50	Yes	Yes*
29/09/2017	Nelspruit	Mpumalanga	25°28'21.9"S 30°59'29.8"E	Public garden	*Citrus aurantium*	10	Yes	No
29/09/2017	Nelspruit	Mpumalanga	25°28'45.8"S 30°59'38.0"E	colony	*Citrus sinensis*	20	No	No
03/10/2017	Letsitele	Limpopo	23°52'01.0"S 30°23'20.0"E	Privat garden	*Murraya exotica*	1	No	No
03/10/2017	Letsitele	Limpopo	23°52'07.0"S 30°23'29.6"E	Privat garden	*Murraya exotica*	3	No	No
03/10/2017	Letsitele	Limpopo	23°51'44.7"S 30°23'01.9"E	Commercial	*Citrus sinensis*	50	No	No
03/10/2017	Tzaneen	Limpopo	23°55'11.8"S 30°13'58.4"E	Private garden	*Citrus limon*	3	No	No
03/10/2017	Tzaneen	Limpopo	23°50'24.0"S 30°18'07.2"E	Commercial	*Citrus sinensis*	150	No	No
04/10/2017	Tzaneen	Limpopo	23°55'11.8"S 30°13'58.4"E	Organic commercial	*Citrus sinensis*	150	Yes	No
04/10/2017	Tzaneen	Limpopo	23°54'42.1"S 30°13'28.0"E	Organic commercial	*Citrus sinensis*	150	Yes	No
04/10/2017	Tzaneen	Limpopo	23°53'14.7"S 30°19'44.8"E	Organic commercial	*Citrus sinensis*	150	Yes	No
04/10/2017	Tzaneen	Limpopo	23°55'06.3"S 30°13'59.5"E	Organic commercial	*Citrus sinensis*	150	Yes	No
04/10/2017	Tzaneen	Limpopo	23°55'07.9"S 30°13'55.4"E	Organic commercial	*Citrus sinensis*	150	Yes	No
04/10/2017	Nkowankowa-C	Limpopo	23°53'24.4"S 30°19'40.7"E	Private garden	*Citrus limon*	2	No	No
04/10/2017	Nkowankowa-C	Limpopo	23°53'27.9"S 30°19'39.6"E	Private garden	*Citrus limon*	3	Yes	No

04/10/2017	Nkowankowa-C	Limpopo	23°53'25.6"S 30°19'42.1"E	Private garden	*Citrus limon*	1	No	No
04/10/2017	Nkowankowa-C	Limpopo	23°53'19.3"S 30°19'43.8"E	Private garden	*Citrus limon*	2	No	No
04/10/2017	Nkowankowa-C	Limpopo	23°53'31.0"S 30°19'45.3"E	Private garden	*Citrus limon*	1	Yes	No
05/10/2017	Tzaneen	Limpopo	23°50'13.7"S 30°09'37.8"E	Private garden	*Citrus limon/reticulata*	5	Yes	Yes*
05/10/2017	Tzaneen	Limpopo	23°47'56.8"S 30°26'11.0"E	Private garden	*Citrus limon*	5	No	No
05/10/2017	Tzaneen	Limpopo	23°47'54.1"S 30°26'07.9"E	Private garden	*Citrus limon*	50	No	No
05/10/2017	Pretoria	Gauteng	25°44'52.8"S 28°15'32.1"E	Experimental farm	*Citrus limon*	10	No	No
05/10/2017	Pretoria	Gauteng	25°44'52.1"S 28°15'33.6"E	Experimental farm	*Citrus limon*	25	Yes	Yes*
09/10/2017	Pretoria	Gauteng	25°45'40.6"S 28°14'12.5"E	Private garden	*Citrus sinensis*	4	No	No
09/10/2017	Pretoria	Gauteng	25°45'40.6"S 28°14'12.5"E	Private garden	*Citrus limon*	3	Yes	Yes
09/10/2017	Pretoria	Gauteng	25°44'31.3"S 28°18'45.7"E	Private garden	*Murraya exotica*	1	Yes	No
09/10/2017	Pretoria	Gauteng	25°44'21.4"S 28°16'24.7"E	Public garden	*Citrus aurantium*	10	No	No
10/10/2017	Pretoria	Gauteng	25°44'30.2"S 28°18'48.6"E	Private garden	*Citrus limon*	5	Yes	No
20/10/2017	Pretoria	Gauteng	25°45'30.5"S 28°14'31.4"E	Public garden	*Citrus sinensis*	3	No	No
01/11/2017	Pretoria	Gauteng	25°44'31.3"S 28°18'45.7"E	Private garden	*Citrus limon*	1	Yes	No
17/11/2017	Pretoria	Gauteng	25°49'55.6"S 27°53'20.3"E	Public garden	*Citrus sinensis*	5	No	No
09/12/2017	Pretoria	Gauteng	25°50'50.0"S 28°22'14.5"E	Private garden	*Citrus limon*	6	Yes	Yes*



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      F   L   S   T   N   H   K   I   I   G   T   L   Y   F   F   L   G   I   W   S
61   GGA CTT TTA GGT TTA TCT TTA AGA ATA ATT ATC CGA TTA GAA TTA AGA CAA TCG TCC TCT
      G   L   L   G   L   S   L   S   M   I   I   R   L   E   L   S   Q   S   S   S
121  TTA TTA ATG AAT GAT CAA ATT TAC AAT ACA ATT GTT ACT GCT CAT GCT TTT ATT ATA ATT
      L   L   M   N   D   Q   I   Y   N   T   I   V   T   A   H   A   F   I   M   I
181  TTT TTT ATA ACA ATA CCT ATT ATT ATT GGA GGA TTT GGA AAT TGA TTA ATT CCT TTA ATA
      F   F   M   T   M   P   I   I   I   G   G   F   G   N   W   L   I   P   L   M
241  ATT GGT GCT CCA GAT ATA GCA TTT CCT CGA CTT AAT AAT TTA AGT TTT TGA TTA TTA ATT
      I   G   A   P   D   M   A   F   P   R   L   N   N   L   S   F   W   L   L   I
301  CCA TCT TTA TAT TTA TTA ATT ATA AGT AGT TTA TTA GAT ATA GGA GTT GGG ACA GGA TGA
      P   S   L   Y   L   L   I   M   S   S   L   L   D   M   G   V   G   T   G   W
361  ACA GTT TAC CCT CCT TTA TCA AAT GCT ATA TTC CAT AGA GGA TAT TCA GTA GAT ACT GCT
      T   V   Y   P   P   L   S   N   A   M   F   H   S   G   Y   S   V   D   T   A
421  ATT TTT TCT TTA CAT TTA GCA GGA ATT TCA TCT ATC TTA GGA GCA ATT AAT TTC ATT ACA
      I   F   S   L   H   L   A   G   I   S   S   I   L   G   A   I   N   F   I   T
481  ACA ATT CTT AAT ATA CGA AGA TGT ATA TGT AAT TTA GAA AAA ATA CCT TTA TTT GTG TGA
      T   I   L   N   M   R   S   C   M   C   N   L   E   K   M   P   L   F   V   W
541  TCA GTA TTA ATT ACT GCT TTT TTA TTA TTA TTG GCA TTA CCT GTT TTA GCA GGA GCA ATT
      S   V   L   I   T   A   F   L   L   L   L   A   L   P   V   L   A   G   A   I
601  ACT ATA TTA TTA ACA GAT CGA AAT ATA AAT ACT TCT TTT TTT GAT CCA GCC GGG GGA GGA
      T   M   L   L   T   D   R   N   M   N   T   S   F   F   D   P   A   G   G   G
661  GAT CCT ATT TTA TAT CAA CAT TTA TTT TGA TTT TTT GGA CAT CCT GAA GTA
      D   P   I   L   Y   Q   H   L   F   W   F   F   G   H   P   E   V
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Supplementary Figure S1. Nucleotide sequence of COI barcode fragment for *Trioza erytreae* generated in the present work. Deduced amino acid (aa) sequence of the corresponding polypeptide is shown under each triplet. The coding region (cds) of *T. erytreae* COI gene and specific primers position used for the amplification of the barcode fragment –714 bp including the sequence primers– are shown for schematic purposes. Coordinates of COI cds are given with respect to the mitochondrial nucleotide sequence of *T. erytreae* (GeneBank accession number: NC_038142).

Chapter 6

General Discussion

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General Discussion

Currently integrated pest management in Mediterranean citrus relies on conservation biological control with rational use of insecticides (Zappalà, 2010; Magrama, 2014; Mazih, 2015). However, when a new invasive pest is established, the use of broad spectrum insecticides increases and biocontrol IPM strategies can become disrupted. Insecticide use undoubtedly exerts detrimental effects on native beneficial insects thus allowing resurgence of citrus pests that had already been sustainably managed. At present, *T. erytreae* and *D. aberiae* are two of the most threatening invasive pests for Mediterranean citriculture.

Until *D. aberiae* was first detected in Valencia, the mealybug was considered to be a polyphagous secondary pest in its native South Africa, where it went unnoticed for years (Hattingh et al., 1998; Miller and Giliomee, 2011; Steffen et al., 2015). Even though *T. erytreae* has been considered to be a primary pest and HLB vector for many years, no studies about this psyllid have been conducted since the 70s (Catling, 1969; Cocuzza et al., 2017). Thus far, the control of both emerging pests, especially *D. aberiae*, has been based on the use of broad spectrum insecticides, apparently, without even considering the implementation of an IPM program. Throughout this thesis, three approaches have been developed to improve the management of *D. aberiae* and *T. erytreae*: the development of a reliable sampling procedure to precisely define the EEIL for *D. aberiae,* the conservation biological control of *D. aberiae* by assessing the role of indigenous natural enemies, and the classical biological control of *T. erytreae*.

Contrary to other Mediterranean citrus mealybug species, such as *P. citri* or *P. longispinus, D. aberiae* produces serious direct fruit damage, reaching values of more than 80% of fruit. In light of this potential economic loss, the urgent response of the farmers was the use of insecticides with neither threshold nor timing criterion, which has hindered the maintenance of prior IPM in citrus. Within this framework, one of the first steps for the implementation of an IPM program that includes *D aberiae,* is the development of an accurate sampling protocol and the establishment of an environmental and economic injury level (Pedigo, 1986; Groffman et al., 2006; Pedigo and Rice, 2009). Their implementation can lower the overuse of insecticides and enhance natural enemy conservation (Landis et al., 2002 Ehler, 2006). The first step to achieve this goal was to calculate the aggregation pattern of the pest. Our results showed a clumped pattern of *D. aberiae* in all organs with no significant differences between generations on fruit. These results are in accordance with other mealybugs such as *P. maritimus* in vines (Geiger and Daane, 2001), *P. peruvianus* (Beltrà et al., 2013b) in ornamental plants and *R. invadens* in sugarcane (Boavida et al., 1992). In addition, our data showed that fruit damage at harvest was strongly correlated with fruit occupation in spring. Based on these results, 7.1% and 12.58% fruit occupation correspond to the EIL and EEIL, respectively. Since EEIL takes into consideration side effects of insecticide spraying, we recommend this threshold and the sampling of 275 fruits per orchard (~1 hectare) randomly by using a binomial procedure. Contrary to the enumerative sampling protocol, a binomial procedure is easier, less time consuming, and excludes the removal of 275 fruits from the trees to count the mealybugs. The monitoring process should be carried out fortnightly after petal fall because *D. aberiae* damaging period takes place between April and July and insecticidal treatments during the flowering season are forbidden (Martínez-Blay et al., 2018a). Even though this sampling protocol is accurate for the spring season, further research should be conducted to reduce mealybug populations in summer or autumn taking into account late citrus cultivars such as Valencia oranges. These orange varieties still have mature fruit after petal fall and, therefore, should not be sprayed with insecticides which add residues that could remain on the fruit for several weeks. In collaboration with the Universidad Politécnica de València (UPV), we have demonstrated the density of *D. aberiae* in the canopy to be related to its density in corrugated cardboard bands on trunks sampled during spring. Thus, this sampling procedure can be a suitable methodology to determine *D. aberiae* populations (Martínez-Blay et al., 2018b). This sampling approach is already used to estimate other mealybug population levels as well as the role of predators (Browning, 1959; Furness, 1976; Goolsby et al., 2002). In this context, further research might be needed in order to determine whether this could be a reliable sampling protocol for the application of the EEIL. Another technique commonly used for mealybug surveys that might contribute to the sampling protocol of *D. aberiae* is the use of pheromone traps (Serrano et al., 2001; Millar et al., 2002; Zada et al., 2004) in light of the recent identification of the *D. aberiae* sexual pheromone (Vacas et al., 2019).

The value obtained for the EIL was similar to that of *P. kellyanus*, another invasive pest that causes direct fruit damage after petal fall (Navarro-Campos et al., 2012). In addition, the sampling period for *D. aberiae* fruit occupation may overlap with other citrus pests such as *Aphis spiraecola* Patch (Hemiptera: Aphididae), *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) or *P. kellyanus* (Hermoso de Mendoza etal., 2006; Vassiliou, 2008; Navarro-Campos et al., 2012). Therefore, chemical control could be reduced to one spring insecticide treatment if they overlap.

Contrary to other citrus pseudococcids, *D. aberiae* does not remain in the canopy throughout all its generations. In collaboration with UPV, we have shown that *D. aberiae* displays seasonal movements between the tree canopy, trunk and soil stratum (Martínez-Blay et al., 2018b). During spring, adult females settled in the canopy thereafter they descend down the trunk to lay their ovisacs in the soil. Once first instar nymphs emerge they ascend to the tree canopy (Martínez-Blay et al., 2018a). In this context, although the soil has habitually been neglected in the IPM of other citrus Mediterranean mealybug species, soil-dwelling natural enemies could help manage *D. aberiae*. Soil stratum contains a rich complex of arthropods, especially soil mite predators (Grout and Ueckermann, 1999; Jamieson and Stevens, 2006; Navarro-Campos et al., 2012). Among them, *Gaeolaelaps* (*Hypoaspis*) *aculeifer* is one of the species most employed in biological control (Moreira and Moraes, 2015; Navarro-Campos et al., 2016; Ajvad et al., 2018). It is naturally occurring in citrus orchard mulch (Navarro-Campos et al., 2012; Manwaring et al., 2018) and is also reared and sold by biological control companies (Lenteren et al., 2019). It preys on a wide range of invertebrates such as nematodes, sciarid fly larvae, collembola, other mites, and thrips (Kevan and Sharma, 1964; Ragusa and Zedan, 1988; Gillespie and Quiring, 1990; Lesna et al., 1996; Premachandra et al., 2003; Berndt et al., 2004b), but, it had never been reported as a mealybug predator. For the first time, this predatory mite that spends part of its life cycle in the soil has been demonstrated to be a potential biological control agent for mealybugs. *Gaeolaelaps* (*Hypoaspis*) *aculeifer* also preys on *Dysmicoccus brevipes* Cockerell, *Geococcus coffeae* Green, *Rhizoecus hibisci* Kawai & Takagi, and *Rhizoecus kondonis* (Broza et al., 1995; Godfrey and Pickel, 1998; Huang et al., 2002; Kabi et al., 2016).

Our semi-field study showed that *G. aculeifer* could play an important role in reducing *D. aberiae* tree infestation, preying mostly on *D. aberiae* nymphs. The conservation and proliferation of *G. aculeifer,* as other soil-dwelling predatory mites, should be implemented to improve the biological control of *D. aberiae* as well as the exotic thrips *P. kellyanus* and *Chaetanaphothrips orchidii* (Moulton). The presence of soil-dwelling predatory mites can be enhanced by adding compost (Navarro-Campos et al., 2013) or through the application of mulch (Hurlbutt et al. 1958; Parr et al. 2011). In addition, the positive effect of ground covers should not be overlooked, as ground covers might also increase the presence of these mites as well as other potential predators (Monzó et al., 2011).

Cryptolaemus montrouzieri, widely used as a biological control agent and the main predator of other citrus mealybug species (Kairo et al., 2000; Mani and Krishnamoorthy, 2008; Kaur and Virk, 2012; Kairo et al., 2013), has been also reported to be a predator of *D. aberiae*. Throughout our two-year field study both *C. montrouzieri* and *D. aberiae* population dynamics were synchronised with two main population peaks in spring and summer.

However, even though both prey-predator populations were synchronised, *D. aberiae* fruit damage was not prevented with *C. montrouzieri* presence. More than 70% of fruits had already been damaged by June, after the first peak of both insects. We have discussed several hypotheses that might explain this lack of control. Likely, *D. aberiae* has lower temperature requirements than *C. montrouzieri*; a predator that needs a minimum temperature of 21ºC to feed or lay eggs and 17ºC to complete development (Kairo et al., 2013). This hypothesis is supported by the fact that *D. aberiae* is active during the Mediterranean winter with a male flight occurring in February (Martínez-Blay et al., 2018a, b). Despite the lack of damage control, the use of *C. montrouzieri* in conservation biological control approaches should not be neglected as our results have demonstrated. Its role in managing and reducing *D. aberiae* population levels is always complementary and strengthened by specific mealybug parasitoids (Prokopenko, 1982; Longo and Benfatto, 1987; Moore and Hattigh, 2004; Martínez-Ferrer, 2003; Kairo et al., 2013). In the case of *D. aberiae*, unfortunately, there are no effective naturalised or indigenous parasitoids able to parasitize it because this mealybug species is able to encapsulate the eggs of the parasitoids (Tena et al., 2017). In this context, the most promising biocontrol alternative is to rely on classical biological control. At present, in its origin (South Africa), two new parasitoid species of *D. aberiae* have been described: *Anagyrus aberiae* Guerrieri sp. nov. and *Anagyrus antoniae* Guerrieri sp. nov. (Beltrà et al., 2015; Guerrieri and Cascone, 2018) and are expected to be introduced in Spain. Their introduction, release and potential establishment is the most promising strategy to control *D. aberiae.*

Even though the establishment and spread of the invasive mealybug *D. aberiae* poses an emerging threat for citriculture, the recent detection of the citrus psyllid *T. erytreae* in mainland Europe has the potential to be even more damaging to European citrus. This psyllid has spread from Galicia to the south of Portugal since 2014 (Arenas-Arenas et al., 2018). Neither in Spain nor in Portugal has a natural enemy been found to contain the spread of this psyllid (Cocuzza et al., 2017). Within this framework, classical biological control is the most feasible measure for preventing *T. erytreae* from spreading into the rest of the European citrus producing areas. In its native South Africa, parasitoids are considered the most effective biological control agents due to their host specificity and numerical response (Aubert, 1987; Van den Berg and Greenland, 2000; Tamesse et al., 2002; Cocuzza et al., 2017). Therefore, as a first step for the implementation of a classical biological control program against *T. erytreae,* its native parasitoid complex in South Africa was studied. Our results showed that *T. erytreae* was parasitized by three species of primary parasitoids: the ectoparasitoid *Psyllaephagus pulvinatus,* the endoparasitoids *Tamarixia dryi* and a new species of *Tamarixia,* along with three hyperparasitoids: *Aphidencyrtus cassatus*, *Marietta javensis* and a species of the genus *Aphanogmus*. Parasitism rates varied between 0.40 and 0.70. These rates were similar to the ones obtained by van der Merwe (1923) and Catling (1969b) in South Africa and Swaziland, respectively and Tamesse et al. (2009) in Cameroon. Within the parasitoid complex, *T. dryi* was the most abundant and effective species achieving parasitism rates of 0.90 in some of the sampled sites. This high value of *T. dryi* parasitism rates supported the introduction of this exotic species in Spain and Portugal. Taking this data and previous references from the 70s into account, the Entomology group at IVIA requested the permits to introduce *T. dryi* into Spain. The permit was obtained in December 2017 and the parasitoid was introduced in the Canary Island where, together with our colleagues from Instituto Canario de Investigaciones

Agrarias, the parasitoid has been reared to carry out several assays before the parasitoid was released in the field in 2018. Importantly, one of these assays has demonstrated that *T. dryi* is highly specific. Females did not parasitize any of the 11 non-target psyllid species tested, including five species of the *Trioza* genus (Urbaneja-Bernat *et al.,* 2019). Therefore, the introduction, release and establishment of *T. dryi* in Europe within the classical biological control program of *T. erytreae* should neither affect other psyllid species nor have significant environmental impacts. The Entomology group at IVIA, sent this data to the Spanish Ministry to obtain the permit to release the parasitoid in the Iberian Peninsula. During this period, the Government of Portugal has also contacted IVIA to release the parasitoid in Portugal. Taking into consideration the presence of *T. erytreae* in mainland Portugal, where it has spread up to southern Lisbon, the introduction, mass-production, and release of *T. dryi* in Portugal is urgent.

In case *T. dryi* does not adapt to the diverse European climate zones or displays low parasitism rates, the release of *P. pulvinatus* could be considered as a feasible complementary alternative. However, further studies may need to be conducted prior to its release in order to determine niche occupancy and resource competition between *P. pulvinatus* and *T. dryi*. Moreover, more than 200 species of the genus *Psyllaephagus* are facultative hyperparasitoids (Steinbauer et al., 2014); it is still unknown whether *P. pulvinatus* belongs to this group of parasitoids. Previous studies of *T. erytreae* parasitoids were restricted to population dynamics and species abundance but little is known about parasitoid biology (Catling, 1969b; Mc Daniel and Moran, 1972; Tamesse et al., 2009).

In our study, a new parasitoid species from the genus *Tamarixia* coexisted with *T. dryi* and the hyperparasitoid *A. cassatus*. Our molecular analysis confirmed that this new species has not yet been reported in the Genbank database; it is closely related to *T. dryi*. It could have been named "*Tetrastichus* sp. n." in Western and Eastern Africa and classified as a primary parasitoid (Aubert 1986) or could be an unknown hyperparasitoid named as "*Tetrastichus* sp." in Cameroon, Zimbabwe and Malawi surveys (Mc Daniel and Moran, 1972; Aubert and Quilici, 1988; Tamesse et al., 2002). Its high abundance and the fact that no pupae or larvae of any *Tamarixia* species were attacked by other larvae suggested that our *Tamarixia* sp. is a primary parasitoid. Being that little is known about the biology and parasitic efficacy of this primary parasitoid and the fact that it is still neither fully identified nor described makes its prompt introduction in Europe unfeasible.

Overall, the results of this thesis provide valuable insights and enhance the IPM of *D. aberiae* and *T. erytreae* reducing the use of chemical control and promoting the implementation of biological control.

Chapter 7

Conclusions

CHAPTER 7

Conclusions

7.1 Aggregation patterns, sampling plan, and economic injury levels for the new citrus pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae).

i. *Delottococcus aberiae* showed a clumped aggregation pattern in all tree organs (twigs, leaves and fruits) with no significant differences between the first and second generation on fruits.

ii. Fruit damage at harvest produced by *D. aberiae* was strongly correlated with fruit occupation at the damaging period (spring).

iii. EIL and EEIL were established as 7.1% and 12.1% of occupied fruits respectively sampling 275 fruits using a binomial sampling method or alternatively, 140 fruits with an enumerative method.

iv. Considering these data and the damaging period of *D. aberiae*, we recommend sampling 275 fruits per orchard bimonthly between petal fall and July and spray only when 12% of fruits are occupied by the mealybug.

7.2 The soil mite *Gaeolaelaps* (*Hypoaspis*) *aculeifer* (Canestrini) (Acari: Laelapidae) as a predator of the invasive citrus mealybug *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae): implications for biological control.

i. The soil-dwelling predatory mite *Gaeolaelaps aculeifer* has been recorded as a potential biological control agent against *D. aberiae* when the mealybug spends part of its life cycle on the soil.

ii. *Gaeolaelaps aculeifer* preyed two times more frequently on the first instar nymphs than on eggs of *D. aberiae* and females were more voracious than males.

iii. The presence of *G. aculeifer* halved the infestation levels of *D. aberiae* at semi-field conditions.

iv. Conservation biological control of *G. aculeifer*, as well as other soil mite species, should be enhanced in order to reduce *D. aberiae* population levels on the soil in spring.

7.3 Field evaluation of *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae) as biological control agent of the citrus invasive pest *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae).

i. The seasonal trends of the mealybug destroyer *C. montrouzieri* and its prey *D. aberiae* were synchronised during our two-year field study. Both *C. montrouzieri* and *D aberiae* had two major peaks per year: one in spring and one in the summer season.

However, the predator did not prevent fruit damage produced by *D. aberiae* in spring.

ii. *Cryptolaemus montrouzieri* was not able to regulate the populations of *D. aberiae* within the same year, but the abundance of *C. montrouzieri* in summer affected the increase of *D. aberiae* density the following year.

iii. The utilization of *C. montrouzieri* might be considered as a complementary biological control agent of *D. aberiae* reinforcing a classical biological control program based on the introduction of a specific parasitoid.

7.4 Classical biological control of the African citrus psylla *Trioza erytreae*, a major threat to the European citrus industry.

i. The parasitoid complex of *T. erytreae* in its native South Africa included three species of primary parasitoids: *Tamarixia dryi*, *Psyllaephagus pulvinatus* and another parasitoid of the genus *Tamarixia*. Molecular analysis showed that it is a new species closely related to *T. dryi.*

ii. *Tamarixia dryi* was the most abundant primary parasitoid but its relative abundance varied among sampling sites.

iii. The sex ratio (males/females) of *T. dryi* and *Tamarixia* sp. decreased with *T. erytreae* size and became female biased when psyllid nymphs were larger than 0.6 and 1.2 mm^2, respectively.

iv. Primary parasitoids were attacked by three species of hyperparasitoids, *Aphidencyrtus cassatus*, *Marietta javensis* and a species of the genus *Aphanogmus*. *Aphidencyrtus cassatus* was the most abundant hyperparasitoid and it tended to emerge from large nymphs

v. Overall, our results confirm that *T. dryi* is the most promising parasitoid species to be introduced in Spain to control *T. erytreae*. The presence of native hyperparasitoids in Spain might also affect the efficacy of *T. dryi* as biological control agent of *T. erytreae* as occurs in South Africa.

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