Departament de Ciències Agràries i del Medi Natural

Control biorracional de *Ceratitis capitata* (Wiedemann): Mejora, aplicación y evaluación de la técnica del insecto estéril

Memoria presentada para optar al grado de Doctora Ingeniera Agrónoma por la Universitat Jaume I por

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La mosca Mediterránea de la fruta, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), es una plaga endémica en las áreas de frutales y cítricos del Mediterráneo. Los programas actuales de manejo integran junto al control químico de *C. capitata* técnicas biorracionales como el control autocida o Técnica del Insecto Estéril (TIE). Este método se basa en la suelta de un elevado número de machos estériles competitivos para acoplarse con las hembras salvajes y, de esta forma, disminuir o impedir su descendencia. El objetivo general trazado en esta tesis ha sido ampliar conocimientos en ciertos componentes esenciales que afectan al papel de los machos estériles (actualmente la cepa Vienna-8) liberados en campo, y de este modo contribuir a optimizar el funcionamiento de los programas TIE.

La exposición de los machos estériles de *C. capitata* a aceite de jengibre (GRO, del inglés Ginger Root Oil) por aromaterapia antes de la suelta es una estrategia comúnmente empleada para mejorar su competitividad frente a los machos salvajes en campo. Sin embargo, aplicar GRO por medio de aromaterapia supone un coste elevado por lo que era importante estudiar la eficacia de otras vías de aplicación y/o encontrar otras sustancias de coste asequible. Se ha evaluado vías de aplicación mediante incorporación en dieta, cuyos resultados son similares a la aplicación por aromaterapia. Entre las sustancias alternativas testadas, el linalool y limonene presentes en los aceites de cítricos, fueron seleccionados por su presencia natural en el hábitat de *C. capitata*. De ellos solo el linalool dio resultados similares al GRO en competencia sexual y longevidad de los machos estériles. Estos resultados abren las puertas a futuros ensayos en semi-campo que ayuden a optimizar y definir las dosis y concentraciones de utilización tales que

permitan minimizar el coste de aplicación y al mismo tiempo maximicen la competitividad de los machos estériles Vienna-8.

Una vez liberados en campo, los machos Vienna-8 concurren con otras plagas clave cuyo control reside en la aplicación de plaguicidas cuando superan su umbral económico de daños. Los efectos secundarios de estos tratamientos sobre los machos estériles Vienna-8 liberados no han sido evaluados a pesar de que una posible mortalidad diferencial frente a los machos salvajes podría tener una repercusión importante sobre la eficacia de la TIE. Se expuso a los machos Vienna-8 al residuo fresco de abamectina, etofenprox, etoxazol, aceite mineral, pimetrozina, piriproxifen, clorpirifos y spinosad sobre hojas de clementino tratadas en laboratorio. Los residuos frescos de clorpirifos y spinosad causaron mortalidades superiores al 80% y sus residuos persistieron tóxicos hasta el día 21. Sorprendentemente, clorpirifos y spinosad resultaron más tóxicos y persistentes sobre los machos salvajes que sobre los machos Vienna-8. Para determinar si estos resultados eran debidos a las características intrínsecas de los plaguicidas o a diferencias de comportamiento entre los machos Vienna-8 y los salvajes, se llevó a cabo un experimento de aplicación tópica. Los resultados de las aplicaciones tópicas mostraron el doble de susceptibilidad de los machos Vienna-8 tratados con clorpirifos (RP = 0.425), y susceptibilidad ligeramente menor a spinosad (RP = 1.338) comparados con los machos salvajes. La baja actividad de los machos estériles Vienna-8 comparada con la de los machos salvajes parece explicar el menor riesgo de mortalidad de los Vienna-8 en la exposición a los residuos de los plaguicidas. Estos resultados se deben tener en cuenta en aquellas áreas de aplicación de la TIE donde los tratamientos con clorpirifos están autorizados.

Actualmente, la eficacia de los machos estériles para conseguir cópulas en campo viene indicada por la razón estéril *vs* fértil de machos contada en trampas. Sin embargo, esta razón no tiene en cuenta a las hembras ni el efecto de las cópulas estériles sobre la siguiente generación de plaga. Con el objetivo de mejorar esta medida de la eficacia de las sueltas de machos estériles se realizó la medición de reducción de población, por efecto de distintos ratios de liberación de machos estériles en condiciones de semi-campo, incluyendo también el parámetro de cópula efectiva (presencia de esperma estéril en las hembras). En un primer experimento, el número de machos Vienna-8 liberados mostró una relación positiva con la proporción de esperma Vienna-8 identificado en las espermatecas así como negativa con la de fruta dañada. Posteriormente, se realizaron experimentos similares en campo que cubrieron el rango de condiciones climáticas del Levante en España. La eficacia de los distintos ratios estéril *vs* fértil de machos liberados mediante la proporción del esperma Vienna-8 identificado molecularmente se modelizó frente a la temperatura. Además, se observó una relación positiva entre la identificación molecular de esperma Vienna-8 en las espermatecas y la reducción de descendencia de la población salvaje comparada con un área control sin liberación de machos estériles.

La identificación de esperma estéril es un proceso costoso en tiempo y recursos económicos, pero que ha sido útil para medir la eficacia de los machos estériles. Se ha procedido a validar la exclusividad de los marcadores de esperma Vienna-8 en muestras de machos salvajes procedentes de poblaciones distribuidas por todo el mundo. El siguiente paso fue mejorar el protocolo de laboratorio, ya que el original requería

tres días de trabajo para procesar 30 muestras. Con la mejora obtenida fue posible analizar 96 muestras en 2 días de trabajo disminuyendo así su coste y tiempo de ejecución. Además, esta mejora en el protocolo permitió analizar muestras que tenían un tiempo pre-procesado de conservación en laboratorio superior. El desarrollo de estas nuevas herramientas sirve para conocer más en detalle el papel de los machos Vienna-8 en campo y de este modo, pueden contribuir a mejorar la eficacia de los programas TIE.

La mosca mediterrània de la fruita, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), és una plaga endèmica de les àrees productores tant de fruita dolça com de cítrics del Mediterrani. Els programes actuals de gestió de *C. capitata* integren el mínim ús del control químic, amb tècniques bio-racionals com són el control autocida o Tècnica de l'Insecte Estèril (TIE). Aquest mètode es basa en la solta d'un elevat nombre de mascles estèrils competitius per a acoblar-se amb les femelles salvatges i, d'aquesta forma, disminuir o impedir la seua descendència. L'objectiu general traçat en aquesta tesi ha sigut ampliar coneixements en certs components essencials que afecten al paper dels mascles estèrils alliberats en camp (actualment la soca Vienna-8), i d'aquesta manera contribuir a optimitzar el funcionament dels programes TIE.

L'exposició dels mascles estèrils de *C. capitata* a oli de gingebre (GRO, de l'anglès Ginger Root Oil) per aromateràpia abans de la solta és una estratègia comunament emprada per a millorar la seua competitivitat enfront dels mascles salvatges en camp. No obstant açò, aplicar GRO per mitjà d'aromateràpia suposa un cost elevat. Per tant era important estudiar l'eficàcia d'altres vies d'aplicació i/o trobar altres substàncies de cost mes assequible. S'ha avaluat vies d'aplicació mitjançant incorporació en dieta, els resultats de la qual són similars a l'aplicació per aromateràpia. Entre les substàncies alternatives testades, el linalool i limonene presents en els olis de cítrics, van ser seleccionats per la seua presència natural en l'hàbitat de *C. capitata*. D'ells solament el linalool va donar resultats similars al GRO en competència sexual i longevitat dels mascles estèrils. Aquests resultats obrin les portes a futurs assajos en semi-camp que ajuden a optimitzar i definir les dosis i concentracions d'utilització tals que permeten minimitzar

el cost d'aplicació i al mateix temps maximitzen la competitivitat dels mascles estèrils Vienna-8.

Una vegada alliberats en camp, els mascles Vienna-8 concorren amb altres plagues clau, el control de les quals resideix en l'aplicació de plaguicides quan superen el seu llindar econòmic de danys. Els efectes secundaris d'aquests tractaments sobre els mascles estèrils Vienna-8 alliberats no han sigut avaluats a pesar que una possible mortalitat diferencial enfront dels mascles salvatges podria tenir una repercussió important sobre l'eficàcia de la TIE. Es va exposar als mascles Vienna-8 al residu fresc d'abamectina, etofenprox, etoxazol, oli mineral, pimetrozina, piriproxifen, clorpirifos i spinosad sobre fulles de clementí tractades en laboratori. Els residus frescos de clorpirifos i spinosad van causar mortalitats superiors al 80% i els seus residus van persistir tòxics fins al dia 21. Sorprenentment, clorpirifos i spinosad van resultar més tòxics i persistents per als mascles salvatges que per als mascles Vienna-8. Per a determinar si aquests resultats eren deguts a les característiques intrínseques dels plaguicides o a diferències de comportament entre els mascles Vienna-8 i els salvatges, es va dur a terme un experiment d'aplicació tòpica. Els resultats de les aplicacions tòpiques van mostrar el doble de susceptibilitat dels mascles Vienna-8 tractats amb clorpirifos (RP = 0.425), i susceptibilitat lleugerament menor a spinosad (RP = 1.338) comparats amb els mascles salvatges. La baixa activitat dels mascles estèrils Vienna-8 comparada amb la dels mascles salvatges sembla explicar el menor risc de mortalitat dels Vienna-8 en l'exposició als residus dels plaguicides. Aquests resultats caldria tenir-los en compte en aquelles àrees d'aplicació de la TIE on els tractaments amb clorpirifos estan autoritzats.

Actualment, l'eficàcia dels mascles estèrils per aconseguir còpules en camp ve indicada per la raó estèril *vs* fèrtil de mascles obtinguda a partir de les captures entrampes. No obstant açò, aquesta raó no té en compte a les femelles ni l'efecte de les còpules estèrils sobre la següent generació de plaga. Amb l'objectiu de millorar aquesta mesura de l'eficàcia de les soltes de mascles estèrils es va realitzar el mesurament de reducció de població, per efecte de diferents ràtios d'alliberament de mascles estèrils en condicions de semi-camp, incloent també el paràmetre de còpula efectiva (presència d'esperma estèril en les femelles). En un primer experiment, el nombre de mascles Vienna-8 alliberats va mostrar una relació positiva amb la proporció d'esperma Vienna-8 identificat en les espermateques així com negativa amb la de fruita danyada. Posteriorment, es van realitzar experiments similars que van cobrir el rang de condicions climàtiques del Llevant a Espanya. L'eficàcia dels diferents ràtios estèril *vs* fèrtil de mascles alliberats mitjançant la proporció de l'esperma Vienna-8 identificat molecularment es va representar en un model enfront de la temperatura. A més, es va observar una relació positiva entre la identificació molecular d'esperma Vienna-8 en les espermateques i la reducció de descendència de la població salvatge comparada amb un àrea control sense alliberament de mascles estèrils.

La identificació d'esperma estèril és un procés costós en temps i recursos econòmics, però que ha sigut útil per a mesurar l'eficàcia dels mascles estèrils. S'ha procedit a validar l'exclusivitat dels marcadors d'esperma Vienna-8 en mostres de mascles salvatges procedents de poblacions distribuïdes per tot el món. El següent pas, va ser millorar el protocol de laboratori, ja que l'original requeria tres dies de treball per a processar 30

mostres. Amb la millora obtinguda va ser possible analitzar 96 mostres en 2 dies de treball disminuint així el seu cost i temps d'execució. A més, aquesta millora en el protocol va permetre analitzar mostres que tenien un temps pre-processament de conservació en laboratori superior. El desenvolupament d'aquestes noves eines servirà per a conèixer més detalladament el paper dels mascles Vienna-8 en camp i d'aquesta manera, poden contribuir a millorar l'eficàcia dels programes TIE.

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is a pest that is endemic to the citrus and fruit-producing areas of the Mediterranean region. The current control programs integrate *C. capitata* chemical control with other biorational control strategies in which the Sterile Insect Technique (SIT) is included. This method consists in releasing a large number of sterilized males that are able to adequately compete with wild males in mating with wild females, consequently resulting in reducing or eliminating production of offspring. The overall objective of this thesis has been to enhance the knowledge related to certain essential components that affect the role of released sterile males (the Vienna-8 strain is currently being used) in the field and, in turn, contribute to the optimization of SIT programs.

Exposure of *C. capitata* sterile males to Ginger Root Oil (GRO) through aromatherapy before release is a common strategy employed to improve sterile male competitiveness with wild males in the field. However, GRO aromatherapy application involves elevated costs that have led to the evaluation of the efficiency of other ways of application and/or the discovery of other compounds with more reasonable costs. GRO exposure through feeding has been evaluated and results showed similar effectiveness as that of aromatherapy. Among the alternatives tested, linalool and limonene (present in citrus oil) were selected due to their presence in the natural habitat of *C. capitata*. Of these two, only linalool gave similar results compared to GRO in terms of sexual competitiveness and lifespan of sterile males. These results open the door to future semi-field trials that may help to optimize and define the rates and

concentrations to be used that allow for minimizing application costs while maximizing the competitiveness of Vienna-8 sterile males.

Once released in the field, Vienna-8 males have to withstand the control measures used against other key pests whose control relies upon the application of pesticides when economic thresholds are exceeded. The side effects of these treatments might have upon released Vienna-8 sterile males have not been addressed despite the fact that a differential mortality between sterile and wild males could have significant repercussions on the effectiveness of an SIT program. Vienna-8 males were exposed to clementine mandarin leaves treated in the laboratory with fresh residues of abamectin, etofenprox, etoxazole, petroleum spray oil, pymetrozine, pyriproxyfen, chlorpyrifos and spinosad. Chlorpyrifos and spinosad were the only residues that caused mortality greater than 80% and remained toxic beyond 21 days. Surprisingly, these pesticides resulted more toxic and persistent for wt than for Vienna-8 males. To determine whether these results could be attributed to intrinsic characteristics of the pesticides or to behavioral differences among Vienna-8 and wt males, a topical application trial was conducted. Results concluded that Vienna-8 males were twice as susceptible as wt to chlorpyrifos (RP = 0.425), whereas their susceptibility to spinosad was slightly lower (RP = 1.338). We hypothesize that the lower activity of Vienna-8 males relative to wt ones can explain the lower risk observed for Vienna-8 males in the residual tests. Our results should be taken into account when planning area-wide Sterile Insect Technique programs against *C. capitata* especially in those areas where treatments with chlorpyrifos are approved.

The current method to determine whether sterile males are successfully mating in the field is by looking at the ratio of sterile *vs* fertile males present in captured traps. However, this ratio does not take into account the number of females nor the effect that successful sterile matings will have on future generations. In order to improve the accuracy of the measurement of the effect of sterile male releases, the effect of differing sterile male release rates on population reduction under semi-field conditions were studied as well as a new parameter which indicates successful sterile mating by analyzing the presence of sterile male sperm in wild female spermathecae. In an initial experiment, the number of released Vienna-8 males showed a positive correlation with the proportion of Vienna-8 sperm identified in the spermathecae as well as a negative correlation with damaged fruit. Additional, similar experiments were conducted under field conditions to encompass the range of climatic conditions present in the Eastern region of Spain. The effectiveness of the different fertile:sterile male release ratios (based on Vienna-8 sperm identification) was modeled against temperature. Additionally, a positive relationship between molecular Vienna-8 sperm identification in the spermathecae and a reduction in the subsequent wild offspring was observed when compared with a control area without sterile male releases.

The identification of sterile male sperm is a costly and time-consuming process, but it has proved useful in determining the effectiveness of sterile male releases. The uniqueness of Vienna-8 sperm markers in different *C. capitata* populations from around the world was validated. The next step was to improve laboratory procedures considering that the original protocol required 3 days to process 30 samples. The improvements to the

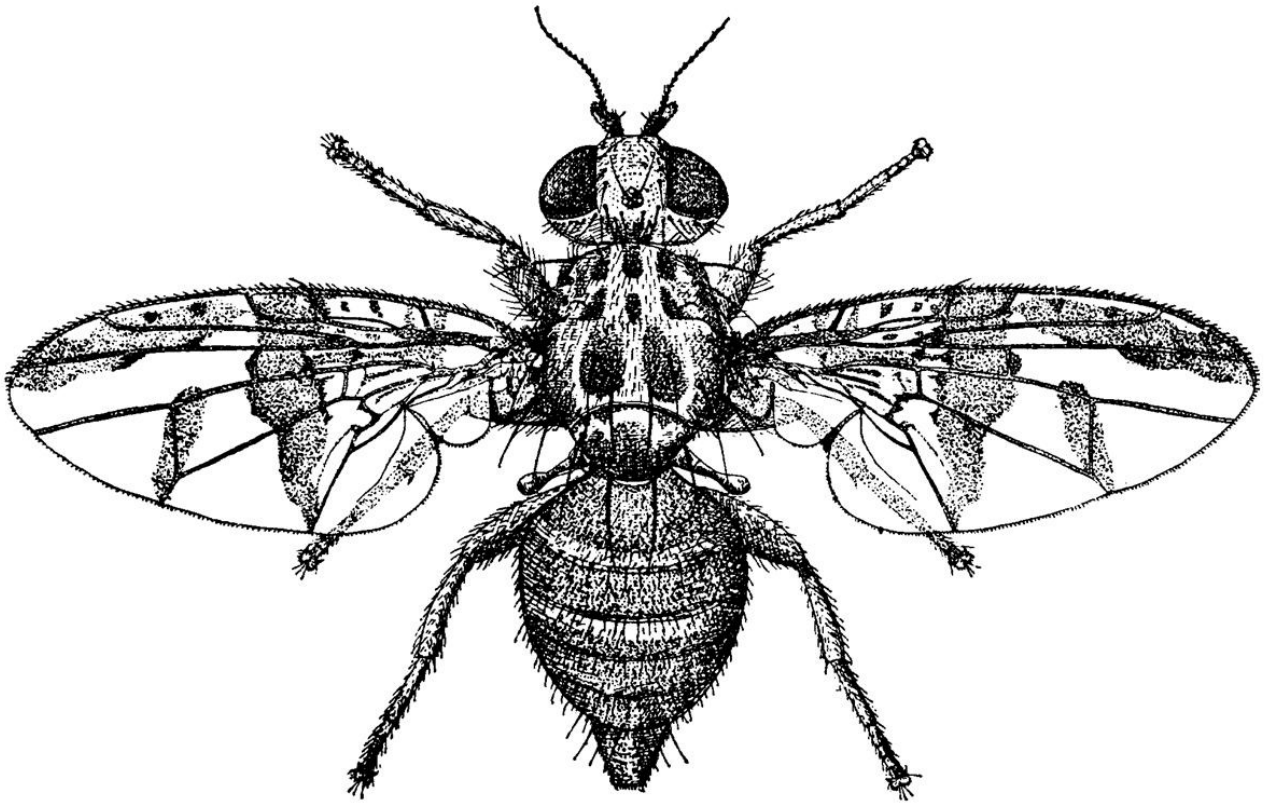
procedure allowed for 96 samples to be processed in 2 days therefore reducing costs and time needed. Additionally, this improvement in the procedure allowed samples to be analyzed that had a longer conservation time in laboratory prior to be processed. The development of these new tools will serve to better understand the role of Vienna-8 males in the field and in turn, can contribute to improving the overall effectiveness of SIT programs.

Capítulo 1

Chapter 2

Capítulo 1

1. Introducción general



1.1. Gestión integrada de tefrítidos

Las verdaderas moscas de la fruta pertenecen a la familia Tephritidae (Diptera: Tephritidae), la cual con más de 5.000 especies descritas es uno de los grupos más diversos de dípteros o moscas verdaderas. Muchas de las especies de esta familia son de importancia clave en los cultivos frutícolas. Los tefrítidos de mayor importancia por su impacto como plaga se engoblan dentro de cinco géneros: *Anastrepha*, *Bactrocera*, *Dacus*, *Ceratitis* y *Rhagoletis* (White y Elson-Harris 1992, De Meyer et al. 2008, Malacrida et al. 2007) (Figura 1.1.). Los tefrítidos se consideran plagas con un importante impacto económico por las siguientes características (Klassen y Curtis 2005):

- Presentan un ciclo de vida multivoltino con una capacidad reproductiva explosiva.
- Capacidad de infestar un gran número de hospederos.
- Habilidad de dispersión a largas distancias de forma natural como adultos, o de forma indirecta movidos dentro del hospedero vegetal infestado (en forma larvaria). Los adultos tienen la habilidad de poder sobrevivir varios meses bajo condiciones climáticas desfavorables.

Estas características, a su vez, dificultan el manejo de la plaga.

Durante el siglo pasado, los métodos de protección de cultivos han sufrido una transformación muy intensa en los países occidentales. De una fase inicial de agricultura de subsistencia se pasó a la agricultura de explotación en la que se recurría de manera sistemática a la aplicación intensiva y rutinaria de fitosanitarios de síntesis. Por ejemplo, el control de la mosca

Mediterránea de la fruta, *Ceratitis capitata* (Wiedemann), considerada una plaga endémica en las áreas frutícolas Mediterráneas (Malacrida et al. 2007), se basó en la aplicaciones cebo por vía aérea y terrestre de plaguicidas organofosforados. En España, estas aplicaciones se han venido haciendo principalmente con insecticidas organofosforados hasta hace relativamente poco tiempo (Chueca et al. 2007, CEE 1991). Con este tipo de manejo, las reinfestaciones de los cultivos por la plaga se repetían continuamente y con ellas los tratamientos con plaguicidas sin que se llegase a manejar satisfactoriamente a la plaga ya que cada brote se trataba de forma puntual y descoordinada. Por lo tanto, se incurría en pérdidas económicas debido al deficiente manejo de la plaga. Además, a partir de mediados de la década de los sesenta, comenzaron a hacerse evidentes los efectos negativos causados por el abuso de plaguicidas sobre el medio ambiente y la salud humana. Por ello, se inició un cambio en el concepto de protección de cultivos con el cual ya no se buscaban solamente la eficacia sobre las plagas sino también su bondad desde el punto de vista económico, ecológico y toxicológico. Además, se dio prioridad al empleo de elementos naturales de regulación y se introdujo el concepto de umbrales de tolerancia de plagas por parte de los cultivos. Más recientemente, la necesidad de cambio en los métodos de manejo de plagas se ha visto agravada por el desarrollo de resistencias a plaguicidas. Este ha sido el caso de la mosca de la fruta *C. capitata* sobre la cual se ha detectado resistencia a malatión en poblaciones naturales (Ortego et al. 2005, Magaña et al. 2007). La aparición de resistencias provoca la disminución en la eficacia de los plaguicidas y por lo tanto un aumento en el número de tratamientos necesarios para controlar a la plaga.

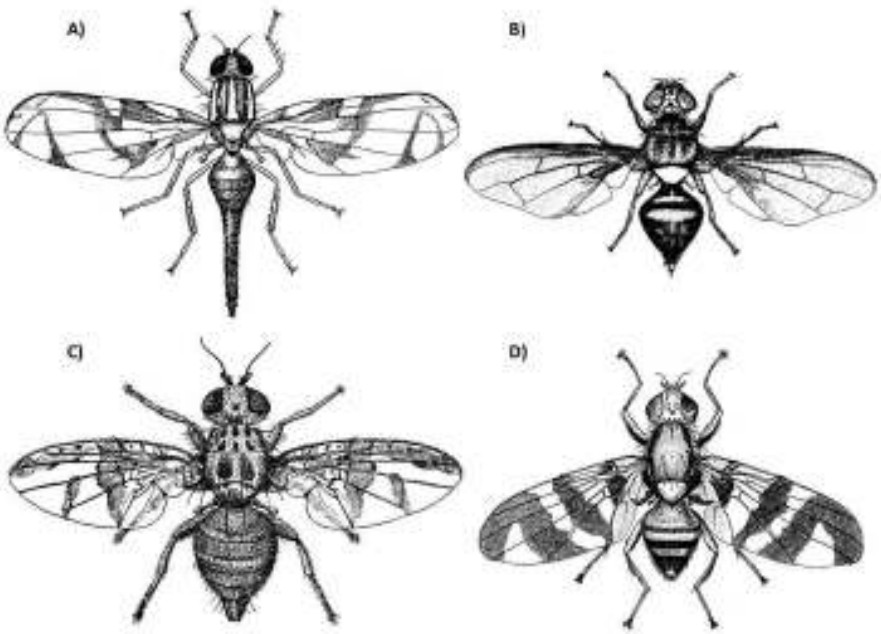


Figura 1.1. Moscas de la fruta de la familia Tephritidae. **A)** La mosca Mejicana de la fruta, *Anastrepha ludens* Loew. **B)** La mosca de Queensland, *Bactrocera tryoni* (Froggatt). **C)** La mosca Mediterránea de la fruta, *Ceratitis capitata* (Wiedemann). **D)** La mosca de la nuez, *Rhagoletis juglandis* Cresson. Fuente: Slingerland y Crosby. 1914. Manual of fruit flies, The MacMillan Company.

La Gestión Integrada de Plagas (GIP) es una estrategia de control que consiste básicamente en la aplicación racional de una combinación de medidas biológicas, biotecnológicas, químicas, culturales, o de selección del material vegetal, de modo que la utilización de productos fitosanitarios se limite al mínimo necesario (Tena et al. 2011). De forma generalizada las técnicas que actualmente se emplean para el manejo de las moscas de la fruta son el control químico, el control biológico, los métodos de trampeo y la Técnica del Insecto Estéril (TIE) (Urbaneja et al. 2012). La decisión de emplear uno u otro método, así como la integración entre ellos residen en

las condiciones particulares de cada especie y área de cultivo. Para el caso de plagas como las moscas de la fruta que presentan las características descritas anteriormente, los programas de manejo integrado alcanzan su óptimo de eficacia cuando se aplican a gran escala mediante una estrategia conjunta y coordinada de todos los agentes responsables de gestionar la plaga (Klassen y Curtis 2005). Klassen (2005) define la GIP a gran escala como la GIP aplicada contra toda la población de una plaga presente en un área geográfica delimitada, siendo esta área de una superficie lo mínimamente grande o protegida por una zona de amortiguación tal que la dispersión natural de la plaga sólo ocurre dentro del área de aplicación. La GIP a gran escala permite el empleo de métodos de control especializados y potentes como son la TIE, el uso de trampeo a gran escala o ciertos programas de sueltas inundativas de parasitoides que generalmente no son efectivos cuando se aplican en parcelas individuales.

En España, la mosca de la fruta *C. capitata* representa un serio problema principalmente para el cultivo de cítricos el cual se extiende a lo largo de toda la Comunidad Valenciana. La Conselleria de Agricultura, Pesca y Alimentación de la Generalitat Valenciana inició en 2003 un Plan Integral de Actuación contra la Mosca de la Fruta en la Comunidad Valenciana que propugna el desarrollo de métodos biorracionales, no contaminantes, para el control de la plaga en cítricos, basando fundamentalmente toda la estrategia de control en el uso de la TIE (Primo-Millo et al. 2003). La aplicación de la GIP a gran escala que incluye la TIE ha sido muy satisfactoria para el control de las moscas de la fruta tal y como se refleja en la diversidad de programas que se han aplicado o aplican para la prevención, supresión o erradicación de estas plagas (Hendrichs 2000). El

programa TIE llevado a cabo en el sur de Japón contra la mosca del melón, *Bactrocera cucurbitae* Coquillett, finalizó a mediados de los 90 (siglo XX) con la erradicación de esta mosca de las islas de Okinawa y de otros archipiélagos nipones. En Australia, la TIE fue aplicada con éxito para erradicar a la mosca de Queensland, *Bactrocera tryoni* (Frogatt) del oeste del país. Además, en este país se realizan sueltas preventivas de machos estériles para proteger a las zonas frutícolas del movimiento estacional de esta plaga. El programa TIE contra *C. capitata* en California, aplicado desde 1994, ha permitido evitar brotes de la plaga así como evitar daños significativos sobre la producción de frutales desde su instauración. La aplicación del programa GIP contra *C. capitata* a gran escala en la Comunidad Valenciana es relativamente reciente y tanto su eficacia en campo como su óptima integración merecen especial atención. En los capítulos de esta tesis se han abordado estos aspectos en lo relativo a la TIE.

1.2. *Ceratitis capitata* (Wiedemman) (Diptera: Tephritidae)

1.2.1. Clasificación taxonómica

De acuerdo con Richards y Davies (1984), la clasificación taxonómica es la siguiente:

- Clase: Insecta
- Orden: Diptera
- Suborden: Brachycera
- Familia: Tephritidae (= Trypetidae)
- Género: *Ceratitis*

- Especie: *Ceratitis capitata*

1.2.2. Biología y Ecología

La metamorfosis de los tefrítidos y dípteros en general es del tipo holometábola o completa. Presentan un ciclo biológico con cuatro estados de desarrollo: huevo, larva, pupa y adulto. Las hembras adultas depositan los huevos (Figura 1.2.) bajo la corteza de los frutos dentro de los cuales transcurre el estado larvario. La larva, una vez desarrollada, salta del fruto y realiza la pupación en el suelo o en el sustrato disponible.

Figura 1.2. Hembra de *Ceratitis capitata* al realizar la puesta de huevos.

El **huevo** de *C. capitata* tiene forma ovoidea, su tamaño medio es de 0,2 mm de ancho y 1 mm de largo. Es de color blanquecino en el momento de la puesta y vira después a tonos amarillentos (Ros 1988) (Figura 1.3. A). La **larva** es de color blanco o amarillento, pudiendo presentar manchas rojizas o anaranjadas debidas a la presencia de alimentos en su tubo digestivo. Es ápoda, con la parte cefálica situada en el extremo más agudo del cuerpo. Efectúa dos mudas hasta alcanzar su completo desarrollo. El gancho del aparato bucal diferencia las tres fases larvarias que presenta: en la $\mathbf{L_1}$ el

gancho no presenta dientes desarrollados, en la **L_2** muestra dos dientes bien patentes y en la **L_3** sólo muestra el diente exterior del gancho. La longitud media de la L_3 es de 7-9 mm (White y Elson-Harris 1992) (Figura 1.3. B). La **pupa** tiene forma elipsoidal, con la superficie lisa. Su color vira del blanquecino al marrón oscuro conforme aumenta la edad de la pupa. Su longitud media es de 4-4,3 mm de longitud y 2-2,4 mm de ancho (Thomas et al. 2001, Chueca 2007) (Figura 1.3. C).

Figura 1.3. Estados de desarrollo de *Ceratitis capitata*. **A)** Huevo. **B)** Larva sobre sustrato de pupación empleado para la cría masiva de la mosca. **C)** Pupas en salvado fino de trigo procedente de la cría masiva.

El **adulto** emerge por la parte antero-distal de la pupa. El imago distiende el ptilinio empujando para abrir el pupario de forma transversal lo que permite su emergencia del pupario (Richards y Davies 1984). Su cuerpo muestra en el tórax bandas de colores amarillo, blanco y negro. En la zona dorsal del tórax tiene pelos. Las alas son transparentes, con manchas ahumadas y bandas amarillentas. En el abdomen presenta bandas transversales de color gris y pardo. Tienen los ojos iridiscentes. La longitud del adulto es de entre 4 y 5 mm. Los adultos de *C. capitata*, como se observa para el resto de dípteros, sólo presentan dimorfismo sexual en el estado adulto. Los machos, como carácter de dimorfismo sexual, poseen

unas quetas supra-orbitales negras con forma de espátula en la parte distal (Figura 1.4.). Además, la forma de estas quetas supra-orbitales es característica de cada especie del género *Ceratitis* siendo uno de los caracteres que permiten identificarlos y diferenciarlos del resto de especies de tefrítidos de importancia agronómica. La hembra, posee un prominente oviscapto retráctil de forma triangular que utiliza para perforar los frutos y realizar la puesta (Mau y Kessing 1992) (Figura 1.2.).

Figura 1.4. Macho de *Ceratitis capitata*. Las flechas señalan las quetas supra-orbitales negras con forma de espátula que sólo se observa en las antenas de los machos de esta especie.

Después de la emergencia, los adultos de *C. capitata* se alimentan principalmente de azúcares y proteínas. Los adultos adquieren estos alimentos de secreciones, néctar y exudados de savia de las plantas, de fruta podrida, melaza de homópteros, insectos moribundos o defecaciones de pájaros (Christenson y Foote 1960). Una vez alcanzada la madurez sexual, la cual tiene lugar entre los 7-11 días de edad en los machos y entre los 8-12 días en las hembras (Shelly et al. 2007a), se inicia el periodo de cópula. Los machos se juntan para copular y agrupados forman lo que

comúnmente se conoce con la palabra inglesa *lek* donde emiten feromonas sexuales (Figura 1.5. A). Las hembras son atraídas a los *leks* y después de un proceso de cortejo por parte del macho eligen una pareja para la cópula. El cortejo incluye el aleteo de las alas, movimientos de la cabeza, del abdomen, y la producción de sonidos (Prokopy y Hendrichs 1979). La cópula dura una media de 2-3 horas (Whittier et al. 1992) durante las cuales tiene lugar la transferencia de esperma (Seo et al. 1990, Taylor et al. 2001) (Figura 1.5. B). Las preferencias de la hembra para elegir a un macho como pareja de cópula parecen estar basadas en características relacionadas con el rendimiento del macho (cantidad y calidad de feromona, comportamiento durante el cortejo) (Briceño y Eberhard 1998) así como con la apariencia externa del macho (simetría del cuerpo, estado de las alas) (Hunt et al. 1998).

Figura 1.5. A) Agregación ("*lek*") de machos sobre las hojas de un árbol. **B)** Pareja de *Ceratitis capitata.*

Los principales factores que afectan al ciclo biológico de los tefrítidos son la humedad, la temperatura, la luz, la disponibilidad de alimento, los

enemigos naturales y los simbiontes (Bateman 1972). El desarrollo del huevo, larva y pupa cesa a temperaturas menores de 10ºC. Las hembras no realizan la puesta a temperaturas inferiores a los 16ºC. En condiciones de temperatura favorables (24-26ºC) la duración de la fase de huevo, larva y pupa dura 1,5-3, 6-10, y 6-13 días, respectivamente. Cuando las condiciones ambientales no son favorables debido a las bajas temperaturas el tiempo de desarrollo de los estadios se extiende. Aproximadamente el 50% de los adultos mueren durante los 2 primeros meses de vida. Algunos adultos pueden vivir incluso hasta los 6 meses, o incluso más bajo condiciones favorables de alimento, agua y temperaturas bajas (Christenson y Foote 1960, Thomas et al. 2001, Duyck y Quilici 2002).

La estrategia de vida de *C. capitata* incluye cambiar de especie hospedero a lo largo del año (ver apartado 1.2.3.) ya que la larva se desarrolla solo en el interior de frutos maduros. Por lo tanto, los adultos sobreviven mucho tiempo en el campo siendo capaces de dispersarse rápidamente cuando no hay frutos maduros disponibles en el área en la cual se encuentran. En el Levante Español (Tarragona, Valencia e Ibiza) se han observado de 5 a 8 generaciones anuales basándose en el seguimiento del vuelo de los adultos capturados en trampas. Esta población presenta entre 1 y 2 picos máximos de población (sólo en verano, o en verano y en otoño) en función de la presencia o ausencia de hospederos alternativos a los cítricos (Martínez-Ferrer et al. 2007).

1.2.3. Distribución y hospederos

Ceratitis capitata es una especie que muestra elevada polifagia, distribución geográfica, y adaptabilidad. Es una de las plagas más importantes que

afecta a la fruticultura de muchos países debido al amplio rango de hospederos sobre los que se desarrolla (Liquido et al. 1991, Aluja y Mangan 2008) siendo el mango (*Mangifera indica*), el melocotonero (*Prunus persica*), el naranjo (*Citrus sinensis*) y el mandarino (*Citrus reticulata* sensu stricto) sus hospederos preferentes (EPPO 2012).

La mosca Mediterránea de la fruta es un insecto originario de África (Malacrida et al. 1998) que se distribuye en las regiones templadas, subtropicales y tropicales del planeta (EPPO 2012) (Figura 1.6.).

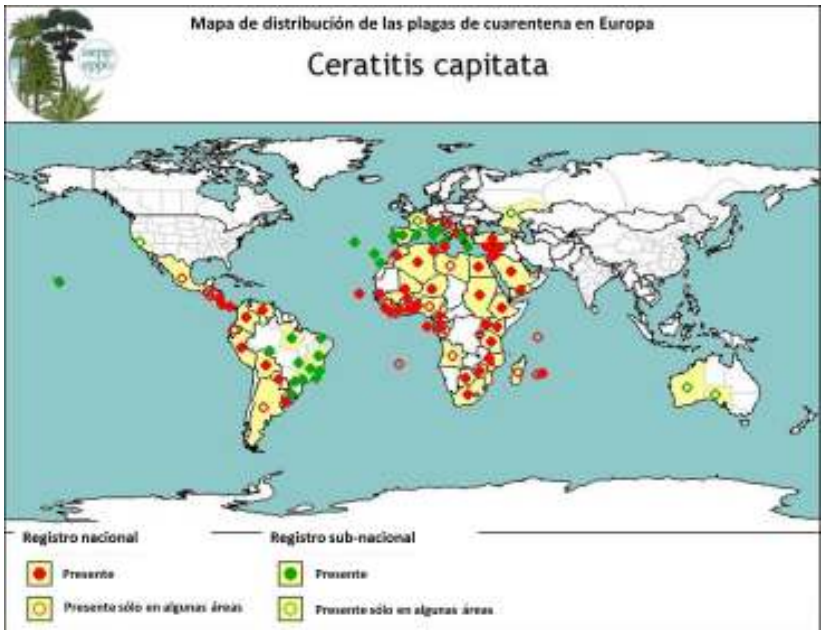


Figura 1.6. Mapa de distribución mundial de *Ceratitis capitata*. Fuente: EPPO 2012.

Actualmente se encuentra presente en gran parte del continente africano a donde se ha extendido desde las zonas tropicales del este sub-sahariano donde se considera su centro de origen (Malacrida et al. 1998). Fuera de África, se encuentra distribuida en América (Centroamérica, Méjico,

California, Hawaii y América del sur excepto en Chile y Surinam), Asia (Arabia Saudí, Iraq, Israel, Jordania, Líbano, Siria y Yemen), Europa (en todos los países de la cuenca Mediterránea, en las islas Azores, Canarias y Madeira, en Portugal, Bulgaria, sur de Rusia y Suiza), y Oceanía (oeste y sur de Australia) (EPPO 2012).

A pesar de no ser originaria del Mediterráneo, se la conoce como mosca Mediterránea de la fruta porque su distribución en esta región es antigua, amplia, y es en esta cuenca donde su incidencia económica se ha hecho más patente. Su supervivencia es mayor en las zonas costeras del Mediterráneo (Mavrikakis et al. 2000). En España se encuentra distribuida por toda la costa este y sur del país, zona dominada por una amplia variedad de cítricos en coexistencia con otros frutales (albaricoqueros, caquis, ciruelos, higueras, azufaifos, melocotoneros, nísperos) (Martínez-Ferrer et al. 2010). El éxito de *C. capitata* en la colonización de zonas de clima mediterráneo se atribuye a su capacidad para adaptarse (pasar el invierno como larva dentro del fruto o como pupa en el suelo) y/o para reinvadir estas regiones ya que presentan inviernos templados (Papadopoulos et al. 1996, Israely et al. 1997, Peñarrubia et al. 2012).

1.2.4. Daños e importancia económica

Ceratitis capitata, es la mosca de la fruta de la familia Tephritidae que presenta una distribución más amplia y probablemente la que constituye una plaga más importante (White y Elson-Harris 1992). En Europa, más de 300.000 hectáreas de cultivos de cítricos y 500.000 de otros frutales son susceptibles a *C. capitata* (Eurostat 2012). En España, la mosca Mediterránea de la fruta afecta a más de 270.000 hectáreas de cítricos y a

alrededor de 300.000 de frutales no cítricos (MAGRAMA 2010). La importancia económica y social de esta especie en nuestro país radica en el enorme peso de la producción de los cítricos dentro del sector agrario español, y en particular en el de la Comunidad Valenciana. La importancia de esta plaga ha aumentado desde mediados de la década de los 90 (siglo XX) debido a la expansión de especies extra-tempranas de cítricos (satsumas y clementinas) que son más susceptibles al insecto al madurar en septiembre cuando los niveles poblacionales de la plaga son elevados (Martínez-Ferrer et al. 2007).

Los daños ocasionados por *C. capitata* pueden ser tanto directos como indirectos. Por un lado, los daños que afectan directamente al fruto son:

- La picadura de puesta de los huevos causa un pequeño orificio en la superficie del fruto que desencadena una reacción necrótica a su alrededor lo que hace que pierda valor comercial (Figura 1.7. A).
- Los agujeros de puesta realizados por la hembra y los agujeros de salida de la L_3 (Figura 1.7. B) sirven de entrada a microorganismos en el interior del fruto que junto a la descomposición de la pulpa provocada por la actividad alimenticia de la larva dan lugar a reacciones que favorecen los procesos de oxidación y maduración prematura del fruto (Figura 1.7. C) lo que provoca su caída del árbol.

Los países libres de la plaga imponen protocolos de cuarentena y de exportación muy restrictivos como consecuencia de la importancia de esta plaga (Enkerlin 2005). Por lo tanto, los países con presencia de plaga han de sumar a las pérdidas económicas de tipo directo las cuantiosas pérdidas derivadas de la aplicación de estos protocolos. En particular, hay que tener en cuenta que España es el principal exportador de cítricos del mundo. De

hecho, en 2001 se produjeron pérdidas cifradas en unos 300 millones de euros debido al bloqueo a la exportación de clementinas españolas por parte de Estados Unidos (Castañera 2003).

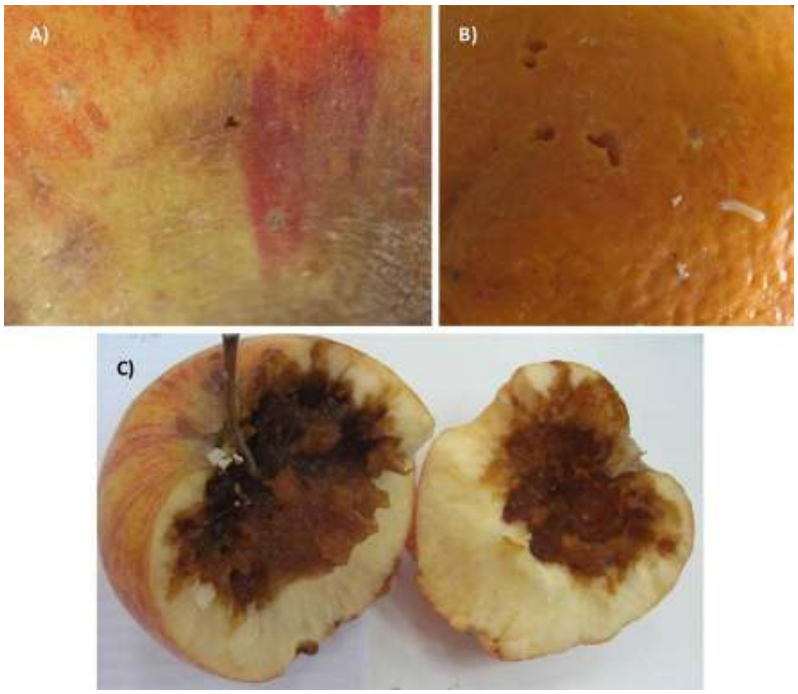


Figura 1.7. Daños provocados por *Ceratitis capitata*. **A)** Orificio de puesta en manzana. **B)** Orificio de salida del fruto de la larva L_3 en naranja. **C)** Descomposición de una manzana por daño directo de *C. capitata*.

1.2.5. Métodos de control

En el ámbito de la producción de los cultivos la estrategia de protección de plagas empleada se basa fundamentalmente en el conocimiento del agroecosistema. Principalmente en la identificación, en el conocimiento de la biología, y en la importancia económica de las plagas así como de sus

enemigos naturales. Basándose en esta información indispensable se seleccionan los métodos de control idóneos para que el manejo de las plagas sea eficaz y a su vez tenga un mínimo impacto negativo sobre el agroecosistema.

1.2.5.1. Manejo cultural

Los métodos culturales practicados tradicionalmente en la Comunidad Valenciana consisten en la eliminación de los frutos dañados o caídos y en la eliminación de árboles reservorio de la plaga. La caída progresiva del precio de los cítricos en los últimos años ha tenido como consecuencia el abandono de muchos huertos lo cual ha producido un aumento elevado de la población de la mosca y por lo tanto, de los daños provocados por *C. capitata*. La recolección y posterior enterrado tanto de los frutos caídos como de los maduros que quedan en el árbol sin comercializar es una práctica recomendable. Esta práctica evita el desarrollo de las larvas que puedan estar en su interior y consecuentemente disminuye la población de moscas así como evita que las hembras encuentren un hospedero donde ovipositar (Chueca 2007). De especial atención son los frutos dañados o caídos de árboles frutales que se encuentran aislados cerca de las parcelas comerciales. Estos frutales (principalmente higueras y nísperos) son hospederos del insecto y suelen ser el foco de infestación de las parcelas comerciales (Tena et al. 2011).

1.2.5.2. Control biológico

Actualmente en España la acción de los enemigos naturales no es suficiente para controlar por completo los daños producidos por *C. capitata*, lo cual

no significa que no realicen un papel importante en la disminución de las poblaciones presentes en campo (Urbaneja et al. 2012).

Los primeros métodos de lucha biológica desarrollados para combatir a *C. capitata* se basaron principalmente en el control biológico clásico con parasitoides (Beitia et al. 2007). Los mejores resultados con este método se obtuvieron en Hawai a mediados del siglo XX con la introducción de los himenópteros *Diachasmimorpha tryoni* (Cameron), *Diachasmimorpha longicaudata* (Ashmed) y *Fopius arisanus* (Sonan) (Hymenoptera: Braconidae) (Wong et al. 1991). Hasta la fecha, los diversos intentos de importar parasitoides exóticos en España han tenido distintos resultados pero en ningún caso han conseguido mantener a la plaga por debajo de su umbral económico de daño. Entre los parasitoides autóctonos los más abundantes son los pteromálidos: *Pachycrepoideus vindemmiae* (Rondani) y *Spalangia cameroni* Perkins. Ambos son parasitoides de pupas (Beitia et al. 2003, 2007). El Instituto Valenciano de Investigaciones Agrarias (IVIA) mantiene en desarrollo una línea de investigación que estudia la posibilidad de emplear parasitoides como agentes de control de *C. capitata* para ampliar el abanico de métodos de control disponibles dentro del Plan Integral de Actuación contra la Mosca de la Fruta (Castañera 2007). A finales del año 2002 se importaron las tres especies de parasitoides con las que mejores resultados se han obtenido en otros países: *D. tryoni*, *D. longicaudata* y *F. arisanus*. *Diachasmimorpha tryoni* y *D. longicaudata* son parasitoides de larvas de tercer estadio de la mosca mientras que *F. arisanus* lo es de los huevos del díptero (Beitia et al. 2003, Castañera 2003). Sabater-Muñoz et al. (2009) han llevando a cabo ensayos en laboratorio, semi-campo y campo para determinar el potencial de estas tres especies

como agentes de control en España. Adicionalmente, se está evaluando la posibilidad de su liberación en combinación con la de machos estériles (Beatriz Sabater, IVIA, comunicación personal).

Las líneas de investigación actuales también incluyen el estudio del control biológico de *C. capitata* ejercido por los depredadores polífagos presentes en el suelo del cultivo (Castañera 2007). Los depredadores polífagos locales son componentes de los agroecosistemas cuyo manejo y conservación puede contribuir a la reducción de las poblaciones de *C. capitata*. Como depredadores de larvas y pupas de moscas de la fruta se ha citado a hormigas, carábidos, estafilínidos y arañas (Urbaneja et al. 2006). En la Comunidad Valenciana, Monzó et al (2010) demostraron la actividad depredadora de una especie de araña, *Pardosa cribata* Simon (Araneae: Lycosidae), sobre las pupas de *C. capitata* así como del carábido *Pseudophonus rufipes* (De Geer) (Coleoptera: Carabidae) sobre los adultos recién emergidos del pupario (Monzó et al. 2011a). El control biológico por conservación mediante el favorecimiento de refugios (manejo de cubiertas vegetales) o la disposición de alimento de manera artificial (adición de polen sobre el cultivo) son algunos de los ejemplos que pueden potenciar el papel de estos artrópodos beneficiosos sobre el control biológico de *C. capitata* y es el siguiente paso a estudiar dentro de este ámbito (Jonsson et al. 2008, Jacas y Urbaneja 2010).

El papel de los microorganismos entomopatógenos tales como las especies de hongos *Metarhizium anisopliae* (Metschnicoff), *Beauveria bassiana* (Balsamo), *Paecilomyces fumosoroseus* (Wize) y la bacteria *Bacillus thuringiensis* (Berliner) presentan actividad entomopatógena sobre larvas, pupas y/o adultos de *C. capitata*. Los estudios de semi-campo y campo

llevados a cabo hasta la fecha han demostrado un elevado potencial infectivo de estos entomopatógenos los cuales además de provocar la muerte del insecto pueden causar efectos secundarios sobre la reproducción y ser transmitidos horizontalmente entre individuos por vía sexual (Castillo et al. 2000, Dimbi et al. 2003, Quesada-Moraga et al. 2006, Vidal-Quist et al. 2010). La posibilidad de integrar esta técnica con la liberación de machos estériles, de forma que éstos sirvan de vectores iniciales del entomopatógeno hacia las poblaciones de mosca en el campo se encuentra en fase de estudio donde está demostrado resultados positivos (Toledo et al. 2007, San Andrés et al. 2010). En España, formulados de *B. bassiana* están registrados y autorizados para su uso contra *C. capitata* (MAGRAMA 2012a).

1.2.5.3. Control químico

Tal y como se ha citado en el apartado 1 del presente capítulo, el control de *C. capitata* en España se ha venido realizando principalmente con aplicaciones aéreas y terrestres de plaguicidas organofosforados (malatión en los últimos años) combinados con atrayentes alimenticios (proteína hidrolizada). Esta práctica no ha mostrado ser capaz de mantener a la plaga por debajo de su umbral económico de daño y sí ha mostrado tener efectos nocivos sobre la salud humana (Flessel et al. 1993), el medio ambiente (Mañosa et al. 2001) y la fauna útil (Urbaneja et al. 2009) así como originar resistencia al malatión en poblaciones de *C. capitata* (Ortego et al. 2005, Magaña et al. 2007).

Actualmente, recurrir a tratamientos con productos fitosanitarios dentro de un programa de GIP de *C. capitata* no es el sistema más recomendable ni

deseable. Lo indicado es recurrir a su empleo únicamente cuando los daños no puedan ser evitados por medio de otros métodos alternativos. Existe una gran variedad de compuestos registrados en España para el control de *C. capitata* mediante tratamientos terrestres localizados (Tabla 1.1.). De la lista de compuestos registrados, spinosad, fosmet y lambda cihalotrin son los más selectivos para los enemigos naturales (parasitoides y depredadores) presentes en el ecosistema donde está implantado el programa GIP contra *C. capitata* (Williams et al. 2003, Urbaneja et al. 2009). Por lo tanto, se les ha considerado la alternativa menos perjudicial para remplazar el uso del malatión.

Tabla 1.1. Productos fitosanitarios registrados para el control químico de *Ceratitis capitata* en España. Fuente: MAGRAMA (2012a).

Modo de Acción	Materia Activa
Inhibidor de la síntesis de la quitina	Lufenuron
Inhibidores de la acetilcolinesterasa	Metil Clorpirifos
	Fosmet
Moduladores del canal sodio	Betaciflutrin
	Lambda cihalotrin
	Etofenprox
	Deltametrina
Antagonista del receptor de nicotínico acetilcolinesterasa	Spinosad
	Imidacloprid
Desconocida (multisitio)	Azadiractina
Repelente	Caolín

Se ha de tener en consideración que recientemente Couso-Ferrer et al. (2011) han demostrado moderada resistencia cruzada a fosmet y a lambda cihalotrin en las poblaciones de *C. capitata* resistentes a malatión.

1.2.5.4. Trampeo

Los métodos de trampeo son una de las alternativas de control biorracional incluídas en el Plan Integral de Actuación contra la Mosca de la Fruta en la Comunidad Valenciana. La atracción de adultos de *C. capitata* a las trampas se realiza mediante atracción visual (diseño de la trampa) y olfativa (atrayentes alimenticios y/o sexuales) (Epsky et al. 1995, Heath et al. 1997). Un avez atraídas, las moscas mueren por la acción de un compuesto insecticida presente en la trampa. La eficacia del trampeo depende del sistema compuesto por el tipo de trampa, el atrayente y el insecticida empleados. Existe una gran variedad de posibilidades en el mercado. Además, factores como el tipo de cultivo, las condiciones climáticas, y el nivel de población de plaga presente en campo influyen en el papel del trampeo dentro del control de la plaga (Peñarrubia-María 2010).

Por un lado, en la Comunidad Valenciana se emplea el trampeo para el seguimiento de las poblaciones. Este tipo de trampeo sirve para detectar la presencia de la mosca, para obtener información de los niveles de las poblaciones en campo, y para crear barreras entre áreas infestadas y áreas libres de la plaga (Martínez-Ferrer et al. 2007). Las trampas Nadel cebadas con la paraferomona sexual Trimedlure, que atrae principalmente a los machos, es la trampa usada actualmente para el seguimiento de poblaciones de *C. capitata*. Se emplea una densidad de trampas superior a 1 trampa cada 200 hectáreas (GVA 2012).

El trampeo masivo es otro de los método de trampeo empleados, en este caso para reducir considerablemente la población de *C. capitata* mediante la captura de adultos. En la Comunidad Valenciana se aplica en las áreas que requieren una especial protección por su alta densidad de variedades de cítricos extra-tempranas, por ser inaccesibles mediante los tratamientos convencionales a base de insecticidas, por su alto valor ecológico, por encontrarse cerca de áreas urbanas, o por tratarse de frutales aislados (Primo Millo et al. 2003). Se emplean trampas Tephri (Sorygar S.L., Madrid, Spain) cebadas con un atrayente alimenticio de tres componentes (acetato amónico, trimetilamina y putrescina) [Biolure Tripack MedFly Lure® (Suterra Corporate, Bend, OR, USA) en la campaña 2012] que atrae principalmente a hembras y, en menor medida, a machos. Las capturas de machos estériles son superiores a las de machos salvajes en las trampas que contienen este tipo de atrayente (Midgarden et al. 2004). Estas trampas se distribuyen con una densidad de 40-50 por hectárea en las áreas de cultivo de caqui, uva, y de variedades extratempranas y tempranas de cítricos (Navarro-Llopis et al. 2008, GVA 2012).

El trampeo masivo denominado de “atracción y muerte” se emplean en las parcelas de cultivo ecológico. Son trampas desechables que realizan atracción selectiva de la mosca mediante la utilización de feromonas específicas. Una vez en la trampa, la mosca entra en contacto con un insecticida al alimentarse de un cebo alimenticio compuesto por proteínas hidrolizables. Las moscas en esta técnica de trampeo no quedan atrapadas dentro de la trampa. Al inico del desarrollo de las trampas y los atrayentes de “atracción y muerte” esta característica se consideró un inconveniente para establecer la eficacia de la técnica ya que se hacía necesario comparar

el nivel de fruta infestada en la misma parcela entre distintas campañas o comparar el nivel de infestación con parcelas próximas no tratadas (Katsoyannos y Papadopoulos 2004). A pesar de este inconveniente inicial, las mejoras desarrolladas en la técnica han demostrado su eficacia en campo principalmente sobre poblaciones aisladas, pequeñas, y poco densas de *C. capitata* (El-Sayed et al. 2009, Navarro-Llopis 2009). Además, esta característica también ofrece ventajas ya que las trampas no se saturan de moscas muertas por lo que su frecuencia de recambio y mantenimiento es baja (Navarro-Llopis 2009). En la Comunidad Valenciana en 2012 se han distribuído 200 trampas por hectárea de estas trampas en las parcelas de agricultura ecológica de cítricos y frutales (GVA 2012).

El empleo del trampeo masivo minimiza los efectos negativos sobre el medio ambiente y los enemigos naturales además de no dejar residuos en los frutos (Lux et al. 2003, Leblanc et al. 2010).

El diclorvos (DDVP) es el insecticida que se ha venido utilizando hasta la fecha en todas las trampas excepto en las de "atracción y muerte" donde se emplea un piretroide. La directiva Europea 91/414 EEC (CEE 1991) propuso retirar este producto del mercado por lo que se están estudiando materias activas alternativas. Una autorización excepcional permite el uso del DDVP en cultivos de cítricos, frutales y uva de mesa hasta el 14 de octubre de 2012 mientras se estudia su remplazo por otros formulados eficaces para la captura masiva y seguimiento de *C. capitata* en campo (MAGRAMA 2012b). Hasta la fecha, una formulación de deltametrina ha mostrado ser buena candidata a remplazar al DDVP en el trampeo de *C. capitata* (Ros et al. 2005).

1.2.5.5. Control autocida o Técnica del Insecto Estéril (TIE)

El control autocida o TIE es un método de control de plagas que consiste en la suelta periódica en campo de un gran número de machos esterilizados de la especie diana para que copulen con las hembras de la población salvaje y de este modo éstas pongan huevos inviables, lo cual, provocará un descenso de la población en las siguientes generaciones (Knipling 1955). Tal y como se ha introducido en el apartado 1, la TIE es uno de los métodos en los que se basa el Plan Integral de Actuación contra la Mosca de la Fruta en la Comunidad Valenciana diseñado para la gestión integrada de la plaga a gran escala (Primo-Millo et al. 2003). Cuando el objetivo del manejo de la plaga es reducir la población es más eficiente y económico aplicar la TIE sobre niveles bajos de población. Por ello, la TIE se aplica junto a otros métodos de control disponibles para *C. capitata* con los cuales no se han demostrado incompatibilidades hasta la fecha (Argilés y Tejedo 2007, Navarro-Llopis et al. 2011). El programa TIE en la Comunidad Valenciana se aplica sobre 152.500 hectáreas y tiene la capacidad de producir alrededor de 540 millones de pupas de machos estériles semanales (Argilés y Tejedo 2007, GVA 2012).

Aunque el concepto de la TIE es sencillo, su implementación es compleja. Para la aplicación eficaz de la TIE se han de cumplir unos requisitos (Lance y McInnis 2005):

- La cría en masa, esterilización, y suelta a gran escala de una cantidad de machos en campo tal para que el ratio (estéril:fértil) que sobrevuela el área tratada sea lo suficientemente elevado.

- Los machos estériles deben ser capaces de competir eficazmente en el campo con los machos salvajes.

El programa TIE en la Comunidad Valenciana ha sido, a grandes rasgos, el objetivo de esta tesis doctoral. Por ello, en el apartado 3 se explican en profundidad los puntos que son críticos durante la aplicación de la TIE para que ésta sea implementada con éxito.

1.2.5.6. Quimioesterilización

El método de quimioesterilización consiste en reducir las poblaciones del insecto plaga por contacto con una sustancia química que reduce la fertilidad o fecundidad de sus individuos y/o de los individuos de la misma especie con los que se apareen. Los individuos adultos de *C. capitata* son atraídos a las trampas quimioesterilizantes por medio de atrayentes sexuales y/o alimenticios. Cuando la mosca entra en contacto con el cebo también lo hace con la sustancia quimioesterilizante lufenuron, un inhibidor de la síntesis de la quitina (Navarro-Llopis et al. 2004).

La gran ventaja para reducir la población de plaga del lufenuron radica en la anulación de la eclosión de los huevos puestos por hembras de *C. capitata* que han sido expuestas directamente al mismo o que han sido copuladas por machos expuestos a este insecticida (Casaña-Giner et al. 1999). La transmisión horizontal de machos a hembras, ya que no quedan atrapados en la trampa, también se ha probado obteniéndose así buenos resultados con densidades poblacionales de mosca elevadas (Navarro-Llopis et al. 2007). Además, no deja residuo en los frutos, hasta la fecha no se ha observado ningún efecto perjudicial sobre la fauna útil (Navarro-Llopis et al. 2007), y es compatible con la TIE (Navarro-Llopis et al. 2011).

En la actualidad, este sistema está siendo comercializado por la empresa Syngenta con la denominada unidad de control Address®. Se recomienda colocar de 20-24 trampas de este tipo por hectárea (GVA 2012).

1.2.5.7. Control post-cosecha

Los tratamientos post-cosecha contra *C. capitata* se aplican para evitar la dispersión del insecto dentro de los frutos en forma de larva cuando éstos se exportan a áreas libres de la plaga.

El primer tratamiento post-cosecha que recibe la fruta es la identificación automatizada de picaduras cuando se realiza la clasificación comercial (Castañera 2003). Además, en el caso de España, los frutos cítricos exportados a países como EEUU, Japón o Australia han de recibir tratamientos cuarentenarios en post-cosecha para seguir protocolos dictados por el país importador. Actualmente, los tratamientos cuarentenarios post-cosecha se basan en la aplicación de frío mientras la fruta está en tránsito hacia el mercado de destino. El tratamiento se aplica manteniendo los frutos a una temperatura y durante un tiempo determinados que varía en función del país importador (Palou et al. 2007). El frío puede variar las características sensoriales y físico-químicas de la fruta por lo que en estos momentos se encuentran en evaluación distintas alternativas basadas mayoritariamente en cuarentena con atmósferas insecticidas, con calor, y con radiaciones ionizantes durante periodos de exposición al frío reducidos.

1.3. Técnica del Insecto Estéril

El descubrimiento de que la radiación ionizante (rayos X o gamma) inducía mutaciones letales dominantes, que acumuladas en el genoma del esperma de los machos evitaban la producción de descendencia viable, y por lo tanto inhibía el crecimiento de la población plaga, fue aplicado a gran escala por primera vez en 1954 para erradicar al gusano barrenador del ganado *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) en Estados Unidos, Méjico y América Central (Klassen y Curtis 2005). Desde entonces, se han implementado eficazmente muchos programas de control que integran la TIE para la gestión de entre otras, las moscas de la fruta, moscas tse tse (Diptera: Glossinidae), el gusano rosado *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) o la polilla del manzano *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Klassen y Curtis 2005). En España, la lucha autocida contra *C. capitata* se utilizó por primera vez en Tenerife, Murcia y Granada (Mellado et al. 1974) durante la década de los 60 y principios de los 70 (siglo XX), aunque los resultados obtenidos no fueron los esperados por diversas dificultades técnicas y de aplicación (Moner et al. 1988, Domínguez 1989).

Desde finales de los 70 (siglo XX), el desarrollo de líneas de *C. capitata* de sexado genético (GSS, de sus siglas en inglés) ha sido uno de los factores clave que ha favorecido el aumento del éxito en la aplicación de la TIE ya que ha permitido mejorar los métodos de cría masiva. Las líneas de sexado genético de *C. capitata* se desarrollaron a principios de los 90 (siglo XX) por la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO, de sus siglas en inglés) junto al Organismo Internacional de la Energía Atómica (IAEA, de sus siglas en inglés) (Klassen y Curtis 2005). Estas líneas

se caracterizan por tener dos marcadores genéticos, *temperature sensitive lethal* (*tsl*) (sensibilidad letal a la temperatura) y *white pupa* (*wp*) (pupa blanca), y una translocación Y-autosoma que hace que la mutación sea heredable y ligada al sexo que permite la eliminación de las hembras (en estado de huevo) durante el proceso de cría masiva (Rössler 1979, Franz y Kerremans 1994, Cáceres 2002). En el programa TIE de la Comunidad Valenciana en la actualidad se emplea la línea Vienna-8 (V8) GSS *tsl wp* cuyas características genéticas distintivas son:

1. Posee una mutación inducida que le confiere sensibilidad letal a la temperatura (*tsl*). El tratamiento de los huevos con temperaturas elevadas mata todos los zigotos femeninos lo que permite la producción sólo de machos (Figura 1.8. A).
2. Una mutación natural que hace que las pupas de las hembras sean de color blanco (*wp*) mientras que las de los machos son marrones (Figura 1.8. B).

Mediante el empleo de las líneas GSS se ha disminuido el coste de producción de grandes cantidades de machos estériles al eliminar las hembras al inicio del proceso, incrementando también la eficacia del programa respecto a liberaciones de líneas bisexuales (machos y hembras).

Desde 2007 se encuentra en funcionamiento en Caudete de las Fuentes (Valencia) una Biofábrica para producir pupas estériles de *C. capitata*. Durante el proceso de cría masiva en la Biofábrica, los huevos son sometidos durante 24 horas a 24ºC de temperatura y durante 24 horas más a 32ºC en medio líquido oxigenado para eliminar a las hembras. Las larvas se desarrollan sobre una dieta artificial compuesta de: azúcar, pulpa de remolacha deshidratada, levadura de cerveza como fuente de proteínas y

agua. Como conservantes de la dieta se adiciona ácido clorhídrico y benzoato de sodio. Las pupas obtenidas, sólo de machos V8, se tiñen con fluoresceína (Day-Glo® Color Corp., Cleveland, OH, USA) antes de ser irradiadas lo que permite la identificación de los machos V8 cuando se capturan en campo en trampas para el seguimiento de las poblaciones. La irradiación de las pupas se realiza en condiciones de hipoxia para reducir daños durante el proceso. A las pupas se les aplica irradiación beta mediante un acelerador de electrones a una dosis de 105 ± 10 Gy (IONISOS Ibérica S.A., Tarancón, Cuenca). Las pupas irradiadas son enviadas al Centro de Evolución de machos estériles localizado en el IVIA (Moncada, Valencia). En este centro se rompe la hipoxia de las pupas irradiadas y se mantienen en jaulas de evolución donde emergen los adultos que son alimentados con azúcar hasta alcanzar la edad de suelta en campo (3 días). Los machos estériles se liberan en campo sobre grandes áreas mediante el uso de avionetas (Argilés y Tejedo 2007).

Figura 1.8. Cría masiva de la línea de sexado genético Vienna-8 *tsl wp* de *Ceratitis capitata*. **A)** Baño a 32ºC con aireación forzada donde se seleccionan los huevos machos resistentes al calor para la cría masiva. **B)** Pupas de hembras (blancas) y machos (marrones) de la cepa Vienna-8 *tsl wp* sin seleccionar por calor en el estado de huevo.

A lo largo del proceso de funcionamiento de los programas TIE se pone de manifiesto que todavía existen puntos críticos donde es necesario desarrollar nuevas herramientas para mejorar su eficacia, lo cual puede servir para mejorar el coste efectivo de los programas (Gurr y Kvedaras 2010, Franz y Robinson 2011). La eficacia de los machos estériles debe basarse en 3 criterios (McInnis et al. 1994):

1. Su calidad antes y después de ser liberados en campo.
2. Su nivel de esterilidad. Basado en la eclosión de los huevos y en la identificación de esperma estéril.
3. El grado de reducción observado en la población diana basado en las capturas en trampas y en la descendencia medida en la fruta.

1.3.1. Parámetros de calidad

La existencia de métodos de cría masiva disponibles es uno de los factores que permite la aplicación económicamente eficiente de la TIE (Lance y McInnis 2005). El número de insectos que se puede criar determina la dosis de liberación y el tamaño del área de liberación que se puede alcanzar. Por ello, un progama TIE con un sistema de cría eficiente funciona mejor cuanto más bajo sea el nivel de plaga en campo ya que podrá lograr ratios de recaptura más altos o tratar un área más grande (Argilés y Tejedo 2007).

La cría masiva así como las operaciones de manejo llevadas a cabo hasta que los machos estériles se sueltan en campo pueden afectar negativamente a su calidad. La calidad de los machos estériles es el factor más importante para el éxito de un programa de gestión integrada a gran escala que integre la TIE. La competencia sexual es el rasgo más importante que determina la calidad de los machos estériles. Además, la calidad también incluye otros rasgos tales como la supervivencia, la capacidad de dispersión, así como otros rasgos relacionados con el vigor de los machos estériles (Itô y Yamamura 2005).

La mayoría de las Biofábricas diseñadas para la aplicación de la TIE sobre tefrítidos llevan a cabo procesos estandarizados para medir la calidad de su producción de moscas estériles. Los protocolos del manual de control de calidad publicado por la FAO/IAEA/USDA (2003) indican cómo medir e interpretar los resultados obtenidos en laboratorio del proceso de irradiación, peso de las pupas, porcentaje de emergencia y habilidad de vuelo de adultos, longevidad bajo estrés, sex ratio y tiempo de emergencia de los lotes de pupas, nivel de esterilidad obtenido, y compatibilidad en

cópula de los machos con las hembras salvajes (Cáceres et al. 2007). Una disminución de la calidad se suele compensar con el aumento de la dosis de suelta. Este aumento se suele combinar con diferentes estrategias para mejorar la competitividad sexual que son implementadas durante el procesado previo a la suelta de los machos (ver apartado 1.3.1.1.). La competitividad sexual de los machos estériles es un factor más importante que los ratios alcanzados en campo (Itô y Yamamura 2005).

El otro aspecto de importancia, de valoración más compleja, es la calidad para actuar que los machos estériles muestran expuestos a factores de campo. Este efecto es difícil de medir en laboratorio. Los protocolos desarrollados y validados al respecto, aunque pendientes de estandarización, indican cómo medir el rendimiento en la cópula sobre árboles enmallados así como la dispersión y supervivencia de los machos estériles mediante la suelta y recaptura en trampas situadas en campo (FAO/IAEA/USDA 2003, Cáceres et al. 2007).

1.3.1.1. Mejora pre-suelta de la competencia sexual de los machos estériles

Existe un considerable campo de prácticas aplicables previamente a la suelta para mejorar la eficiencia de la TIE sobre las moscas de la fruta (Pereira et al. 2012). Cabe destacar los estudios centrados en la exposición de los machos estériles a suplementos alimenticios (Yuval et al. 2002), hormonales (Teal et al. 2000), y semioquímicos (Shelly 2001) así como la modificación de los métodos de manejo y suelta (Shelly et al. 2012).

Los estudios sobre machos estériles de *C. capitata* han demostrado que la adición de proteína en su dieta de adulto acelera su maduración y mejora

su rendimiento sexual mientras que la utilización de aromaterapia (vapor) con distintos aceites esenciales, sobre todo el de jengibre, ha demostrado un significativo aumento de su competencia sexual. Además, la manipulación de su flora bacteriana es objeto de estudio ya que ha demostrado contribuir al buen estado y al rendimiento sexual de los machos estériles (Ben-Yosef et al. 2008, Ben-Ami et al. 2009, Gavriel et al. 2011).

La incorporación de proteína a la dieta de los adultos depende en cada uno de los programas TIE contra *C. capitata* del conocimiento específico que se tiene de las necesidades nutricionales de los machos, de la compensación entre el aumento de rendimiento sexual obtenido y la disminución de supervivencia observada. Del mismo modo influyen los costes añadidos que implica y los beneficios que aporta. En algunos casos, la adición de proteína ha demostrado disminuir la supervivencia y la dispersión de los machos liberados. Aunque esta desventaja desaparece cuando el ratio de proteína en la dieta disminuye. Por ello, la dosis de proteína y el modo de suministro óptimos están siendo objeto de estudio actualmente (revisado en Pereira et al. 2012).

Está demostrado que el α-copaeno (Nishida et al. 2000, Shelly 2001, Shelly et al. 2004a, Briceño et al. 2007) aumenta la competitividad sexual de los machos. El aumento de la competitividad sexual observada en los machos estériles de *C. capitata* expuestos a aceites de raíz de jengibre (GRO, de sus siglas en inglés) (Shelly 2001), de cítricos (Kouloussis et al. 2012) y del árbol del té (Shelly et al. 2008a) se atribuye a su contenido en α-copaeno. La exposición por aromaterapia a GRO en los programas SIT que se encuentran

actualmente en marcha es una práctica ampliamente extendida (Pereira et al. 2012).

Ceratitis capitata es una plaga asociada al cultivo de cítricos en las áreas Mediterráneas (Franco et al. 2006, Jacas et al. 2010). Ioannou et al. (2012) han observado que el contenido de los volátiles linalool y el limonene en el aceite de naranja tienen un efecto contrario sobre la oviposición de las hembras de *C. capitata*. El linalool tiene un efecto disuasorio mientras que el limonene estimula la oviposición. Los machos salvajes de *C. capitata* se muestran menos atraídos hacia naranjos modificados para expresar bajos niveles de limonene (Rodríguez et al. 2011). Estudios previos han demostrado un efecto positivo sobre la actividad de cópula de los machos estériles cuando son expuestos a aceites de cítricos y a mezclas de sus componentes volátiles (Kouloussis et al. 2012) aunque todavía no se han identificado en concreto qué componentes son los responsables de esta mejora observada.

1.3.1.2. Evaluación post-suelta de la supervivencia de los machos estériles

La habilidad de los machos estériles para sobrevivir en campo así como para dispersarse para alimentarse, copular y protegerse desde el punto donde son soltados es crítica para el éxito de los programas TIE de tefrítidos (FAO/IAEA/USDA 2003, Calkins y Parker 2005, Vreysen 2005).

La frecuencia de las sueltas de machos estériles depende de cada especie y varía de acuerdo a su supervivencia. La longevidad de los machos estériles puede disminuir debido a un efecto secundario del proceso de cría masiva, de la genética de la cepa criada, del proceso de irradiación, o de los métodos de manipulación y suelta empleados (Lance y McInnis 2005).

Cuando la supervivencia de los machos estériles en campo disminuye es necesario aumentar el número de machos soltados para mantener los ratios deseados en campo (Dowell et al. 2005, Vreysen 2005). Una vez en campo el movimiento de los machos estériles puede influir sobre su supervivencia. Los estudios más recientes de suelta y recaptura muestran que los machos estériles de la línea Vienna de *C. capitata* pueden dispersarse igual que los machos salvajes pero que su dispersión está muy determinada por el método empleado para soltarlos en campo (Paranhos et al. 2010, Gavriel et al. 2012). Respecto a cómo la dispersión de los machos estériles afecta a su supervivencia también se ha de tener en cuenta su interacción con otras estrategias de control empleadas contra las plagas. La captura en trampas de las cuales los machos estériles V8 no son diana se ve afectada por la dieta de adultos suministrada antes de ser soltados. San Andrés et al. (2009) demostraron que los machos V8 alimentados sólo con azúcar se mueven en búsqueda de una fuente de proteína y caen en trampas que contienen una fuente de proteína hidrolizada como cebo atrayente. Otra vía de estudio dentro de este aspecto esta abierta desde que Hendrichs et al. (2007) demostraron que la agilidad de los machos estériles de *C. capitata* para evitar depredadores es menor que la de los machos salvajes.

1.3.2. Evaluación post-suelta de las cópulas estériles

A diferencia de otros insectos para los cuales la TIE ha sido aplicada con éxito, *C. capitata* pertence al grupo de especies que presentan un complejo sistema de apareamiento basado en la agregación de machos en un *lek*, dentro del cual la hembra selecciona pareja escogiendo entre el total de los

machos (salvajes y estériles) en cortejo (Hendrichs et al. 2002). El rendimiento sexual de los machos estériles dentro de este sistema es fundamental para el éxito de la TIE.

Yuval et al. (2002) definen la secuencia de eventos que rigen el acoplamiento y que finalizan en la fertilización de los huevos, desde el punto de vista del macho, determinada por los umbrales nutricionales, la competición intra-sexual o la elección de la hembra. En concreto definen 7 etapas fundamentales en el éxito de la fertilización de un huevo: elección de un *lek*, emisión de feromona, cortejo, cópula, transferencia de esperma y almacenaje, fertilización de huevos y prevención o retraso de reacoples en las hembras. La consecución de la transferencia de esperma y su almacenaje, la fertilización de los huevos y la inhibición de los reacoples en las hembras son fundamentales para el éxito de los machos una vez que son aceptados por la hembra como pareja de cópula (Yuval et al. 1996, Mossinson y Yuval 2003). Los factores que influyen en la transferencia eficaz de esperma durante la cópula son las condiciones ambientales y el biotipo de la mosca (Papadopoulos et al. 2010).

Los tests diseñados por la FAO/IAEA/USDA (2003) se usan para obtener índices de competencia sexual de los machos estériles mediante la observación directa de las cópulas obtenidas en árboles enmallados. Actualmente, los programas TIE evalúan la eficacia de la sueltas en campo mediante el ratio estéril:fértil de machos capturados en las trampas distribuídas para el seguimiento de las poblaciones (Calkins y Parker 2005, Itô y Yamamura 2005, Vreysen 2005). Un elevado ratio estéril:fértil capturado indica un buen funcionamiento del programa TIE (McInnis et al. 1994, Rendón et al. 2004, Shelly et al. 2007b). Desde los años 90 (siglo XX)

se han desarrollado herramientas basadas en el uso de muestras de hembras para evaluar el éxito de las cópulas de los machos estériles en campo. De las herramientas disponibles, un primer grupo emplea hembras vivas para medir la eclosión de sus huevos o para diseccionarlas e identificar al microscopio el origen del esperma contenido en sus espermatecas (Jang et al. 1999, Katsoyannos et al. 1999, McInnis 1993, McInnis et al. 1994). El segundo grupo de herramientas disponibles para moscas de la fruta, desarrolladas a partir del siglo XXI, emplean métodos moleculares para identificar el origen del esperma contenido en las espermatecas (San Andrés et al. 2007a, Fritz et al. 2010). Las herramientas moleculares muestran más potencial para ser implementadas en los programas TIE debido a que emplean menos tiempo y tienen más posibilidades para aplicarse a gran escala.

1.3.3. Evaluación post-suelta de la eficacia para reducir la población plaga

Sobre la eficacia de la TIE influye tanto el éxito de los machos estériles en las cópulas como su traducción en una reducción de la población plaga (McInnis et al. 1986).

La poliandria (múltiples cópulas) de las hembras de *C. capitata* es un factor que puede disminuir el éxito de la TIE. La importancia de la cantidad de esperma transferido durante la cópula por los machos estériles depende de cuánto influye sobre la recópula de las hembras. En los machos, la capacidad de inhibir la recópula de las hembras depende principalmente del esperma y de las proteínas producidas en las glándulas accesorias que forman parte del semen de la mosca ya que tienen capacidad para influir sobre la receptividad de la hembra (Mossinson y Yuval 2003). La cópula

estéril falla en reducir la reproducción de la hembra cuando inhibe deficientemente la recópula. En estos casos existe mayor posibilidad de que la hembra busque otros machos para copular (Pérez-Staples et al. 2012). La irradiación esterilizante que reciben los machos afecta a la espematogénesis en todas las especies de tefrítidos. Por este motivo los machos estériles de *C. capitata* tienen menor capacidad que los salvajes para inhibir la recópula de las hembras (Vera et al. 2003, Kraaijeveld y Chapman 2004, Gavriel et al. 2009). Recientemente, Morelli et al. (2012) han demostrado en laboratorio que machos estériles expuestos a GRO tienen similiar capacidad que los salvajes para inhibir la recópula. La edad de los machos es también un factor clave ya que afecta a la espermatogénesis. Los machos estériles más jóvenes son menos capaces de inhibir la recópula de las hembras (Shelly et al. 2007a).

La descendencia obtenida de las hembras expuestas a los machos estériles es el dato más verosímil para medir el éxito de los machos estériles si se tienen en cuenta los factores descritos anteriormente que afectan al rendimiento de las cópulas. La técnica comúnmente empleada para medir la descendencia en campo es el muestreo de fruta en un área donde se realizan sueltas de machos estériles y el posterior conteo de la eclosión de los huevos presentes en ella (Wong et al. 1986, McInnis et al. 1986, 1994, Rendón et al. 2004, Shelly et al. 2005). Sin embargo, este procedimiento es bastante tedioso y requiere mucho tiempo y personal para evaluarlo por lo que su optimización es todavía necesaria.

1.4. Objetivos

El objetivo general del trabajo que se presenta en esta tesis doctoral se encuadra en las líneas de actuación para la mejora del control de la mosca Mediterránea de la fruta, *C. capitata,* mediante técnicas biorracionales. La mayoría de los programas de gestión integrada de *C. capitata* a gran escala que se llevan a cabo en la actualidad se basan en la aplicación del control autocida o TIE. Los programas TIE incluyen una serie de componentes clave que deben funcionar óptimamente para asegurar su éxito. Dado el peso de esta técnica en el control de la plaga, se plantearon los siguientes objetivos con el fin de optimizar su aplicación:

1. Evaluar nuevas rutas de exposición al GRO así como el potencial del linalool y el limonene para mejorar la competencia sexual de los machos estériles de la línea Vienna-8 de *C. capitata*.
2. Evaluar los posibles efectos secundarios de los tratamientos plaguicidas sobre los machos estériles Vienna-8. Analizar si existe un efecto negativo de la cría y el manejo previo a la suelta que implique una mortalidad diferencial entre los machos Vienna-8 y los salvajes.
3. Validar el método molecular de identificación de esperma estéril Vienna-8 en hembras para medir la eficacia de la TIE en campo en presencia de fruta disponible para la puesta.
4. Estudiar la posibilidad de relacionar la actividad de cópula de los machos estériles Vienna-8 liberados medida molecularmente con la reducción de la población en campo de la siguiente generación de *C. capitata*.

5. Mejorar los protocolos empleados para la detección molecular de esperma estéril Vienna-8 en hembras para permitir su aplicación en los programas TIE a gran escala.

Chapter 2

2. Alternatives to ginger root oil aromatherapy for improved mating performance of sterile *Ceratitis capitata* (Diptera: Tephritidae) males

Una versión de este capítulo ha sido publicada:

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2.1. Introduction

In Mediterranean fruit fly (*Ceratitis capitata*) Sterile Insect Technique (SIT) programmes, millions of male flies are mass-reared, sterilized, marked, and released into the field. The success of an SIT programme in controlling Mediterranean fruit fly depends greatly upon the mating performance of the released sterilized males. The economic effectiveness of SIT programmes requires mass-rearing and continuous and timely releases (Dyck et al. 2005); thus the millions of flies produced daily should achieve sexually maturity quickly, be sexual competitive and survive long enough to induce sterility on wild populations. The mass-rearing process with its inherent overcrowding conditions can lead to under-competitive and short-lived males, which must be released at overflooding ratios (Shelly et al. 1994, Briceño and Eberhard 1998, Cayol 2000, Gaskin et al. 2002, Franz 2005). Therefore, much research has addressed sterile male mating competiveness during recent years in order to implement cost-effective SIT programmes (Barry et al. 2003a, Shelly and Villalobos 2004, Liedo et al. 2007, Papadopoulos et al. 2007, Shelly et al. 2007c, San Andrés et al. 2009).

Several natural attractants have been shown to increase the mating success of males through aromatherapy (Shelly and McInnis 2001, Shelly et al. 2005, 2007d), one of which is ginger root oil (GRO, *Zingiber officinale* Roscoe). This oil contains in low proportion a natural attractant of *C. capitata* males, α-copaene, capable of stimulating mating behaviour (Nishida et al. 2000, Shelly 2001, Shelly et al. 2004a, Briceño et al. 2007). Aromatherapy with GRO is currently being used to improve mating competitiveness of released males in many SIT programmes, including USA

and Western Australia (Shelly et al. 2006a) and Spain (R. Argilés, TRAGSA, S.A., Spain, unpublished results). Nevertheless, its use has increased programme costs in terms of infrastructure, personnel and handling time requirements. For exposure, 24 h prior to release, at least three independent holding rooms are needed for each batch of *C. capitata* (pre-exposure, exposure, post-exposure/chilling room). The first objective of this work was to explore other options with a view to reducing costs at fly emergence and release facilities, such as the addition of essential oils directly to the post-teneral diet, which would reduce the need for independent holding rooms and personnel required to allocate the flies to each treatment room.

Despite the advantages of using GRO, other chemical cues, also involving the α-copaene, produced by host plants or fruit exudates (such as citrus, guava, mango, grapefruit, or fig) could trigger host-plant searching or feeding behaviours that detract from the main objective of releasing these males, *i.e.* to track and mate with wild females (Warthen and McInnis 1989, Shelly and Villalobos 2004, Papadopoulos et al. 2007, Shelly et al. 2007c). Katsoyannos et al. (1997) reported the effect of chemicals from citrus fruit juice and peels on *C. capitata* male and female mating behaviours. Other authors (Papadopoulos et al. 2001a, 2006, Jang 2002, Shelly 2004, 2009, Shelly et al. 2004b, 2008b) have reported similar enhancement of sterile males' mating competitiveness by other essential oils, plant or fruit exudates. In all cases, the sesquiterpene α-copaene is present in these plant extracts and is considered the component responsible for arousing mating behaviour. However, other compounds present in those essential oils are also likely to have an effect on the mating behaviour of the Mediterranean

fruit fly, and deserve further research (Jang et al. 1989, Warthen and McInnis 1989), which is one of the objectives of this work.

The monoterpenes limonene and linalool are major components of orange oils, compounds also cited as common in other types of citrus and the male Mediterranean fruit fly sex pheromone (Papadopoulos et al. 2001a, and references therein). More recent studies have shown that females prefer males with a specific scent from host plants, preferably *Citrus* species (Shelly 2004, Papadopoulos et al. 2006, Shelly et al. 2007c). Thus, the second objective of this study concerns the identification of other natural sources of Mediterranean fruit fly attractants, which were studied to determine whether they could replace GRO as a mating competitiveness enhancer in sterile males.

In summary, the aims of this work were: 1) to provide a feasible alternative to GRO aromatherapy by means of incorporating GRO into the post-teneral diet, and 2) to identify other molecules to improve the mating performance of the released sterile males.

2.2. Material and methods

2.2.1. Strains and rearing conditions

The wild-type strain (wt) adults were obtained from a laboratory colony (generation XI and XII) housed at the Generalitat Valenciana (GVA)-IVIA emergence facility (Moncada, Spain), which is refreshed twice annually with wild individuals from field-infested fruits. The new individuals are introduced in a new setup colony cage in the same number as those from the colony strain, producing a small bottleneck in the population, and

allowing random mating with the resulting introduction of the new individuals' genes into the colony. Every two years, this colony is completely replaced by wild individuals. Sterile males of the Vienna-8 (V8) temperature sensitive lethal (*tsl)* Genetic Sexing Strain (GSS) mix 2002 strain (also named GS1/D53 or T(Y;5-30C) (Franz 2002)), currently under production at the mass-rearing facility in Caudete de las Fuentes, Valencia Province, Spain, were obtained two days prior to fly emergence, as marked and irradiated pupae.

After emergence, about 1,000 adult wt females, wt males (<24 h old) or V8 males were separated by sex and strain into perspex (poly(methyl methacrylate) or Plexiglas®) cages (20 x 20 x 20 cm) with 250 individuals per cage. Adults were maintained at 25 ± 4ºC, 75 ± 5% RH and 14:10 h (L:D) photoperiod (photophase started at 06:00 h including a one hour dawn and dusk period) in an environmental chamber. Female wt adults were fed a mixture of sugar and hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; w:w) whereas males (wt or V8) were fed only sugar. Water and diet were provided *ad libitum*.

2.2.2. Effect of GRO-supplemented post-teneral diet

2.2.2.1. Mating experiment

Four treatments of sterile males were compared: i) GRO-supplemented diet (GROd); ii) GRO-supplemented diet plus aromatherapy (GROda); iii) control diet plus aromatherapy (GROa); and, iv) control diet without aromatherapy (control). The control diet consisted of 10 g of sugar mixed with 1.5 ml of 20% sugar (w/v) to obtain a similar texture as the GROd. The GROd

consisted of 10 g of sugar complemented with 1.5 ml of 16.7% (v/v) GRO in 20% sugar (w/v) (GUINAMA, Valencia, Spain) to resemble a sugar block. The GRO dose in the diet was determined by previous assays in which mortality was not affected (data not shown). Periodic surveillance of the experimental treatment cages showed that males were in contact with, and fed on, the GROd (Figure 2.1. A). For all the aromatherapy treatments (GROa and GROda), 1 d before testing, sterile V8 males were exposed to GRO for 3 h (dose of 310 $\mu l/m^3$) in the forced-ventilation exposure room of the fly emergence and release facility. Virgin wt females and males, and sterile males were kept apart in different rooms to prevent any pheromone and/or aromatherapy effects.

For all the mating experiments, wt females were 10 d old, wt males 7 d old and sterile V8 males 3 d old (Cáceres et al. 2007, Shelly et al. 2007a, Shelly 2011). The mating arena consisted of perspex cages (30 x 40 x 30 cm) with ventilation openings. Fifty males wt and 50 sterile V8 (1:1) were placed in the mating arena first (8:00-8:30 h photophase started at 06:00 h including a one hour dawn) and left to settle for 15 min, then 50 females were introduced, comprising the final ratio (1:1:1) tested. In each arena, observations for mating pairs were carried out continuously for 3 h removing each couple as formed by gentle soaking into 50 ml vials; after this time, the arena were supervised discontinuously (each 15-20 minutes) for additional 3 h after which any remaining uncoupled female was discarded. All vials were annotated with copula starting time and supervised during 3 h to assess copula completion. After copula completion (Taylor et al. 2000), the male type in each pair was determined by the presence (sterile) or absence (wt) of fluorescent dye (Dyck et al. 2005).

Mating experiments were done with four replicates and repeated twice (8 replicates in total for each treatment).

2.2.2.2. Longevity

Longevity experiments were carried out only with sterile males subjected to the same four treatments. The experimental arena consisted of ventilated plastic cylinders (16 cm high x 13 cm in diameter) that were placed in an environmental chamber (25 ± 4 ºC, 75 ± 5% RH, and 16:8 h (L:D) photoperiod including a one hour dawn and dusk period). Forty 3 d old sterile males were confined per container and a total of four containers per treatment were tested. Longevity was assessed under no stress feeding scenario (sugar and water *ad libitum*) (Figure 2.1. B).

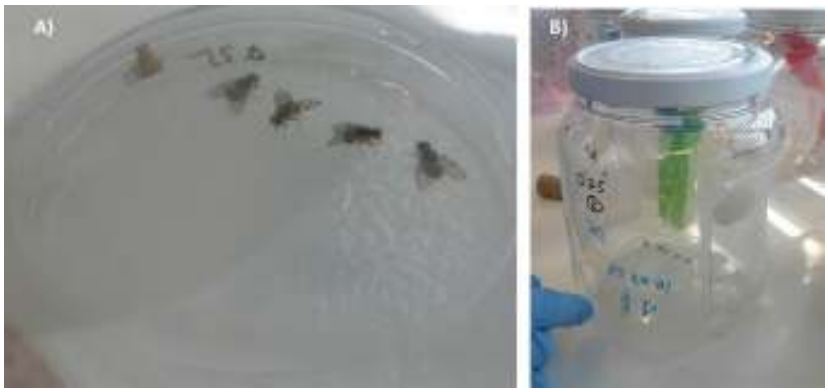


Figure 2.1. A) Sterile Vienna-8 males feeding on the GRO-supplemented diet (GROd). **B)** Experimental arena for longevity tests.

For all the experiments, the arena was checked daily at the same time (13:00 h), until post-treatment day 15 (18 days of adult male), and dead males were recorded and removed.

2.2.3. Effect of aromatherapy with essential oils

2.2.3.1. Mating experiment

The aroma-deprived control was compared with three aromatherapy treatments: i) GRO (as described above); ii) Limonene (Limonene 145, ≥ 99.0%, sum of enantiomers, FLUKA, Seelze, Germany); and, iii) Linalool (± Linalool, ≥ 95.0% (GC), FLUKA). For the aroma treatments, 1 d before starting the assay, flies were exposed to essences for 3 h at a dose of 310 μl/m^3. The GRO treatment was conducted in the forced-ventilation exposition room (143 m^3) of the fly emergence and release facility as described. Whereas Limonene and Linalool treatments were conducted in small rooms (one of 24 and two of 33.8 m^3) without forced ventilation, so, to assure aroma scenting by the flies, 2 μl of each essential oil to a dose of 310 μl/m^3 was deposited in a piece of filter paper and introduced inside of a gauze-covered cup in each cage (room size was also take into account to achieve the dose of 310 μl/m^3). Exposure was conducted simultaneously in different rooms from those used to hold all other flies, thus avoiding inadvertent exposure of the control and aroma-deprived males to essential oils. The four treatments, with four replicates per treatment, were compared for mating success as previously described.

2.2.3.2. Longevity

The longevity assay was carried out with sterile males subjected to the same treatments including the aroma-deprived control. The experimental arena was set up as described above under the same feeding scenario. In the same manner, mortality of sterile males was daily counted at the same

hour (13:00 h) until post-treatment day 15 (18 days of adult male age). Dead males were recorded and removed from the arena.

2.2.4. Statistical analysis

In all mating experiments, the percentage of sterile matings was calculated based on the total number of matings observed (matings sterile + wild) in each cage. The average percentage of sterile matings obtained from the total number of cages that set each treatment was compared among treatments using one-way ANOVA with Tukey test for multiple comparisons ($P < 0.05$). In longevity tests, the number of sterile males dead recorded each day on each experimental arena was analyzed by the Kaplan-Meier survival analysis. Within the Kaplan-Meier procedure cumulative survival functions (combination of survival time and survival chance) were compared among treatments by using Breslow test was performed to determine differences in survival curves among treatments. The Bonferroni method was used to determine statistically significant differences among treatments after the Breslow tests. Breslow tests compare the number of terminal events actually observed to the number of expected terminal events, which is calculated from the number at risk and the number of deaths at each observation point. SPSS 10.05 was used for all statistical analyses (SPSS, Chicago USA).

2.3. Results

2.3.1. Effect of GRO-supplemented post-teneral diet

2.3.1.1. Mating test

Mating competitiveness of 3 d old sterile males exposed to any of the three treatments was significantly higher than the control (F = 12.64; df = 3, 30; P < 0.0001) (Figure 2.2.). The number of sterile males mating when GRO was added to the post-teneral diet (GROd), did not differ from those exposed only to GRO aromatherapy (GROa). However, when GRO aromatherapy was combined with a GRO-supplemented diet (GROda), a significant higher number of sterile matings were observed, compared with the treatment where males were only exposed GROa and with the control without GRO exposure.

2.3.1.2. Longevity

Sterile male survival was not affected by the addition of GRO to the post-teneral diet (GROd and GROda) or presented as aromatherapy (GROa) when compared to control males (χ^2 = 1.942; df = 3; P = 0.585) (Figure 2.3.). Fifteen days after GRO exposure, a high proportion of sterile males survived as did the no GRO exposed sterile males (GROd 81.1%, GROda 83.5%, GROa 83.0%, and control 87.1%).

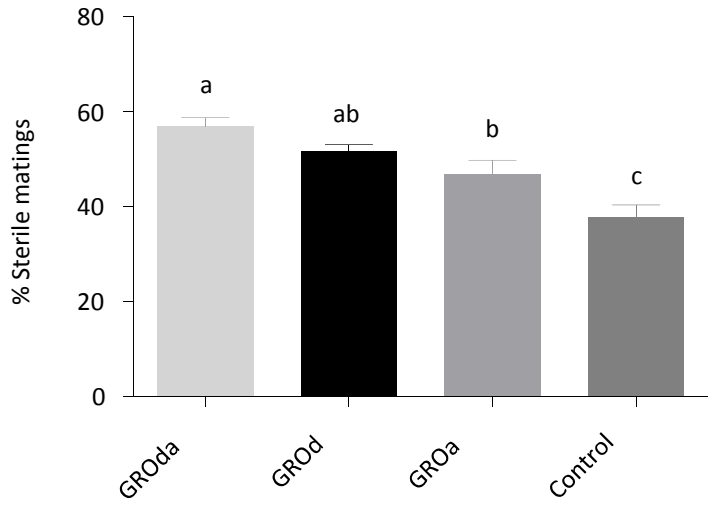


Figure 2.2. Percentage of matings (mean ± SE) achieved by 3 d old sterile Vienna 8 *tsl* Mediterranean fruit flies exposed to ginger root oil aromatherapy (GROa), GRO-supplemented diet (GROd), combined treatment of GRO aromatherapy and GRO-supplemented diet (GROda) and untreated control; in competition with laboratory wild-type males at a 1:1 ratio.

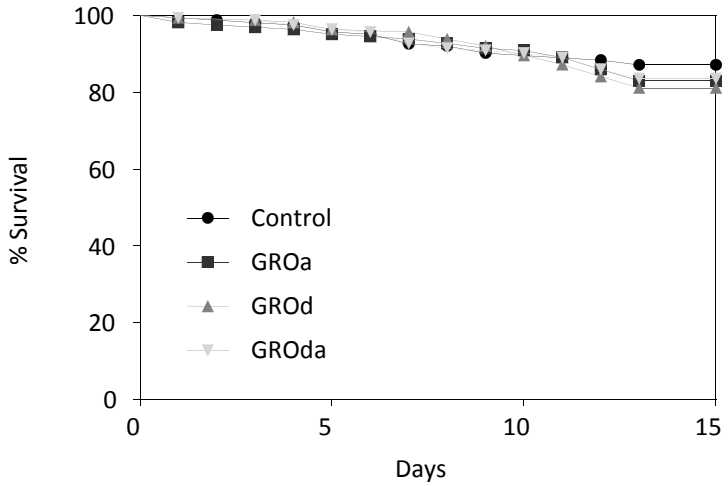


Figure 2.3. Survival (mean percentage) of 3 d old sterile *Ceratitis capitata* Vienna 8 *tsl* males subjected to aromatherapy with ginger root oil (GROa), GRO-supplemented diet (GROd), combined GRO aromatherapy and GRO-supplemented diet (GROda), and a control (no GRO exposure) in a post-treatment feeding regime of no-stress (with *ad libitum* access to water and sugar).

2.3.2. Effect of aromatherapy with essential oils

2.3.2.1. Mating test

The number of 3 d old sterile males that mated was significantly higher in the GRO and linalool aroma treatments when compared to the limonene and control treatments (F = 18.790; df = 3, 15; P < 0.0001) (Figure 2.4.).

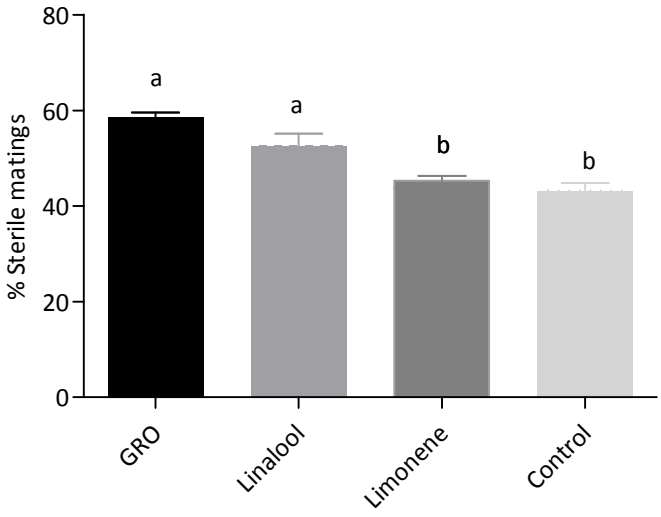


Figure 2.4. Percentage of matings (mean ± SE) achieved by 3 d old sterile Vienna 8 *tsl* Mediterranean fruit flies exposed to aromatherapy with ginger root oil (GRO), limonene, linalool and control; in competition with laboratory wild-type males at a 1:1 ratio.

2.3.2.2. Longevity

Breslow tests within the Kaplan-Meier survival analysis for 3 d old sterile males did not reveal significant differences in survival among treatments when adults had access to water and sugar (no stress χ^2 = 8.073; df = 3; *P* = 0.045) (Figure 2.5.). Survival rates for all treatments throughout the 15 days period were higher than 80%.

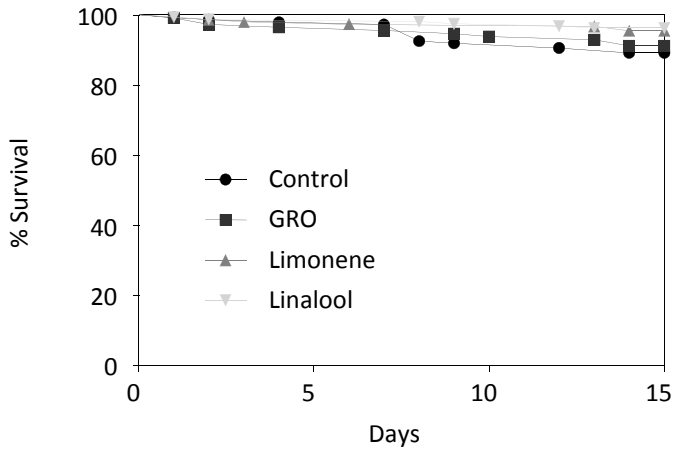


Figure 2.5. Survival (mean percentage) of 3 d old sterile *Ceratitis capitata* Vienna 8 *tsl* males subjected to aromatherapy with ginger root oil (GRO), limonene, linalool, and a control (no aromatherapy exposure) in a post-treatment feeding regime of no-stress (with *ad libitum* access to water and sugar).

2.4. Discussion

In this laboratory trial we found that adding GRO to the post-teneral diet combined with GRO aromatherapy enhanced mating success of treated sterile males (56.9% *vs* 37.7% of sterile male matings in the control). GRO in the post-teneral diet alone led to an increase in matings similar to GRO aromatherapy alone (51.7% and 46.8%, respectively). This contrasts with previous studies conducted in field cages that showed no positive effect on mating success either by GRO-supplemented larval diet or by allowing the pre-release males to be in contact (not feeding) with GRO (Shelly 2001, Shelly et al. 2008c). Nevertheless, our results showed an additive positive effect of GRO-supplemented diet when males were also subjected to GRO

aromatherapy. None of the GRO treatments tested in this study produced a detrimental effect on longevity of the treated males, in accordance with other studies (Shelly et al. 2007d, San Andrés et al. 2009).

Despite its positive effects on mating performance in the laboratory, the GRO-supplemented pre-release diet increases costs as more GRO (~ 3x) is needed than in the aromatherapy alone. On the other hand, when compared to aromatherapy, this cost is offset by the reduction in handling time and associated personnel costs, and costly upgrading and management of compartmentalized release facilities. Further research must be performed to evaluate the effect on the male sexual performance of the studied treatments when tested under semi-natural conditions (field cages) and also to define what GRO concentration is needed in the adult diet to improve sterile male mating rates.

In addition, it is necessary to consider that host-plant volatiles in release areas could interfere with the interactions between α-copaene (from the GRO) and enhanced mating competitiveness in sterile males. As stated by several authors (Nishida et al. 2000, and references therein), a rendezvous role of tephritidae host-plant volatiles is evident, increasing local female traffic and thus influencing lek formation. This attraction has mainly been attributed to the sesquiterpene α-copaene; however, other trace chemicals (sesquiterpenes, monoterpenes, etc.) in these host plants also seem to be of importance (Jang 2002, Shelly 2004, 2009, Shelly et al. 2008b). As demonstrated in previous works, wild females are more likely to visit leks or host plants harboring males, when these males have acquired scent from citrus, guava or papaya (Nishida et al. 2000, Jang 2002, Shelly and Villalobos 2004, Papadopoulos et al. 2007).

Bearing in mind these plant chemical cues, alternatives to GRO were tested as aroma-therapeutic agents in the present study to evaluate their possible effects on the mating success of sterile males. Limonene and linalool were chosen due to their influence on mating behaviour in males (found in pheromones produced by calling males, by Jang et al. 1989) and tested with a view to understanding which component(s) of the citrus oils is/are responsible for enhanced mating (Papadopoulos et al. 2001a, Shelly et al. 2004b, 2008b, Kouloussis et al. 2012). In response to this second research question, we obtained different results in mating performance with the tested components of orange oil. Sweet orange oil (GUINAMA, obtained from cold pressure of fruits) was included in the pre-test to determine effect of one natural mixture of limonene and linalool, and the results in sterile mating enhancement were resembling to the obtained with GRO aromatherapy (in the same dose and application procedure; data not shown). The monoterpene limonene, the primary volatile emitted from citrus oils; did not seem to affect the mating performance of treated males (45.3% *vs* 43.3% of sterile matings in the aroma-deprived control), whereas surprisingly, linalool enhanced mating performance similar to those obtained with GRO exposure (ca. 52.6% *vs* 58.6%, figure 2.4.), which coincided with results from other studies (eg. Shelly et al. 2004a). Previous investigations (Salvatore et al. 2004, Chang et al. 2009) have reported toxic effects of linalool application on adult survival or female oviposition preference; however, in our study, treatment with this component was not linked to any detrimental effect on longevity. To our knowledge, this is the first time that this component has been used successfully to boost male competitiveness with promising results, suggesting that it could be implemented in SIT programmes. Despite that in one recent work

(Kouloussis et al. 2012) only a small advantage to increase matings was observed in linalool treated males. Further work is needed to establish the appropriate application dose and test the effect under semi-natural conditions.

As stated before, the use of an essential oil that is already present in the programme area (*i.e.* linalool) may mean that exposed sterile males do not need to locate these chemical sources in the environment, thus reducing the time and energy costs associated with searching. It follows that these males might devote more time/energy to tracking wild females, which is the main objective of SIT programmes, thereby representing an advantage over GRO. However, all these associated behaviours (chemical and protein sources, and female search) deserve further research. Furthermore, the application of aromatherapy with linalool from locally available sources is advantageous to the citrus industry as it utilizes a by-product. In this way, the study of Kouloussis et al. (2012) shows that males exposed to different citrus fruits (commercial oils or wounded fruits) and to different doses of sweet orange oil, presented a significant mating advantage. Even more, some males were often observed in apparent attempt to feed on these oils, thus assessing the advantage of citrus oils or mixture of compounds over GRO. These results agree with the presented ones, despite the applied doses were lower and oil sources and exposure methods were different in our study. Therefore, further work should be needed to establish an application dose, to select the more convenient and economically affordable linalool source (citrus oil from local industry) and its effect under natural conditions. Concerning cost reduction, the cost of aromatherapy in the *Comunitat Valenciana* SIT programme represents only 0.001% of its

total cost (calculated for current GRO aromatherapy, R. Argilés (TRAGSA, Spain)). In the *Comunitat Valenciana* SIT programme, GRO and the tested essential oils are currently supplied by foreign manufacturers; consequently, the actual availability of local producers of these oils, mainly the proposed linalool would represent a limited cost reduction.

In conclusion, our laboratory experiments showed that 1) a GRO-supplemented diet gave results comparable to GRO aromatherapy, which could result in a net reduction in programme costs; and 2) linalool aromatherapy improved mating performance in sterile males, and could serve as viable alternative to GRO aromatherapy. With respect to SIT programme success by boosting sterile male performance through aromatherapy, further studies are needed to find the best and most economically viable variety of citrus oils that contain higher percentages of linalool (including also studies of dose and exposure methodology optimization as in Kouloussis et al. 2012 and Paranhos et al. 2012). In particular, field cage studies under semi-natural conditions and further field studies should be conducted to compare the induced sterility in nature.

Chapter 3

3. Side effects of selected pesticides used in citrus on sterile males of *Ceratitis capitata*: differential toxicity with wild males

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

Juan-Blasco M., B. Sabater-Muñoz, R. Argilés, J.A. Jacas, F. Ortego y A. Urbaneja. Side effects of selected pesticides used in citrus on sterile males of *Ceratitis capitata*: differential toxicity with wild males.

3.1. Introduction

Fruit flies are considered insects of economic importance (White and Elson-Harris 1992). Particularly in Mediterranean citrus growing areas, the Mediterranean fruit fly or Medfly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), causes serious direct damage triggering important economic losses in both production and export trade (Delrio 1986, Enkerlin and Mumford 1997). *Ceratitis capitata* control in Spain has primarily been based on ground and aerial treatments of organophosphate insecticides, especially malathion bait sprays (Chueca et al. 2007, Urbaneja et al. 2009). However, the intense use of malathion has triggered resistance to this insecticide on wild *C. capitata* field populations in this area (Magaña et al. 2007). In addition, malathion-resistant populations showed moderate levels of cross-resistance to other pesticides used to replace it, as spinosad and lambda-cyhalothrin (Couso-Ferrer et al. 2011).

Current management of *C. capitata* in citrus in the Mediterranean basin includes the integrated use of chemical and biological controls, the Sterile Insect Technique (SIT), and mass-trapping (Castañera 2003, Martínez-Ferrer et al. 2012). Since 2007, an area-wide SIT (aw-SIT) program against *C. capitata* based on the release of sterile Vienna strain males has been implemented in 150,000 ha of citrus growing areas in the Region of Valencia (eastern Spain) (Argilés and Tejedo 2007, San Andrés et al. 2007a). An aw-SIT production facility located 60 km west of the city of Valencia, with a capacity to mass-rear 540 million male pupae per week, is in charge of producing the flies necessary to cover this area. Sterilized Vienna-8 (V8) males emerge to adults and are maintained in the aw-SIT emergence facility until they reach sexual maturity (Argilés and Tejedo 2007). The release of

V8 sexually mature males using aircrafts facilitates the application of aw-SIT over the expansive citrus area where *C. capitata* is distributed in the Region of Valencia (Moltó et al. 2007). The area under aw-SIT in Valencia includes a large number of cultivated citrus species where other pests exist (Jacas et al. 2010). In this mosaic of citrus crops the most important damaging pests besides *C. capitata* are the aphids *Aphis gossypii* Glover and *A. spiraecola* Patch (Hemiptera: Aphididae), the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae), and the California red scale *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) (Jacas et al. 2010, Tena and García-Marí 2010, Urbaneja et al. 2012). When citrus key pests exceed their economic thresholds, pesticides are usually applied (Jacas et al. 2010). To avoid exceeding intervention thresholds, management efforts are focused on enhancing the conservation of either native or naturalized beneficial arthropods, such as predators and parasitoids, which are relevant natural enemies of these key pests (Jacas and Urbaneja 2010). Indeed, the need for the evaluation of the impact of pesticides and their residues on the most important natural enemies has been long recognised in citrus IPM (Jacas and Garcia-Marí 2001, Michaud 2003, Michaud and Grant 2003, Suma et al. 2009, Planes et al. 2012, Vanaclocha et al. 2012). The interaction between SIT and other pest management approaches should be considered and, therefore, the impact of pesticides on beneficial V8 sterile males should be assessed (Gurr and Kvedaras 2010). The risk assessment of pesticides is determined by the intrinsic toxicity of the product and the risk associated with exposure of the insect (Desneux et al. 2007). There is no information supporting the idea that sterile males of *C. capitata* respond to pesticides in a different way relative to wild ones. San Andrés et al. (2009) found high mortality of sterile V8 males on proteinaceous malathion and spinosad

baits under laboratory conditions. Nevertheless, it is not known if pesticides recommended for IPM in citrus against other pests could cause unexpected mortality on released V8 males and as a result, cause a reduction in the expected efficacy of the SIT program.

In this study, the residual effect of selected pesticides on the sterile V8 strain of *C. capitata* males was evaluated under laboratory conditions. The mortality of the V8 males was assessed for 0, 7, 14, 21 and 28 day-old residues of the most common pesticides used against key pests in Spanish citrus (Table 3.1.). Additionally, those pesticides that resulted insecticidal to V8 males were tested by both contact and topical application on wild-type *C. capitata* males to ascertain a possible differential mortality.

3.2. Material and methods

3.2.1. Pesticides

The active substance, manufacturer, trade name, authorized dose in citrus, and target pest of the pesticides used in this study are reported in Table 3.1. All chemicals tested were selected from the list of pesticides included in the Integrated Production Guidelines for Citrus Integrated Pest Management (IPM) in Spain (Urbaneja et al. 2012). These pesticides were selected based on their action against different citrus key pests. The concentration tested was the highest recommended by the manufacturer and authorized by the Spanish Ministerio de Agricultura, Alimentación y Medio Ambiente.

Table 3.1. Pesticide formulations evaluated in the present study.

Active Substance	Trade Name	Manufacturer	Authorized dose in citrus[a] (ml/l)	Target Pest
Abamectin 1.8% [EC] W/V	Cable©	Ender Iberica S. A.	0.30-0.40	Leafminers and Mites
Chlorpyrifos 48% [EC] W/V	Dursban 48©	Dow Agrosciences LTD (UK)	1.50-2.00	Aphids, Scales and Thrips
Etofenprox 30% [EC] W/V	Trebon 30 LE©	Certis Europe BV (Spanish branch)	0.40	Aphids
Etoxazole 11% [SC] W/V	Borneo©	Sumitomo Chemical Agro Europe S. A. S.	0.13-0.50	Mites
Petroleum Oil 83%[EC] W/V	Volck miscible©	Agrodan S. A.	10.00-15.00	Aphids, Mites and Scales
Pymetrozine 25% [WP] W/W	Plenum 25 WP©	Syngenta Agro S. A.	0.40	Aphids
Pyriproxyfen 10% [EC] W/V	Juvinal 10 EC©	Sumitomo Chemicals CO. LTD.	0.25-0.75	Scales
Spinosad 48% [SC] W/V	Spintor 480 SC©	Dow Agrosciences LLC (USA)	0.25-0.30	Lepidopters and Thrips

[a] MAGRAMA (2012a). W: Weight, V: Volume, EC: Emulsifiable concentrate, SC: Suspension concentrate, WP: Wettable powder

3.2.2. Sterile males

Pupae of the sterile males of V8 temperature sensitive lethal (*tsl*) Genetic Sexing Strain (GSS) mix 2002 produced at the mass-rearing facility located at Caudete de las Fuentes (Valencia, Spain) were irradiated at 105±10 Gy dose two days prior to fly emergence. The irradiation treatment was applied under hypoxia in an electron accelerator plant (IONISIOS S.A.) located in Tarancón (Cuenca, Spain). After emergence, adult V8 males <24 hours old were separated in Perspex cages (20 × 20 × 20 cm) with a ventilated lid (16 × 16 cm) containing 250 individuals per cage. Cages were maintained in a climatic chamber at 25 ± 1ºC, 60 ± 10% RH and 16:8 h (L:D) photoperiod until use in the experiments. Two day-old V8 males were exposed for 3 h to an aromatherapy treatment with Ginger Root Oil (GRO) [Zingiber officinale Roscoe (Zingiberaceae)] (Lluch Essence S.L., El Prat de Llobregat, Barcelona, Spain). The aromatherapy treatment was conducted by placing the V8 males inside the Perspex cages described above along with a piece of filter paper impregnated with 2.5 µl of GRO placed inside of a gauze-covered cup. The cages were located in a 10 m^3 room without forced-ventilation. Water and sugar, the standard pre-release diet in the Spanish SIT program, were supplied *ad libitum* to V8 males before and during the experiments. V8 males were 3 days-old when used in the trials, which corresponds with the release age in the Spanish SIT program. Three day-old sterile males treated with GRO have shown to be good competitors against sexually mature wild males. (Shelly et al. 2007a, Juan-Blasco et al. 2012a).

3.2.3. Wild-type males

Male adults of the wild-type (wt) strain used in the experiments were obtained from a colony (generations XI and XII) maintained at the *C. capitata* emergence facility of the IVIA (Moncada, Valencia, Spain). This colony is refreshed twice annually with wild individuals obtained from field-infested fruit. (Juan-Blasco et al. 2012a). To obtain a cohort of adults for the experiments, wt males <24 hours old were separated in Perspex cages as before and maintained under the same environmental conditions. Wt adult flies, which are assumed to have access to sugar and protein supplies in the field, (San Andrés et al. 2009) were fed with a mixed diet of sugar and hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; w:w) and water *ad libitum* before and during the experiments. To evaluate the side effects of pesticides on sterile releases, lethal toxicity was evaluated at the age when V8 and wt males are supposed to compete for matings with wt females. Therefore, 5 day-old wt adults were used in the trials. Five day-old wt males of the laboratory colony used in this study have shown to be competitors as effective as sexually mature wt males 7 to 11-day-old (Shelly et al. 2007a) in previous laboratory mating experiments (data not shown).

3.2.4. Contact application trials

To evaluate the contact toxicity of pesticides on both strains of *C. capitata* males, an extended laboratory method was used (Sterk et al. 1999, Urbaneja et al. 2009). Clementine (*Citrus clementina* Hort. ex Tan. cvar. Nules) leaves were collected from 2-year old trees grown in a pesticide-free greenhouse. The petiole of these leaves was inserted into a 1.5 ml microcentrifuge vial (NIRCO S.L., Barberà del Vallès, Spain) containing a

solution of Triton X-100® (Sigma-Aldrich, Saint Louis, MO, USA) 3% (vol/vol) in water to maintain leaf turgidity during the experiments. Leaves were sealed inside the tubes with plasticine (Plastilina Jovi JOVI, S.A. Barcelona, Spain). Pesticides (Table 3.1.) were applied to the upper face of the leaves using a Potter Spray Tower (Burkard Scientific Ltd, Uxbridge, UK). Two millilitres of the solution of each product were sprayed at 150 kPa resulting in a 2 mg/cm^2 deposit per leaf. A distilled water spray control was included as a positive control. Although spinosad is not authorized in citrus, this product was included in the experiment as a negative control because of its lethal effect on wild strains of *C. capitata* (Adán et al. 1996). To date, field populations of *C. capitata* resistant to malathion have showed no cross-resistance to spinosad in bait formulation, which is the authorized formulation against *C. capitata* in citrus in Spain (MAGRAMA 2012a). Treated leaves were individually transferred and attached with plasticine to the bottom of an experimental unit consisting of a polypropylene (PP) cage (15 × 7 × 10 cm) with a ventilated lid (12 × 8 cm) (Figure 3.1. A). Twenty adult males were introduced in each cage with the use of an entomological aspirator. Ten cages (replicates) were used for each pesticide residue. The diet corresponding with the strain used as described above, and water were provided *ad libitum* during the experiment. The experimental units were kept inside a climate chamber at 25 ± 1ºC, 60 ± 10% RH and 16:8 h (L:D) photoperiod. Different residues of the selected pesticides [0 day-old (fresh), 7, 14, 21 and 28 day-old] were assayed in a sequential way. Pesticides that resulted in mortality lower than 30% were considered not toxic (IOBC category 1, see 3.2.6. Data Analysis) and were no further assayed. Treated leaves used in the 7, 14, 21 and 28-day old residue trials were stored at 25 ± 4ºC, 75 ± 5% RH in complete darkness. The effectiveness of spinosad

residues on citrus leaves is quickly reduced when exposed to ultra-violet light (DowElanco 1996). Therefore, the storage in darkness allowed the persistence of the pesticide on the treated leaves and this allowed us to simulate the worst case scenario for sterile males. To conduct the contact application trial, mortality of V8 males was first evaluated with all the pesticides listed in the Table 3.1. Subsequently, wild-type male mortality was evaluated for those pesticides that had resulted toxic to V8 males. Mortality was assessed 6 days after initial exposure to treated leaves (Urbaneja et al. 2009). Flies were considered dead if they were ataxic (remained on their backs, unable to walk, with no further sign of movement when gently touched with a fine brush) (Magaña et al. 2007).

3.2.5. Topical application trials

Chlorpyrifos and spinosad, the pesticides that showed contact toxicity on V8 males compared to the positive control, were topically applied to V8 and wt *C. capitata* males. Flies, separated by strain, were placed in acrylic dishes (8.9 cm diameter, 46 mm high) with a ventilation area (2 cm diameter) where they were anesthetized with CO_2 (for no longer to 25 seconds) to facilitate the topical application of the pesticides. A synergistic effect of CO_2 on the toxicity of neurotoxic pesticides has been described (Viñuela 1982). To date, synergistic toxicities between the pre-treatment with CO_2 and the lethal effect of chlorpyrifos and spinosad on *C. capitata* have not been described. However, because we were interested in the relative toxicity of the same product against V8 and wt males, this possible synergistic effect was considered irrelevant. Pesticides were diluted in acetone (synthesis grade) (Scharlau S.L., Sentmenat, Spain) to obtain a range of doses. Each fly

was treated with a 0.5 µl drop of an acetone solution of the pesticide or acetone alone (control) on the notum of the thorax of each fly by using an automatic microapplicator 900X (Burkard Manufacturing Co., Hertfordshire, United Kingdom). Three to 6 different doses of chlorpyrifos and spinosad with a partial lethal response on flies (0% < mortality < 100%) were selected. Once treated, flies were placed in the experimental unit consisting of acrylic dishes (8.9 cm diameter, 2.3 cm high) with a ventilation area (2 cm diameter) (Figure 3.1. B). Ten male flies, separated by strain, were confined per unit. Three replicates were performed for each pesticide concentration. The experimental units were kept in an environmental chamber at 25 ± 1ºC, 60 ± 10% RH and 16:8 h (L:D) photoperiod. Mortality was recorded after 48 h (Magaña et al. 2007). Flies were considered dead as above.

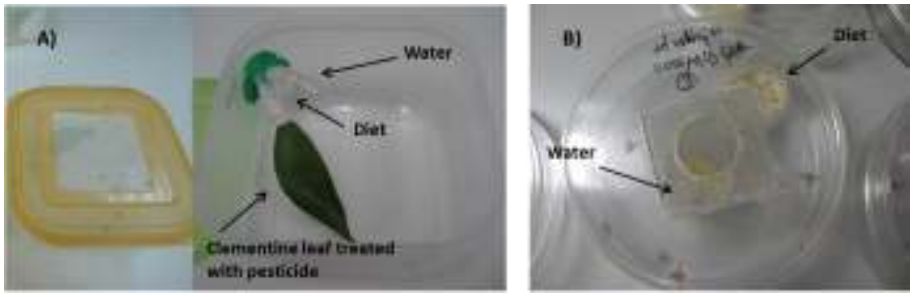


Figure 3.1. A) Experimental unit used in contact application trials. **B)** Experimental unit used in topical application trials.

3.2.6. Data Analysis

In the contact application trials, male mortality was compared by the non-parametric Kruskal-Wallis test because data could not be fit to a normal distribution due to the presence of extreme values. Mortality data of V8 males was subjected to the same analysis except for data from the 28 day-

old residue where only two treatments were compared. A separate non-parametric pair-wise Mann-Whitney U test ($P < 0.05$) comparison was carried out in this case. The same analysis was applied when significant differences were found among treatments for those subjected to Kruskal-Wallis test.

The Schneider-Orelli formula (Bakr 2007) was used to calculate the effect of pesticides only on V8 males mortality because of its condition of biological control agents used against *C. capitata*. These results were interpreted according to International Organisation for Biological and Integrated Control (IOBC)-Working Group "Pesticides and Beneficial Organisms" standards (Sterk et al. 1999) when significant differences were found between the treatment and the control ($P < 0.05$). This classification includes four categories: 1) harmless, mortality lower than 30%; 2) slightly harmful, between 30-79%; 3) moderately harmful, between 80-99%; and 4) harmful, mortality higher than 99%.

In the topical application trials, the mortality data of V8 and wt males were used to determine susceptibility to chlorpyrifos and spinosad by estimating Lethal Dose 50 (LD_{50}) with a 95% Confidence Limit (CL). The mortality was analysed with probit analysis using the computer program POLO-PC (LeOra Software 1997). Lines adjusted for each pesticide were forced to equality and, when if this failed, to parallelism using the same software (Robertson et al. 2007). A chi-square test was used to prove the goodness-of-fit in all cases (Finney 1971).

3.3. Results

3.3.1. Contact application trials on Vienna-8 males

Six days after exposure to a fresh residue of abamectin, etofenprox, etoxazole, petroleum spray oil, pymetrozine, and pyriproxyfen V8 males showed low mortality rates similar to those of males exposed to the water-treated leaves (Table 3.2.). However, residues of chlorpyrifos and spinosad resulted in significantly higher mortalities than positive control for all ages tested (Table 3.2.). According to IOBC categories, fresh residues of abamectin, etofenprox, etoxazole, petroleum spray oil, pymetrozine, and pyriproxyfen resulted harmless (IOBC 1) to V8 males. The fresh residues of chlorpyrifos and spinosad on leaves caused 92.9 and 83.6% of mortality, respectively, and were classified as moderately harmful (IOBC 3). Seven and 14 day-old residues of chlorpyrifos resulted in 41.9 and 37.7% of mortality, respectively. The same age residues of spinosad caused 66.3 and 43.6% of mortality, respectively. All these results resulted in a slightly harmful classification (IOBC 2). The exposure to 21 day-old chlorpyrifos residue caused 18.6% population reduction compared to positive control males and accordingly, the pesticide was classified harmless (IOBC 1). The 21 day-old residue of spinosad caused 31.6% reduction in the exposed V8 population and thus resulted slightly harmful (IOBC 2). Twenty-eight day-old spinosad residue was finally classified as harmless (IOBC 1) for V8 males (Table 3.2.).

3.3.2. Contact application trials on wt males

Mortality rates of wt males exposed to the residues of chlorpyrifos and spinosad were significantly different than those of positive control (Table

3.3.). The fresh residue of chlorpyrifos showed the highest lethal effect and caused a significantly different population reduction compared to spinosad, 99.6 and 93.2% respectively. The toxicities of 7, 14, 21 and 28 days-old residues of chlorpyrifos were similar to those of spinosad and both pesticides were significantly different from positive control (Table 3.3.). The residual effect of both, chlorpyrifos and spinosad, lasted longer on wt males than V8 males (Table 3.2. and 3.3.).

3.3.3. Topical application trials on Vienna-8 and wt males

The probit lines adjusted to data obtained for chlorpyrifos and spinosad for both V8 and wt males are shown in Table 3.4. The susceptibility of V8 males to chlorpyrifos (LD_{50} = 0.052) was higher when compared to wt (LD_{50} = 0.120), whereas for spinosad it was slightly lower (LD_{50} = 0.132 for V8 and 0.100 for wt). For each pesticide, the lines could be satisfactorily forced to parallelism (slope = 5.412 ± 0.780; χ^2 = 0.725; df = 1; P = 0.395 for chlorpyrifos, and slope = 3.173 ± 0.417; χ^2 = 3.147; df = 1; P = 0.076 for spinosad). However, the test of equality between strains was rejected for both insecticides (χ^2 = 30.98; df = 2; P < 0.0001 for chlropyrifos and χ^2 = 7.088; df = 2; P = 0.029 for spinosad), indicating that the differences in susceptibility were significantly different. The relative potency (RP) of chlorpyrifos-treated wt males compared to chorpyrifos-treated V8 males resulted 0.425 (confidence interval: 0.367-0.507) and that of spinosad 1.338 (confidence interval: 0.939-1.830)

Table 3.2. Percent mortality (mean ± SE) of Vienna-8 sterile *Ceratitis capitata* males after 6 days of exposure to different residues plus a water-treated control. The corrected mortality (Abbott) is shown between brackets.

	Residue Age				
Treatment	**Fresh**	**7-d-old**	**14-d-old**	**21-d-old**	**28-d-old**
Abamectin	3.4 ± 1.0c				
	(-)				
Chlorpyrifos	93.3 ± 4.2a	44.4 ± 9.9a	41.4 ± 11.5a	23.0 ± 6.4a	
	(92.9)	(41.9)	(37.7)	(18.6)	
Etofenprox	9.3 ± 1.7c				
	(-)				
Etoxazole	6.6 ± 1.6c				
	(-)				

Table 3.2. (Cont.)

	Residue Age				
Treatment	**Fresh**	**7-d-old**	**14-d-old**	**21-d-old**	**28-d-old**
Petroleum oil	10.6 ± 5.4c				
	(-)				
Pymetrozine	10.1 ± 2.1c				
	(-)				
Pyriproxifen	8.3 ± 2.5c				
	(-)				

Table 3.2. (Cont.)

	Residue Age				
Treatment	**Fresh**	**7-d-old**	**14-d-old**	**21-d-old**	**28-d-old**
Spinosad	84.5 ± 3.1b (83.6)	67.8 ± 8.0a (66.3)	46.9 ± 7.7a (43.6)	35.3 ± 11.9a (31.6)	30.5 ± 4.5a (17.8)
Water	5.7 ± 2.0c	4.2 ± 1.6b	5.9 ± 1.5b	5.4 ± 1.7b	15.4 ± 3.4b
Statistics	$H_8 = 52.268$ $P < 0.0001$	$H_2 = 18.122$ $P < 0.0001$	$H_2 = 15.321$ $P < 0.0001$	$H_2 = 8.098$ $P = 0.017$	$U_1 = 17.500$ $P = 0.025$

Different letters within each set of columns indicate significantly different values of mortality ($P < 0.05$).

Table 3.3. Percent mortality (mean ± SE) of the wild-type *Ceratitis capitata* males after 6 days exposure to different residues plus a water-treated control. The corrected mortality (Abbott) is shown between brackets.

	Residue Age				
Treatment	**Fresh**	**7-d-old**	**14-d-old**	**21-d-old**	**28-d-old**
Chlorpyrifos	99.7 ± 0.3a (99.6)	85.3 ± 2.7a (83.4)	77.3 ± 6.0a (72.8)	37.8 ± 7.6a (36.6)	28.5 ± 8.7a (25.4)
Spinosad	94.0 ± 1.8b (93.2)	84.1 ± 6.1a (82.1)	89.5 ± 4.3a (87.4)	43.7 ± 11.2a (42.7)	60.1 ± 16.3a (58.9)
Water	11.2 ± 2.8c	11.1 ± 4.0b	16.6 ± 2.9b	1.9 ± 1.0b	4.1 ± 2.2b
Statistics	$H_2 = 23.769$ $P < 0.0001$	$H_2 = 18.826$ $P < 0.0001$	$H_2 = 20.101$ $P < 0.0001$	$H_2 = 17.884$ $P < 0.0001$	$H_2 = 13.013$ $P = 0.001$

Different letters within each set of columns indicate significantly different values of mortality ($P < 0.05$).

Table 3.4. Probit lines fitted to the susceptibility experiments of the Vienna-8 and wild-type strains of *Ceratitis capitata* males to topical applications of chlorpyrifos and spinosad.

Pesticide	*C. capitata* strain	n	Slope ± SE	df	χ^2	*P*	LD_{50}[a] (95% CL)	RP (95% CL)
Chlorpyrifos	V8	222	6.098 ± 1.402	10	14.019[b]	0.172	0.052 (0.041-0.062)	
	wild-type	179	4.708 ± 1.143	10	6.041[b]	0.811	0.120 (0.100-0.134)	0.425 (0.367-0.507)
Spinosad	V8	251	2.698 ± 0.466	13	15.517[b]	0.276	0.132 (0.094-0.168)	
	wild-type	161	4.361 ± 0.961	9	10.379[b]	0.321	0.100 (0.078-0.129)	1.338 (0.939-1.830)

[a] Lethal Dose 50 (LD_{50}). expressed in micrograms of chlorpyrifos or spinosad per gram fresh weight of insect (µg/gr) for topical trials.

[b] Good fit of the data to the probit model ($P < 0.05$).

3.4. Discussion

Information about the acute toxicity that pesticides cause to beneficial sterile males is necessary for the implementation of aw-IPM programs; this study provides some of this necessary information. Apart from chlorpyrifos and spinosad, pesticides tested in this study resulted harmless to V8 males. The toxicity observed with the pesticides abamectin, etofenprox, etoxazole, petroleum spray oil, pymetrozine, and pyriproxyfen indicated that they could be used in combination with V8 sterile male releases. A striking result was that obtained with etofenprox which is approved for the control of *C. capitata* in citrus (authorized dose of 1 ml/l) (MAGRAMA 2012a). Etofenprox did not result toxic by contact to V8 males (Table 3.2.). Nevertheless, this insecticide is used against *C. capitata* mixed with protein baits, targeting flies primarily by ingestion. Malathion-resistant wild strains of *C. capitata* have showed moderate levels of cross-resistance by ingestion to the pyrethroid lambda-cyhalothrin which has the same mode of action as etofenprox (Couso-Ferrer et al. 2011). In the present study, we tested etofenprox at the recommended dose against aphids in citrus (0.40 ml/l) which showed low lethal effects on V8 males. It should be kept in mind that sublethal doses of pesticides can affect beneficial males decreasing their performance (Desneux et al. 2007) as well as causing the appearance of resistance to this pyrethroid (Couso-Ferrer et al. 2011). Sublethal effects of pesticides involve changes in physiology and behavior of beneficials which in V8 males may compromise their longevity, feeding, dispersal and mating ability (Mazzi and Dorn 2012). Therefore, sublethal effects and pyrethroid resistance in *C. capitata* should be monitored in citrus areas where aphids are frequently treated with etofenprox. Our results provide baseline

information to optimize the selection of chemical treatments under the aw-IPM programs against *C. capitata* that include SIT. However, further research should include examination of pesticide sublethal effects on the mating behavior and performance of V8 sterile males to completely confirm compatibility of these pesticides with SIT releases.

Both, chlorpyrifos and spinosad tested in formulation at authorized doses against other concurrent key pests were toxic by contact to V8 and wt males. Chlorpyrifos is the most commonly used pesticide for chemical control of key pests in Spanish citrus. However, it is not authorized against *C. capitata*. Spinosad (in bait formulation) is one of the pesticides recommended against *C. capitata* in Spain (MAGRAMA 2012a). Contact activity trials showed lower susceptibility to chlorpyrifos and spinosad of the sterile V8 males compared with wt males, and the activity of aged resiudes of both pesticides lasted longer on wt males. However, when the effects were compared on both male strains in the topical application trials, the susceptibility of V8 males compared to wt for chlorpyrifos was higher and slightly lower for spinosad. These results were not in complete agreement with those of the extended laboratory trial. In the case of spinosad, differences in intrinsic toxicity among the two populations could explain why irradiated V8 males were more susceptible than wt ones. However, in the case of chlorpyrifos different mobility traits of both male strains could explain the differences observed. Sterile males have demonstrated different foraging, dispersal and flight ability compared to non-irradiated males due to the mass-rearing, handling, and irradiation procedures (Wong et al. 1982, Hendrichs et al. 2002, Barry et al. 2003b, Barry et al. 2003c). This result led us to hypothesize that behavior is playing

a major role in the risk of chlorpyrifos to V8 males. V8 males, due to low fitness-related traits, might have less frequent contact with chlorpyrifos and therefore have a lower risk of mortality compared to wt males. However, a low dispersal of sterile males could have an important cost regarding lek participation and encounters with *C. capitata* females (Plant and Cunningham 1991). Moreover, how contact with pesticides could affect sterile male dispersal and sexual performance should be linked to the pre-release procedures (e.g. mating improvement techniques, release method), the nutrition regime, and the sterile male strain (Shelly et al. 2006b, Shelly and Edu 2009, McInnis et al. 2012) which are not considered here.

Spinosad bait treatments are considered compatible with natural enemies in IPM against tephritids in the field (Vargas et al. 2001) although negative effects have been found on certain parasitoids species (Williams et al. 2003, Michaud 2003, Urbaneja et al. 2009, Suma et al. 2009). Besides, this pesticide (not in bait formulation) is regularly sprayed on fruit crops adjacent to citrus orchards and some of them are considered alternative host plants for *C. capitata* (e. g. apples, apricots, figs, peaches) (Martínez-Ferrer et al. 2006, Escudero-Colomar et al. 2008, Peñarrubia-María et al. 2012). Our results indicate that wt and V8 *C. capitata* males are almost as susceptible to spinosad treatments (excluding those applied with bait) as wt males and that the acute toxicity of the pesticide on both male strains persists for more than 20 days. In those crops where SIT is concurrent with spinosad bait treatments, the attention should be focussed in the pre-release feeding regime of the sterile males (Vargas et al. 2001, Barry et al. 2003b, San Andrés et al. 2009).

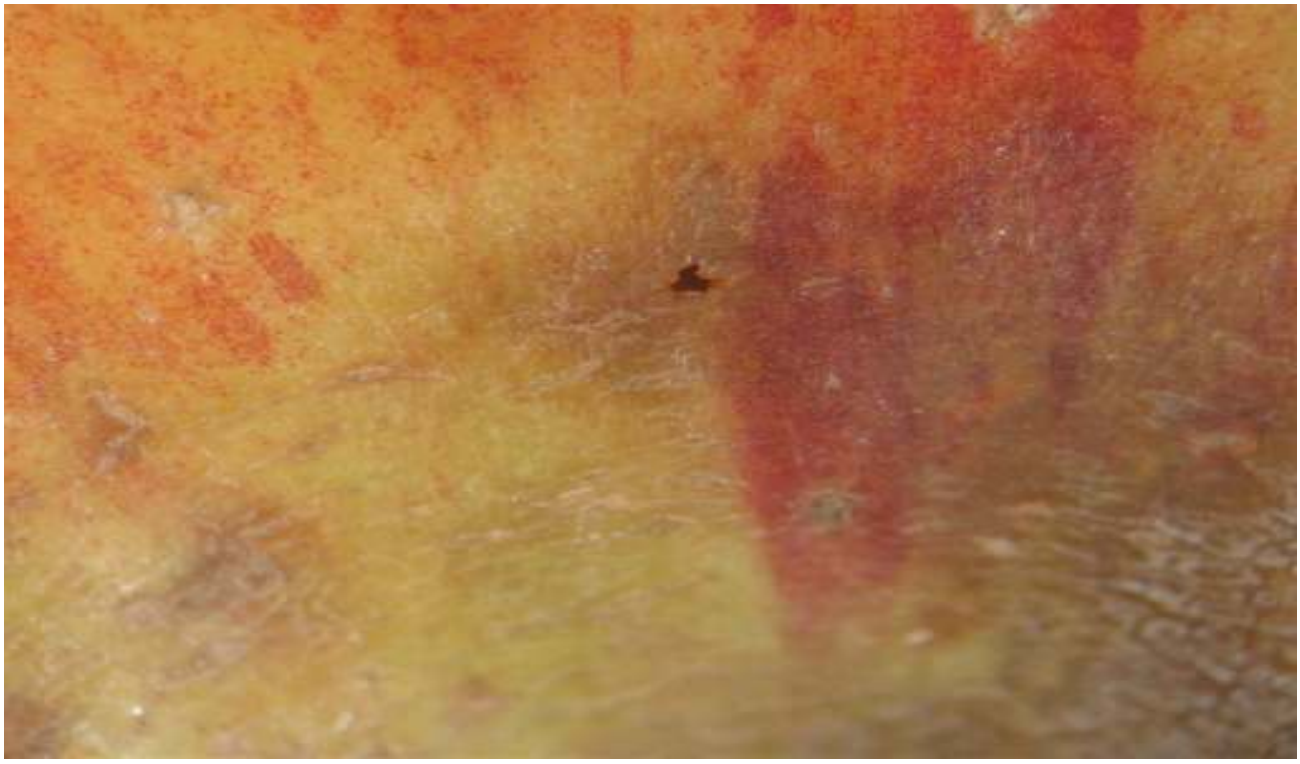
This is the first report documenting chlorpyrifos acute toxicity against beneficial sterile *C. capitata* males. In addition, this study reveals the importance of exposure to assess the risk of V8 males to chlorpyrifos. The available information about susceptibility of wt strains of *C. capitata* to chlorpyrifos is limited. Raga and Sato (2006) obtained high lethal times (LT_{50}) of spray applications of chlorpyrifos on 1-2 day-old wt males. Due to methodological differences, our results cannot be directly compared with those of Raga and Sato (2006). Moreover, the risk of developing resistance to chlorpyrifos is likely since *C. capitata* has developed resistance to other organophosphates (Magaña et al. 2007). Furthermore, negative side-effects of chlorpyrifos against key natural enemies in citrus has been already documented (Jacas and García-Marí 2001, Urbaneja et al. 2012). Chlorpyrifos is the most widespread pesticide used in Spanish citrus growing areas against *A. aurantii*, one of the citrus key pests in Spain, and Kelly's citrus thrips, *Pezothrips kellyanus* (Bagnall) (Thysanoptera: Thripidae) an invasive pests recently recorded in Spain, when both pests exceed their economic thresholds (Tena et al. 2009, Urbaneja et al. 2012). Our results point at the urgent need to (1) minimize the use of chlorpyrifos in Spanish citrus orchards and (2) implement alternative non-chemical control methods especially against key pests.

To sum up, our results provide relevant information about the persistence and acute toxicity of the residues of some of the most widely used pesticides in Spanish citrus orchards and elsewhere against V8 sterile. This information will be useful in making decisions about V8 male release dates in aw-SIT programs. Most products resulted slightly toxic to these beneficial insects. However, the lethal effects of both spinosad and, especially,

chlorpyrifos are indicative of the urgency to identify and implement more selective control alternatives compatible with SIT.

Chapter 4

4. Molecular tools for sterile sperm detection to monitor *Ceratitis capitata* populations under SIT programmes



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4.1. Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is an important pest species reported in more than 400 host plant species (Liquido et al. 1991, Aluja and Mangan 2008), which is distributed throughout the world (EPPO 2012). Furthermore, this species is considered a key pest of quarantine importance in many countries. The Sterile Insect Technique (SIT) has been successfully implemented against many insect pests and disease vectors, such as fruit flies and mosquitoes (Klassen and Curtis 2005). In Spain, where this fly is considered a major pest of citrus orchards, an ongoing area-wide Sterile Insect Technique (aw-SIT) program against *C. capitata* was initiated in 2003 (Argilés and Tejedo 2007) covering at present an area of 150,000 ha (R. Argilés, unpublished). Mass trapping, biological control, selective pesticide applications, chemosterilization and area-wide Sterile Insect Technique (aw-SIT) are used in combination (Navarro-Llopis et al. 2004), as part of an Integrated Pest Management program. The combination of all these management strategies is aimed at reducing *C. capitata* populations and consequently the presence of infested fruit and its economic implications.

The SIT is based on area-wide releases of large numbers of sterile males whose purpose is to decrease the target insect population by mating with wild females which produce unfertile eggs (Knipling 1955). The success of the aw-SIT depends on the production and release of quality sterile male insects to increase the ability of sterile males to achieve mates with wild females once released (Calkins and Parker 2005). In Spain, since the implementation of the aw-SIT program, a quality control protocol for production of sterile *C. capitata* males is routinely followed in the

laboratory. It includes measuring features such as flight ability, dispersion, longevity and mating success (Argilés and Tejedo 2007, FAO/IAEA/USDA 2003). In addition, field cage tests were carried out when the new Vienna-8 (V8) sterile male strain was incorporated into the program and are carried out once per year to test the performance of sterile V8 males against wild males (Cayol et al. 1999, FAO/IAEA/USDA 2003, Calkins and Parker 2005). However, to optimize current SIT programs, it would be beneficial to conduct periodical monitoring of the sexual behavior of sterile males under different ratios of wild: sterile males in field cages (McInnis et al. 1986, Vera et al. 2003). Additionally, field cage tests should include processes to assess the overall competitiveness of sterile males based on the production of the following *C. capitata* generation (McInnis and Wong 1990, Jang et al. 1999, FAO/IAEA/USDA 2003).

At present, the most commonly used method to measure the efficacy of aw-SIT programs is an indirect method based on a monitoring trap network distributed throughout the release area, where the population of sterile and wild insects is estimated weekly (Vreysen 2005). A positive balance (ratio) towards sterile males counted in monitoring traps is considered a success of the releases and hence of the SIT program (McInnis et al. 1994, Rendón et al. 2004, Shelly et al. 2007b). However, this estimation does not provide information on the actual number of mated females with sterile males, which is the real target of SIT. In this sense, some more precise approaches intended to assess the efficacy of sterile males by determining the egg hatchability of eggs collected in artificial laying devices placed in the field (Jang et al. 1999, Katsoyannos et al. 1999) and, by counts of stored sperm in trapped females (McInnis 1993, McInnis et al. 1994). The major

drawback of both methods is that they require live trapping of females and laboratory facilities for handling of the progeny. Consequently, they are time consuming with limited practical application to aw-SIT programs (Vreysen 2005). Recently, San Andrés et al. (2007a) developed a molecular technique that allows the identification of the origin of *C. capitata* sperm (wild or sterile V8 strain) present in the spermathecae of wild females. This technique could be used to directly measure the sterile male mating success in the field, and hence, to measure the success of the ongoing SIT program.

The aim of this work was to validate a field cage protocol to simulate the real scenario where, once released, sterile V8 males should result in the control of a wild population. Firstly, a laboratory test to address the mating activity of V8 males under different male ratios was carried out. In this assay different fruit host were considered. The field cage protocol tested included the evaluation of V8 male mating activity and its consequences in the decrease of the following *C. capitata* generation under different wild: sterile male release ratios.

4.2. Materials and methods

4.2.1. Strains and rearing conditions

The wild-type strain (wt) adults of *C. capitata* was obtained from a laboratory colony (generation VI) housed at the GVA-IVIA emergence facility (Moncada, Valencia, Spain). The refreshment of the colony is carried out annually with wild flies collected from field-infested fruit. Sterile male puparia of the Vienna-8 (V8) temperature sensitive lethal (*tsl)* Genetic

Sexing Strain (GSS) mix 2002 strain, currently under production at the GVA mass-rearing facility (Caudete de las Fuentes, Valencia, Spain) were obtained two days prior to fly emergence. These puparia were marked with pink fluorescent dye (Day-Glo® Color Corp., Cleveland, OH, USA) so that released adults resulted in being marked with the dye by contact with the puparia during emergence. V8 pupae were irradiated under hypoxia using beta irradiation at a dose of 105±10 Gy, since at these doses the digestive physiology and the longevity of irradiated medflies is not affected (San Andrés et al. 2007b).

4.2.2. Tests in laboratory cages

A total of 600 <24 h old adult wt females, 600 wt males, and 3,000 sterile V8 males were maintained separately in Perspex cages (20 × 20 × 20 cm) with one ventilated window (16 × 16 cm) per cage in the lid. Each cage contained a maximum of 250 individuals. To simulate the real scenario, wt adult flies were fed with a mixed diet of sugar and hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; w:w) since it is assumed that they have access to sugar and protein supplies in the field (San Andrés et al. 2009). V8 males were fed with sugar, which is used as standard pre-release diet in the Spanish SIT program. Water and the corresponding diets were provided *ad libitum*. Wt adult flies were separated by sex in different rooms to prevent any effect due to contact with sex pheromones from the opposite sex until used (9 day-old for the wt females and 7 day-old for the wt males) that would interfere with posterior mating performance during the tests. Rooms were maintained at 25 ± 4ºC, 75 ± 5% RH with natural light. V8 males were maintained at 25 ± 4ºC, 75 ± 5% RH and complete

darkness in the emergence facility to simulate actual pre-release conditions until used (3 day-old). Likewise, sterile V8 males were subjected to aromatherapy treatment with Ginger Root Oil (GRO) [*Zingiber officinale* Roscoe (Zingiberaceae)] (dose of 320 µl/m^3) for 6 h in a large room (149 m^3) in which the GRO aroma was dispersed by air fans to enhance mating competitiveness (Juan-Blasco et al. 2012a). Laboratory tests were conducted in polyethylene (PE) cages (40 × 30 × 30 cm) with a ventilated area (21 × 15 cm) in the lid. Treatments consisted of applying different (wt female:wt male:sterile V8 male) ratios in individual cages. The ratios tested were 1:1:0, 1:1:1, 1:1:5, 1:1:10, and 1:1:20. These treatments consisted of 10 wt females, 10 wt males, and 0, 10, 50, 100, or 200 sterile V8 males. These treatments were performed on three different host fruit: apple (*Malus domestica*) cv. Royal Gala, clementine mandarin (*Citrus clementina* Hort. es Y. Tan.) cv. Marisol, and sweet orange (*Citrus sinensis* (L.) Osbeck) cv. Washington Navel. All fruit were of commercial quality. Three replicates were tested for each treatment and fruit host combination. On the first day of the test, 10 fruit were introduced per cage. A polypropylene (PP) tube (50 ml) full of water was attached to the base of the cage with plasticine (Plastilina Jovi JOVI, S.A. Barcelona, Spain) as water source. Afterwards, males (wt and V8) and females (wt) were introduced into the cages with an entomological aspirator. Males were introduced 15-30 minutes prior to females to allow them to scatter in the cage (FAO/IAEA/USDA 2003). The cages were kept for 48 hours in a climate-controlled room at 25 ± 4ºC, 75 ± 5% RH, and 16:8 h (L:D) photoperiod. After this period, flies were cold anesthetized (10ºC for 2 minutes) to facilitate the collection of the females and the fruit without affecting the survival of the eggs laid (Christenson and Foote 1960).

4.2.3. Test in field cages

After emergence, <24 h old adult wt females, wt males, and sterile V8 males were handled as described above. Prior to introduction into the field cages, the insects were handled in the following manner: newly emerged wt females (approximately 1,500) and wt males (approximately 900), were maintained in PP jars (1000 ml) with a ventilated area of 9 × 8 cm (Figure 2.1. B). A density of 50 individuals (females or wt males) per jar was used. Approximately 7,000 newly emerged sterile V8 males were maintained in PP jars (4000 ml) with a ventilated area of 12 × 11 cm with 250 sterile males (V8) per jar (Figure 4.1.).

Figure 4.1. Jars used to maintain Vienna-8 males from emergence to introduction into field cages.

All flies were kept under the same feeding regime and environmental conditions as above. V8 males were exposed to GRO as previously described. The field test was conducted in twenty-four 17 year-old trees

located in a commercial clementine mandarin orchard in Les Alqueries (Castelló, Spain). Trees were kept inside screened anti-thrips cages (3 × 3 × 3 m) with a zippered-door (1.5 m high) in one of the sides. The experimental trees were arranged in two rows of 12 connected cages (one side shared between adjacent cages) (Figure 4.2. A). The same five treatments (wt female:wt male:sterile V8 male ratios) described in the laboratory test were used. Treatments consisted of 50 wt females, 50 wt males, and 0, 50, 250, 500, and 1000 sterile V8 males, respectively. Based on the laboratory assay previously described, apples were selected as sentinel hosts for oviposition. The apples were individually fitted inside a PE mesh (holes of 1 cm diameter) and were randomly hung within the tree canopy. Ten sentinel apples were offered per tree. The tree cages were kept free of pests, and weeds were removed before the test following IPM guidelines (Urbaneja et al. 2012), though pesticide treatments were avoided. The ground community of arthropods naturally occurring in Spanish citrus orchards (Urbaneja et al. 2006, Monzó et al. 2011a, 2011b) were not prevented in the experimental tree cages. Three replicates were conducted per treatment with a random distribution of the replicates among the cages. The test started in the morning (≈ 15 min after sunrise) by hanging the 10 sentinel apples in each tree. Afterwards, males (wt and sterile V8) and females (wt) were released into the cages. Males were introduced 15-30 minutes prior to females to allow them to disperse and establish territories (FAO/IAEA/USDA 2003). After a 12 hour period (≈ 15 min before sunset) a Tephri trap (Sorygar S.L., Madrid, Spain) baited with the female-targeted attractant Biolure Tripack MedFly Lure® (Suterra Corporate, Bend, OR, USA) and 1/6 of a tablet of the insecticide dichlorvos (DDVP) (Suterra España Biocontrol S.L., Cerdanyola del Vallès, Spain) was

hung on each tree (Figure 4.2. B). The apples and the traps were removed two days later. The field test was carried out during July 2009.

Figure 4.2. A) Row of experimental trees inside screened cages. **B)** Experimental set up inside the field cages trees [The trap showed in this picture is the Mosquitrap (SanSan Prodesing S.L., Valencia, Spain) model].

4.2.4. Evaluation of laboratory and field tests

Two parameters were studied: 1) percentage of sterile mates by PCR in trapped females; and 2) reduction of the following generation, as number of puparia per fruit. For the first parameter, females were collected and stored in 70% ethanol at room temperature until processing. The sperm ID method of San Andrés et al. (2007a) was followed. In Brief, this method consists of: the extirpation of the female spermathecae under binocular lens followed by DNA extraction. This DNA was screened by PCR using the specific marker CcYsp which detects male sperm (wt or V8), followed by PCR with the specific marker Ccmt which detects presence of DNA from *C. capitata* in the sample, and finally, a digestion with the *HaeIII* enzyme of

the Ccmt PCR product. The digestion with *HaeIII* determines sperm identity, wt or V8 type, in the sample (Figure 4.3.). For the second parameter, the host fruit was placed individually in polystyrene cups (1 liter) during the period of time required for the development of the immature stages from egg to pupation. The bottom of each cup was filled with 100 ml of vermiculite (Europerlita Española S.A., Rubí, Spain) as pupation substrate, and covered with a mesh to avoid larvae jumping out of the cup. Cups were kept at 25 ± 4ºC, 75 ± 5% RH with natural light. The number of puparia inside each cup was counted weekly for 1 month. Additionally, this parameter provided information about fruit damage. Apples with ≥ 1 puparia were considered as damaged fruit.

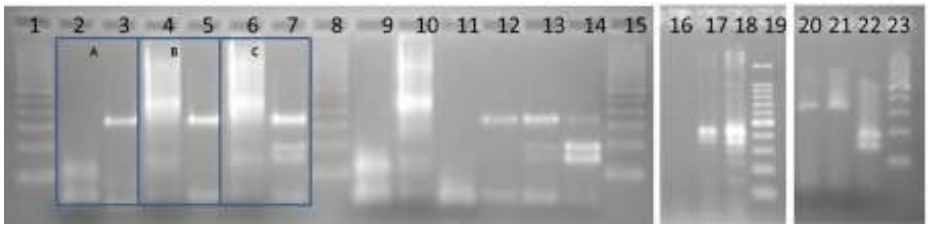

Figure 4.3. Amplification patterns for sperm ID in female spermathecae DNA extracts. Amplification pattern with markers CcYsp and Ccmt/*HaeIII* can be used to differentiate: **A)** unmated females (lane 2: negative amplification for CcYsp and lane 3: only one 330 bp band for Ccmt/*HaeIII*), **B)** wild-male mated female (lane 4: positive amplification for CcYsp and lane 5: only one 330 bp band for Ccmt/*HaeIII*), and **C)** Vienna-8 mated female (lane 6: positive amplification for CcYsp and lane 7: three bands of 330, 190 and 140 bp for Ccmt/*HaeIII*). Controls for routine sperm ID screening consisted on controlled mated females (lanes 9 to 14) or reference strains (wild-type and Vienna-8 (lanes 16 to 23) DNA extracts as available. In controlled mated females controls, those virgin females give negative amplification for CcYsp marker (lane 9), and mated females give positive CcYsp amplification

(lane 10). Ccmt/HaeIII marker negative control (no DNA in PCR reaction) was always included (lane 11), positive controls do include virgin female or wt-male mated female (lane 12), a double wt-male and Vienna-8 mated female (lane 13), and Vienna-8 mated female (lane 14). When using head DNA extracts as PCR controls the patterns obtained with CcYsp resulted negative for wt-female (lane 16), and positive for wt-male and Vienna-8 males (lanes 17 and 18 respectively). When considering the Ccmt/HaeIII with these head extracts, wt-males and females gave a single 330 bp band (lanes 20 and 21 respectively) whereas Vienna-8 male gave two bands of 190 and 140 bp (lane 22). Differences among Ccmt/HaeIII pattern in lane 22 and those in lanes 13 and 14 relies in the presence of 330 bp band originated in the last cases by the presence of female DNA from the spermathecae tissue, bands of 190 and 140 bp are only obtained when Vienna-8 mitochondrial DNA is present. Lanes 1 to 15 and 20 to 23 correspond to 2.5% agarose gel, whereas lanes 16 to 19 correspond to 2% agarose gel. Molecular weight marker (100 bp ladder, Invitrogen) was used for size reference in lanes 1, 8, 15, 19 and 23.

4.2.5. Statistical analysis

For laboratory tests, the detection of sterile V8 sperm in spermathecae of wt *C. capitata* female and F1 puparia obtained from fruit exposed to these females were considered as indicator variables of the mating efficacy of sterile V8 males. A Generalized Linear Mixed Model (GLMM) (Breslow and Clayton 1993) was used to analyze treatment and substrate effects on both variables in laboratory cages. Treatments (wt female:wt male:sterile V8 male ratio) and hosts (apple, clementine mandarin and sweet orange) were included in the model as fixed effects. The interaction between treatment and fruit host was also considered. Selection of the best model was based

on the Akaike Information Criterion (AIC). This criterion revealed that the Normal distribution was appropriate to analyze treatment and host effects.

For field cages, differences among treatments on sterile V8 sperm detections and F1 puparia were compared by one-way ANOVA. If necessary, means were subsequently separated with the least significant difference (LSD) test ($P < 0.05$). V8 sperm detection and F1 puparia values were subjected to the angular transformation and log (x+1), respectively to correct heterogeneity prior to ANOVA. The fitted values of the expected V8 sperm detection and of fruit with oviposition damage were obtained with a Generalized Linear Model (GLM) (McCullagh and Nelder 1989). Treatments were included as fixed effects. The AIC criterion indicated Binomial family as the appropriated model in this case.

4.3. Results

4.3.1. Tests in laboratory cages

The highest number of positive V8 sperm detections were obtained in the spermathecae of the females subjected to the 1:1:20 treatment for all fruit hosts tested. The maximum number of sterile V8 mating, measured as sperm detection, was obtained in cages containing clementine mandarins. In the 1:1:1 treatment, Vienna-8 sperm could be detected only in the citrus species tested (clementine mandarins and sweet oranges). In contrast, it was positive in the treatments 1:1:5, 1:1:10 and 1:1:20 for all fruit host used (Table 4.1.). Overall, the molecular detection of sterile V8 sperm in female spermathecae showed differences among sterile ratios (GLMM normal: $F_{4,24} = 4.143$; $P = 0.011$) and fruit hosts (GLMM normal: $F_{2,24} = 8.504$; $P = 0.002$).

The number of V8 sperm detections in female spermathecae in treatments 1:1:5, 1:1:10 and 1:1:20 showed differences compared to the control treatment 1:1:0 (no sterile males) but did not show differences among treatments with sterile males present. The number of mates achieved in cages with clementine mandarins was significantly different than those obtained in sweet oranges and apples (Table 4.2.). The interaction between the treatment and the fruit host did not have a significant effect on the number of V8 positive *C. capitata* females (GLMM normal: $F_{8,24} = 0.996$; $P = 0.464$).

The maximum number of puparia in the host was obtained from apples. The number of *C. capitata* puparia obtained for the citrus species tested under treatments 1:1:1, 1:1:5, 1:1:10 and 1:1:20 were below 1 pupa per fruit (Table 4.1.). The number of larvae that developed to pupae per fruit followed the same pattern showed by the positive molecular detections of sterile V8 sperm in females and also showed differences among treatments (GLMM normal: $F_{4,27} = 9.523$; $P < 0.0001$) and fruit hosts (GLMM normal: $F_{2,27} = 6.553$; $P = 0.005$). Fewer puparia were obtained for females exposed to treatments 1:1:5, 1:1:10 and 1:1:20 than for those subjected to the 1:1:0 treatment. In addition, the treatment 1:1:1 was also significantly different from 1:1:0 in reducing the number of F1 puparia per fruit. The number of puparia obtained from apples was significantly different to that obtained from females offered clementine mandarins and sweet oranges (Table 4.2.). The interaction between treatment and hosts did not have a significant effect on the number of puparia obtained (GLMM normal: $F_{8,27} = 1.964$; $P = 0.091$).

Table 4.1. Sterile Vienna-8 sperm detections in the spermathecae of *Ceratitis capitata* wild-type females and the number of puparia obtained in fruit exposed to these females in laboratory.

	Vienna-8 sperm detection (percentage ± SE)			Puparia/fruit (mean ± SE)		
[a]Treatment	Apple	Clementine mandarin	Sweet orange	Apple	Clementine mandarin	Sweet orange
1:1:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	10.50 ± 1.50	7.50 ± 3.50	5.00 ± 3.54
1:1:1	0.00 ± 0.00	27.50 ± 11.46	17.78 ± 9.69	9.00 ± 4.73	0.00 ± 0.00	0.00 ± 0.00
1:1:5	12.50 ± 12.50	64.29 ± 10.71	25.71 ± 16.74	1.33 ± 0.67	0.33 ± 0.33	0.33 ± 0.33
1:1:10	22.22 ± 14.70	41.67 ± 12.73	10.00 ± 10.00	1.67 ± 0.88	0.00 ± 0.00	0.00 ± 0.00
1:1:20	23.21 ± 17.34	63.89 ± 7.35	20.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.33	0.00 ± 0.00

[a]Treatments: 1:1:0 (10 wild-type females: 10 wild-type males: 0 Vienna-8 males); 1:1:1 (10 wild-type females: 10 wild-type males: 10 sterile Vienna-8 males); 1:1:5 (10 wild-type females: 10 wild-type males: 50 sterile Vienna-8 males); 1:1:10 (10 wild-type females: 10 wild-type males: 100 sterile Vienna-8 males); 1:1:20 (10 wild-type females: 10 wild-type males: 200 sterile Vienna-8 males).

Table 4.2. Probability values of contrasting by pairs the percentage of sterile Vienna-8 sperm detections in the spermathecae of wild-type *Ceratitis capitata* females and the number of puparia obtained in fruit exposed to these females in laboratory.

Factor	Contrast between factors	Vienna-8 sperm detection		Puparia/fruit	
		t_{24}	[b]*P*	t_{27}	[b]*P*
[a]**Treatment**	1:1:0 *vs* 1:1:1	1.510	0.144	3.335	0.002*
	1:1:0 *vs* 1:1:5	3.310	0.003*	5.002	< 0.0001**
	1:1:0 *vs* 1:1:10	2.386	0.025*	5.082	< 0.0001**
	1:1:0 *vs* 1:1:20	3.458	0.002*	5.399	< 0.0001**
	1:1:1 *vs* 1:1:5	2.050	0.051	1.864	0.073
	1:1:1 *vs* 1:1:10	1.025	0.316	1.953	0.061
	1:1:1 *vs* 1:1:20	2.215	0.037*	2.308	0.029*
	1:1:5 *vs* 1:1:10	0.988	0.333	0.089	0.930
	1:1:5 *vs* 1:1:20	0.159	0.875	0.444	0.661
	1:1:10 *vs* 1:1:20	1.147	0.263	0.355	0.725
Host	Apple *vs* Clementine mandarin	3.755	0.001*	2.819	0.009*
	Apple *vs* Sweet orange	0.402	0.691	3.377	0.002*
	Clementine mandarin *vs* Sweet orange	3.265	0.003*	0.557	0.582

[a]Treatments: 1:1:0 (10 wild-type females: 10 wild-type males: 0 Vienna-8 males); 1:1:1 (10 wild-type females: 10 wild-type males: 10 sterile Vienna-8 males); 1:1:5 (10 wild-type females: 10 wild-type males: 50 sterile Vienna-8 males); 1:1:10 (10 wild-type females: 10 wild-type males: 100 sterile Vienna-8 males); 1:1:20 (10 wild-type females: 10 wild-type males: 200 sterile Vienna-8 males). [b]Values followed by asterisk denote significant differences between factors according to LSD test (*$P < 0.05$, **$P < 0.0001$).

4.3.2. Test in field cages

Mean temperature and relative humidity registered inside the cages during the trial was 26 ± 4ºC and 75 ± 10%, respectively. The experiment on apples under field conditions showed percentages of detection above 50% of V8 sperm in all the treatments where sterile males were released (1:1:1, 1:1:5, 1:1:10 and 1:1:20). In relation to *C. capitata* progeny, puparia were obtained from apples under all the treatments tested. The V8 sperm detections in the analysed females under the treatments 1:1:1, 1:1:5, 1:1:10 and 1:1:20 were higher than those detected in control treatment 1:1:0 (ANOVA: $F_{4,12}$ = 28.239; P < 0.0001). The number of puparia obtained from apples was significantly lower under treatments 1:1:5, 1:1:10 and 1:1:20 than in treatments 1:1:1 and 1:1:0 (ANOVA: $F_{4,14}$ = 7.49; P = 0.005) (Table 4.3.). Furthermore, a positive relationship between the wt:sterileV8 male ratios and the proportion of V8 sperm detections obtained in the spermathecae of females was observed (GLM binomial: P = 0.002) (Figure 4.4.). At low wt female:wt male:sterile V8 male ratios (≤ 1:1:5), the probability of detection of V8 sperm in females trapped was below 50%, whereas the probability increased to values up to 84% when the male ratio in field was above 1:20 (wt:sterileV8) (Figure 4.4.). Interestingly, a negative relationship between the wt:sterile V8 male ratio and the number of damaged fruit was obtained (GLM binomial: P = 0.002) (Figure 4.5.). The percentage of damaged fruit dropped from 61% to 34%when the wt:sterile V8 ratio increased from 1:5 to 1:20.

Table 4.3. Sterile Vienna-8 sperm detections in the spermathecae of wild-type *Ceratitis capitata* females and the number of puparia obtained in fruit (apple) exposed to these females in field cages.

[a]Treatment	[b] Vienna-8 sperm detection (percentage ± SE)	[b] Puparia/fruit (mean ± SE)
1:1:0	0.00 ± 0.00d	229.67 ± 62.89a
1:1:1	55.00 ± 5.00c	183.00 ± 91.22a
1:1:5	64.44 ± 9.88bc	28.33 ± 12.41b
1:1:10	100.00 ± 0.00a	27.67 ± 2.91b
1:1:20	86.67 ± 10.18ab	21.00 ± 11.59b
Statistics	$F_{4,12}$ = 28.239; P < 0.0001	$F_{4,14}$= 7.490; P = 0.005

[a]Treatments: 1:1:0 (50 wild-type females: 50 wild-type males: 0 Vienna-8 males);
1:1:1 (50 wild-type females: 50 wild-type males: 50 sterile Vienna-8 males);
1:1:5 (50 wild-type females: 50 wild-type males: 250 sterile Vienna-8 males);
1:1:10 (50 wild-type females: 50 wild-type males: 500 sterile Vienna-8 males);
1:1:20 (50 wild-type females: 50 wild-type males: 1000 sterile Vienna-8 males).

[b]Values within column followed by different letters indicate statistical differences accordingly to LSD test (P < 0.05).

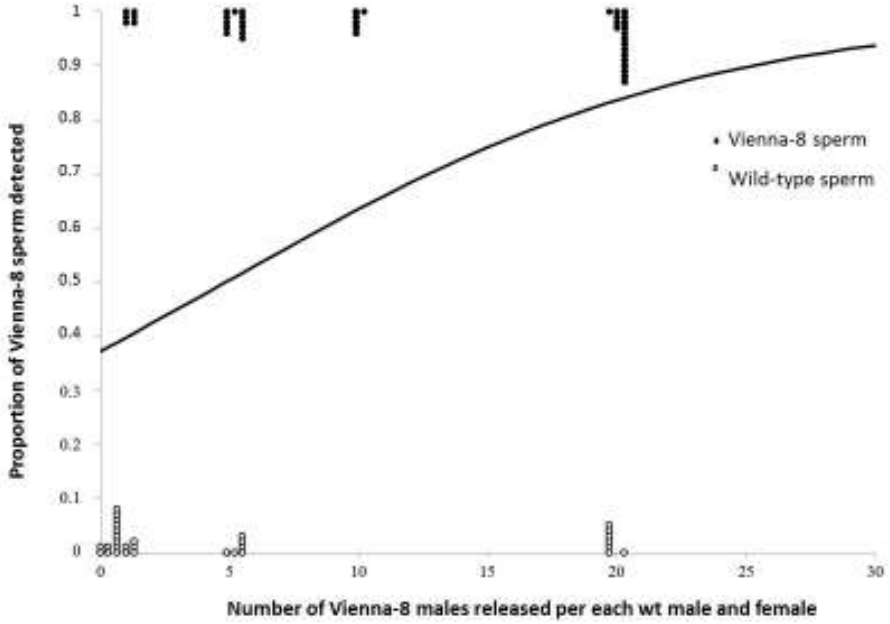

Figure 4.4. Fitted values of detected sterile Vienna-8 sperm in the spermathecae of *Ceratitis capitata* females exposed to different quantities of sterile and wild-type males in field cages. The equation $y = \exp(-0.523151 + 0.10772 \times x) / (1 + \exp(-0.523151 + 0.10772))$ ($P = 0.002$) explains the relation between the number of Vienna-8 sterile males released per wild-type males (x) and the identity of the sperm detected in the spermathecae of *C. capitata* females (y). The dots are the observed values (Vienna-8 sperm: black dots; Wild-type sperm: white dots).

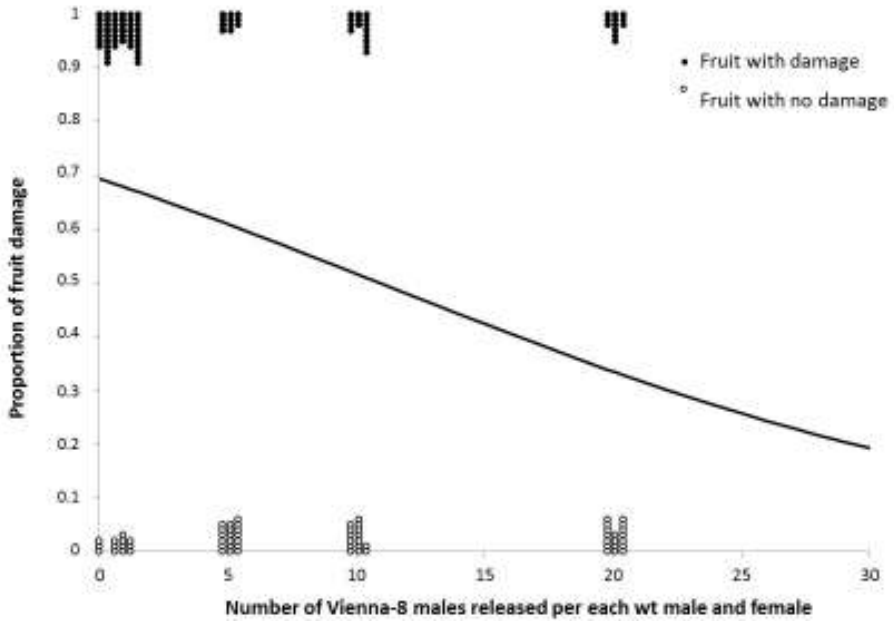

Figure 4.5. Fitted values of damaged apples var. Royal Gala caused by *Ceratitis capitata* females exposed to different quantities of sterile and wild-type males in field cages. The equation $y = \exp(0.81924 - 0.07516 \times x) / (1 + \exp(0.81924 - 0.07516 \times x))$ ($P = 0.002$) explains the relation between the number of Vienna-8 sterile males released per wild-type males (x) and the fruit damage. The dots are the observed values (Fruit with damage: black dots; Fruit with no damage: white dots).

4.4. Discussion

As predicted by the basic principle underlying the SIT (Knipling 1955), we have found that sterile releases became effective when the sterile males outnumbered wild males. Thus, sperm detection by molecular markers in female spermathecae increased progressively when male wt over sterile V8 male ratio exceed 1:1. However, the number of sperm detections in the laboratory was relatively low when compared to the results from the field cage test. This was probably due to overcrowding conditions inside laboratory cages that may have affected courtship success (Briceño and Eberhard 1998). Furthermore, the fruit host used affected the number of sterile V8 mates identified. Higher rates of V8 sperm were found in females offered citrus fruit (clementine mandarin and sweet orange) as compared to apple, probably related to the fact that *C. capitata* populations are commonly found in citrus orchards in Mediterranean countries (Franco et al. 2006, Jacas et al. 2010). This is supported by previous studies that described the positive effect on sterile male mating activity to exposure of citrus volatiles (Kouloussis et al. 2012, Juan-Blasco et al. 2012a, Rodríguez et al. 2011). However, the number of puparia obtained from citrus fruit was low in comparison with apple, as citrus fruit exhibit resistance mechanisms against the larval stage of tephritids due to mechanical barriers and toxic compounds present mainly in the flavedo, albedo and peel of the fruit (Papachristos et al. 2008). This finding is useful to screen the mating competitiveness of sterile V8 males under different wt female:wt male:sterile V8 male ratios and select an appropriate sentinel fruit host for quality control tests aimed at estimating the effectiveness of SIT under field conditions. Papadopoulos et al. (2002) and Zeki et al. (2008) had previously

demonstrated a good population development in apples, which are a main overwintering host crop for *C. capitata* in warmer sites of Israel (Israely et al. 1997), Greece (Papadopoulos et al. 2001b), Italy (Rigamonti 2004), Turkey (Zeki et al. 2008) and Spain (Escudero-Colomar et al. 2008, Peñarrubia-María et al. 2012). Our laboratory results are also in agreement with these observations, as the highest number of puparia was obtained in apples. In addition, the reduction observed in puparia numbers was related to the positive V8 male sperm detections in females.

Field cage experiments proved to be a useful tool to estimate V8 male mating success using the molecular method developed by San Andrés et al. (2007a). It was possible to observe under natural conditions how a relative increase in the number of sterile V8 males released in proportion to wt males lead to an increase in their mating success, measured as V8 male sperm identifications in females, and subsequently in a decrease in fruit damage (Figure 4.4. and 4.5.). Field cage experiments are known to be useful to obtain a detailed understanding of sterile male performance in the complex mating system of lek (male aggregations in mating arenas) species, as is the case of *C. capitata* (Prokopy and Hendrichs 1979, Hendrichs et al. 2002). The mating performance of sterile males includes traits such as the ability to compete in leks (Hendrichs et al. 2002) to effectively transfer sperm to females (Seo et al. 1990, Taylor et al. 2001) and to reduce the risk of female remating (Bonizzoni et al. 2002, Mossinson and Yuval 2003, Vera et al. 2003). Previous studies had stated the capacity of GRO-treated sterile *C. capitata* males to successfully compete with wt males by counting mating pairs in field cages (Shelly and McInnis 2001, Shelly et al. 2004a, Shelly et al. 2007e) and, under laboratory conditions by their capacity to avoid remating

of females (Morelli et al. 2012). In this study, the observed reduction in the number of puparia per fruit and the corresponding reduction of the following *C. capitata* generation, suggests that GRO-treated sterile V8 males were able to efficiently compete in leks, in transfering sperm, and in remating under natural conditions (data not shown). Furthermore, the number of puparia per fruit obtained revealed that the effects of these factors on the *C. capitata* mating scenario in the field did not mask the results of the sterile mates identified with the molecular method. In addition, our field cage test demonstrates that sterile V8 males (≈ 3-6 day-old during the field cage test) were able to evade the risk of mortality and/or disruption of lek activities by other co-ocurring arthropods (i.e., ants, earwigs and spiders were observed during the test inside the tree cages) and therefore were able to achieve mating with females after their release (Hendrichs et al. 2007, Monzó et al. 2010). Good rates of survival after release of sterile males (≈ 4-5 day-old) that were trap-captured in open field had been reported (Paranhos et al. 2010).

Our field cage test has been shown to be a good candidate to be implemented routinely in ongoing SIT programs. The test was useful to estimate the likelihood of a female obtaining sterile mating and the overall competitiveness of these matings based on the quantification of the population of the following *C. capitata* generation. Further research is required to verify if the results obtained with this study are confirmed under different agro-ecological conditions to which sterile V8 males are exposed in the field.

Chapter 5

5. Models to correlate the effect of sterile matings with *Ceratitis capitata* offspring reduction

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5.1. Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) is widely distributed among fruit growing areas throughout the world (EPPO 2012) and has a wide host range (Liquido et al. 1991, Aluja and Mangan 2008). This insect causes direct damage mostly to fruit and therefore it is considered an economically major pest in fruit production (White and Elson-Harris 1992, Malacrida et al. 2007). *Ceratitis capitata* is highly mobile and shows resistance to a wide range of environmental conditions which facilitates its prevalence and ability to reinvade pest-suppressed areas (He and Haymer 1999, Papadopoulos et al. 2001b, Meixner et al. 2002, Epsky et al. 2010, Martínez-Ferrer et al. 2010, Mazzi and Dorn 2012). In addition, *C. capitata* is characterized by a mating system based on complex traits regarding leks, remating and sperm transfer (Burk and Calkins 1983, Hendrichs et al. 2002). Damage caused to fruit and the difficulties in managing this pest are substantial enough to consider the application of large-scale projects focused on reducing or suppressing the populations of this insect (Myers 1998, Hendrichs et al. 2007).

The area-wide integrated pest management (aw-IPM) is a strategy widely applied in several countries to manage *C. capitata* populations throughout the ecosystems (Klassen 2005). In Spain, after years of an aw control program based on insecticide applications, an aw-IPM program has been implemented to reduce the long term costs of its control (Urbaneja et al. 2009, Navarro-Llopis et al. 2011). The application of tactics designed to interfere with mating as a method to reduce insect populations [(e. g., Sterile Insect Technique (SIT)] is a strategy often considered against pest

species of economic importance (Liebhold and Tobin 2008, Yamanaka and Liebhold 2009). Sucessful aw-IPM programs designed against *C. capitata* in Spain and elsewhere rely on the application of the SIT (Hendrichs et al. 2005). The SIT is a control method based on the release of sterile insects to reduce the population of a pest by means of reducing the number of matings between fertile males and fertile females, resulting in a reduction of viable offspring (Knipling 1955). In the context of aw-IPM, the population reduction obtained in economically important pest populations should vastly outweigh the cost of establishing a SIT program which needs mass-rearing of sterile insects and regular aw releases (Mumford 2005, Nagel and Peveling 2005). Therefore, tools to accurately measure how sterile males interact with wild populations of *C. capitata* at the field scale are needed in order to apply this technique successfully and cost effectively (Vreysen 2005).

The fact that for SIT to be a viable management technique, the success of mating interference must be measured, makes the evaluation of SIT efficacy a complex aim to achieve (Katsoyannos et al. 1999, Vreysen 2005, Yamanaka and Liebhold 2009). Currently, SIT programs obtain information about the density of the target wild population using a monitoring trap network distributed throughout the release area. This trap network is also used to measure the relative abundance of sterile flies using mark and recapture methods. Finally, program managers use these data to assess the number of sterile males for release (theoretical wild type:sterile male ratios) that will be effective to reduce pest population (Calkins and Parker 2005, Itô and Yamamura 2005, Vreysen et al. 2005). However, the quality of the released sterile males in the field, specifically their sexual

competitiveness, is much more important than the overflooding ratio (Barclay 2005, Itô and Yamamura 2005). Due to their dependence on sterile males reproductive performance, ongoing SIT programs should regularly measure the sexual competitiveness of sterile strains competing with wild strains in field-cages (FAO/IAEA/USDA 2003, Calkins and Parker 2005). Moreover, environmental variables play a determinant role in other biological traits of fruit flies such as mating behavior, population dynamics, breeding areas, distribution, or movement patterns (Christenson and Foote 1960, Mazzi and Dorn 2012). Therefore, the development of more effective monitoring tools including environmental and behavioral ecology traits such as sexual competitiveness, could greatly contribute to avoid aw-SIT control failures (Hendrichs and Hendrichs 1990, Vargas et al. 1983, Yamagishi et al. 1993, Papadopoulos et al. 2001b, Barry et al. 2004, Itô and Yamamura 2005, Malacrida et al. 2007).

To date, the impact of the sexual competitiveness of the sterile males on offspring has been indirectly measured only by counting the inherent sterility of eggs in fruit and the wild:sterile ratio in traps in the release area (Wong et al. 1986, McInnis et al. 1994, Rendón et al. 2004, Shelly et al. 2007e). Nowadays, the use of molecular techniques expand the possibilities to reliably evaluate the role of sterile males in the Thephritidae *Anastrepha suspensa* (Loew) and *Bractocera dorsalis* (Hendel) under natural conditions (Fritz et al. 2010, Aketarawong et al. 2011). A Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method has been developed to measure the sexual competitiveness of sterile *C. capitata* males based on identifying the sperm content [wild or sterile Vienna-8 (V8) male] in the spermathecae of field trapped females (San Andrés et al.

2007a). Models could help us to understand the mating behavior of the sterile males released in the environment and therefore their impact on wild populations. Given that offspring reduction by the SIT is a function of the number of matings between sterile males and wild females (McInnis et al. 1994), an additional step was obtained by Juan-Blasco et al. (2012b) (see *Chapter 4* in this document) which validated the San Andrés et al. (2007a) method which related the relative increase of mating success of V8 males with a decrease in fruit damage.

The purpose of this study was to clarify which environmental factors determine the mating activity of sterile males after release and then, to include these relevant environmental factors in new more efficient tools to further improve the evaluation of SIT efficacy. As a consequence, the main objective of this research has been to model the population reduction of *C. capitata* due to the mating success of sterile males using sterile mating data obtained under field conditions.

5.2. Material and methods

The study was conducted from June 2010 to June 2011 in Moncada (Valencia, Spain) where *C. capitata* is a prevalent pest (Urbaneja et al. 2009) under an aw-SIT program. Ten field cage tests following the same handling methods, experimental set-up, and evaluation described in Juan-Blasco et al. (2012b) (see *Chapter 4* in this document) were carried out every ~ 30 days under different environmental conditions.

5.2.1. Field cage site

The field trial was conducted in an experimental clementine mandarin [(*Citrus clementina* Hort. es Y. Tan.) cv. Clemenules] orchard. The site included eighteen, 32-year old clementine mandarin trees distributed in a row. Each tree was equipped with an anti-thrips screen cage (3 × 3 × 3 m) with a zippered-door (1.5 m high) on one of the sides of the cage. These trees were drip-irrigated and fertilized, weeds were mechanically removed and pesticide treatments were avoided. Trees were pruned to prevent the canopies from touching the walls of the cages or the soil. The physical barrier Tangle-Trap® (Bioestimulantes Agrícolas S.L., Massalfassar, Valencia, Spain) was sprayed monthly on the trunk to exclude ants (Juan-Blasco et al. 2011) due to the high number of individuals observed on the trees prior to the trial.

5.2.2. Environmental conditions

Two data logger instruments (mod. Testo 175-H2, Testo Ltd., Alton, Hampshire, UK) were randomly placed in the canopy of two trees of the selected row to measure mean temperature and relative humidity inside the cages during the test (Table 5.1.). Daily minimum and maximum temperatures, hours of daylight (number of hours from sunrise to sunset), cold hours (hours below 7ºC), precipitation and evapotranspiration during the test were obtained from the IVIA Irrigation Technology Service Moncada station (STR-IVIA) (UTMX: 23368.000, UTMY: 4385233.000, 58 m asl) located 150 m south from the caged trees site (Table 5.1.).

Table 5.1. Mean values of the environmental parameters included in our study during the field cage tests (two days per assay).

Date	Mean Temperature (Temp)* (ºC)	Maximum Temp (ºC)	Minimum Temp (ºC)	Relative Humidity* (%)	Hours of daylight[a] (h)	Cold Hours[b] (h)	Precipitation[c] (mm)	Evapotranspiration[c] (mm)
Jun-2010	25	31	17	61	13	0	0	5
Sep-2010	27	32	16	60	12	0	0	5
Nov-2010	16	24	8	61	9	0	0	2
Dec-2010	9	13	4	86	5	9	2	1
Jan-2010	8	17	3	87	7	10	0	1
Feb-2011	7	16	3	75	8	14	0	1
Mar-2011	10	16	2	51	10	7	0	2
Apr-2011	18	23	9	67	12	0	0	3
May-2011	23	24	10	65	13	0	0	4
Jun-2011	22	25	13	71	11	0	2	4

[*] Measured inside field cages.
[a] Number of hours from sunrise to sunset.
[b] Number of hours under 7ºC.
[c] Total precipitation.
[c] Evapotranspiration of reference, calculated using Penman-Monteith method.

5.2.3. Insect strains

The insects used in the trials were obtained from the SIT mass-rearing and emergence facilities located in Caudete de las Fuentes (Valencia, Spain) and in Moncada (Valencia, Spain), respectively. The mass-rearing facility of Caudete de las Fuentes is designated to mass-rear the sterile V8 temperature sensitive lethal (*tsl)* Genetic Sexing Strain (GSS) mix 2002 strain of *C. capitata* from egg to pupa. During the mass-rearing process, sterile V8 puparia are marked with pink fluorescent dye (Day-Glo® Color Corp., Cleveland, OH, USA). As a consequence, male adults result marked with the dye during emergence. Sterile V8 puparia are irradiated under hypoxia at a dose 105±10 Gy. The SIT emergence facility hosts the emergence of the sterile male adults and their rearing until field release. In the present study, sterile V8 males were separated after emergence in two types of polypropylene (PP) jars; 4000 ml jars with a ventilated area of 12 × 11 cm with 250 males per jar, and 1000 ml jars with a ventilated area of 9 × 8 cm with 50 males per jar. V8 males were fed with sugar and water *ad libitum*, the standard pre-release diet in the Spanish SIT program. Moreover, to simulate actual pre-release conditions at the emergence facility, V8 males were maintained in complete darkness and were subjected to aromatherapy treatment with Ginger Root Oil (GRO) [*Zingiber officinale* Roscoe (Zingiberaceae)] (Lluch Essence S.L., Prat de Llobregat, Barcelona, Spain) to enhance mating competitiveness (Juan-Blasco et al. 2012a). The SIT emergence facility also hosted a laboratory colony of the wild-type strain (wt) of *C. capitata* refreshed annually with wild flies collected from field-infested fruit. Wt newly emerged males and females [generation V-IX (year 2010); generation III-VI (year 2011)] were separated

by sex in 1000 ml PP jars as before. Jars containing wt flies were maintained at 25 ± 4ºC, 75 ± 5% RH with natural light in different rooms to prevent any effect due to contact with sex pheromones from the opposite sex. Wt adult flies were fed *ad libitum* with water and a mixed diet of sugar and hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; w:w) to simulate field natural food sources (San Andrés et al. 2009).

5.2.4. Field cage test

The first day of the test, 10 sentinel apples (commercial quality) were individually fitted inside a polyethylene (PE) mesh (holes of 1 cm diameter) and randomly hung within the canopy of each caged tree. Soon after, *C. capitata* males (wt and sterile V8) were released inside the cages and 15-30 minutes later females (wt) were introduced. Five different treatments or *C. capitata* ratios 1:1:0, 1:1:1, 1:1:5, 1:1:10, and 1:1:20 (wt female:wt male:sterile V8 male) were considered. Treatments consisted of 50 wt females, 50 wt males, and 0, 50, 250, 500, or 1000 sterile V8 males, respectively. A female-control treatment 1:0:0 (no males) was included. Three replicates per treatment were included in each test. Replicates were randomly distributed among the eighteen cages. V8 males were 3 day-old which corresponds with the release age in the Spanish SIT program, to simulate real field conditions (Juan-Blasco et al. 2012a). Wt males and wt females were 7 and 9 day-old, respectively, when are considered to be sexually mature (Shelly et al. 2007a). Twelve hours after the release of the females, a Mosquitrap (SanSan Prodesing S.L., Valencia, Spain) baited with BioLure Tripack MedFly Lure® (Suterra Corporate, Bend, OR, USA) and with 1/6 of a tablet of the insecticide dichlorvos (DDVP) (Suterra España

Biocontrol S.L., Cerdanyola del Vallès, Spain) was hung on each tree. The apples and the trap were removed from trees two days after the start of the test. The baited traps used in every test were hung back in the trees between tests to capture flies that remained alive inside the caged trees.

5.2.5. Capture of wt females in traps

Females captured in the traps were used to study the mating activity of V8 males following the method developed by San Andrés et al. (2007a). Females were collected from traps and stored in 70% ethanol at room temperature until their spermathecae were dissected under binocular microscope.

5.2.6. Male sperm identification (Sperm ID)

The DNA from each spermatheca was extracted to identify the origin of its sperm content by means of PCR. Two PCRs with the specific markers CcYsp and Ccmt, and a digestion with the *HaeIII* enzyme determined sperm identity (wt or V8) (Figure 4.3.).

5.2.7. F1 generation

The apples exposed to females under the different treatments in the caged trees were individually placed in isolated cups with vermiculite (Europerlita Española S.A., Rubí, Spain) as pupation substrate. Cups were maintained at 25 ± 4ºC, 75 ± 5% RH with natural light. The number of puparia obtained from each fruit was counted weekly for 1 month.

5.2.8. Statistical analysis

The number of females captured in traps, the number of mated females that of those whose spermathecae contained V8 sterile sperm, and the number of F1 puparia per fruit were counted for each tree throughout the ten field cage tests as described before. These values were the basis of the model developed to explain the mating efficacy of V8 males in the field. As a first step, these values were expressed as proportions per tree. Subsequently, treatments were compared with a chi-square test for homogeneity of proportions. To investigate if the mating efficacy of V8 males in the field was significantly affected by the treatment and environmental variables, a generalized linear mixed model (GLMM) (Breslow and Clayton 1993) was performed for each measurement. GLMMs were chosen because data were not normally distributed and because allowed us to incorporate random effects. The incorporation of random effects served to analyze the variation among mating efficacy more than quantify the exact effect of each determinant variable. The month in which each test was carried out was considered the random effect in all the GLMMs due to the presence of immeasurable effects in the field which would affect the set of data obtained. Treatments (wt female:wt male:sterile V8 male ratio) and environmental variables were included as fixed effects. For the F1 puparia, an additional GLMM was obtained considering the proportion of positive V8 sperm detections in spermathecae and environmental variables as fixed effects. Selection of the best model was based on the Akaike Information Criterion (AIC). Binomial GLMM were used to model the number of females captured in traps and

the detection of sterile V8 sperm in spermathecae meanwhile Poisson GLMM were used to analyze the F1 puparia.

Correlations among the environmental variables recorded during the days of the tests were studied to include the influence of these factors on the activity of the flies. It was possible to obtain simplest models by including only one of the correlated variables when two or more variables resulted correlated. It was also analyze the correlation between the females mated per tree and the females mated with V8 males per tree.

The predicted values of F1 puparia (determined from positive V8 sperm detections and environment data) were used to calculate the percentage of *C. capitata* population reduction in treated trees using Abbott's formula (Abbott 1925). The population reduction was used to estimate the impact of release sterile males (treatments 1:1:1, 1:1:5, 1:1:10, and 1:1:20) compared to control (treatment 1:1:0). In addition, a logarithmic regression was performed to further investigate the relationship between the percentage of *C. capitata* population reduction and the proportion of V8 sperm positive detections in the field.

All statistical analyses were performed using the software R (R Development Core Team 2011).

5.3. Results

5.3.1. Environmental conditions

The range of environmental data registered along the tests is shown in Table 5.1. Mean temperature measured during the tests was strongly correlated with cold hours (r = - 0.80; P < 0.0001), minimum temperature (r

= 0.91; $P < 0.0001$), maximum temperature ($r = 0.84$; $P < 0.0001$), hours of daylight ($r = 0.72$; $P < 0.0001$), evapotranspiration ($r = 0.88$; $P < 0.0001$), and relative humidity ($r = -0.59$; $P < 0.0001$). Precipitation ($r = -0.05$; $P < 0.0001$) was marginally correlated with mean temperature. Relative humidity and precipitation were poorly correlated as well ($r = 0.23$; $P < 0.0001$).

5.3.2. Capture of wt females in traps

Throughout the field cage tests, 709 wt females were captured in traps when they were exposed to the different male ratios (wt:V8). Females were captured under the entire range of observed temperatures from 7ºC to 27ºC. The maximum number of females captured in a single trap was 36 in the treatment 1:0:0 at a mean temperature of 16ºC. The mean number of captures per tree is shown in Table 5.2. A. The GLMM analysis revealed that treatment, mean temperature, and relative humidity ($P < 0.0001$) had a significant effect on the number of females captured, whereas precipitation ($P > 0.05$) did not. The expected number of captures was higher under the treatment 1:0:0 compared to treatments 1:1:0, 1:1:1, 1:1:5, 1:1:10, and 1:1:20 ($P < 0.0001$). Under all treatments tested, the probability to capture wt females in traps gradually increased with temperature (Figure 5.1.).

5.3.3. Sperm ID

347 females out of the 709 captured proved positive for molecular sperm detection (Table 5.2. B). V8 sperm was detected in females (n = 127) exposed to treatments where sterile males were released (1:1:1, 1:1:5, 1:1:10, and 1:1:20) (Table 5.2. C). The detection of V8 sperm in females was

strongly correlated to the number of females mated (wt or V8 male) per tree under the treatments 1:1:1 (r = 0.67; P < 0.0001), 1:1:10 (r = 0.76; P < 0.0001) and 1:1:20 (r = 0.89; P < 0.0001) (Figure 5.2.). However, this relation was not strong in the treatment 1:1:5 (r = 0.31; P < 0.0001). No significant differences were not found between treatments 1:1:10 and 1:1:20 in the number of positive V8 sperm detections (χ^2 = 8.805; df = 7; P = 0.267). The analysis of the spermathecae content indicated that V8 matings were determined by treatment and mean temperature (P < 0.0001) and were not influenced by relative humidity (P > 0.05) and precipitation (P > 0.05). The probability to detect V8 sperm increased with mean temperature in all treatments where V8 males were released. At a mean temperature of 9ºC and below, the probability of obtaining positive V8 sperm detections in treatments 1:1:10 and 1:1:20 was below 50%. Contrarily, V8 sperm detection was above 80% when mean temperature reached 22ºC (Figure 5.3.).

5.3.4. Puparia from F1 generation

Puparia were only obtained in treatments where females were exposed to males (wt or V8) and this proves that no wild males entered the cages during the assays. No puparia were obtained from fruit for mean temperatures between 7ºC and 10ºC (Table 5.2. D). Treatment and mean temperature had a significant effect on the number of puparia obtained (P < 0.0001). As expected, the highest number of puparia was predicted in treatment 1:1:0 compared to treatments 1:1:1, 1:1:5, 1:1:10 and 1:1:20 (P < 0.0001). Furthermore, significant differences from homogeneity were found among ratios where V8 males were released (Chi-square test, P < 0.0001).

For all treatments, the number of puparia was increased when mean temperature rose. Under all the temperatures tested, the number of puparia predicted in treatment 1:1:10 and 1:1:20 were 3 and 7-fold lower, respectively, compared to treatment 1:1:0. The estimations indicated less than 1 puparia/fruit at temperatures below 14ºC independently of the male ratio (wt:V8) used. For treatment 1:1:20, the probability of obtaining less than 1 puparia/fruit was estimated for mean temperatures up to 19ºC (Figure 5.4.).

Table 5.2. Mean number of *Ceratitis capitata* females captured in traps **(A)**, proportion of mated females identified by means of molecular sperm detection in their spermathecae **(B)**, proportion of V8 sperm detection **(C)**, and number of F1 puparia obtained from fruit exposed to females **(D)** at different fly ratios under different mean temperatures inside field-cages.

A)

	Females captured in traps (mean ± SE)					
Mean Temperature (ºC)	**Control**	**Treatment (wild female:wild male:sterile V8 male ratio)**				
	1:0:0	**1:1:0**	**1:1:1**	**1:1:5**	**1:1:10**	**1:1:20**
7	4.00 ± 1.53	0.67 ± 0.67	1.33 ± 0.33	4.00 ± 1.53	0.33 ± 0.33	1.00 ± 0.58
8	4.00 ± 1.53	1.33 ± 0.33	2.67 ± 1.76	1.50 ± 0.41	2.00 ± 0.58	0.33 ± 0.33
9	5.33 ± 2.19	2.33 ± 0.88	1.50 ± 0.41	2.33 ± 0.88	2.00 ± 0.58	3.50 ± 1.22
10	0	0.33 ± 0.33	0	1.67 ± 0.88	0.33 ± 0.33	1.00 ± 0.58
16	26.33 ± 8.21	2.33 ± 1.33	3.33 ± 0.88	3.33 ± 0.88	0.67 ± 0.33	4.50 ± 0.41
18	15.00 ± 5.72	5.50 ± 2.86	1.50 ± 0.41	2.00 ± 1.00	3.00 ± 0.82	3.33 ± 1.45
19	15.50 ± 4.50	11.33 ± 8.88	4.00 ± 2.52	2.67 ± 0.33	3.00 ± 1.15	3.33 ± 1.45
23	9.33 ± 4.63	10.00 ± 1.53	10.00 ± 1.15	3.33 ± 0.33	2.67 ± 1.20	8.00 ± 2.08
25	4.00 ± 0.00	1.33 ± 0.33	4.00 ± 1.73	4.00 ± 2.31	3.67 ± 0.88	4.67 ± 0.88
27	12.33 ± 3.53	1.67 ± 0.33	1.33 ± 0.88	1.00 ± 0.00	1.00 ± 0.00	1.67 ± 1.20

B)

	Mated females (percentage ± SE)					
Mean Temperature (ºC)	**Control**	**Treatment**				
	1:0:0	**1:1:0**	**1:1:1**	**1:1:5**	**1:1:10**	**1:1:20**
7	0	0	50.00 ± 28.87	0	0	25.00 ± 25.00
8	0	0	28.33 ± 14.81	0	8.33 ± 8.33	0
9	0	33.33 ± 33.33	16.67 ± 16.67	25.00 ± 25.00	31.19 ± 4.52	42.38 ± 11.38
10	-	0	-	33.33 ± 33.33	0	33.33 ± 33.33
16	0	93.33 ± 6.67	82.22 ± 9.69	100	100	91.67 ± 8.33
18	0	100	80.30 ± 15.38	83.33 ± 16.67	100	88.89 ± 11.11
19	0	100	100	100	100	100
23	0	100	86.39 ± 3.41	80.56 ± 10.02	66.67 ± 33.33	87.78 ± 6.19
25	0	100	95.24 ± 4.76	100	100	100
27	0	66.67 ± 33.33	33.33 ± 33.33	100	66.67 ± 33.33	87.50 ± 12.50

C)

	Vienna-8 sperm detection (percentage ± SE)					
Mean Temperature (ºC)	**Control**	**Treatment**				
	1:0:0	**1:1:0**	**1:1:1**	**1:1:5**	**1:1:10**	**1:1:20**
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	100	66.67	0	100
10	-	0	-	50.00	0	33.33 ± 20.41
16	0	0	25.00 ± 14.43	30.00 ± 30.62	100	41.67 ± 28.87
18	0	0	0	80.00 ± 20.41	46.15	77.78 ± 14.70
19	0	0	8.33 ± 3.70	12.50 ± 11.11	100	70.00 ± 8.68
23	0	0	38.46 ± 11.22	37.50 ± 33.33	71.43 ± 12.25	95.24 ± 3.33
25	0	0	9.09 ± 8.33	16.67 ± 5.10	63.64 ± 26.67	78.57 ± 11.60
27	0	0	0	100	0	75.00 ± 13.61

D)

	Puparia/fruit (mean ± SE)					
Mean Temperature (ºC)	**Control**	**Treatment**				
	1:0:0	**1:1:0**	**1:1:1**	**1:1:5**	**1:1:10**	**1:1:20**
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
16	0	61.65 ± 0.20	14.03 ± 1.34	14.73 ± 5.97	21.37 ± 13.60	7.90 ± 3.29
18	0.40 ± 0.33	46.57 ± 19.75	50.97 ± 6.22	15.57 ± 4.14	10.23 ± 2.85	5.17 ± 2.37
19	0	22.13 ± 8.94	13.23 ± 2.76	9.20 ± 5.32	1.23 ± 0.43	2.03 ± 1.31
23	0	70.60 ± 20.95	39.90 ± 13.49	14.63 ± 5.80	9.10 ± 5.28	10.53 ± 1.91
25	0	43.72 ± 5.14	54.97 ± 8.11	25.67 ± 7.57	28.60 ± 10.98	8.63 ± 1.56
27	0	10.70 ± 0.82	7.77 ± 3.77	3.53 ± 1.64	4.90 ± 2.48	0.83 ± 0.47

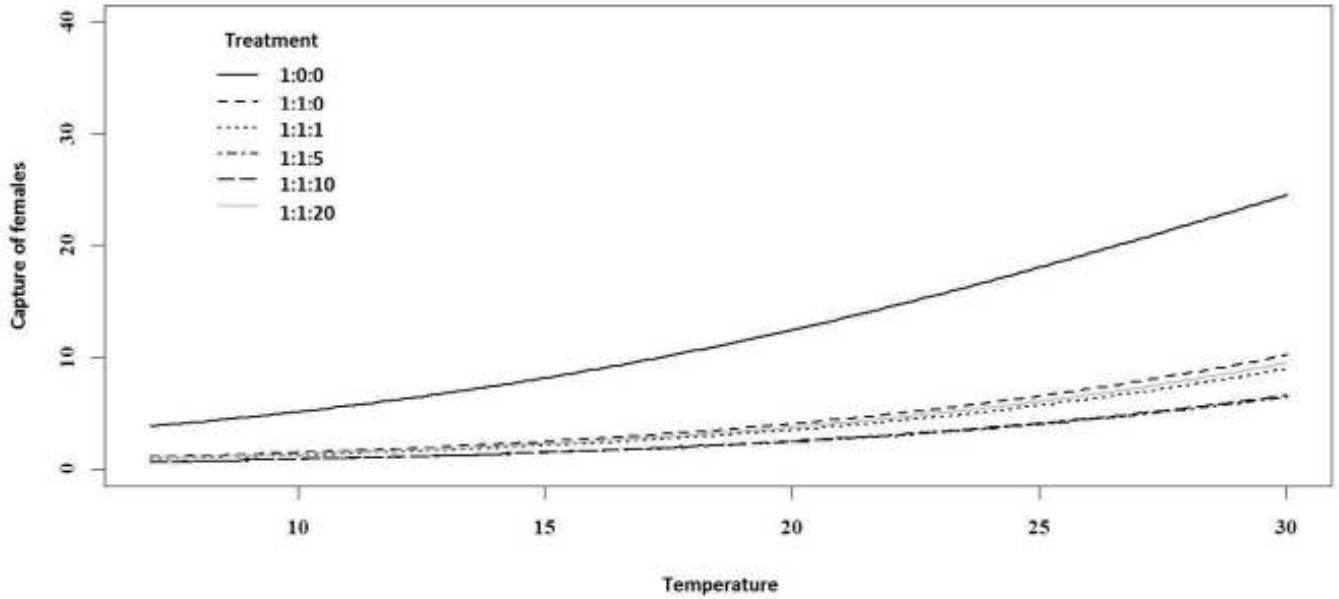

Treatment
1:0:0
1:1:0
1:1:1
1:1:5
1:1:10
1:1:20
Capture of females
0
10
20
30
40
10
15
20
25
30
Temperature

Figure 5.1. Probability of capture *Ceratitis capitata* females in traps at different treatments consisting of fly ratios of 1:0:0, 1:1:0, 1:1:5, 1:1:10, and 1:1:20 (number indicate relative ratios of wild female:wild male:sterile Vienna-8 males) and in relation to temperature and relative humidity. For each treatment the number of released females was 50 individuals, with the corresponding males according the above established ratios. The female capture (*y*) probability is calculated by the formula $y = 50 \times e^{(-7.32 + 0.11 \times \text{Temperature} + 0.06 \times \text{Relative Humidity} - 1.33 |_{1:1:0} - 1.48 |_{1:1:1} - 1.87 |_{1:1:5} - 1.85 |_{1:1:10} - 1.41 |_{1:1:20})} / (1 + e^{(-7.32 + 0.11 \times \text{Temperature} + 0.06 \times \text{Relative Humidity} - 1.33 |_{1:1:0} - 1.48 |_{1:1:1} - 1.87 |_{1:1:5} - 1.85 |_{1:1:10} - 1.41 |_{1:1:20})})$.

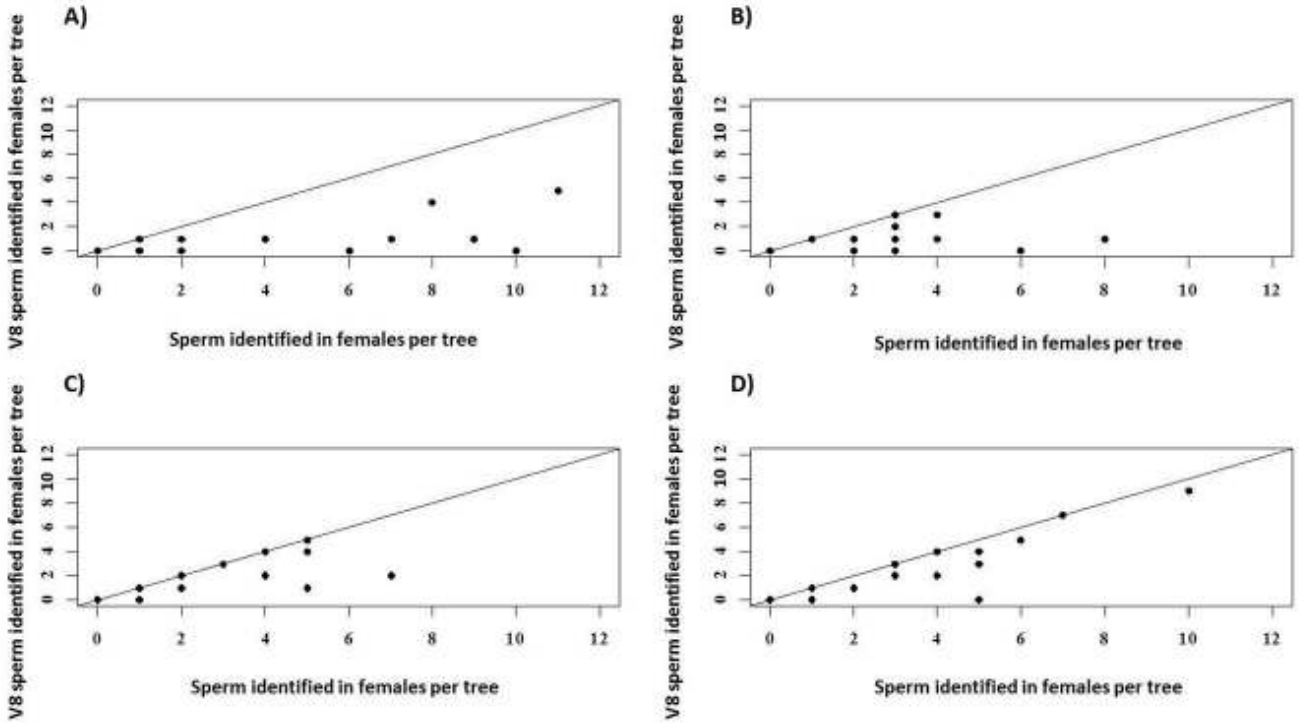

A)
B)
C)
D)
V8 sperm identified in females per tree
Sperm identified in females per tree
0 2 4 6 8 10 12

Figure 5.2. Relationship between the number of females mated per tree (wild-type or Vienna-8) and the number of females mated with Vienna-8 males per tree under treatments **A)** 1:1:1, **B)** 1:1:5, **C)** 1:1.10 and **D)** 1:1:20 (wild-type female:wild-type male:sterile Vienna-8 male).

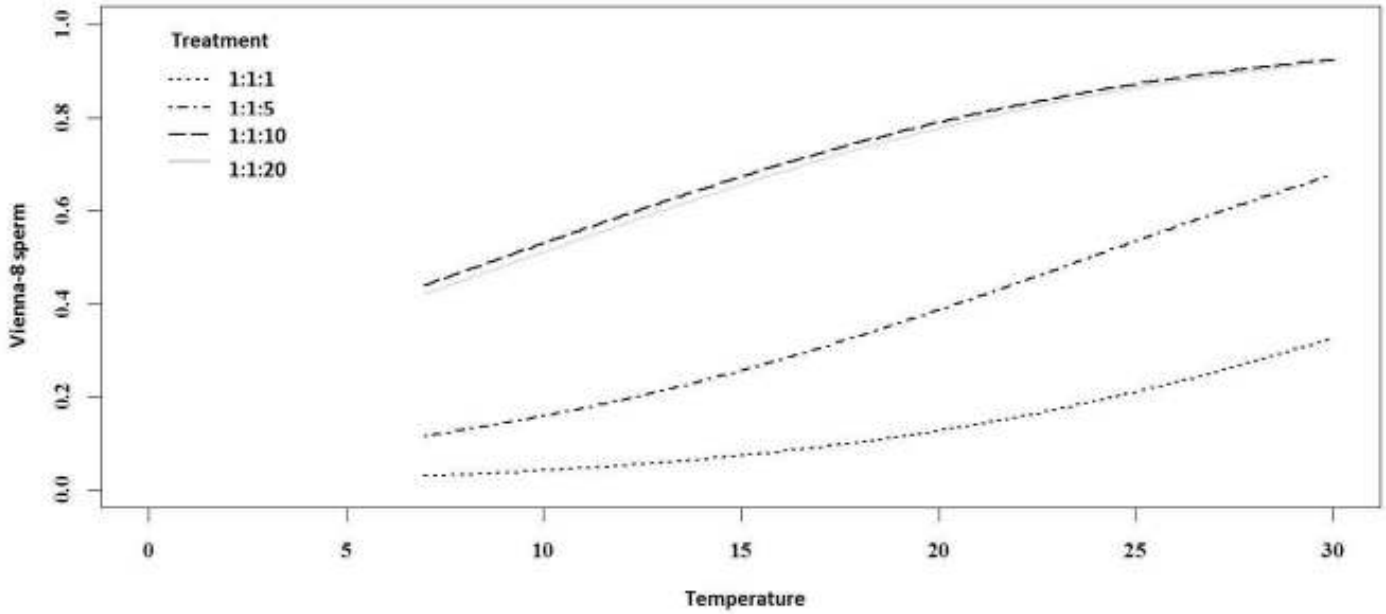

Treatment
1:1:1
1:1:5
1:1:10
1:1:20
Vienna-8 sperm
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
20
25
30
Temperature

Figure 5.3. Proportion of sterile Vienna-8 sperm detections in spermathecae of *Ceratitits capitata* females at different fly ratios 1:0:0, 1:1:0, 1:1:5, 1:1:10, and 1:1:20 (wild female:wild male:sterile Vienna-8 male) and in relation to temperature. For each treatment the number of released females was 50 individuals, with the corresponding males of each type according to the above indicated ratios. The probability of obtain a proportion of Vienna-8 sperm ID (*y*) is calculated by the formula $y = e^{(-4.34 + 0.12 \times \text{Temperature} + 1.46 |_{1:1:5} + 3.25 |_{1:1:10} + 3.18 |_{1:1:20})} / (1 + e^{(-4.34 + 0.12 \times \text{Temperature} + 1.46 |_{1:1:5} + 3.25 |_{1:1:10} + 3.18 |_{1:1:20})})$.

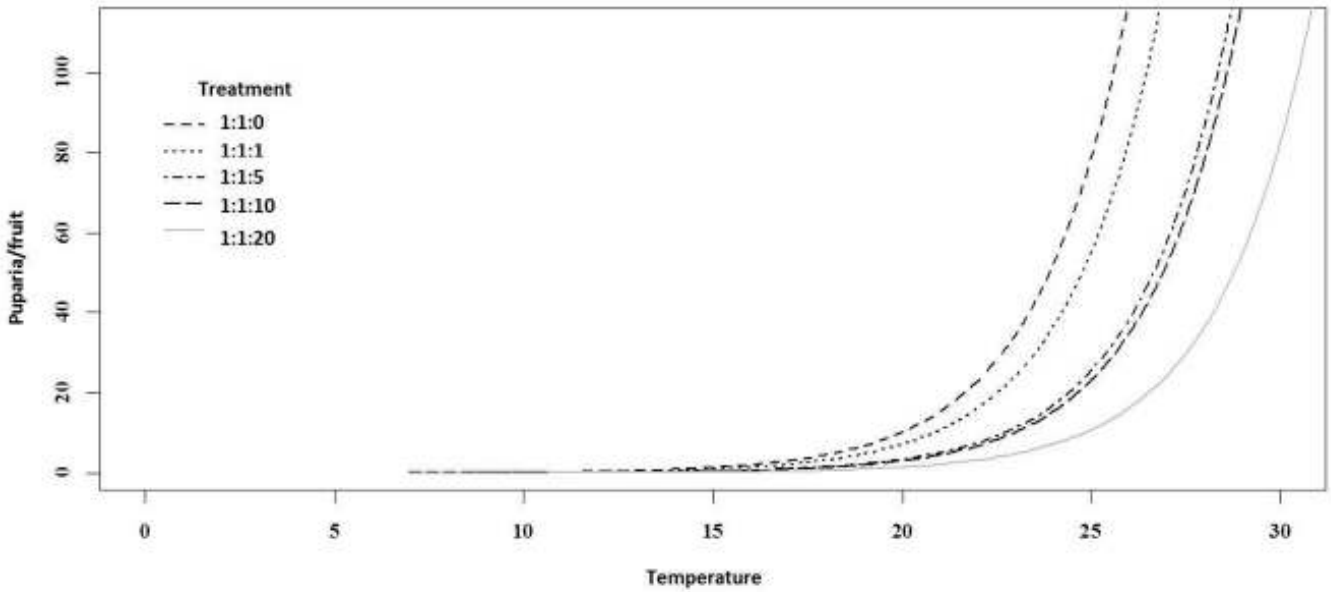

Treatment
1:1:0
1:1:1
1:1:5
1:1:10
1:1:20
Puparia/fruit
0
20
40
60
80
100
0
5
10
15
20
25
30
Temperature

Figure 5.4. Number of *Ceratitis capitata* puparia obtained from sentinel apple fruits exposed to different fly ratios 1:0:0, 1:1:0, 1:1:5, 1:1:10, and 1:1:20 (number indicate relative ratios of wild female:wild male:sterile Vienna-8 males) and in relation to temperature. For each treatment the number of released females was 50 individuals, with the corresponding males of each type according the above indicated ratios. The probability of obtain pupae per fruit (*y*) is calculated by the formula $y = e^{(-5.87 + 0.41 \times \text{Temperature} - 0.35 |_{1:1:1} - 1.13| - 1.23|_{1:1:5} - 1.99|_{1:1:10} - 1.99|_{1:1:20})}$.

5.3.5. Predicting the offspring

The number of *C. capitata* F1 puparia was estimated based on the proportion of V8 positive detections ($P < 0.0001$) and the mean temperature ($P < 0.0001$) (Figure 5.5.). This relationship, though, showed that high numbers of V8 matings at higher temperatures resulted in low puparia reductions. A logarithmic regression was used to show the relationship between V8 sperm detection and population reduction in a treated area compared to a control area exposed to the same environmental conditions (Figure 5.6.). The corrected percentage of *C. capitata* population reduction was strongly related to the proportion of V8 sperm detected in the captured females. When V8 sperm detection in females analyzed reached 50%, the predicted population reduction was 52%. The maximum population reduction predicted was 78% which occurred when positively detected V8 sperm was found in 100% of the females captured.

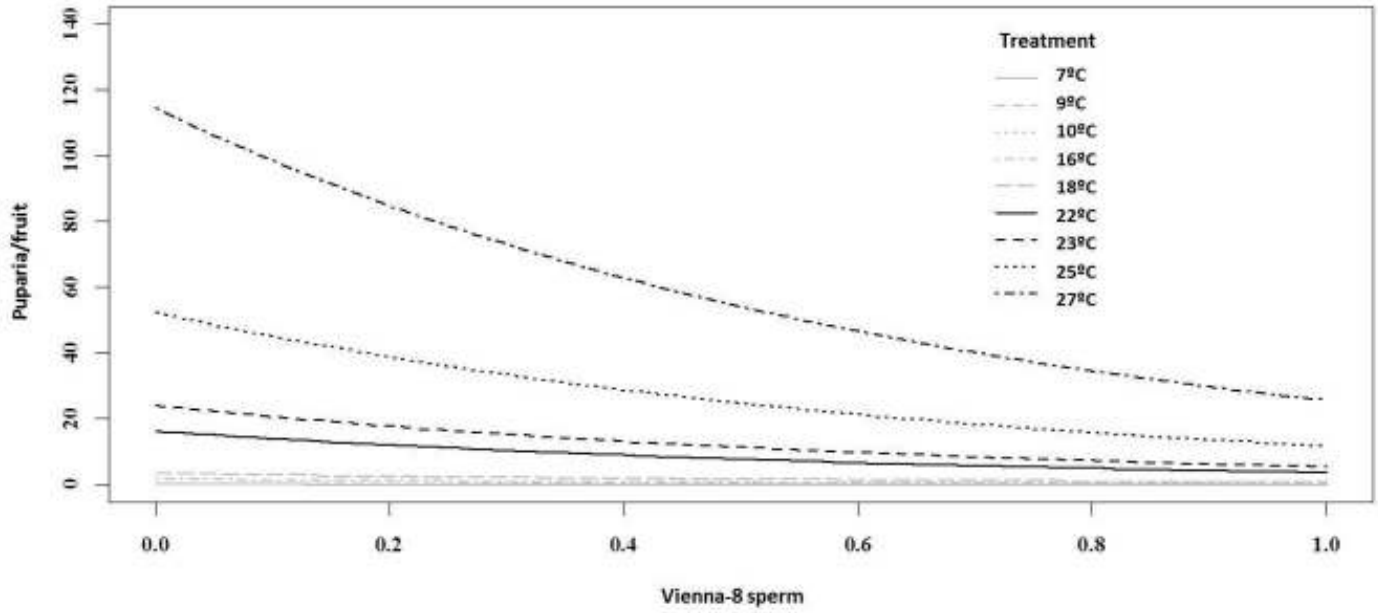

Treatment
7ºC
9ºC
10ºC
16ºC
18ºC
22ºC
23ºC
25ºC
27ºC
Puparia/fruit
0
20
40
60
80
100
120
140
0.0
0.2
0.4
0.6
0.8
1.0
Vienna-8 sperm

Figure 5.5. Probability to obtain *Ceratitis capitata* puparia/fruit at different temperatures and in relation to the proportion of molecular Vienna-8 sperm detected in spermathecae. The number of puparia (*y*) is calculated by the formula $y = e^{(-5.83 - (1.50 \times \text{Proportion of positive V8 detections}) + (0.39 \times \text{Temperature}))}$.

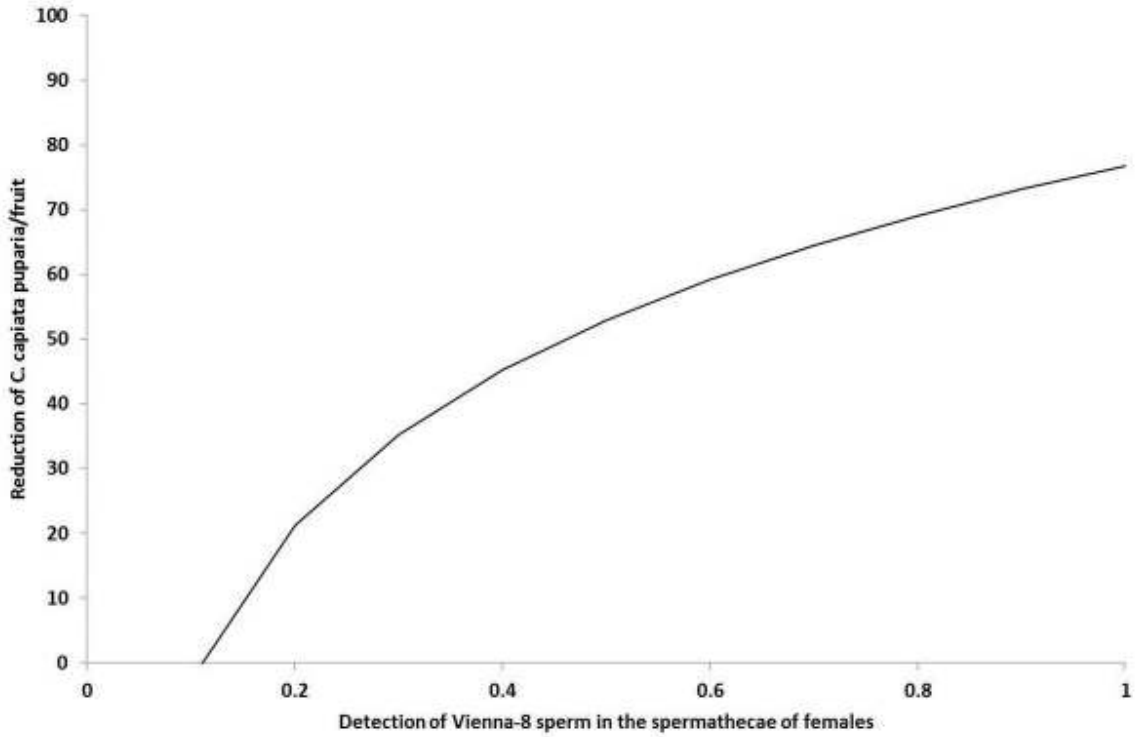

100
90
80
70
60
50
40
30
20
10
0
Reduction of C. capiata puparia/fruit
0
0.2
0.4
0.6
0.8
1
Detection of Vienna-8 sperm in the spermathecae of females

Figure 5.6. Reduction of *Ceratitis capitata* puparia (%) obtained in an SIT area in relation to the variation in the proportion of molecular Vienna-8 sperm ID in spermathecae. $y = 34.50 \ln(x) + 76.80$; $R^2 = 0.939$.

5.4. Discussion

The use of newly available investigative tools such as modeling and molecular analysis has increased our ability to measure the efficacy of natural enemies and therefore, to enhance the success of control programs (Mills and Kean 2010). The success of sterile insects to control target populations is closely related to their mating performance in natural conditions. The principles of population and behavioral ecology determine the mating behavior of insect species. Therefore, these concepts should be understood for the successful application of the SIT (Itô and Yamamura 2005). Moreover, mating behavior in natural populations of insects has been predicted by environmental and demographical parameters (Warburg and Yuval 1997, Thompson et al. 2011). In agreement with previous works, our results demonstrate the influence of environmental factors on the traits that involve reproductive success in insects (Lance and McInnis 2005). In the case of SIT releases in the field, both sterile V8 sperm presence in spermatheca and offspring of *C. capitata* females could be predicted using two variables: (1) ratio of wt females:wt males:V8 males and (2) temperature. Additionally, the number of females captured in traps was also affected by relative humidity. However, the influence of relative humidity in the final balance of the model was low. Our model was developed for a Mediterranean climate with low variations in relative humidity (Table 5.1.). Therefore, relative humidity should be taken into account in SIT programs applied in other climatic areas where relative humidity variations could play a more important role.

The females captured in traps were a suitable field source of information about the mating success of V8 males. The number of females caught was

higher in the control ratio 1:0:0 where no males were released compared to treatments that included the release of males (1:1:0, 1:1:1, 1:1:5, 1:1:10 and 1:1:20) under all the environmental conditions tested. *Ceratitis capitata* female catches are influenced by temperature and relative humidity in Mediterranean citrus agroecosystems (Miranda et al. 2001, Navarro-Llopis et al. 2008). Previous studies have showed a decrease in the number of female catches for temperatures ≤ 10ºC, and at low population levels compared to high ones when traps contain food-attractants (Miranda et al. 2001, Martínez-Ferrer et al. 2012). It is possible that the male sexual stimulus caused females to be less interested in feeding (Hendrichs and Hendrichs 1990) and therefore, in traps, which were baited with food attractants. The interpretation of these declines in the number of insects trapped is complex though, because they are affected by numerous (often unknown) factors such as age and physiological state (Vreysen 2005). Trap biases or behavioral response of the insects to the trap are two factors that could play a role in the females catches (Katsoyannos et al. 1999, Rendón et al. 2004, Vreysen 2005). It is known that trap density is critical and need to be adjusted based on factors that include trap and lure/attractant efficiency, climate and geographical location, and type and presence of host. Indeed, trapping is a dynamic process that changes according to survey objectives and control applications (IAEA 2003). Further investigations should explore the efficacy of V8 males using the method proposed on the adjustment of trap density, sampling timing, and to adapt the molecular method to the volume of females trapped at aw-field scale.

Classic mathematical models have linked the sexual competitiveness of released insects to the wt:sterile ratio obtained from field traps (Knipling

1955, Vreysen et al. 2005). Sterile V8 matings measured using molecular markers showed a higher success rate of male performance with increasing wt:V8 ratios. This result demonstrates that V8 males were as competitive as wt males in sexual and other quality-related parameters (Holbrook and Fujimoto 1970, Barry et al. 2003c, Lance and McInnis 2005, Vreysen 2005). V8 males were inferior competitors at temperatures below 9°C than when temperatures were warmer. The effect of decreasing the temperature at which males are reared in the emergence facility (e.g. from 24ºC to 15ºC) some days before their actual release in the field could improve their competitiveness. This improvement would also imply an increase of their life cycle and therefore of the cost of the program (McInnis et al. 2012). As a consequence, further studies are necessary before implementing this type of changes. The temperature-ratio models obtained (Figures 5.3. and 5.4.) provide useful information to evaluate and therefore, to support the decision-making process to improve the efficacy of SIT programs currently underway. The differences between the analysis of field samples and the expected proportion of matings could serve to detect failures of V8 releases in real-time. Similarly, they could be used to detect deviations in the actual wt:V8 male ratios present in the field. Both models, together with the V8 matings-temperature model (Figure 5.5.) highlight the influence that temperature has on the mating, oviposition activities and offspring of *C. capitata* (Bateman 1972, Hendrichs and Hendrichs 1990, Warburg and Yuval 1997, Duyck and Quilici 2002). For example, at temperatures below 15ºC, less than 1 puparia per fruit was predicted even under the 1:1:0 ratio, whereas at temperatures above 19ºC, 1 or more puparia per fruit were predicted even if 100% of the spermathecae contain V8 sperm. This result can be taken as indicative of the higher frequency of female remating at

high temperatures, a phenomenon that would occur even if wt:V8 male ratio increased. On the other hand, the release of higher numbers of V8 males at low temperatures could be a poor use of resources that could be saved to increase the fitness of the sterile males (Pereira et al. 2012). These observations also may assist in improving the management of low levels of pest population at the end of the winter, and pest outbreaks in summer in Mediterranean climates, which could be satisfactorily managed by implementing mass-trapping (Martínez-Ferrer et al. 2012) or chemosterilization (Navarro-Llopis et al. 2011).

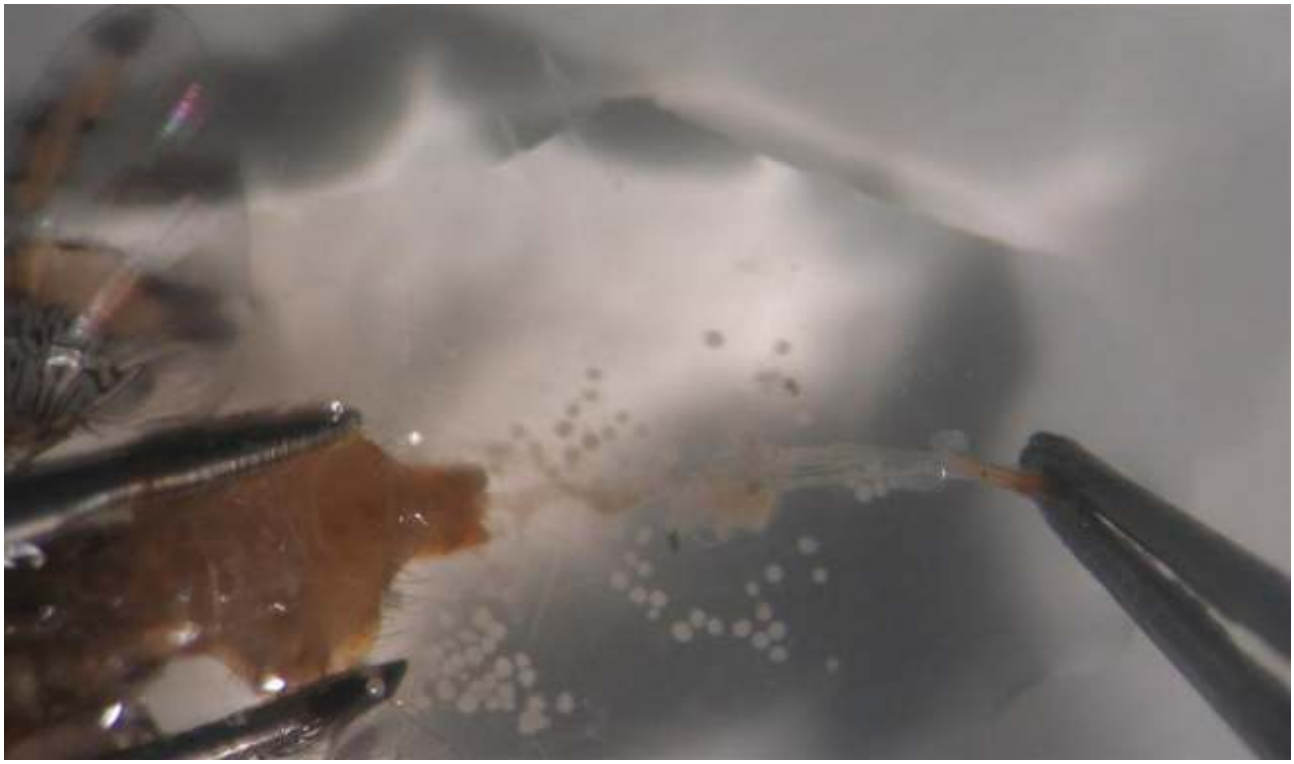
Quantifying the pest offspring reduction in a treated area compared to control area where no sterile males are released constitutes the most powerful straightforward tool to assess progress in SIT programs (Waterhouse et al. 1976, Vreysen 2001) since the reproductive potential of the pest in treated and control areas is determined by the same biological, ecological and environmental factors (Hendrichs et al. 2002). *Ceratitis capitata* offspring reduction in a SIT-treated area was predicted using the V8 mating success measured directly with molecular markers under Mediterranean climatic conditions (Figure 5.6.). This model helped to refine the observed overestimations when only ratios and temperature were considered. The maximum offspring reduction estimated was 78%. Therefore, these results confirm the significant influence that traits not specifically considered here can cause increases in the *C. capitata* population under field conditions. These include traits such as remating (Hendrichs and Hendrichs 1990, Bonizzoni et al. 2002), high mobility, and dispersal, or ability to recolonize areas from alternative hosts (He and Haymer 1999, Papadopoulos et al. 2001b, Meixner et al. 2002, Epsky et al.

2010, Martínez-Ferrer et al. 2010). Furthermore, information about the spermathecal content of females not recaptured remains unknown. The possibility to include these parameters to construct the model would qualitatively improve it as a tool to predict the impact of SIT on wild populations.

The molecular method has proved to be a good indicator to predict *C. capitata* offspring reduction related with V8 mating success in the field. This method has allowed the development of less laborious and tedious tools to evaluate the releases with regards to both efficiency and cost effectiveness in SIT programs. Logically, the essential next step is to validate the models developed using female samples from wild populations. Additionally, this method provides new opportunities for other environmental conditions or fruit fly species under SIT.

Chapter 6

6. Improving the sterile sperm ID method for its implementation in the AW-SIT program against *Ceratitis capitata* (Diptera: Tephritidae) in Spain



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

Juan-Blasco M., A. Urbaneja, V. San Andrés, P. Castañera y B. Sabater-Muñoz. Improving the sterile sperm ID method for its implementation in the AW-SIT program against *Ceratitis capitata* (Diptera: Tephritidae) in Spain.

6.1. Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) is a key destructive pest in citrus around the world. Native of sub-Saharan Africa is now established in almost all temperate regions and has the potential to spread further still due to its huge host range, its ability to skip the uncomfortable season and even its ability to spread into new territories after an unplanned man-droved introduction (White and Elson-Harris 1992, De Meyer et al. 2004, 2008, Malacrida et al. 2007).

The Sterile Insect Technique (SIT) is the basis of medfly environmentally-safe management procedures in many citrus production areas with the presence of this pest species and the key of eradication programs in countries on which this species has a quarantine status (as USA, Guatemala, Japan or Australia) (Hendrichs et al. 2002). Nowadays, the evaluation of SIT programs against fruit flies relies in the recapture ratio of sterile *versus* fertile males, without taking into consideration the real target of SIT that are the wild females (Dyck et al. 2005). Moreover, the actual monitoring system based on fluorescent dye presence (to establish the recapture male type ratio) is being questioned due to the dye cost, its implication in human health and its intrinsic error rate (dye can be lost by time and environmental conditions and can be transferred to wild-type flies) (Hagler and Jackson 2001). Other methods as egg hatchability or sperm head size measurement (McInnis 1993, McInnis et al. 1994, Katsoyannos et al. 1999, Rendón et al. 2004) are direct methods to assess the mating success of released sterile males, but both are tedious and time consuming methods, which also requires great laboratory spaces for maintain alive the captured

females and their progeny, and personnel to handle all these flies for the tests.

The application of molecular genetic methods to Control Programs has been of interest since early 90's (Hoy 2000). However, its application to fruit flies control started late in the 90's and has been mainly focused on the pathway analysis of medfly introductions by means of obtaining molecular genetic data to the knowledge of probable geographic source(s), to characterize the release strains or to characterize the new in development strains, or to follow the dispersion of released flies of mixed sexes (Gasparich et al. 1997, Silva et al. 2003, Bonizzoni et al. 2004, Malacrida et al. 2007, Barr 2009, Isasawin et al. 2012). In 2007 (a), San Andrés and coworkers (our group) developed a PCR-RFLP diagnostic method to identify the sterile sperm in the female spermathecae. A study that opened the door to a new wave in SIT against fruit flies, the incorporation of the real target of SIT, the wild females, into the evaluation of the success of the released males. In 2010 Fritz and coworkers added a new step, to identify and quantify sperm DNA by PCR (based on microsatellite analysis) in sperm storage organs, but applied to another tephritid species, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae) the Caribbean fruit fly. In this case, microsatellites where chosen due to the lack of a characterized and selected strain for only-male production and release, which renders difficult to identify released flies from natural populations. Several later works have focused in the sperm marking of medfly by transgenic technology with the aim of its use for effective monitoring of SIT programs (Schetelig et al. 2006, Scolari et al. 2008). The aim of releasing transgenic flies was to detect directly (under microscopy) the genetic nature of captured females and

their stored sperm, reducing the cost of analysis (neither dissection nor rearing). Despite the advances in strain and sperm marking in the medfly, the regulatory government processes limit the release of transgenic animals and render this methodology inapplicable at this moment (Bossin et al. 2006, Knols et al. 2007, Mumford 2012). More recently, Isasawin et al (2012) also used microsatellite DNA markers for the evaluation of aw-SIT against *Bactrocera dorsalis*, but focused on the dispersion and identification of released flies, not in the identification of sterile sperm in wild-fertile females. In both cases, cost and implementation of microsatellites remain as principal drawback for its use in aw-SIT programs. Thus the only method to determine the sperm ID in medfly females at this moment is the San Andrés et al. protocol (2007a). However its application to aw-SIT has two major drawbacks, the personnel cost and the analysis time gap (as outlined in this work). Therefore, it was advisable to develop a modification of the San Andrés protocol to establish a world-wide adapted protocol for the evaluation of aw-SIT programs against the medfly. In this way, the universality of the markers should be checked and upgraded if needed by testing medfly samples from all around the globe. Furthermore, the sperm ID protocol should be simplified to allow the high-trough put analysis of samples from aw-SIT programs. Both approaches are the objectives of this work.

6.2. Material and methods

6.2.1. Medfly samples to test universality of sperm ID markers

Samples have been requested to worldwide SIT facilities or laboratories working with *C. capitata* (Table 6.1.). Each sample consisted in at least 100

individuals of each gender for the wild-type flies, and at least by 50-100 males of each sterile strain [almost all based in the Vienna temperature sensitive lethal (*tsl*) series with the Egypt mitochondrial haplotype (IAEA 2006)].

Table 6.1. Medfly samples, collector and number of individuals received.

Country (Locality)	**Collector/Supplier**	**No. Individuals of each gender (wt and sterile strain)**
Argentina	D. Segura	100
Australia (Perth, West Australia)	R. Magliani	100
Brazil (Medfly facility)	A. Malavasi	100
Brazil (Petrolina, Pernambuco)	B.J. Paranhos	100
Guatemala (Antigua)	C. Cáceres	100
Egypt (Giza)	IAEA Staff	35
Israel	Y. Gazit	50
Morocco (Agadir)	H. Aboussaid	150
Morocco (Marrakech)	H. Aboussaid	50
South Africa (Hex River)	B. Barnes	50
Tunisia (Bigerte, Taprelsa)	S. Elfekih	30
Portugal (Madeira)	L. Dantas	105
Greece (Magnisia)	N. Papadopoulos	48
Greece (Creta (Lab))	A. Economopoulos	100

6.2.2. Medfly strains and rearing conditions for mating assays

The wild-type strain (wt) adults were obtained from a laboratory colony (generations XIV to XVII, 2010-11) housed at the GVA-IVIA emergence facility (Moncada, Spain). Sterile males of the Vienna-8 (V8) (*tsl*) Genetic Sexing Strain (GSS) mix 2002 strain were produced at the mass-rearing facility in Caudete de las Fuentes (Valencia, Spain) and transferred as pupae after irradiation to the GVA-IVIA emergence facility. After emergence, adult wt females, wt males (<24 h old to assure female virginity) or V8 males were separated by sex and strain into poly-methyl methacrylate cages (20 x 20 x 20 cm) with 100 individuals per cage. Adults were kept in different rooms to prevent any pheromone effect prior assay at 25 ± 4ºC, 65 ± 10% RH with natural light until achieving the 'release' age (10 d-old for the wt females and 7 d-old for the wt males). V8 males were maintained at 25 ± 4ºC, 75 ± 5% RH in complete darkness (no photoperiod) in a different environmental chamber until achieving the release age (3 d-old) to resemblance pre-release conditions in the SIT facility. Wt adults were fed with a mixture of sugar and hydrolysed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; w:w) (protein enriched diet) whereas sterile males were fed with sugar (protein deprived diet). *Ceratitis capitata* wt adults at field have a diet based mainly in sugar and protein (San Andrés et al. 2009). Sugar is the pre-release diet of sterile males in the Spanish SIT facility. Water and diet was provided *ad libitum*. V8 males were subjected to aromatherapy treatment with Ginger Root Oil (GRO) 24 h prior release age, in the same room and at the same time that those sterile males for release in aw-SIT-program (Juan-Blasco et al. 2012a).

6.2.3. Mating assays

Mating assays were conducted to validate the PCR-RFLP sperm ID method in females. The mating arena consisted of methacrylate cages (30 x 40 x 30 cm) with ventilation openings. Fifty males (wt or V8 depending on cross) were placed in the mating arena first (8:00-8:30 h, photophase started at 06:00 h including a one hour dawn) and left to settle for 15 min, then 50 females were introduced, comprising the final ratio (1:1) tested (as for the sterile males quality control, FAO/IAEA/USDA 2003). In each arena, observations for mating pairs were carried out continuously for 3 h removing each couple as formed by gentle soaking into 50 ml plastic vials; after this time, the arena were supervised discontinuously (each 15-20 minutes) for additional 3 h after which any remaining uncoupled female was discarded. All vials were annotated with copula starting time and supervised during 3 h to assess copula completion. Only females mated for ≥ 3h were used. After this time all vials were frozen to kill all the flies. For each vial the male type was confirmed by the presence (sterile) or absence (wt) of fluorescent dye (Dyck et al. 2005). Mating experiments were repeated several times to obtain a significant number of mated females for all the DNA extractions.

The validation of the sperm ID method on these females was analysed at different days after death to assess performance of the method over time and thus, to resemble the real scenario of capture in field which include different sampling times. Killed females were placed in a Tephri-trap® (Sorygar S.L., Madrid, Spain) and kept in an environmental chamber (SANYO 560 MLR) at 25 ± 4ºC, 75 ± 5% RH and 16:8 h (L:D) photoperiod for 0, 7, 28

or 56 days. After these 'capturing'-period females were processed depending on DNA extraction protocol.

6.2.4. Female storage for DNA extraction protocol

Mated 'captured' females were stored for additional 0, 7, 28 or 56 days either in 70% ethanol at room temperature or dry at -20ºC, depending on spermathecae DNA extraction protocol. Two new methodologies were tested to improve sperm ID protocol: sonication and membrane spermathecae imprinting. For sonication freshly (dissected immediately after copula completion) or 7 d-old mated 'captured' female samples were used. Three different kinds of vials were tested to performance the sonication: i) 1.5 ml centrifuge tubes (ref. 200400 Deltalab S.L., Barcelona, Spain), ii) 0.2 ml thin-wall PCR strip-tubes (ref. 4095.2N Deltalab S.L., Barcelona, Spain), and iii) 96-well PCR plates (ref. 72.1978.202 Sarstedt AG & Co. Nümbrecht, Germany). For the spermathecae membrane imprinting method, 0, 7, 28 and 56 d-old mated 'captured' females were used (in 96-well PCR plates). For each female batch, two DNA preservation methods were tested: i) females preserved in 70% ethanol at RT, and ii) dry females at -20ºC; in both cases for additional 0, 7, 28 and 56 days.

6.2.5. DNA extraction protocols and PCR conditions

For mitochondrial *Hae*III genotyping, DNA extraction was performed using only the head or thorax of each fly by the 'Salting out' method (Sunnucks and Hales 1996). Each sample was tested with the Ccmt-*HaeIII* marker to obtain the RFLP pattern as described previously (San Andrés et al. 2007a).

For molecular sperm ID, the mated 'captured' females were dissected and spermathecae subjected to DNA extraction following three different methods described below;

(i) The *San Andrés and coworkers* (2007a) method based on the 'Salting out' method to extract individually the DNA of a spermathecae in a 1.5 ml centrifuge tube.

(ii) The *sonication DNA extraction method* consisting in sonication application of 10 pulse per second during 1 min followed by a second round of 1 min with a 30 sec of stop in ice bath in between rounds, to a vial (1.5 ml tube, 0.2 ml PCR strip or 96-well plate) containing the female spermathecae in 100 µl of sonication buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA pH 8.0, 0.1% SDS). Sonicated vials were immediately frozen till use for PCR. Sonication was performed in an ultrasonic water bath of 0.9 litters (Fungilab SA, Barcelona, Spain; HF-frequency 35 kHz).

(iii) *Spermathecae membrane imprinting*. The dissected spermathecae were crushed in 0.25 mm^2 nylon membrane (Hybond N+, Amersham-GE Healthcare, UK) with the aid of a plastic pestle (ref. Z359947 Sigma-Aldrich Co., St Louis, MO, USA). Each imprinted membrane was deposited in a 96-well plate and covered with 100 µl of extraction buffer (0.1 M Tris-HCl, 50 mM NaCl, 0.1% Triton, 1 M Glycine, 1 mM EDTA pH 8.0, 1% DTT, 0.1 mg/ml Proteinase K). DNA extraction was performed in a thermocycler (Eppendorf Mastercycler gradient) for 60ºC during 60 min followed by 95ºC for 15 min.

After this, the plates were spin-down and frozen (-20ºC) till amplification. DNA concentration and purity was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific Inc., Massachusetts, USA).

Sperm ID markers CcYsp and Ccmt-*HaeIII* were tested by PCR as described (San Andrés et al. 2007a) using 1, 3, 5 or 10 µl of DNA extract depending of the extraction method (i, ii or iii) and marker. All PCR products were electrophoresed in 2% agarose gel, and visualized under UV after staining with ethidium bromide, whereas the PCR-RFLP with *HaeIII* was analysed in 2.5% agarose gel.

6.2.6. Statistical analysis

Sperm identification was coded as 1 (positive) or 0 (negative) for detection or absence of sterile sperm (CcYsp marker and Ccmt-*HaeIII*) and was considered as indicator variable. A Generalized Linear Model (GLM, McCullagh and Nelder 1989) was used to analyse the differences between the effect of extraction protocol [San Andrés et al. (2007a) *vs* membrane imprinting method] (fixed effect) in sterile sperm detection. The sample processing before to DNA extraction (the conservation times and preservative methods) were included as fixed factors. Selection of the best model was based on the Akaike Information Criterion (AIC). This criterion revealed that the Binomial distribution with log-link was appropriate to analyse sterile sperm identification by the two extraction protocols.

6.3. Results

6.3.1. Universality of Ccmt-*HaeIII* marker

All the tested Vienna *tsl* strains in use in the several aw-SIT programs (Table 6.1.) give a positive *Hae*III-RFLP pattern, as all the tested strains (Vienna-6, Vienna-7, Vienna-7 Madeira, Vienna-8 and Vienna-8 mix 2002-2004) maintain the Egypt mitochondrial haplotype (Figure 4.3.). None of the tested medfly wild-type samples give a positive RFLP pattern. With the exception of the Creta laboratory strain which in fact corresponds to the original Egypt *wp* (white pupa) strain (A. Economopoulos personal communication after analysis).

6.3.2. Improvement of the sterile sperm ID protocol

The *San Andrés et al. (2007a) sperm extraction method* was followed as control and as a source of positive controls for PCR.

The *sonication protocol* resulted in a fast and dirty (low A260/280 values) protocol with an average DNA concentration of 2.70 ± 1.10 ng/µl (mean ± SE) and A260/280 absorbance ratio of 0.61 ± 0.30 (mean ± SE). When extracting more than one sample per time the percentage of sterile sperm positive detection decreased till 0.67 ± 0.13% (mean ± SE) or as less as 0.43 ± 0.13% (mean ± SE) when 7-d mated 'captured' females where used as source of spermathecae tissue. These results were also affected by the vial material used to perform sonication (Table 6.2.).

Table 6.2. Number (percentage ± SE) of successful sperm identification in Vienna-8-mated females spermathecae extracts by sonication. Sonication was performed in three kinds of vials (normal 1.5ml centrifuge tubes, 0.2ml thin-wall PCR tubes, and 96-well PCR plates). Mated females were dissected immediately after copula completion or after a period of 7 d kept at 25ºC to resemble the field monitoring system.

Sonication performed in	Freshly dissected	7 d post-mating
1.5 ml tubes (n= 10)	10 (1.00 ± 0.00)	9 (0.90 ± 0.10)
0.2 ml PCR tubes (n=24)	18 (0.75 ± 0.15)	-
96-well PCR plate (n=40)	27 (0.67 ± 0.13)	17 (0.43 ± 0.13)

-, not performed

The *membrane imprinting*, is giving best results with an average DNA concentration of 81.31 ± 74.61 ng/µl (mean ± SE) and A260/280 absorbance ratio of 0.45 ± 0.10 (mean ± SE). The small A260/280 ratio in this extraction protocol is mainly due to the presence of Proteinase-k in the extraction buffer, which is not removed prior analysis, but is heat-inactivated. The A260 values are of 1.54 ± 0.20 (mean ± SE) indicating the presence in great quantities of DNA and RNA in the samples. Sterile sperm was detected even at 56 d after copula completion (1.00 ± 0.00%) (Table 6.3.). In overall, the molecular detection of sterile sperm by membrane imprinting showed no significant differences with the San Andres et al. (2007a) method (GLM binomial: P = 0.195). Sterile sperm detection decreased greatly with conservation and preservation time (GLM binomial: $P < 0.0001$ and P = 0.034, respectively) (Table 6.3.). When considering only the sterile sperm detection by the membrane imprinting method no significant differences were observed between the 70% ethanol and -20ºC preservative methods (GLM binomial: P = 0.990 and P = 0.990, respectively) compared to

unpreserved samples of the same age. But when considering the same conservation and preservation time the sterile sperm detection was significantly affected by the elapsed time from copula completion to analysis (GLM binomial: $P = 0.0067$ and $P < 0.0001$, respectively).

This protocol, *membrane imprinting*, allowed performing the extraction of 96 samples at the same time by using PCR plates in a hands-off system that allows continuing with the spermathecae extirpation process. This compared to the thirty 1.5 ml vials used per run (limited by the centrifuge rotor size) in the San Andrés protocol with a hands-on time requirement for two hours renders the *membrane imprinting* extraction method more affordable for an aw-SIT evaluation system.

Table 6.3. Percentage (mean ± SE) of successful sterile sperm ID of sterile male mated females subjected to different post-mating timing (7, 28 and 56 days) and with different preservation methods (ethanol or -20ºC) over the time (for 0, 7, 28 and 56 days) following San Andrés et al (2007a) dissection protocol or membrane imprinting.

Female treatment	***Membrane imprinting***		**San Andrés**	
(post-mating days, preservation days and @ method)	Mean ± SE	n	Mean ± SE	n
1 (7 d, 0 d)	1.00 ± 0.00	12	0.90 ± 0.10	10
2 (28 d, 0 d)	1.00 ± 0.00	12	0.75 ± 0.13	12
3 (56 d, 0 d)	1.00 ± 0.00	12	-	-
4 (7 d, 7 d @ 70%)	1.00 ± 0.00	13	1.00 ± 0.00	10
5 (7 d, 7 d @ -20ºC)	0.60 ± 0.16	10		-
6 (7 d, 28 d @ 70%)	0.69 ± 0.13	13	1.00 ± 0.00	9
7 (7 d, 28 d @ -20ºC)	0.69 ± 0.13	13		-
8 (7 d, 56 d @ 70%)	0.64 ± 0.15	11	0.40 ± 0.16	10
9 (7 d, 56 d @ -20ºC)	0.82 ± 0.12	11	-	-
10 (28 d, 7 d @ 70%)	0.91 ± 0.09	11	-	-
11 (28 d, 7 d @ -20ºC)	0.82 ± 0.12	11	-	-
12 (28 d, 28 d @ 70%)	0.42 ± 0.15	12	-	-
13 (28 d, 28 d @ -20ºC)	0.67 ± 0.14	12	-	-
14 (28 d, 56 d @ 70%)	0.56 ± 0.15	13	-	-

-, not performed.

6.4. Discussion

6.4.1. Universality of Ccmt-*HaeIII* marker

In none of the samples a partial RFLP was obtained as predicted by Barr et al. (2009) which detected sequencing errors and mismatches in the mitochondrial DNA of the medfly from samples around the world. So, the

absence of positive Ccmt-*HaeIII* pattern in samples from all the medfly SIT regions tested validates the Sperm ID marker Ccmt-*HaeIII* for its implementation in the studied regions. The marker developed by San Andrés et al. (2007a) for Vienna sperm identification is still reliable.

6.4.2. Improvement of the sterile sperm ID protocol

As previously mentioned, in order to evaluate the success of the SIT program against *C. capitata* in Spain it was advisable to test for the presence of sterile sperm in the captured wild-females. However and due to the high number of flies to be tested an alternative or a modification to the San Andres et al. (2007a) extraction protocol was required to handle all these flies in a reasonable time frame.

The *sonication protocol* resulted in a simple, fast and cheap method but in contrast results were dirty. False negatives increased with sample age (age determined as time elapsed from mating event to spermathecae dissection) and with vial used to perform the extraction (1.5ml tube, 0.2ml thin-wall PCR tube or 96-well PCR plate). These results contrast with the sonication results obtained by Fritz et al. (2010), but in this case, sonication was used to remove maternal cells from spermathecae content in order to determine the origin of sperm (single or multiple mated females). Sonication was successfully used to extract high quality DNA from museum preserved vouchers, as a way for not destroying these valuables specimens (often unique specimens) (Hunter et al. 2008). Our results are in agreement with the overall 66% amplification efficiency of Hunter and coworkers (2008). However, we obtained variable results depending on vial used. As for the SIT program evaluation requirements, a high-throughput technology should

be desirable, to decrease sample processing time, and the use of single tubes to perform DNA extraction are limiting its use.

The *membrane imprinting method* simplifies the procedures, and arose as the best method to perform analysis of all aw-SIT monitoring traps samples than the San Andrés et al. (2007a) protocol, mainly by the reduced handling time required. The San Andrés et al. (2007a) was tried to upgrade by performing DNA extraction on 96 deep-well plates to reduce handling time. But due to the size of spermathecae, homogenization system and centrifuge type an increment of lost samples was achieved which rendered this method not reliable (data not shown). Further research should be done to upgrade the homogenization and extraction protocol with 96-well plates to overcome this limitation. One key point that also was highlighted with this work was the DNA degradation or a decrease in amplification efficiency of samples (irrespectively of the preservation method used to protect sperm DNA) for more than 28 days. This result was also in accordance with the reduced DNA concentration and purity observed in dried samples field collected and stored in 70% ethanol prior sample manipulation for microsatellite analysis of tephritid flies (Maxwell et al. 2011). A reduced DNA yield may result in an increment of false negative results, due to a not amplification of sterile male DNA.

In overall, our results using the membrane imprinting method indicate that *C. capitata* females captured after being in monitoring traps for 7 to 56 days are useful samples to assess mating success of released sterile males and thus to assess the efficacy of the SIT program. However, special care should be taken to preserve sperm DNA whilst not processing the samples (Table

6.3.). Future studies should examine a way to avoid spermathecae dissection, as it remains as the time limiting step.

7. Conclusiones generales

1. La competitividad en cópula de los machos estériles de *C. capitata* frente a los machos salvajes se mejora mediante su exposición a aceites esenciales (aromaterapia con linalool o aceite de jengibre, o aceite de jengibre en dieta) antes de ser liberados en campo. Sin embargo, antes de implementar su uso a gran escala sería necesario ajustar la dosis óptima de aplicación y probar la competitividad en cópula de los machos tratados en condiciones de semi-campo.

2. Los machos Vienna-8 considerados como enemigos naturales están sometidos a los efectos secundarios de los tratamientos plaguicidas empleados contra otras plagas. El contacto de los machos Vienna-8 a los plaguicidas abamectina, etofenprox, etoxazol, aceite mineral, pimetrocina y piriproxyfen no resulta en mortalidades significativas. Sin embargo, si que presentan elevada mortalidad tanto por contacto como aplicados tópicamente a los plaguicidas clorpirifos y spinosad. Estos resultados tienen que tenerse en cuenta durante las liberaciones y tratamiento a gran escala.

3. La evaluación del éxito en cópulas de los machos Vienna-8 soltados en campo en los programas TIE se puede realizar mediante la aplicación de un método molecular que identifica la presencia de esperma Vienna-8 en las espermatecas de las hembras salvajes.

4. Se ha obtenido un modelo matemático que permite predecir en campo la reducción que se obtendrá de la población de *C. capitata* a partir de la medida de las cópulas de los machos Vienna-8 en las hembras capturadas en trampas, integrando los factores de ratios de liberación y condiciones climáticas.

5. Para favorecer la aplicación a gran escala de la TIE, se ha puesto a punto un protocolo de laboratorio nuevo que permite la identificación de esperma para la escala de muestras capturadas en un programa TIE de gran envergadura.

Como conclusión general, los resultados, así como los modelos obtenidos, abren nuevas posibilidades para la utilización de herramientas útiles en tiempo real, para la toma de decisiones acerca de la liberación de machos Vienna-8, así como la evaluación de su eficacia.

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