El Colegio de la Frontera Sur

Desempeño de una cepa genéticamente modificada de la mosca del Mediterráneo, *Ceratitis capitata* (Wied.) para su uso en la TIE.

TESIS
presentada como requisito parcial para optar al grado de
Doctor en Ciencias en Ecología y Desarrollo Sustentable
Con orientación en Agroecología y Manejo de Plagas

Por

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para obtener el grado de **Doctor en Ciencias en Ecología y Desarrollo Sustentable**

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TABLA DE CONTENIDO

Resumen

La utilización de organismos genéticamente modificados puede contribuir a mejorar la eficiencia de la Técnica del Insecto Estéril (TIE). Recientemente se han desarrollado cepas de la mosca del Mediterráneo, que portan secuencias de ADN que codifican para la expresión de proteínas fluorescentes. Una de estas cepas fluorescentes es la #1260_F-3_m-1 (VIENNA 8 1260), desarrollada a partir de la cepa VIENNA 8, que porta genes que permiten la separación de hembras y machos. La expresión de fluorescencia, producto de la inserción, puede ser aprovechada como marcador para dar mayor eficiencia a la TIE, al identificar en campo de forma precisa el origen de los individuos. Por ser insectos transgénicos el uso de la cepa VIENNA 8 1260 puede tener un costo biológico en su desempeño, el riesgo de transmitir el transgen de fluorescencia a poblaciones silvestres de esta especie y debe cumplir con requisitos de bioseguridad para su liberación en campo. Se realizó una serie de ensayos para determinar la viabilidad del uso de la cepa transgénica en programas que aplican la TIE. Se documentó una mejora generacional, gradual, en el desempeño de la cepa en cría masiva, alta estabilidad y conservación de la fluorescencia del 100%. La competitividad sexual bajo condiciones de jaula de campo fue menor para VIENNA 8 1260 que para la cepa actualmente en uso (VIENNA 8 D53-). Las dosis de irradiación requeridas para la esterilización fueron similares para ambas cepas; sin embargo, para minimizar el riesgo de la trasmisión vertical hacia la población silvestre, se recomiendan dosis mayores a 140 Gy. Se evidenció recópula en hembras, precedencia en el uso de esperma a favor del macho con que recopulan y un mayor desempeño (fitness) del esperma de machos no fluorescentes. No se observó trasmisión horizontal del transgen fluorescente hacia el parasitoide *Fopius ceratitivorus* durante 16 generaciones.

Palabras clave: Tephritidae, transgénico, cepas de sexado genético, fluorescencia, VIENNA 8 1260.

CAPÍTULO I

Introducción

Las moscas de la fruta son plagas que limitan el desarrollo frutícola y consecuentemente el crecimiento económico de los países. Dentro de estas plagas, la mosca del Mediterráneo, *Ceratitis capitata* (Wiedemann) ocasiona serios daños en la fruticultura alrededor del mundo, atacando a una considerable cantidad de especies de frutales (Liquido et al. 1991, Copeland et al. 2002). Para combatirla se han creado programas de control que emplean técnicas eficientes y convenientes. Una de ellas es la Técnica del Insecto Estéril (TIE) (Knipling 1955), la cual ha resultado ser un método de control eficaz y amigable con el medio ambiente dentro del enfoque de Manejo Integrado de Plagas en Areas Extensas (MIP-AE) (Hendrichs et al. 1995, Dyck et al. 2005). La TIE consiste en liberar en campo grandes cantidades de insectos estériles, que se aparearán con individuos silvestres para que la descendencia sea inviable (Knipling 1955, 1979). Los insectos que se liberan son criados masivamente en ambientes controlados y esterilizados por radiación gama en estado de pupa, utilizando comúnmente fuentes radioactivas, como Cobalto-60 ó Cesio-137 (Calkins y Draz 1988, Parker y Mehta 2007).

Los programas de control de plagas que aplican la TIE necesitan que las cepas de insectos utilizadas proporcionen garantías biológicas y técnicas (Irvin et al. 2004), debido a que el éxito en el control dependerá de su condición física, por lo que es importante considerar los antecedentes genéticos y de cría masiva de las cepas utilizadas (McInnis et al. 2002). El desarrollo de nuevas y mejores cepas ha sido posible por la aplicación de conocimientos y técnicas de genética cada vez más novedosas (Morrison et al. 2010, Robinson et al. 2004). Por ejemplo, a finales del siglo pasado, empleando genética clásica, se desarrollaron cepas de sexado genético (CSG) de *C. capitata* que permitieron la separación de machos y hembras (Fisher 1998, Franz 2005). Estas cepas, como la VIENNA 8, no son consideradas Organismos Geneticamente Modificados (OGM) o transgénicos, ya que en su creación no se usó material genético de otras especies, sino translocaciones e inversiones de secuencias de sus propios cromosomas (Franz 2005), fenómenos que puede ocurrir naturalmente cuando las polimerasas del ADN copian y reparan los genomas.

Las primeras cepas de sexado genético se caracterizaron por una inestabilidad genética (Cáceres et al 2004) ya que, por el mecanismo natural de recombinación, mostraron una

tendencia a incrementar la frecuencia de individuos que habían perdido el mecanismo de sexado genético. Para incrementar la estabilidad genética, la cepa VIENNA 8 cuenta con la inversión D53, descrita por FAO/IAEA (2001) y citada posteriormente por otros autores (Cáceres et al. 2004, Papanicolaou et al. 2016), que comprende la región de los genes *wp* y *tsl*, impidendo que se produzca recombinación ya que las polimerasas del ADN no pueden translocar esta secuencia invertida, incrementando así la estabilidad genética de la cepa.

Los programas de control de *C. capitata* actualmente emplean la CSG VIENNA 8, la cual es criada masivamente en laboratorios alrededor del mundo (Franz 2005) ó materiales muy relacionados como la VIENNA 8 sin la inversión D53 (VIENNA 8 D53-), que es la que se tiene actualmente en uso en el programa de control Guatemala-México. El sistema de sexado genético (SSG) se basa principalmente en el gen de sensibilidad letal a la temperatura (*tsl,* por sus siglas en inglés) que está localizado en el brazo derecho del cromosoma autosómico 5, próximo al gen que determina el color blanco de la pupa (*wp*, por sus siglas en inglés) y que es utilizado como un segundo marcador morfológico (Kerremans y Franz, 1994; Kerremans y Franz, 1995). Alelos de estos genes fueron transferidos del cromosoma 5 al cromosoma sexual Y mediante la translocación 101, de forma que los machos de esta cepa son heterocigotos para ambos marcadores (*tsl*$^{+}$*/tsl*; *wp*$^{+}$*/wp*) lo que les confiere color de pupario café y resistencia al tratamiento térmico (sobreviviendo a temperaturas de 34^{o}C), mientras que las hembras tienen cariotipo normal y son homocigotas (*tsl/tsl*; *wp/wp*), lo cual permite que su pupario sea de color blanco y sean susceptibles al tratamiento térmico (expresan un efecto letal cuando se someten a temperaturas por arriba de 34^{o}C, particularmente en el estadio de huevo). La eliminación de las hembras se realiza mediante la exposición de los huevos a una temperatura de 34^{o}C por 12 horas, lo que provoca la activación del gen *tsl* en su estado homocigoto (Cáceres et al. 2000). Estudios en campo demostraron la conveniencia de liberar solamente machos estériles de *C. capitata* (Rendón et al. 2000) debido al mayor control que se ejerce sobre las poblaciones de la plaga, respecto a la liberación conjunta de machos y hembras estériles (Hendrichs et al. 1995, Hendrichs et al. 2002).

Con el propósito de incorporar nuevos atributos en los insectos estériles, se ha recurrido a técnicas de ingeniería genética (Hagler y Jackson 2001, Hoy 2003). Estas biotecnologías han producido, en períodos de tiempo relativamente cortos, insectos genéticamente modificados que representan un uso más eficiente de especies útiles, así como alternativas para el control de diversas plagas de importancia en la agricultura, en la salud animal y humana (Robinson et al. 2004, Alphey 2007, Scolari et al. 2011). Ejemplo de ello son modificaciones en las moscas de la fruta del género *Anastrepha* (Meza et al. 2011), *C. capitata* (Scolari et al. 2008), gusano de seda, *Bombyx mori* (Tan et al. 2013), gusano barrenador del ganado, *Cochliomyia hominivorax* (Allen et al. 2004) y mosquitos como *Aedes aegypti* (Alphey y Andreasen 2002, Irvin et al. 2004) y *Anopheles stephensi* (Catteruccia et al. 2003).

La literatura sobre cepas transgénicas de insectos reporta que estas fueron desarrollados a partir de la tecnología basada en transposones, por ejemplo: *piggyBac, P*, *Minos*, *mariner/Mos1* o *Hermes* (Irvin et al. 2004, Robinson et al. 2004, Marrelli et al. 2006, Scolari et al. 2011). Las implicaciones evolutivas de los transposones son discutidas por Syvanen (1984); otros vectores incluyen virus y plásmidos (Brown, J. 2003). La introducción o inserción de componentes de organismos donantes se puede realizar por una técnica llamada microinyección en líneas germinales, usando transposones como vehículo para recombinar en el genoma (intercambiar material genético). Los transposones son elementos de ADN que únicamente cuando sus terminales invertidas repetidas (ITR – Inverted Terminal Repeats) están intactas y acompañado a ambos lados de elementos específicos de ADN, son capaces de integrar el material genético que portan, dentro de otro genoma, todo esto a través de la intervención de una enzima (transposasa). Estos vectores son del tipo no-autónomo, lo que implica que son estables en ausencia de proteínas (transposasas) exógenas al organismo (Alphey y Andreasen 2002, Rasgon y Gould 2005). Los componentes de inserción y los organismos donantes pueden ser diversos como las proteínas fluorescentes de invertebrados marinos. Por ejemplo, la superfamilia GFP (proteína fluorescente verde) obtenida de la medusa bioluminiscente *Aequorea victoria*, o DsRed (proteína fluorescente roja), originalmente derivada de un coral de la clase Anthozoa, y las proteínas fluorescentes de la superfamilia EGFP (proteína fluorescente verde mejorada) (Prasher et al. 1992, Finokiet et al. 2007).

Una revisión de las proteínas fluorescentes (PF) disponibles como marcadores es presentada por Stepanenko et al. (2011) y sobre los métodos de detección de PF por Bin Wu et al. (2011).

Las moscas de la fruta modificadas con genes de marcaje de fluorescencia, como la VIENNA 8 1260, cuando son excitadas por la iluminación de la longitud de onda apropiada muestran fluorescencia bajo filtros UV específicos. La primera generación de EGFP mostraba picos de fluorescencia en un rango de 395-475 nm, el cual se ha mejorado a un solo pico de absorción en 488 nm (Greb 2012), por lo que la presencia de estas proteínas en su cuerpo puede ser aprovechada como marcador para dar una mayor eficiencia a la TIE (Handler y Harrell 2001, Scolari et al. 2008).

En el caso de *C. capitata* se introdujo en su genoma un fragmento de ADN que da origen a la fluorescencia, de tal manera que a partir de cruces de dos marcadores moleculares con la cepa VIENNA 8, se construyó una nueva cepa fluorescente, denominada #1260_F-3_m-1 (VIENNA 8 1260). Ambos marcadores (DsRed controlado por el promotor “medfly polyubiquitin” y EGFP controlado por el promotor “medfly β2-tubulin”) fueron desarrollados por M. Schetelig, E. Wimmer y F. Scolari (Scolari et al. 2008). La cepa VIENNA 8 1260 expresa fluorescencia roja (586 nm) en el cuerpo de todos los estadios de su ciclo de vida y fluorescencia verde (509 nm) en testículos y esperma. La fluorescencia roja del cuerpo de la cepa VIENNA 8 1260 puede contribuir a una mayor eficiencia de la TIE, ya que puede facilitar y mejorar la identificación de los insectos estériles capturados en trampas en el campo. Esta superioridad comparativa es debido a que el sistema de identificación de los machos estériles de la cepa VIENNA 8, que actualmente se tiene en los programas que aplican la TIE, se basa en el pintado con colorantes en forma de polvo Day-glo®, de Day Glo Color Corp, (Cleveland, OH) (Robinson et al. 2004). El polvo colorante se mezcla, a razón de 2 g/l, con las pupas antes de ser irradiadas esperando que al emerger los adultos se impregnen (Schroeder y Mitchell 1981, Hagler y Jackson 2001). Sin embargo, un mal procedimiento de marcaje, un exceso de humedad ambiental o la eventual degradación de los polvos del cuerpo de los insectos estériles por efectos del ambiente demanda un acucioso chequeo, pues un falso positivo (mosca estéril no marcada) puede provocar costos operacionales

innecesarios para los programas de control (Enkerlin et al. 1996, Cáceres et al. 2004). Como ventaja complementaria por el uso de machos VIENNA 8 1260 está la expresión de fluorescencia en el esperma que permite confirmar el apareamiento de machos estériles con hembras silvestres y revelar conocimientos básicos sobre transferencia, almacenamiento, uso, procedencia y competencia de esperma (Scolari et al. 2008).

El uso de insectos OGM, como la cepa VIENNA 8 1260, en una estrategia de control de plagas tiene como prerrequisito que en su genoma tengan una mínima carga detrimental, de tal manera que el costo en su aptitud o desempeño sea bajo, y que no exista riesgo de una transferencia no controlada del transgen por el mecanismo de transferencia horizontal (TH) o transmisión vertical (TV). La TH, también llamada transferencia lateral (TL), ocurre entre especies o cepas distantes (Silva y Kidwell, 2000), en contraposición de la TV que ocurre normalmente de padres a hijos y generaciones subsecuentes, dentro de una especie (Brown J. 2003). Este riesgo ha sido discutido en forma general por Keese (2008) y más específicamente por Yoshiyama et al. (2001) al considerar la posible TH entre un insecto huésped y su parasitoide.

La transferencia horizontal de genes es más común en bacterias y plantas (Syvanen 1994; Arber 2014) incluyendo algas (Raymond y Blankenship 2003), pero ha sido ampliamente documentada en insectos (Daniels y Strausbaugh 1986, Robertson 1995, Robertson y Lampe 1995, Bushman 2002, Lampe et al. 2003, Sormacheva et al. 2012, Nakabachi 2015) y se ha demostrado que es el mecanismo responsable de la difusión de la resistencia a antibióticos en bacterias (De la Cruz y Davies 2000). Se han identificado especies de bacterias entomopatogénicas capaces de mediar la TH en insectos (Li et al. 2001, Acuña et al. 2012). Se ha evidenciado que los organismos poseen una serie de mecanismos naturales que previenen la TH (Matic et al. 1996, Nielsen 1998, Nielsen et al. 1998, Kurland 2005, Thomas y Nielsen 2005) y que la transferencia horizontal de genes es menos frecuente entre grupos evolutivamente distantes (Fraser et al. 2007). Incluso se considera que la TH es un mecanismo natural que ha sido clave en la evolución de los organismos procariotas (Gogarten et al. 1999) y se ha asociado a mecanismos simbióticos en eucariotas (Keeling y Palmer 2008), incluyendo insectos (Husnik et al. 2013).

El uso de cepas transgénicas supone una serie de ventajas para los programas de control (Wimmer, 2005), las cuales podrían significar una reducción de costos y mayor eficacia en el monitoreo, además de proporcionar un mejor control por mitigar varios aspectos del proceso de cría que pueden impactar negativamente en los insectos estériles (Alphey, 2007). Sin embargo, en contraposición se plantea el costo biológico en la aptitud o desempeño de los individuos transgénicos, debido al potencial efecto negativo de la carga del transgen (genes exógenos) y por la alteración de la función original del gen (mutagenesis de la inserción) en el organismo portador (Marrelli et al. 2006). Por lo que, al pretender usar la cepa transgénica VIENNA 8 1260 en un programa de control surge la pregunta: ¿Es viable el uso de cepas transgénicas de *Ceratitis capitata*, como VIENNA 8 1260, en los programas de control que emplean la TIE?

En el caso de mosquitos, Marelli et al. (2006) propone medir el desempeño a través de la sobrevivencia y la reproducción, analizando parámetros como fecundidad, fertilidad, producción de larvas, tasa de desarrollo, emergencia de adultos, proporción de machos y competitividad sexual. Aunque los estudios de mosquitos genéticamente modificados no son nuevos (McDonald et al. 1977, Petersen 1977, Asman et al. 1981, Lorimer 1981), los estudios realizados por Catteruccia et al. (2003), Irvin et al. (2004) y Moreira et al. (2004), reportaron que el desempeño de mosquitos transgénicos es afectado negativamente por la endogamia, por numerosas mutaciones recesivas, y por la fijación de alelos.

Una estrategia para la construcción de cepas transgénicas homocigotas con aceptable aptitud o desempeño es la de generar muchas líneas durante el proceso de construcción de las cepas, con el objetivo seleccionar las líneas que tengan mejor desempeño (Scolari et al. 2011). Encontrar líneas homocigotas estables y con desempeño aceptable es posible dado que la transgénesis *per se* no siempre es detrimental y porque las mutaciones causadas por la mutagénesis de una inserción es un evento probabilístico (Marrelli et al. 2006). La cepa VIENNA 8 1260 de *C. capitata* fue seleccionada de entre cuatro líneas homocigotas desarrolladas por Scolari et al. (2008). En pruebas preliminares de laboratorio, los machos VIENNA 8 1260 fueron exitosos en competitividad sexual, logrando un 47 % de las cópulas totales con hembras silvestres, sin diferencia

significativa respecto a las cópulas de los machos silvestres. Estos ensayos evidenciaron la ausencia de una carga negativa del transgen sobre el desempeño en habilidad de cópula y rapidez para la misma, sobre la longevidad y la capacidad de generar descendencia aún en competencia con machos silvestres. Para verificar la condición homocigota de VIENNA 8 1260 se emplearon tres técnicas moleculares que incluyen la técnica de la hibridación tipo Southern, la amplificación por PCR reverso dirigida a las secuencias de los sitios de integración (TTAA) que flanquean la construcción insertada y la amplificación por PCR multiplex de las terminales del vector usado, piggyBac (Scolari et al. 2008).

La decisión de implementar cepas GM, como la VIENNA 8 1260, en un programa de control debe ser sustentada científicamente, para determinar: factibilidad de cría a escala masiva, desempeño de los machos estériles en el campo (Robinson et al. 2002, Robinson et al. 2004, Scolari et al. 2011), conveniencia operacional (Cáceres et al. 2004). La cepa VIENNA 8 1260 cuenta con información previa a este estudio que sugiere su potencial utilización. Sin embargo, esta información fue generada a pequeña escala. De ahí que se sugiere realizar evaluaciones a nivel de cría masiva y en jaulas de campo, estableciendo competencia entre machos transgénicos y machos silvestres de poblaciones locales (Scolari et al. 2008).

El objetivo general de la presente investigación, fue el de comparar el desempeño, en laboratorio y campo, de la cepa VIENNA 8 1260 con la cepa VIENNA 8 D53- criada masivamente en la Planta El Pino del Programa Moscamed Guatemala, con una capacidad de producción de hasta 3,000 millones de machos estériles por semana (MM/semana), en la actualidad, una producción conjunta Guatemala-Mexico superior a 1,600 millones/semana (1,100 en la bioplanta El Pino y 500 millones/semana en la bioplanta de Metapa, Mexico, a partir de huevo tratado térmicamente en El Pino). También se compara con la cepa VIENNA 8, con la cual comparte la inversión D53, para descartar que los efectos atribuidos al transgen de fluorescencia en VIENNA 8 1260 en realidad se deban a la presencia de la inversión D53. Fue parte de los objetivos, evaluar la TV y TH, por la aplicación de dosis de irradiación y la cría de un parasitoide específico de *C. capitata*, respectivamente. Se espera que los resultados de esta comparación

aporten información con rigor científico para determinar el potencial y riesgo de la cepa VIENNA 8 1260 en algún programa operativo (Rendón et al. 2009).

La presente tesis se organizó de acuerdo a los objetivos específicos del presente estudio, que fueron: 1) evaluar el desempeño de machos y hembras de la cepa VIENNA 8 1260 en cría a escalas pequeña, media y masiva, así como la competencia de los machos en condiciones de campo (Capítulo II); 2) evaluar el efecto de diferentes dosis de irradiación en la esterilidad y seguridad biológica, determinando la dosis óptima que evite la TV del transgen (Capítulo III) y 3) determinar la probalidad de la TH, utilizando para este caso la cercana relación huésped-parasitoide de *Ceratitis capitata* (Wied) con *Fopius ceratitivorus* Wharton (Capítulo IV). Al final se incluye un capítulo que resume los resultados y sus implicaciones.

CAPÍTULO II

Desempeño de una cepa genéticamente modificada de la mosca de la fruta, *Ceratitis capitata* (Wied.) para su uso en un programa de manejo integrado de plagas en áreas extensas (MIP-AE) que emplea la técnica del insecto estéril (TIE).

Los contenidos de esta sección se encuentran en el siguiente artículo:

Ramírez-Santos et al. 2016. Performance of a Genetically Modified Strain of the Mediterranean Fruit Fly (Diptera: Tephritidae) for Area-wide Integrated Pest Management with the Sterile Insect Technique.

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Performance of a Genetically Modified Strain of the Mediterranean Fruit Fly (Diptera: Tephritidae) for Area-wide Integrated Pest Management with the Sterile Insect Technique

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Abstract

The genetically modified strain of *Ceratitis capitata* VIENNA 8 1260 has two morphological markers that exhibit fluorescence in body and sperm. To asses the feasibility of its use in area-wide integrated pest management (AW-IPM) programs using the Sterile Insect Technique (SIT) its rearing performance and quality control profile under small, medium and large scales was evaluated, as well as in field cages. The VIENNA 8 1260 strain had a lower yield than the control strains, VIENNA 8 with D53 inversion (VIENNA 8) and without D53 inversion (VIENNA 8 D53-). At mass rearing scale, yield gradually increased in three generations without reaching the control strains values. The VIENNA 8 1260 strain was stable in the genetic sexing mechanism (>99.9 %) and expression of fluorescence (100 %). In field cages the VIENNA 8 1260 males reduced the mating potential of wild males in the same magnitude as the VIENNA 8, when evaluated in independent cage tests. However, the relative sterility index (RSI) and the strain male relative performance index (S_nMRPI) of VIENNA 8 1260 males were significantly lower than those of the VIENNA 8. There were no significant differences in longevity of these strains. The potential application of the VIENNA 8 1260 in AW-IPM programs is further discussed.

Key words: genetic sexing, fluorescence, mass rearing, sexual competitiveness, VIENNA 8 1260.

Introduction

Fruit flies are pests that severely limit the development of fruit growing and, as a consequence, economic growth of countries is affected. The Mediterranean fruit fly, *Ceratitis capitata* (Wied.), causes damage to several species of fruits worldwide. Over 400 species of plants have been reported as *C. capitata* hosts (Copeland et al. 2002). Because of its economic impact, area-wide integrated pest management programs (AW-IPM) have been established (Henrichs et al. 2002). The Sterile Insect Technique (SIT) is an effective method to control this pest, used as a complementary tool within the AW-IPM approach (Enkerlin 2005). This technique includes the mass rearing and field release of large amounts of sterile males that will mate with the wild females, thus eliminating their reproductive capacity (Knipling 1955, 1979). The principles of the SIT have remained through the years and its viability has been widely demonstrated with the control of several pest species (Dyck et al. 2005).

Several modern biotechnologies have been incorporated to increase its effectiveness (Robinson and Hendrichs 2005, Franz and Robinson 2011). By the end of the past century and using classic genetic techniques, genetic sexing strains (GSS) were developed in order to allow the separation of *C. capitata* males and females (Fisher 1998, Franz 2005). This breakthrough significantly improved the control of the pest (Hendrichs et al. 1995, Rendón et al. 2000a, Hendrichs et al. 2002). The VIENNA 8 GSS allows practical separation of males and females, based on a reciprocal translocation between the chromosome that determines the male sex (Y) and an autosomal chromosome (5) that carries the wild type alleles to the markers or mutations used to build up the genetic sexing mechanism based on the sensibility to temperature (*tsl*) and pupa color (*wp*) (Rossler 1979, Kerremans and Franz 1994). This strain includes a movement of alleles of the *wp* and *tsl* genes from the autosomic chromosomes number 5 to the Y chromosome, namely the translocation

101, which confers the males their brown puparia color and resistance to thermal treatment (Franz 2005). A practical application of the *tsl* gene is that by the thermal treatment of the eggs (24 hrs at 34^{o}C) all females are eliminated, thus producing a male-only colony.

Due to the combination of these two genes in VIENNA 8, females have white puparia and may be eliminated by the thermal treatment of the eggs, while males express brown puparia and would be resistant to thermal treatment. In each generation, a small number of individuals called *recombinants* would tend to reverse these genetic traits: some females would emerge from brown pupae and be resistant to thermal treatment, while some males would emerge from white pupae and be susceptible to thermal treatment, as a result of a genetic instability described by Cáceres *et al.* (2004) who also summarizes the measures to maintain the lowest level of recombination in mass rearing, through the design of more stable genetic sexing strains, the use of the Filter Rearing System (FRS), among other solutions.

The D53 inversion was an improvement to the GSS (Franz, 2005), introduced after the publication by Caceres *et al.* (2004) that describes all GSS available by that time. By inverting the DNA sequence in the *wp* and *tsl* region of the chromosome, it prevents that the DNA polymerases responsible for the DNA reparation produce recombinants, resulting in a strain with a higher genetic stability. The VIENNA 8 D53- strain mass reared at El Pino facility, Guatemala, lacks this D53 inversion, representing a step previous to the development of the VIENNA 8 strain (with D53 inversion).

Recently, genetic engineering techniques have been used with the purpose of introducing new features in *C. capitata* and produce stable, genetically modified strains (GMS), in relatively short periods of time (Schetelig 2009). Such is the case of the #1260_F-3_m-1 (VIENNA 8 1260) strain that has fluorescent body and sperm. This GMS was developed from the GSS VIENNA 8, due to

the incorporation of two molecular markers: DsRed, controlled by the Medfly polyubiquitin promoter and EGFP, controlled by the Medfly β2-tubulin promoter (Scolari et al. 2008). The male's sperm and testicles express the green fluorescence color and the body (in both sexes) expresses red fluorescence through all life cycle stages. The appearance of these colors under ultraviolet light with the proper wavelenght and filters allows visualizing the fluorescence trait under a stereoscope, in a practical way. So, we can take advantage of this kind of fluorescent proteins as markers within the SIT (Handler 2000, Hagler and Jackson 2001, Handler and Harrell 2001). The red fluorescence in the body of the VIENNA 8 1260 males may contribute to facilitate the monitoring of the sterile males captured in traps (Robinson et al. 2004).

Currently, the males without fluorescence (i.e. VIENNA 8) must be marked with powder dyes (Schroeder and Mitchell 1981, Hagler and Jackson 2001) such as Day-Glo ® from DAYGLO Color Corp. (Cleveland, OH), with some degree of failure risk (1-5 %) in the marking of the body due to its degradation, mainly in the wet, rainy season. This uncertainty demands a thorough examination of the trap-recovered males because a false positive (unmarked sterile male) may cause unnecessary operational costs to the AW-IPM programs (Enkerlin et al. 1996, Cáceres et al. 2004).

The use of genetically modified strains supposes a series of advantages for the AW-IPM programs (Robinson et al. 2004, Wimmer 2005, Alphey 2007). But it also may have a cost in terms of performance of the transgenic individuals due to the probable negative effects of the transgene (endogenous genes) or the alteration of the function of the original gene (insertion mutagenesis) (Irvin et al. 2004, Marrelli et al. 2006). A viable strategy is the generation of several lines during the construction of the GMS, a procedure that allows the selection of the best lines to generate homocigotic transgenic strains with an acceptable profile (Scolari et al. 2011). It is posible to find stable homocigotic lines with an adequate performance because the transgenesis *per se* is not

always detrimental and the mutagenesis caused by a DNA sequence insertion is a probabilistic event (Marrelli et al. 2006). The VIENNA 8 1260 strain was selected amongst four lines and, in order to verify its homocigotic condition, three molecular techniques were used, including a test specific for DsRed and a characterization by multiplex PCR (Scolari et al. 2008). The process of genetic stabilization of the fluorescence transgene is described by Schetelig et al. (2009).

As it has been done with other insects species (Catteruccia et al. 2003, Allen et al. 2004, Irvin et al. 2004, Facchinelli et al. 2013, Harvey-Samuel et al. 2014) and strains of fruit flies (Rendón et al. 2000, McInnis et al. 2002, Cáceres et al. 2004, Meza-Hernández and Díaz-Fleischer 2006), it is imperative to assess the performance of the VIENNA 8 1260 strain under mass rearing and field cage conditions. The goal of this study was to evaluate the performance and stability of the VIENNA 8 1260 GMS under masss rearing conditions at three scales of magnification, as well as the mating competitiveness and longevity of the males in field cages. This knowledge may serve to determine the potential use of the strain as part of the SIT in AW-IPM programs against *C. capitata*.

Materials and Methods

The performance of the VIENNA 8 1260 and VIENNA 8 D53- (from El Pino Medfly laboratory, Guatemala) strains was evaluated based on their yield and quality at small, medium and large scales. The longevity and sexual competitiveness of both strains *vs* wild males was tested in field cages (with double biosafety measures to avoid escapes). The stability of the VIENNA 8 1260 strain was measured by the expression of the fluorescence gene and recombination, expressed as the proportion of females emerging from brown puparia (type 1a) reared from thermally treated

eggs, thus resistent to thermal treatment (type 1b) (Franz 2005). The yield and quality of the VIENNA 8 1260 strain, including percent recombination, was also compared to the VIENNA 8, that in addition to the 101 translocation carries the D53 inversion of a chromosome segment that includes the *tsl* and *wp* genes (Franz 2005). These insects were obtained from a small laboratory colony. A second evaluation of the VIENNA 8 1260 vs VIENNA 8 strains was conducted to rule out the possibility that the differences already documented in the VIENNA 8 1260 vs VIENNA 8 D53- were due to the presence of the D53 inversion in the VIENNA 8 1260 strain and not to the effects of the fluorescence transgene.

This study was conducted at the San Miguel Petapa (SMP) laboratory from the Moscamed Program, 15 km to the south of Guatemala city (N 14º 29´ 2", W 90º 36´ 53"), confined to an area that is officially recognized as an Arthropod containment level 2 quarantine facility. The rearing conditions were: temperature 24 ± 2°C, relative humidity 65 ± 5 % and photoperiod of 12:12 hrs L:D, with a 1,500 lux illumination. The field tests were set in a 600 m^2 area within the SMP gardens, in 0.5 mesh plastic net cages of three types: A) circular cage (CC) of 3 m in diameter x 2 m tall; B) rectangular longevity cage (RLC) of 1 x 1 x 1.40 m; and C) big square cage (BSC) of 10 x 10 x 5 m. The CC and RLC type cages were installed within the BSC to increase biosafety and avoid the escape of GMS insects. Inside each CC and RLC cages a young coffee plant (*Coffea arabica* L.) was enclosed. During the evaluation period the external temperature oscilated between 22 and 28° C, relative humidity between 55 and 70 % and natural illumination between 500 and 3500 lux.

Yield and Quality. The performance of the VIENNA 8 1260 (generation 36) and VIENNA 8 D53- (generation 73 by January 2015) strains was compared through four generations: 1) filter (P generation), 2) initiation (F1), 3) injection (F2) and 4) release (F3), corresponding to the stages of the Filter Rearing System (FRS) described by Fisher and Cáceres (2000). Larval diet used at all

scales was a mixture of sugar, yeast, wheat flour, sodium benzoate, hydrochloric acid, water and sugar cane baggasse or corn cob, a modification of the standard Seibersdorf diet described by Braga et al. (2006). Eggs for the small and medium scales were not thermally treated, thus allowing the production of males and females. At large scale, eggs were thermally treated at 34°C for 12 hrs to produce only males (Fisher 1998). The fecundity (number of eggs/female) and fertility (percent egg hatch), as well as the egg to pupae and egg to adult conversion rates were measured. Quality was determined through pupal weight; pupal color proportion, adult emergence, flight ability and longevity (FAO/IAEA/USDA 2003, QC manual version 6, May 2014). Both strains, VIENNA 8 1260 and VIENNA 8 D53- were obtained from colonies maintained in the laboratory. Originally, these were provided by the Joint FAO/IAEA Insect Pest Control Subprogramme (IPC) in Seibersdorf, Austria (Scolari et al. 2008, Franz et al. 2005) a part of the Food and Agriculture (NAFA) division of the FAO/IAEA whose activities may be traced back to the year 1964.

The small scale consisted of placing 1000 eggs on top of 85 g of diet (ca. 12 eggs/g) for their larval development. The first three generations (P, F1 and F2) were evaluated for each strain (VIENNA 8 1260 and VIENNA 8 D53-), with 19 to 20 replicates per strain per generation. For egg counting, eggs were aligned on wet black filter paper with the help of a fine paintbrush and observed under a Leica L2 stereoscope. The yield was determined by counting the total number of larvae/pupae recovered from the diet.

The medium scale included units of 1 kg of larval diet with 0.6 ml of eggs (~28,000 eggs/ml, 16,800 eggs per tray) collected from mass-rearing egging cages (0.77 x 0.71 x 0.05 m) with the following female:male ratios: 1:1 for the P generation, 2:1 for F1 and 4:1 for F2. The number of units were: 25 for the P generation, 50 for the F1 and 100 for the F2. Three replicates were done for each generation. Fecundity and fertility was recorded on each generation.

For the large scale, the eggs collected from the F3 generation from the mass-rearing egging cages (with a 5:1 female: male ratio) were thermally treated for the production of only males. The units consisted of standard trays with 5 kg of larval diet and 5.5 ml of eggs (~154,000 eggs). Three replicates, each of 0.6 ton (120 trays) of larval diet were done. Yield was measured in two ways: a) as larval recovery (LR) that relates the volume (liters) of larvae collected per kg of diet and b) dividing the total number of pupae collected (MM=millions of males) by the amount of diet (ton), thus obtaining yield in MM/ton. At this scale half of the pupae collected was irradiated and compared to the non-irradiated half, in order to document the effects of irradiation on the insect quality for the different strains.

Comparison of VIENNA 8 1260 and VIENNA 8 Strains. To rule out the possibility that in the VIENNA 8 1260 *vs* VIENNA 8 (without inversion) the documented effects could be due to the presence of the D53 inversion in the VIENNA 8 1260 strain, the yield and quality of this GMS was compared against VIENNA 8, including the genetic sexing mechanism and performance of both strains, following the same procedure as the VIENNA 8 1260 vs VIENNA 8 D53- comparison, but for just one generation.

Stability of the VIENNA 8 1260 Strain. At the three rearing scales, the stability of the strain was referred to two aspects: a) percentage pupae and adults that expressed fluorescence in body parts and sperm; and b) percentage of recombinant females and proportion of pupae colors produced from thermally treated eggs from which the male only colony was produced.

At small scale, the expression of fluorescence was recorded in all the pupae collected. At medium and large scales, samples of 5 ml of pupae were taken. Pupae and adult fluorescence was detected under a Leica® MZ-FL III stereoscope with GFP Plus filter for green fluorescence in testicles and

Texas Red for red fluorescence in pupae and adult body. From the total of pupae and adults obtained, 56 random samples were analyzed at the ECOSUR biotechnology laboratory, by PCR using two primers: 1260DsRedf (GCTCCTCCAAGAACGTCATC) and 1260DsRedr (TGGTGTAGTCCTCG TTGTGG) developed by Macrogen Inc (Rockville, MD) to detect the presence or absence of the genetic markers of fluorescence.

Sexual Competitiveness. The mating indexes of the VIENNA 8 1260 and VIENNA 8 D53- irradiated/sterile males were measured in independent cage tests against wild males, competing to mate with wild females. The wild males and females were obtained from Medfly infested coffee berries (*Coffea arabica* L.) collected in the southeastern region of Guatemala.

The irradiation procedures were as follows: 48 hrs before the adult emergence (11 days old) the pupae were placed in plastic bags and sealed to produce hypoxia. After 2 hrs, pupae from both VIENNA 8 1260 and VIENNA 8 D53- strains were irradiated with a Cesium-137 source (Isomedix Inc. Husman model 521 series 004 irradiator, Whippany, NJ 07981) at a central dose of 145 Gy. The wild insects were not irradiated. Soon after emergence, the adults were sorted by sex, placed in 0.30 x 0.30 x 0.30 m plexiglass cages and confined in different rooms under laboratory conditions. The males were transferred in groups of 25 individuals to 1 liter plastic containers and provided with water and a 3:1 sucrose: yeast mixture *ad libitum*. A maturation time period of 5 to 6 days was allowed for laboratory males and 10 days for wild males and females. To differenciate the males, a different color of Day-Glo® dye was added to the pupae of each strain, at 2 g/liter (=4 g/kg). As a secondary mechanism of differentiation, 48 hrs prior to the start of the tests, a different color of vegetable pigment was added to the adult food of each strain. The wild adults were not marked.

Five treatments were set in order to compare mating competitiveness between strains: (T1): in each field cage, at 06:00 hrs, 50 males from each laboratory strain and 50 wild males were released, an hour later 50 wild females were also released; (T2): control with 150 wild males and 50 wild females in each cage; (T3): 50 males from the VIENNA 8 1260 strain, 50 wild males and 50 wild females per cage; (T4): 50 VIENNA 8 D53- males, 50 wild males and 50 wild females per cage; (T5): control with 100 wild males and 50 wild females in each cage. All cages were observed for 3 hrs and each mating couple was collected in a 2 cm in diameter plastic vial and identified with the order of mating and type of male.

For each laboratory strain, mating competitiveness was calculated through: 1) the proportion of matings, 2) the strain male relative performance index (S_nMRPI) that measures the competition of males from two strains (S_1 and S_2), within the same cage, against wild males ($S_1MRPI=S_1W/WW+S_1W+S_2W$ or $S_2MRPI=S_2W/WW+S_1W+S_2W$) which is an adaptation of the Relative Mating Performance Index (RMPI) applied when there is only one kind of laboratory male in competition with wild males (FAO/IAEA/USDA, 2003) and 3) the Relative Sterility Index (RSI) proposed by McInnis et al. (1996).

Longevity. The longevity of sterile males from the VIENNA 8 1260 y VIENNA 8 D53- strains was compared in field cages. One hundred males from each strain were released in each RLC, placed inside the BSC. Liquid diet (water and sucrose) was provided as a source of food to 56 cages (20 for VIENNA 8 1260 males + 36 for VIENNA 8 D53- males), while 75 cages (40 for VIENNA 8 1260 males + 35 for VIENNA 8 D53- males) were kept without food. The number of live adults was recorded every 12 hrs until the last male died.

Data Analysis. To determine the significance of the differences detected in the arithmetic means of the dependent variables (yield and quality parameters, fluorescence expression and recombination) in each treatment, the data were analyzed using a 1-way or 2-way ANOVA with replications, accordingly with the presence of one or two factors and its levels in each test, with the InfoStat/P software (Di Rienzo et al. 2010). The level of significance was fixed in 5 % ($p<0.05$).

To evaluate the effects of the strains and generations on the yield, the variable 'eggs to pupae conversion' was selected. A linear regression analysis was done between MM/ton and the generation. The number of viable eggs was calculated by multiplying fecundity by fertility. With the longevity data (number of live adults) the live proportion (l_x) was calculated at different times and the mean life (time required to reach 50 % mortality) was estimated by the proportional triangles analysis in a spreadsheet.

Results

Yield and Quality

Small Scale. No differences were found in the yield or quality of the different generations (P, F1 and F2) of each strain, but a significant difference was detected in the total number of pupae and adults of the VIENNA 8 1260 *vs* VIENNA 8 D53- strains (Table 1) regardless of the generation. For all the yield variables evaluated, the VIENNA 8 D53- had a higher average than the VIENNA 8 1260 strain, as shown for the egg to pupae conversion rate (Fig. 1A). At this scale, the VIENNA 8 D53- strain produced 40 % more adults as compared with the VIENNA 8 1260. No differences were found when analyzing the pupae color (white, brown) or percentage of recombination of the different strains.

Medium Scale. The females of the VIENNA 8 D53- had a higher fecundity than the VIENNA 8 1260 females in all the generations evaluated (Table 2). The analysis of the fecundity by fertility product showed that the VIENNA 8 D53- females produced a higher number of viable eggs per female per day (23) than the VIENNA 8 1260 females (14). The average conversion of eggs to pupae was significantly lower for VIENNA 8 1260 (0.44) as compared with the VIENNA 8 D53- (0.63) (Table 2). There were differences in egg to pupae conversion between strains in each generation (Fig. 1B). Significant differences between strains were also recorded for the percentage of the pupal color (59.86 % females for VIENNA 8 D53- and 56.50 % females for VIENNA 8 1260) and pupal weight of VIENNA 8 D53- *vs* VIENNA 8 1260 for both males (7.55 mg *vs* 7.12 mg) and females (8.46 mg *vs* 7.62 mg). The percentage of adult emergence and 'males from white pupae' recombinants showed no differences due to the strains or generations, but there was a significant difference in the 'females from brown pupae': the VIENNA 8 1260 strain showed a lower percentage of this type of recombinants in all generations.

Large Scale. There were significant differences between strains in larval yield (RL), egg to pupae conversion rate and yield (MM/ton) (Table 3). The egg to pupae conversión was 46 % lower for VIENNA 8 1260 (0.20) as compared with the VIENNA 8 D53- (0.37) (Fig. 1C). The average yield for VIENNA 8 1260 was 6.54 MM/ton and 12.03 MM/ton for VIENNA 8 D53-. However, the regression analysis shows that the VIENNA 8 1260 yield increases almost 2 MM/ton per generation and suggests that a similar yield could be achieved after 5 generations (Fig. 2).

Significant differences were detected in pupal weight and longevity (mean life –hrs). VIENNA 8 1260 had lower pupal weight and longevity than VIENNA 8 D53- (irradiated or not irradiated). No differences between strains were detected for the percentage emergence or flight ability (Table 3). For both strains, the thermal treatment was effective in eliminating the females,

producing >99 % males. For VIENNA 8 1260, the percentage recombinants 'females from brown pupae' (1a-type) and resistent to thermal treatment (1b-type) was zero. Irradiation reduced the percentage emergence, but no differences were detected due to the strain.

Performance of the VIENNA 8 1260 *vs* VIENNA 8 Strain.

At small scale, significant differences in yield were observed between VIENNA 8 1260 and VIENNA 8, 20 % less adults were recovered from the VIENNA 8 1260 strain (Table 1). The egg to pupae conversion rate shows a similar trend (Fig. 1A). No differences were found for the percentage of pupal color, percentage of adult emergence (males and females) and percentage recombinants, which was very low for both strains.

At medium scale, the females of both strains produced the same amount of eggs per day. However, the fertility (percentage hatching) was significantly higher in VIENNA 8, producing more viable eggs per female per day (14.62) than VIENNA 8 1260 (13.20) (Table 2). The egg to pupae conversion rate was significantly higher in VIENNA 8 (0.56) *vs* VIENNA 8 1260 (0.45) (Fig. 1B). There was no difference in the quality of the strains, including the percentage of recombination.

At large scale, the VIENNA 8 strain had a higher yield than the VIENNA 8 1260 strain (Table 3). The egg to pupae conversion rate was 12 % lower for VIENNA 8 1260 than for VIENNA 8 (Fig. 1C). No differences were detected in the quality parameters evaluated, including percentage recombination. An average yield of 9.3 MM/ton was estimated for the VIENNA 8 1260 vs 10.6 MM/ton for the VIENNA 8. For both strains the thermal treatment was effective in eliminating the females, thus producing > 99 % males.

Stability of the VIENNA 8 1260 Strain.

Stability of the Fluorescence Expression. In all the pupae and adults of each VIENNA 8 1260 generation, a 100 % fluorescence expression was registered. There was a perfect correlation between pupae and adult body red fluorescence, so that detecting it in pupae means that it will be expressed in adults. These adults also showed 100 % green fluorescence expression in testis. The PCR analysis confirmed the presence of red fluorescence markers in all the pupae which correlated with the adult phenotypic expression of red and green fluorescence.

Stability of the Genetic Sexing Mechanism. No differences in the percentage of brown pupae or the percentage of the 'females from brown pupae' recombinants were detected when comparing both strains at small, medium or large scale (Table 3).

Sexual Competitiveness.

The percentage of matings by wild males was significantly greater than those by the males from the VIENNA 8 1260 and VIENNA 8 D53- strains (Fig. 3). However, the percentage of matings by wild males was reduced by 42.4 % when competing with males from both laboratory strains in the same cage (T1 *vs* T2). When competing in independent cages, the VIENNA 8 1260 males reduced 26.3 % the percent of matings of wild males (T3) and the VIENNA 8 D53- reduced the mating of wild males by 28 % (T4). The strain male relative performance index (S_nMRPI) was higher for VIENNA 8 D53- (0.27) than for VIENNA 8 1260 (0.13). The relative index of sterility (RSI) of the VIENNA 8 D53- males (0.28) was higher than that of the VIENNA 8 1260 males (0.19).

Longevity.

No differences were detected in the longevity of VIENNA 8 1260 and VIENNA 8 D53-, but the addition of food had a significant effect in increasing the longevity of both strains. With liquid diet, the average life was 5.17 days *vs* 1.93 days without liquid diet. Survival (l_x) curves are shown in Fig. 4.

Discussion

The results showed that the VIENNA 8 1260 strain had lower yields and quality parameters as compared with the VIENNA 8 D53- strain, at the 3 evaluation scales. However, the VIENNA 8 1260 was stable in terms of recombination and had a trend to increase its yield at an approximate rate of 2 MM/ton per generation, so that it could be expected that in 2-3 generations more it might reach yield levels similar to those of the VIENNA 8 D53- strain, reared at El Pino Medfly facility from the Moscamed Program in Guatemala. Additionally, it expressed fluorescence in 100 % of the pupae and adults, confirmed by the presence of genetic markers detected by the PCR, results that emphasize the reliability of this internal marking mechanism.

At small and medium scale, the VIENNA 8 1260 strain produced less males than the VIENNA 8 D53- strain, both with (30 %) and without thermal treatment (33 %). This reduction is due to the effect of the strain and may be the result of the genetic modifications by the insertion of the transgene; the thermal treatment is not a key factor. But these differences may also be related to the initial size of the VIENNA 8 1260 population and its environmental conditions which may limit the effects registered for mass rearing. This adaptative process have been documented for other Mediterranean fruit fly strains (Leppla et al. 1983, Cáceres et al. 2004, Liedo et al. 2007). It is probable that the process is favoured because at El Pino facility mass rearing mechanisms, such as

the FRS, the egg producing cages and the amplification system for larvae and pupae production, would promote a higher performance by constant selection of high quality biological material under controlled environments where nutritional elements are offered without restriction to the selected individuals, allowing them to gradually express their maximum biological potential.

The VIENNA 8 1260 strain had a greater stability in the genetic sexing mechanism, as it had a slightly lower percentage recombination as compared with the VIENNA 8 D53- strain. While this difference was not significant at $p<0.05$, it is important to consider that at full scale in any mass rearing facility, millions of insects will be produced each day, thus increasing the probability that even small differences will become significant by the number of repetitions at this scale. The difference in the stability of the strains may be explained by the presence of the D53 inversion that the VIENNA 8 1260 strains carry in its genome (Franz 2005), being it developed from the VIENNA 8 strain (Scolari 2008). As previously stated, the presence of the D53 inversion avoids that the DNA polymerase enzymes copy sequences from other chromosomes right in the inverted region where the *tsl* and *wp* genes are located, eliminating the natural process of recombination. The zero recombinants values for the VIENNA 8 1260 females from brown pupae (1a-Type) and resistant to thermal treatment (1b-Type), reared from thermally treated eggs are comparable to the values reported earlier for the VIENNA 8 strain (Franz 2005). This stability is expected from strains that are to be used in mass rearing for AW-IPM programs that rely in the SIT.

The differences in yield and quality in favor of the VIENNA 8 D53- were lower than those of the VIENNA 8, when each strain was compared with the VIENNA 8 1260. The yield of the VIENNA 8 1260 at the three scales, expressed as the egg to pupae conversion rate was slightly lower than the VIENNA 8, from eggs with or without thermal treatment. The difference may be related to the genetic modification by the insertion of the transgene (Scolari et al. 2011 y Marreli et al. 2006).

However, this difference does not exclude the potential use of VIENNA 8 1260 in the SIT. The quality of the VIENNA 8 1260 strain was acceptable and equivalent to that of the VIENNA 8 D53- and VIENNA 8 strains, based on the standards set by the FAO/ IAEA/USDA (2003) manual.

The VIENNA 8 1260 strain offers several advantages over the traditional marking procedure with powder dyes to identify the sterile males captured in AW-IPM programs. The main advantages that the VIENNA 8 1260 offers are: a) a higher precision in the sterile insect marking procedure (independent from errors in the calculation of the dye/pupae proportion, humidity in the pupae marking rooms or the application procedure); b) faster, less complex identification of the trap-captured sterile males from the field; c) safer working conditions for the personnel by avoiding the prolongued exposure to powder dyes (Khoury and Abi 2001); d) a significant reduction in the environmental impact and economic cost of all mass rearing, release and field activities related to the use of powder dyes. All of these improvements would bring a higher certainty to the monitoring systems, allowing an unequivocal discrimination between sterile and fertile males in the field (Enkerlin et al. 1996) that could also be verified by genetic means if the need arises. However, it is important to recognize that at its present status, it would be wise to consider the use the VIENNA 8 1260 fluorescence marker as a secondary marking system, combined with a primary marking system such as the use of external fluorescent powders, while it is validated at full scale as the only, primary marking system. At the end, this decision would pertain to the authorities of each SIT Programme as it is stated by the FAO/IAEA/USDA (2003), after considering the risks, advantages and disadvantages of each marking system.

In field cages, the VIENNA 8 1260 strain was able to reduce by 26.3 % the matings of wild males, only 2 % below the reduction documented for the VIENNA 8 D53- strain reared at El Pino and succesfully tested in the USA-Mexico-Guatemala AW-IPM program (Enkerlin et al. 2015), as well

as other SIT programmes for the control of the Mediterranean fruit fly around the world (Shelly and McInnis 2016). The S_nMRPI and RSI were also lower for the VIENNA 8 1260 males *vs* the VIENNA 8 D53- strain. Considering that the sterile males must compete succesfully for wild females in the field, in order to achieve the SIT expected effects (Scolari et al. 2011), it might be necessary to adjust the sterile/fertile overflooding ratio in the field, to compensate the documented differences in the sexual performance of the strains. The lower mating competitiveness of the VIENNA 8 1260 vs wild males is an important issue that has implications on the operational costs of the control program. If this issue is not solved, the use of the VIENNA 8 1260 strain might be unviable and not justifiable in terms of the benefit/cost analysis. This is a common problem in mass-reared strains that can be partly overcome by nutrition, hormones, aromatherapy (Pereira et al. 2013) and selection of best performing males for colony maintenance (McInnis et al. 2002b, Mc Innis et al. 2005, Quintero-Fong et al. 2016).

There was no difference in the longevity values of the males of different strains, either in the 3 laboratory scales tested or in the field cages, between the adults of VIENNA 8 1260 *vs* VIENNA 8 D53-. This confirms that the VIENNA 8 1260 males have the potential to survive in field cages, as it has been demonstrated with the VIENNA 8 D53- strain males. However, the presence of food (liquid diet) was decisive to increase the survival (l_x) of the males from both strains. These findings emphasize the relevance of the nutritional reserves that the sterile males must have at their moment of release in the field (FAO/IAEA/USDA, 2003), because the potential stress conditions (no water or food) would reduce their life expectancy and limit the opportunity of finding and mating with wild females. Our data suggest an overall low survival strains but the particular conditions of the field cages at the moment of the tests might have been a key factor. It is important then to focus on the absence of differences in longevity due to the strain.

The results at a small scale represent the usual size of most of the published experiments, thus allowing a direct comparison. However, medium to large-scale results are the most decisive to assess the potential use of a strain in an AW-IPM program, precisely because of the scale, that reflects in a better way the real conditions and effects of mass rearing.

In spite of the performance differences documented between the VIENNA 8 1260 strain and the VIENNA 8 D53- or VIENNA 8 strains, it is important to point out that these results suggest that the yield and quality are subject to improvement (i.e. by modifying some of the rearing conditions) and that the VIENNA 8 1260 expresses desirable traits, such as the stability in the fluorescence and genetic sexing mechanism (*tsl* and *wp* genes), which makes it a strong candidate to be included in Mediterranean fruit fly SIT, AW-IPM programs.

Our results indicate that the VIENNA 8 1260 strain may be mass-reared and it may reach aceptable yield levels (MM/Ton) and efficiency (egg to pupae and egg to adult conversion rates) under large scale mass rearing conditions. The VIENNA 8 1260 sterile males released in the field have the ability to survive and compete for wild females, inducing sterility in their populations. The stability in the expression of red fluorescence in the body and green fluorescence in the testis of the males offers a series of improvements to the field monitoring activities. Its low level of recombinants, particularly 1a-type (females from brown pupae) plus 1b-type (resistent to thermal treatment) is a remarkable advantage as compared with the strains without inversion, such as the VIENNA 8 D53-. The comparison between VIENNA 8 1260 *vs* VIENNA 8 confirms that the differences documented are partially explained by the D53 inversion while others may be due to the effect of the genetic insertion of the transgene responsible for the fluorescence in VIENNA 8 1260.

In conclussion, the VIENNA 8 1260 strain is a potential candidate for its use in SIT, AW-IPM programs, due to the high stability of the genetic sexing mechanism, the steady expression of

fluorescence through all the generations under study and an acceptable yield and quality with a potential of adapting to the laboratory conditions, with operational costs equivalent to those of other strains under mass-rearing systems. However, if some issues such as the lower sexual competitiveness and yield are not solved, this GMS would not be suitable to replace the current strains used in Medfly SIT programs.

The notation system used in this article to refer to the GMS or GSS (i.e. VIENNA 8 1260, VIENNA 8 and VIENNA 8 D53-) wants to provides a concise and unequivocal way of citing these strains while following the nomenclature already used by authorities in this topic (Cáceres et al. 2004) and more recently by several authors, including Rempoulakis et al. (2016).

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Figures

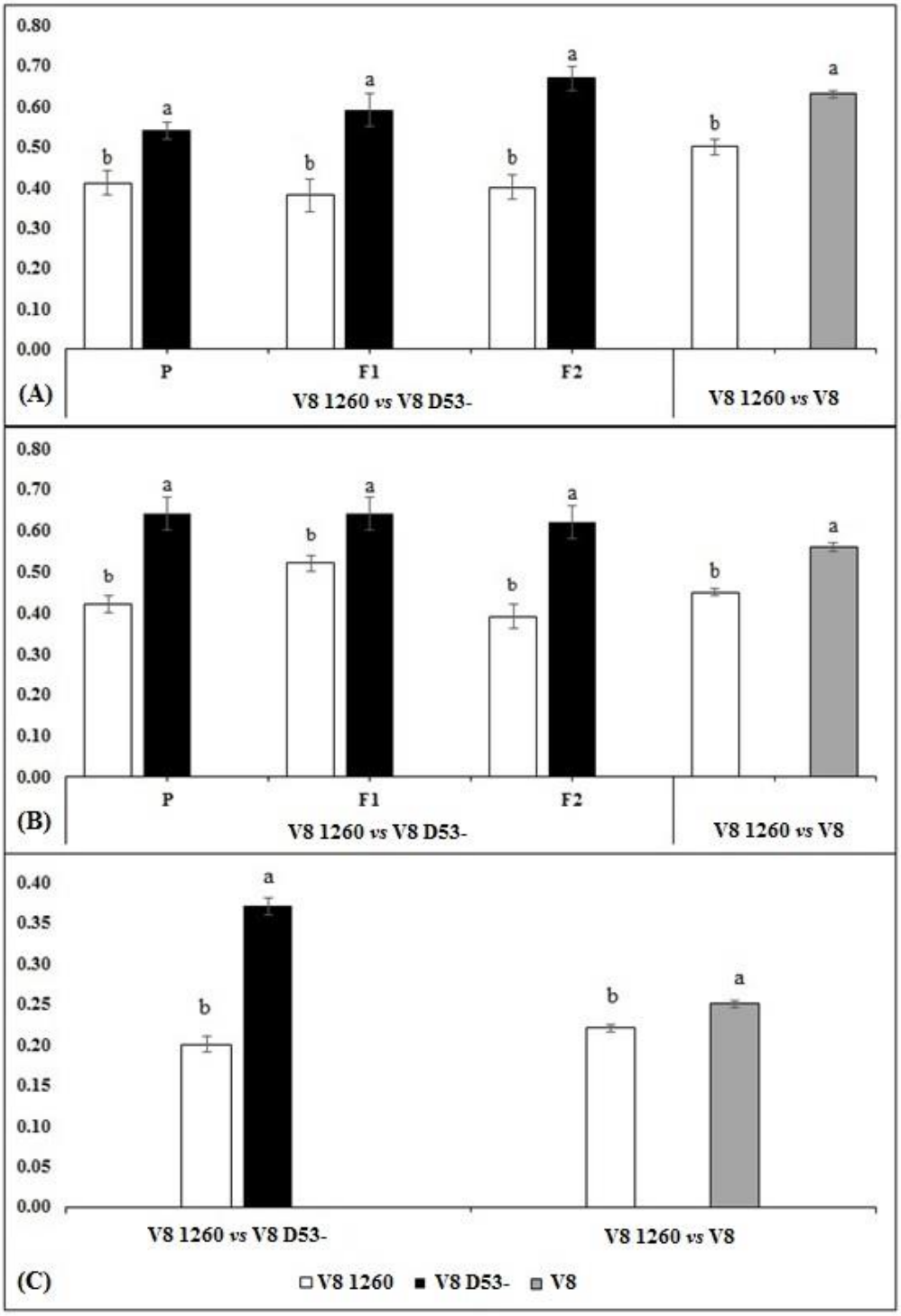

Fig. 1. Egg to pupae conversion rates of the VIENNA 8 1260, VIENNA 8 D53- and VIENNA 8 strains. A) small scale, B) medium scale and C) large (mass rearing) scale.

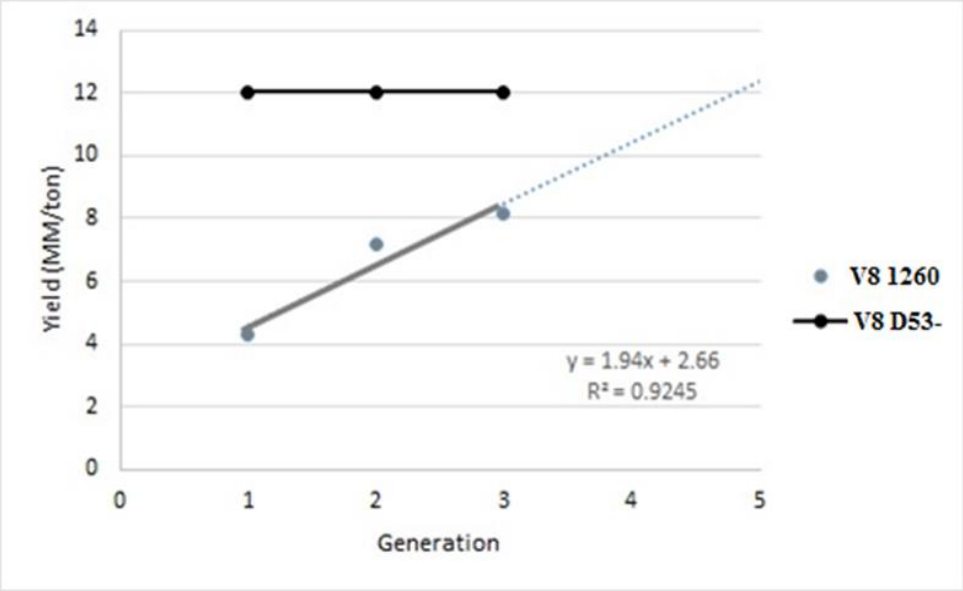

Fig. 2: Regression analysis of yield (MM/ton) *vs* generation for VIENNA 8 1260 (grey dots and line). The average yield of VIENNA 8 D53- is included as a reference (black line).

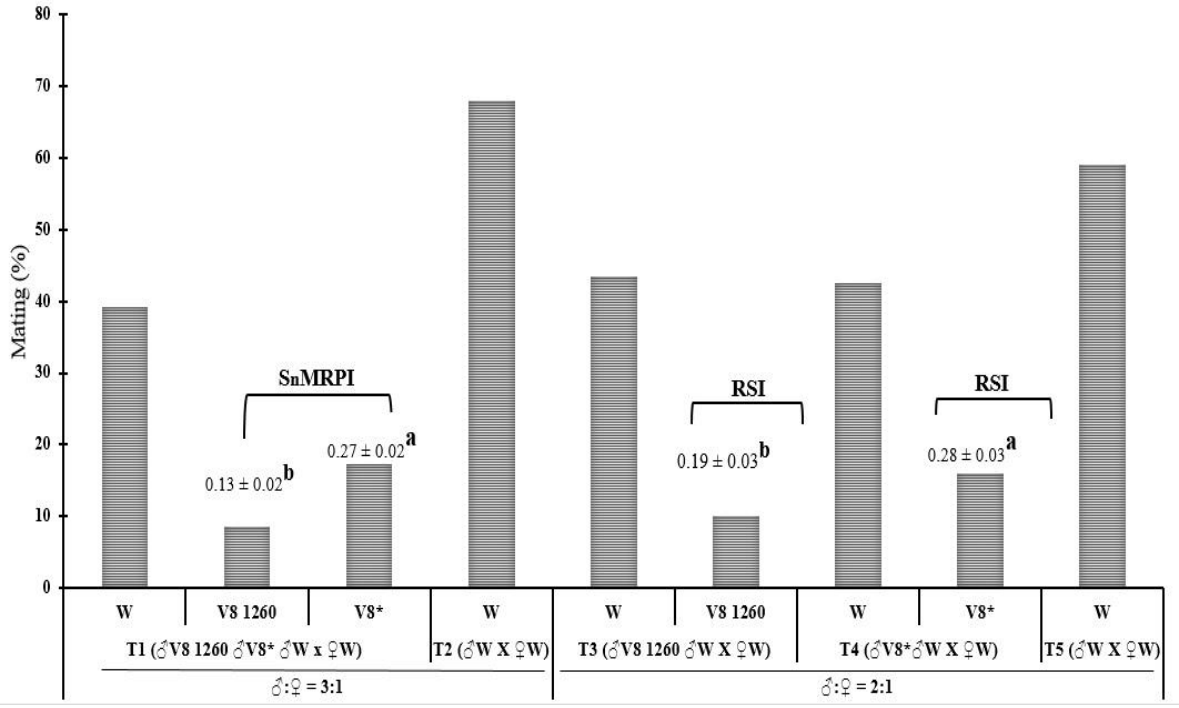

Fig. 3: Percentage of matings of laboratory males (VIENNA 8 1260 and VIENNA 8 D53-) and wild males (W) in competition for wild females with two male:female ratios (3:1 and 2:1). V8* = VIENNA 8 D53-.

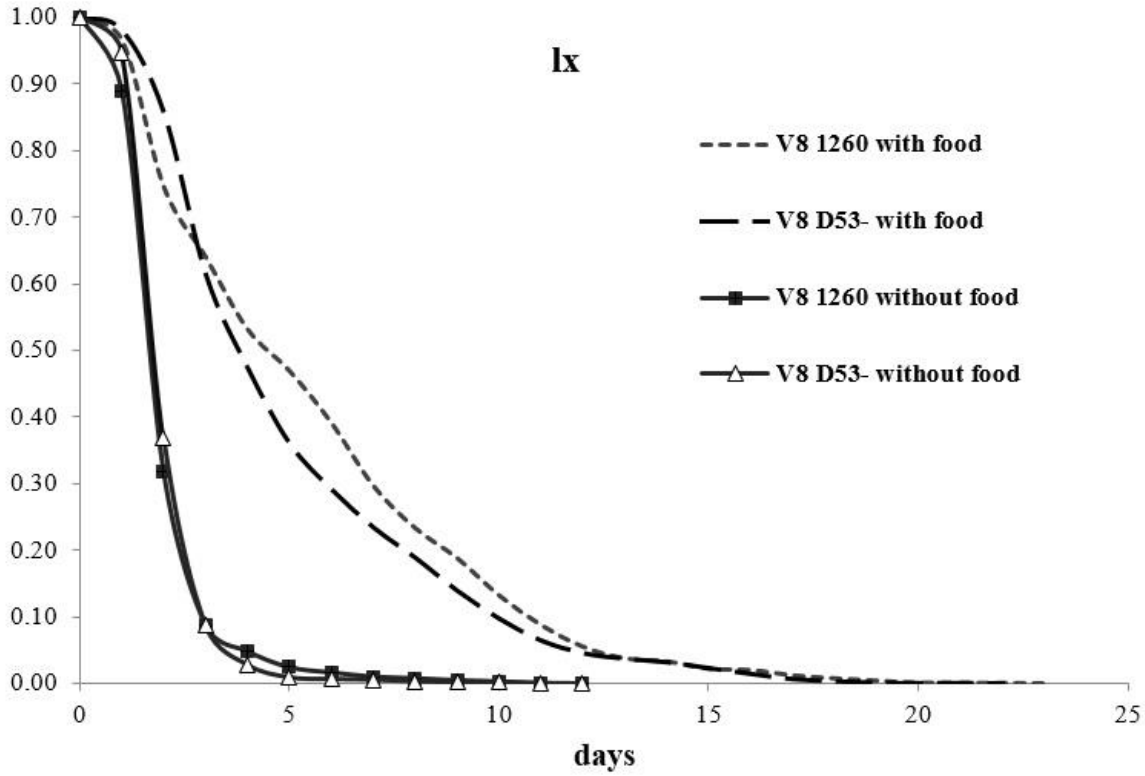

Fig. 4. Survival of VIENNA 8 1260 *vs* VIENNA 8 D53- males in field cages with or without food (liquid diet). lx = proportion of live males through the time.

Tables

Table 1: Average (±SE) yield and quality parameters of the VIENNA 8 1260 strain *vs* VIENNA 8 D53- and VIENNA 8 at small scale, with seeding units of 1000 eggs (without thermal treatment).

	V8 D53-	V8 1260	V8	V8 1260
Egg hatch (%)	82.80 ± 0.95 [a]	63.82 ± 0.91 [b]	77.96 ± 0.57 [a]	64.74 ± 0.89 [b]
Number of brown pupae	330.01 ± 12.30 [a]	221.63 ± 11.85 [b]	331.61 ± 4.51 [a]	273.22 ± 7.35 [b]
Number of white pupae	300.02 ± 10.14 [a]	188.92 ± 9.80 [b]	296.87 ± 5.60 [a]	224.39 ± 9.12 [b]
Brown pupae (%)	53.28 ± 0.88 [a]	46.76 ± 0.88 [a]	52.32 ± 0.30 [a]	51.45 ± 0.29 [a]
White pupae (%)	52.99 ± 0.76 [a]	47.01 ± 0.76 [a]	47.68 ± 0.30 [a]	48.55 ± 0.29 [a]
Egg to pupae conversion	0.60 ± 0.02 [a]	0.40 ± 0.02 [b]	0.63 ± 0.01 [a]	0.50 ± 0.02 [b]
Number of males	310.71 ± 10.95 [a]	187.45 ± 10.58 [b]	289.89 ± 5.15 [a]	239.96 ± 8.39 [b]
Number of females	277.27 ± 9.05 [a]	162.95 ± 8.74 [b]	261.32 ± 4.79 [a]	198.96 ± 7.74 [b]
Males emergence (%)	93.96 ± 1.25 [a]	85.41 ± 1.21 [b]	87.91 ± 1.28 [a]	87.18 ± 0.79 [a]
Females emergence (%)	92.22 ± 1.02 [a]	86.82 ± 0.98 [b]	88.02 ± 0.50 [a]	88.68 ± 0.98 [a]
Egg to adult conversion	0.59 ± 0.02 [a]	0.35 ± 0.02 [b]	0.55 ± 0.01 [a]	0.43 ± 0.01 [b]
R* (males - white pupae)	0.02 ± 0.02 [a]	0.01 ± 0.02 [a]	0.002 ± 0.004 [a]	0.01 ± 0.004 [a]
R* (females - brown pupae)	0.07 ± 0.03 [a]	0.00 ± 0.03 [a]	0.000 ± 0.001 [a]	0.000 ± 0.001 [a]

Paired averages ± standard error with different letters in the same line indicate significant differences ($p<0.05$).
V8 D53- = VIENNA 8 without inversion D53
V8 = VIENNA 8 with inversion D53
V8 1260 = fluorescent VIENNA 8 1260
R* = Recombinants

Table 2: Average (±SE) yield and quality parameters of the VIENNA 8 1260 strain *vs* VIENNA 8 D53- and VIENNA 8 at medium scale, seeding eggs without thermal treatment.

	V8 D53-	V8 1260	V8	V8 1260
Number eggs/♀/day	28.72 ± 1.01 [a]	21.37 ± 0.58 [b]	20.41 ± 0.25 [a]	19.72 ± 0.25 [a]
Egg hatch (%)	80.10 ± 0.70 [a]	63.61 ± 0.42 [b]	71.52 ± 0.90 [a]	66.93 ± 0.90 [b]
Number of viable eggs/day	23.00	13.59	14.62	13.20
Brown pupae (%)	40.14 ± 1.36 [b]	43.50 ± 0.91 [a]	44.96 ± 0.53 [a]	43.87 ± 0.53 [a]
White pupae (%)	59.86 ± 1.36 [a]	56.50 ± 0.91 [b]	56.12 ± 0.53 [a]	55.04 ± 0.53 [a]
Brown pupae weight (mg)	7.55 ± 0.09 [a]	7.12 ± 0.07 [b]	8.14 ± 0.06 [a]	7.97 ± 0.07 [a]
White pupae weight (mg)	8.46 ± 0.09 [a]	7.62 ± 0.08 [b]	8.10 ± 0.04 [a]	8.00 ± 0.04 [a]
Egg to pupae conversion	0.63 ± 0.02 [a]	0.44 ± 0.03 [b]	0.56 ± 0.01 [a]	0.45 ± 0.01 [b]
Males emergence (%)	90.00 ± 1.60 [a]	86.29 ± 1.07 [a]	88.55 ± 1.12 [a]	89.72 ± 1.12 [a]
Females emergence (%)	89.00 ± 1.68 [a]	86.96 ± 1.09 [a]	90.76 ± 1.17 [a]	88.28 ± 1.17 [a]
R* (males – white pupae)	0.11 ± 0.05 [a]	0.05 ± 0.04 [a]	0.002 ± 0.001 [a]	0.004 ± 0.001 [a]
R* (females – brown pupae)	0.04 ± 0.01 [a]	2.7E-03 ± 0.01 [b]	0.001 ± 0.001 [a]	0.001 ± 0.001 [a]

Paired averages ± standard error with different letters in the same line indicate significant differences ($p<0.05$).
V8 D53- = VIENNA 8 without inversion D53
V8 = VIENNA 8 with inversion D53
V8 1260 = fluorescent VIENNA 8 1260
R* = Recombinants

Table 3: Average (±SE) yield and quality parameters of the VIENNA 8 1260 strain *vs* VIENNA 8 D53- and VIENNA 8 at large (mass rearing) scale, seeding eggs with thermal treatment.

	V8 D53-	V8 1260	V8	V8 1260
Eggs hatching (%)	42.69 ± 1.82 [a]	30.78 ± 1.74 [b]	38.25 ± 0.50 [a]	29.42 ± 0.50 [b]
Larval recovery (LR)	0.18 ± 0.01 [a]	0.10 ± 0.01 [b]	0.16 ± 0.001 [a]	0.14 ± 0.001 [b]
Brown pupae (%)	99.83 ± 0.26 [a]	99.39 ± 0.22 [a]	99.22 ± 0.11 [a]	99.05 ± 0.11 [a]
White pupae (%)	0	0	0	0
Brown pupae weight (mg)	7.86 ± 0.06 [a]	7.55 ± 0.04 [b]	7.99 ± 0.05 [a]	7.92 ± 0.05 [a]
Egg to pupa conversion	0.37 ± 0.01 [a]	0.20 ± 0.01 [b]	0.25 ± 0.004 [a]	0.22 ± 0.004 [b]
Irradiated ♂ emergence %	90.74 ± 0.74 [a]	91.44 ± 0.85 [a]	90.44 ± 0.18 [a]	90.23 ± 0.18 [a]
Not irradiated ♂ emergence %	94.10 ± 0.76 [a]	94.85 ± 0.76 [a]	93.75 ± 0.21 [a]	94.26 ± 0.21 [a]
R* (females - brown pupae)	0.02 ± 0.01 [a]	2.8E-03 ± 0.01 [a]	0.001 ± 0.004 [a]	0.001 ± 0.004 [a]
Flight ability (%) irradiated males	87.11 ± 1.28 [a]	82.5 ± 1.39 [a]	86.05 ± 0.72 [a]	86.00 ± 0.72 [a]
Flight ability % not irradiated ♂	91.40 ± 1.25 [a]	87.85 ± 1.25 [a]	89.07 ± 0.89 [a]	89.63 ± 0.89 [a]
Mean life (hrs) irradiated ♂	79.55 ± 1.73 [a]	61.19 ± 1.94 [b]	61.21 ± 1.45 [a]	62.70 ± 1.45 [a]
Mean life (hrs) not irradiated ♂	73.95 ± 1.73 [a]	66.25 ± 1.73 [b]	66.88 ± 0.57 [a]	67.32 ± 0.57 [a]

Paired averages ± standard error with different letters in the same line indicate significant differences ($p<0.05$).
V8 D53- = VIENNA 8 without inversion D53
V8 = VIENNA 8 with inversion D53
V8 1260 = fluorescent VIENNA 8 1260
R* = Recombinants

CAPÍTULO III

Efectos de la dosis de irradiación sobre la inducción de esterilidad y garantías biológicas de una cepa genéticamente modificada de la mosca mediterránea de la fruta, *Ceratitis capitata* (Wied.)

Los resultados de esta sección se encuentran en el siguiente artículo:

Ramírez-Santos et al. 2016. Effect of Irradiation Doses on Sterility and Biological Security in a Genetically Modified Strain of the Mediterranean Fruit Fly (Diptera: Tephritidae).

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Effect of Irradiation Doses on Sterility and Biological Security in a Genetically Modified Strain of the Mediterranean Fruit Fly (Diptera: Tephritidae)

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Abstract

The genetically modified strain of the Medfly, *Ceratitis capitata* (Wied.) VIENNA 8 1260, was developed from the genetic sexing strain VIENNA 8. It has two molecular markers that exhibit red fluorescence in the body and green fluorescence in testicles and sperm. These traits offer a precise tool to discriminate between mass reared and wild males, increasing the effectiveness of SIT. The reproductive performance of the VIENNA 8 1260 and VIENNA 8 without D53 inversion (VIENNA 8 D53-) were compared at different irradiation doses. The general effect of irradiation on VIENNA 8 1260 follows the same patterns documented for VIENNA 8 D53-. Doses of 80 Gy or greater reduced fertility and induced high levels of sterility in wild females. Fecundity reduction was higher in VIENNA 8 1260 than in VIENNA 8 D53- females. Vertical transmission of the fluorescence gene was confirmed up to the F4 generation. Substerilization in the VIENNA 8 1260 could jeopardize the usefulness of the transgenic strain due to the possible vertical transfer of the fluorescence transgene, from the sterile to the wild flies. A biologically safe higher irradiation dose could result in reduced competitiveness of the VIENNA 8 1260 strain. Mating and remating experiments suggest that Medfly females exhibit a relative precedence in the use of the sperm: though both sperms are mixed, sperm from the 2nd mating is spent first. They also suggest a lower fitness of V8 1260 sperm, which is consistent with the lower reproductive performance documented for the V8 1260 strain.

Key words: genetic sexing, fluorescence, sterility induction, remating.

Introduction

Current Mediterranean fruit fly, *Ceratitis capitata* (Wied.) area-wide control programs use a combination of different techniques. One of them is the Sterile Insect Technique (SIT) (Knipling 1955), which is regarded as a reliable and environmentally friendly pest control method (Hendrichs et al. 1995, Dyck et al. 2005). To apply the SIT it is neccesary to release large numbers of sterile males in the field to achive an appropriate sterile: wild (S:W) ratio that will increase the probality of matings between sterile males and wild females. This type of mating produces inviable eggs and reduces population growth rate (Knipling 1955, 1979). The sterile males to be released are mass reared in biofacilities and sterilized at the pupal stage with self contained irradiators using Cobalt-60 or Cesium 137 sources (Calkins and Draz 1988, Parker and Mehta 2007). For successfull application of the SIT, it is required that irradiated males be sexually competitive (Calkins and Ashley 1989) and transfer enough sperm and accessory fluid to reduce the remating frequency of wild females (Favret et al. 1995).

It has been shown that irradiation produces detrimental effects on the quality of sterile male tephritid fruit flies, and this effect is inversely related to the irradiation dose (Barry et al. 2003, Lux et al 2002, Parker and Mehta 2007). It is posible to find optimal irradiation doses that balance the need for sterility induction with an adequate quality of the irradiated insects (Calkins et al. 1994, Orozco-Davila et al. 2015). However, pest density in the field and the quarantine status of the areas to be treated are key elements when deciding on the irradiation dose to be used (Rull et al. 2007). Lower doses might be used in areas with high pest densities or less critical quarantine status, whereas in pest free or preventive releases areas, higher irradiation doses are often preferred. When using a genetically modified strain (GMS), in addition to meeting the standards related to the sterility and quality of the insects, irradiation should also provide security to minimize the risk of vertical transmission of the of the transgene across generations (Irvin et al. 2004).

The #1260_F-3_m-1 (VIENNA 8 1260) is a GMS of *C. capitata* (Wied.) that in addition to the *tsl* and *wp* genetic sexing typical of VIENNA 8 (Kerremans and Franz 1994, 1995; Fisher 1998, Franz 2005) additionally expresses fluorescence (Prasher et al. 1992, Finokiet et al. 2007) of red color in the body of all larval instars and green color in males testicles and sperm (Scolari et al. 2008). The release of VIENNA 8 1260 sterile males into field with fluorescence expression in their bodies has the potential to achieve a higher efficiency in the SIT by facilitating and improving the identification of insects captured in monitoring traps (Handler and Harrell 2001, Scolari et al. 2008), as well as by eliminating the inorganic dyes currently used to mark the sterile males and their operational sequels including, but not limited to: errors in the calculation of the dye/pupae proportion, poor pupae marking due to high humidity or mistakes in the application procedure, time-consuming identification of the trap-captured sterile males from the field, contamination and human health risks related to the prolongued exposure of the workers to powder dyes (Khoury and Abi 2001).

The use of GMS presupposes a series of advantages for pest control programs (Wimmer 2005). However, ecological safety concerns (Scolari et al. 2011) must be carefully considered. When releasing viable GMOs the vertical transmission of genes (i. e. from the transgenic strain to the wild population) is a potential environmental risk (Macer 2005). The SIT represents an opportunity for the safe use of GMOs (Morrison et al. 2010) opening the possibility of properly using GMOs in pest control programs. The absence of females in male-only strains and the reproductive sterility of the transgenic insects in the field act as a double biological safety mechanism that prevents the vertical transmission of the transgenes (Scolari et al. 2011). In the case of the release of transgenic flies such as VIENNA 8 1260, their reproductive sterility must be guaranteed by applying the optimal irradiation dose. In this case, the use of substerilizing doses increases the risk of

introducing the transgene into the wild populations and gradually losing the discriminating power of the fluorescence marker.

Here we explore the viability of using the *Ceratitis capitata* VIENNA 8 1260 GM strain in SIT pest control programs, considering irradiation as a biosafety mechanism. The experiments were designed to: 1) measure the effects of different irradiation doses on the fertility and ability to induce sterility of the VIENNA 8 1260 strain; 2) analyze, under safe, controlled conditions, potential scenarios that might occur if VIENNA 8 1260 males with some degree of fertility were released in the field and 3) evaluate the remating propensity of wild females after mating with VIENNA 8 1260 irradiated males at different doses.

Materials and Methods

The study was divided into four experiments: A) sterility curve of the VIENNA 8 1260 vs VIENNA 8 D53-males exposed to different irradiation doses (50, 80, 110, 140 and 170 Gy); B) effect of the standard 145 Gy dose, used in AW-IPM programs for the control of the Mediterranean fruit fly, on VIENNA 8 1260 vs VIENNA 8 D53- males; C) fluorescence expression in four generations (F1 to F4) of VIENNA 8 1260 irradiated males with substerilizing doses and D) remating of wild females mated with VIENNA 8 1260 males irradiated at different doses and the effect of dose on the production of pupae (with and without fluorescence expression). Within this last experiment, an additional set of females were subject to mating and remating in order to study the precedence of sperm use by the females.

The experimental tests were conducted at the San Miguel Petapa (SMP) laboratory, Guatemala (N 14º 29´ 2", W 90º 36´ 53"), in a biosecure area that meets the US and Guatemala biosafety standards of an officially recognized quarantine, as a level 2 arthropod containment facility. The environmental conditions were: temperature 24 ± 2°C, relative humidity 65 ± 5 % and a 12:12 h

(dark: light) photoperiod with an average lighting of 1500 Lux. The wild and VIENNA 8 1260 flies used in these experiments were provided by the SMP laboratory and the VIENNA 8 D53- were obtained from the standard mass rearing production at El Pino, Guatemala, facility. Initially, the wild insects were collected as larva from infested coffee (*Coffea arabica* L.) berries collected near the Tolimán volcano (N 14º 35´ 00", W 91º 11´ 10"), located 50 km east of Guatemala city. The VIENNA 8 1260 and VIENNA 8 D53- strains were originally provided by the Insect Pest Control Laboratory (IPC) of the joint FAO/IAEA Agriculture and Biotechnology Laboratories at Seibersdorf, Austria.

Insects were irradiated at the pupal stage, two days before adult emergence. The irradiation source used was a self-contained Cesium-137 irradiator (Isomedix Inc. Husman model 521 series 004 irradiador, Whippany, NJ 07981) previously calibrated by Alanine and Gafchromic dosimetries (FAO/IAEA, 2001). The sterility induced into the irradiated males at different doses was estimated by biological dosimetry (FAO/IAEA/USDA, 2003). Fluorescence in pupae and adults was determined by visual observation under a Leica® MZ-FL III stereoscope equipped with UV fluorescence filters: "Texas Red" for red fluorescence from insect bodies and "GFP Plus" for green fluorescence in male testicles and sperm. In order to confirm the fluorescence expression, samples of pupae and adults were analyzed at the Environmental and Agroecological Biotechnology Laboratory (LaBTAA) from El Colegio de la Frontera Sur (ECOSUR), by the PCR with two specific primers (1260DsRedf: GCTCCTCCAAGAACGTCATC and 1260DsRedr: TGGTGTAGTCCTCGTTGTGG) supplied by Macrogen Inc. (Seoul, South Korea) and further detailed in Scolari et al. (2008).

In the first set of experiments that measured the effect of irradiation doses on egg fertility and sterility, the experimental design involved 13 groups of flies: a control (wild male x wild females crosses) and 12 additional treatments resulting from the combination of 2 strains (VIENNA 8 1260

and VIENNA 8 D53-) each at 6 irradiation doses (0, 50, 80, 110, 140 and 170 Gy). In the second set of experiments that measure the effect of a standard 145 Gy irradiation dose on sterility, 4 treatments were made by separately crossing fertile (non-irradiated) and sterile (irradiated) males from each strain (VIENNA 8 1260 and VIENNA 8 D53-) with wild females (WF). In the third set of experiments that measured the expression of fluorescence in offspring, treatments were defined by the cross type and generation. In the fourth set of experiments, the order of mating and remating, and the irradiation doses defined the treatments considered.

Effects of irradiation doses on fertility and egg to adult conversion rates

Two days before adult eclosion, 30 ml pupae samples were placed in plastic bags to produce hypoxia and were irradiated at 5 doses (50, 80, 110, 140 and 170 Gy) plus a control (0 Gy) for each strain. At eclosion, adults were sorted by sex and kept separated. The wild males (WM) and females (WF) used in the crosses were not irradiated. The 15 males (M) irradiated at each dose were placed with WF in a 1:1 ratio within conical cages (0.15 m height x 0.12 m diameter) with water and food (3:1 sucrose: hydrolized yeast) *ad libitum*, and an oviposition device, thus making each cage as an experimental unit. As control treatments, cages were set for the following crosses: VIENNA 8 1260 M (0 Gy) x WF; VIENNA 8 D53- M (0 Gy) x WF; and WM x WF, with the same 1:1 male: female ratio (15:15 in total).

Seven days after adult emergence and after mating, eggs were collected every 24 hrs for 10 consecutive days. These eggs were aligned on wet, black filter paper within 10 cm diameter Petri dishes. After 72 hrs, the percentage of egg hatch was determined. All the eggs were then transferred to 15 cm diameter Petri dishes filled with larval diet: a mixture of sugar, yeast, wheat flour, sodium benzoate, hydrochloric acid, water and sugar cane baggasse or corn cob, a modification of the standard Seibersdorf diet described by Braga et al. (2006). Eleven days later, pupae were carefully extracted and counted; five days later the numbers of emerged adult males and females were

recorded. Fertility (hatched eggs/total eggs x 100), sterility (100 - % fertility), egg to adult conversion (e:a) and female conversion rate for the F1 offspring (FF1) were estimated.

Sterility Induction by VIENNA 8 1260 and VIENNA 8 D53- males irradiated at 145 Gy

In order to determine the ability to induce sterility in VIENNA 8 1260 and VIENNA 8 D53- males irradiated at the standard dose of 145 Gy, the same methodology described in the previous section was applied to the following treatments: 1) irradiated VIENNA 8 1260 M x fertile WF, 2) non-irradiated M VIENNA 8 1260 x fertile WF, 3) irradiated VIENNA 8 D53- M x fertile WF and 4) non-irradiated VIENNA 8 D53- M x fertile WF. For each treatment, 3000 eggs per day were examined to determine percent fertility and percent sterility, as previously described. Egg samples were taken for 10 days for crosses with VIENNA 8 D53- males, and for 11 days for crosses with VIENNA 8 1260 males.

Vertical Transmission of Fluorescence Expression over Generations

A system of crosses between fertile males and females of the VIENNA 8 1260 strain with wild flies was carried out to determine the expression of fluorescence (%F = number of fluorescent individuals/total x 100) in the offspring. Conical cages were used as experimental units with 25 replicates per generation. The crosses were made as follows:

- P Generation: crossing VIENNA 8 1260 M x WF at 1:5 ratio.
- F1 Generation: Three types of crosses were made in a 1:1 ratio: 1) fluorescent VIENNA 8 1260 M x WF; 2) WM x fluorescent VIENNA 8 1260 F and 3) fluorescent VIENNA 8 1260 M x fluorescent VIENNA 8 1260 F.
- F2 Generation: crosses of F1 adults with (+f) and without (-f) fluorescence in a 1M:1F ratio, coming from each one of the 3 previous crosses, as follows: both parents with fluorescence

(M+f x F+f) and both parents without fluorescence (M-f x F-f). These crosses were repeated in the next two generations (F3 and F4).

Eggs from each cross were collected and placed on larval diet 2 days later. Pupae and adults were recovered and the fraction of fluorescent flies was determined. Fluorescence was determined by visual observation under a Leica® MZ-FLIII stereoscope and by the PCR test in pupae and adults.

Remating

Prior to mating, VIENNA 8 1260 males were irradiated in the pupal stage (-48 hrs to adult emergence) at five different doses (50, 80, 110, 140, 170 Gy) plus a control dose of fertile VIENNA 8 1260 males (0 Gy). After the adults reached sexual maturity, fertile wild females (WF) were allowed to mate and remate with irradiated males (M) and fertile wild males (WM) inverting mating order: A) VIENNA 8 1260 M x WF (1^{st} mating) x WM (2^{nd} mating) and B) WM x WF (1^{st} mating) x VIENNA 8 1260 M (2^{nd} mating). These crosses are further explained below.

For the A-type crosses, 100 irradiated VIENNA 8 1260 males (at each dose) were released inside a 30 x 30 x 30 cm plexiglass cage. After 15 min, 100 wild females (WF) were released and allowed to mate. Each mating pair was collected and individually placed in 10 ml plastic vials. All the females that mated for at least 100 min were individually transferred to conical cages and 21 hrs later wild males (WM) in a 1:1 ratio were introduced into these cages. Females were allowed to remate. All the females that remated for at least 100 min were then collected and individually placed in conical cages provided with water and food (3:1 sucrose:hydrolized protein) *ad libitum.*

For the B-type crosses, the same methodology described for A-type crosses was applied but the order of mating was inverted: i. e. fertile, wild males (WM) were first allowed to mate with wild females (WF) and then remating was allowed with VIENNA 8 1260 males irradiated at the five doses previuosly described.

The eggs from each female were collected every 24 hrs for 10 days and aligned on wet, black filter paper. These eggs were incubated for 72 hrs at 100 % relative humidity and 24^{o} C. The fertility percent of egg hatch was recorded. Then, the eggs or larvae were transferred to 15 cm in diameter Petri dishes filled with larval diet (as described above). Pupae were collected 11 days later, counted and observed under specific UV filters (GFP Plus for green fluorescence and Texas Red for red fluorescence) adapted to a Leica® MZ-FL III stereoscope to determine the presence or absence of fluorescence. The same procedure was followed to measure fluorescence expression in the adults. Each one of the remated females together with their eggs were considered a replicate. Percent sterility and fertility, egg to pupae conversion rate and percentage of pupae/adults with expression of fluorescence were the dependent variables measured.

Precedence of Sperm Use by the Medfly Females

Folowing the methodology of the remating experiment described above, an additional set of females were subject to **A-type** VIENNA 8 1260 M x WF (1st mating) x WM (2nd mating) and **B-type** WM x WF (1st mating) x VIENNA 8 1260 M (2nd mating) crosses in order to study the precedence of sperm use by the Medfly females, a process that has been documented at a molecular level by Scolari et al. (2014). A study using a similar experimental design was published by Lee, McCombs and Saul (2003), only that instead of fluorescent and non-fluorescent flies for the mating and re-mating, it uses irradiated and non-irradiated males. Females mated sequentially to irradiated and non-irradiated males had decreased fertility when the second male was irradiated. Females mated to irradiated males recovered fertility when remated to a non-irradiated male. These results suggest that females use preferentially the sperm from the 2nd cross (re-mating), a finding with high relevance for the SIT programmes.

Each female was individually isolated, its eggs were collected on a daily basis and transferred to individual trays with larval diet (one per each female per day) so that at the end, pupae was obtained corresponding to each egg collection from day 1 to day 10. For each female and daily collection, the number of fluorescent (f) and non-fluorescent (f-) pupae was determined by examination under an epifluorescence stereoscope equipped with specific UV filters. A total of 21 females x 10 days = 210 egg (=pupae) collections from the A-type cross and 24 females x 10 days = 240 egg (=pupae) collections from the B-type cross were analyzed.

When a non-fluorescent female mates with a fluorescent VIENNA 8 1260 male, 100% of the F1 pupae should be fluorescent (f). As previously established by Ramirez-Santos et al. (2016), when a VIENNA 8 female mates with a non fluorescent male, 100% of the F1 pupae produced should be non-fluorescent (f-). In the event of A-type and B-type re-mating crosses, a second mating occurs that provides the female with two kinds of sperm and might end in one of three potential outcomes: a) if there is no **precedence** in the use of sperm at all, pupae obtained from day 1 to day 10 egg collections should be mixed (both f and f-) in all collections, b) if there is **absolute precedence**, no mixing of f and f- pupae should occur and females would be able to select which sperm use first, or c) if there is **relative precedence**, though mixing of the sperms (and pupae) might occur each day, females should be able to use preferentially one of the two types of sperm, most probably from the second cross (re-mating), as the study by Lee, McCombs and Saul (2003) suggests. There could be an additional effect of the sperm fitness that could be charactheristic of each strain.

The relative proportion of f and f- pupae would provide additional information: a) when analyzed sequentially (from day 1 to day 10) it would provide clues as to which sperm is used first by the females and b) if equal proportions of f and f- total pupae are produced in bth types of crosses, one might infer that both sperms have equal fitness; unequal proportions might imply differences in

fitness for each sperm, determined either by its quality or quantity transferred during the sequential matings.

Data Analysis

All datasets were checked to determine if normality and homocedascity requirements were fulfilled. When deviations from these were detected, an arcsin √p transformation was applied. Arithmetic means were analyzed by the Scott and Knott test (Balzarini et al. 2008). When significant differences ($P < 0.05$) were detected, additional tests for separation of means were run. Statistical tests were performed with the InfoStat/P software (Di Rienzo et al. 2010).

Results

Effect of irradiation dose on fertility, sterility and egg:adult conversion rate

An inverse relation between dose and fertility was found in both strains when irradiated males mated with wild females (Table 1). Females that mated with non irradiated, fertile males (0 Gy), had a significantly higher fertility as compared with females that mated with irradiated males. Significant differences between the two laboratory strains were detected in fertility at this 0 Gy dose.

In the crosses with irradiated males for both strains, three groups of means were statistically defined: 1) females that mated males irradiated at the lowest dose (50 Gy) had the highest fertility (less than 90 % sterility) and significant differences were detected between the two mass-reared strains (VIENNA 8 D53- M= 19.89±0.85a, VIENNA 8 1260 M= 10.62+0.49b); 2) females that mated males irradiated at 80 Gy (sterility > 90%) had and intermediate fertility level and showed no differences in fertility between strains and 3) females that mated males irradiated at 110, 140 and 170 Gy had the lowest fertility rates without differences between strains. The males treated at 140 Gy and 170 Gy induced more than 99 % sterility.

Egg to adult conversion was significantly higher in the control (0 Gy). At all doses evaluated, the conversion was significantly lower in females mated with VIENNA 8 1260 males (Fig. 1). As the irradiation dose was increased, the egg to adult conversion was reduced. At 110, 140 and 170 Gy doses, offspring production was close to zero (0.003, 0.004, 0.008, respectively for VIENNA 8 1260 and 1, 0.002, 0.002 for VIENNA 8 D53-). The same trend was observed when analyzing the recovery of F1 females per each parental female (F1/P): the higher value (6.84 F1/P) was for females that mated fertile males (0 Gy), followed by the 50 Gy dose (0.85 F1/P), the 80 Gy dose (0.24 F1/P) and the 110, 140 and 170 Gy doses (almost 0 F1/P). All crosses with VIENNA 8 1260 males resulted in fluorescence expression in the offspring at all irradiation doses.

Sterility induction by VIENNA 8 1260 and VIENNA 8 D53- males irradiated at 145 Gy

The fertility of wild females (WF) was significantly lower when mating with non-irradiated VIENNA 8 1260 males than when mating with VIENNA 8 D53- males (Table 2). No differences in the sterility (> 99.99 %) were detected in females after mating either of the two types of males irradiated at 145 Gy. As expected, there was a highly significant difference in the sterility produced by both strains when compared to the non-irradiated (0 Gy) control.

Vertical transmission of fluorescence expression

The vertical transmission experiments showed that 100% of VIENNA 8 1260 males' offspring expressed fluorescence (Table 3). Fluorescence expression was significantly reduced in the F1 crosses. Around 50 % of the offspring expressed fluorescence in the fluorescent (VIENNA 8 1260*) by non-fluorescent (W) crosses, regardless of wether the fluorescence came from the male (VIENNA 8 1260* M, cross 1) or the female (VIENNA 8 1260* F, cross 2). Fluorescence expression in the F1 reached 75 % in the crosses of fluorescent males (VIENNA 8 1260* M) with fluorescent females (VIENNA 8 1260* F). In the F2, F3 and F4 generations fluorescence expression progressively increased in the offspring of fluorescent males (Mf) crossed with

fluorescent females (Ff) while it was zero in the offspring of non fluorescent parents (f-), regardless of the F1 cross from which they originated. This was observed in all 3 types of crossings (Table 3).

Remating by VIENNA 8 1260

There were offspring with and without fluorescence expression in both treatments, A-type cross (VIENNA 8 1260 M x WF x WM) and B-type cross (WM x WF x VIENNA 8 1260 M). Wild males (WM) were able to remate with wild females (WF) that had already mated with VIENNA 8 1260 males and viceversa. When comparing the effect of different irradiation doses (Fig. 3) within each type of cross, the general pattern observed is the same: the higher the dose, the lower the fertility (less pupae produced), i. e. an inverse relation was documented between irradiation dose and fertility. These differences are significant: bars with different letters (a, b) within each cross type are statistically different. When comparing between both types of crosses, the difference in the expression of fluorescence is explained by the order of mating: it was higher when WF mated VIENNA 8 1260 M first. A sperm precedence effect was documented in substerilizing doses (i. e. 50 Gy) where the B-type cross (remating with f males) produced even more f pupae than the A-type cross.

Precedence of Sperm Use by the Medfly Females

The results from this part of experiment are shown in Fig. 4: when analyzed sequentially, from day 1 to day 10, pupae produced in each daily collection from: A) A-type cross (VIENNA 8 1260 M x WF x WM) and B) B-type cross (WM x WF x VIENNA 8 1260 M), show a mixture of f and f- pupae. The number of pupae coming from the 2nd cross (re-mating) is gradually reduced from day 1 to day 10 in both types of crosses. The A-type cross produced a total of 38 % non fluorescent pupae from the remating (Fig. 4C) and the B-type cross produced a total of 11% fluorescent pupae from the remating (Fig 4D). More total pupae (f + f-) is produced in the A-type cross, when females

mate with non-fluorescent males first. Asymmetry was confirmed in the total number of pupae produced from the A-type and B-type crosses.

Discussion

Our results showed that the general effects of irradiation dose on the fertility of wild females mated with irradiated VIENNA 8 1260 males followed the same patterns observed for VIENNA8 D53-: the higher the irradiation dose, the lower the fertility rates recorded in wild females and, consequently, the higher the induction of sterility. In VIENNA 8 1260 the irradiation dose had a significantly higher effect (lower egg to adult conversion) than in the VIENNA 8 D53- strain. However, non-zero fertility rates, although low, at low irradiation doses in the VIENNA 8 1260 GMS have a more critical implication than in non-transgenic flies such as VIENNA 8 D53-. This is because in a GMS these substerilizing doses increase the risk of vertical transmission of the transgene, from the GMS to the wild population.

For the VIENNA 8 1260 and VIENNA 8 D53- males, the 50 and 80 Gy doses may be considered as substerilizing. Even when they had lower fertility than the fertile control (0 Gy), they produced viable offspring with levels of egg to adult conversion that might be considerable through the following generations. By contrast, the 110, 140 and 170 Gy doses showed an adequate induction of sterility, so that they could be regarded as sterilizing doses that guarantee a very low probability of having viable offspring. But in the particular case of the VIENNA 8 1260 males, even the 110 Gy dose would not be recommended for its use in a pest control program as it showed a >1% fertility. Even when there is a low probability that these eggs might reach the adult stage, it represents a risk, considering the biological guarantees that a transgenic strain should offer to the environment (Irvin et al. 2004) while preventing the incorporation of genes into the wild population by vertical transmission (Morrison et al. 2010, Scolari 2011). It is reasonable to offer a higher level

of biosecurity, in the VIENNA 8 1260 males through higher irradiation doses, despite the detrimental effects on sterile male performance (Lux et al. 2002, Barry et al. 2003, Guerfali et al. 2011, Parker and Mehta 2007, Orozco-Dávila et al. 2015). For VIENNA 8 1260 irradiation, 140 Gy, or an intermediate dose between 140 and 170 Gy, will be recommended. It is important to highlight that this interval includes the 145 Gy standard dose used at some Mediterranean fruit fly control programs, while the 100 Gy used at other programs would be a substerilizing dose that should be avoided when using the VIENNA 8 1260 strain. Our results showed that by irradiating the VIENNA 8 1260 GMS at 145 Gy an acceptable level of sterility will be obtained, very similar to the level observed for the VIENNA 8 D53- strain at the same dose.

In addition to providing biological safety, there is another reason to avoid the use of substerilizing doses with fluorescent GMS. When wild females mated with VIENNA 8 1260 males irradiated at substerilizing doses, they were able to produce offspring with fluorescence expression and the frequency of this expression increased in subsequent generations, following a Mendelian pattern of inheritance, as a dominant trait. The advantage of the VIENNA 8 1260 is the unequivocal discrimination of sterile (fluorescent) from wild (non-fluorescent) males, an advantage that would be lost if the fluorescence trangene is transmitted to, and expressed in, the wild population. So, it is very important to highlight that substerilizing doses should be avoided in SIT AW-IPM programs that use VIENNA 8 1260.

The generational expression of fluorescence in the offspring of crosses that involved VIENNA 8 1260 males irradiated at doses below 110 Gy confirms the importance of guaranteeing the sterility of these males before their release into the field. It will be equally important to consider the biosafety in the laboratories where this GMS will be mass reared. If accidentally released, fertile VIENNA 8 1260 flies would have the capacity of transmitting the fluorescence expression to 100% of their offspring. While data suggest that a single release of fluorescent flies would gradually

dilute in the next generations, it is imposible to predict with absolute certainty its behaviour in natural populations without further research to determine if these genes would have a positive, negative or neutral selective value. However, these data suggest that the continuous release of fertile, or even partially fertile flies, either in a SIT program using substerilizing doses or by accidental escapes from a laboratory, they might gradually increase the probability of expressing fluorescence in the wild population with a tendency to recover the fluorescence expression shown by their parents. Our data also show that crosses between nonfluorescent flies (f-) produced by irradiated VIENNA 8 1260 males do not express fluorescence in subsequent generations. This suggests that the transgene was not present and that its phenotypic detection is enough to determine its presence.

The lower fertility of females that mated with VIENNA 8 1260 reduces the risk of accidentally releasing the transgene into wild populations. However, it could also be related to a lower quality or quantity of sperm or accessory fluid from VIENNA 8 1260 males, or simply a poorer sexual performance, that might induce wild females to remate with wild males. While this possibility should not prevent the use of the VIENNA 8 1260 strain in a SIT program, it is an element to be considered as it may require a higher S:W ratio to achieve an adequate level of induced sterility, an issue that would reflect on cost effectiveness. This is why two different types of mating/remating schemes (A-type cross: VIENNA 8 1260 M x WF x WM and B-type cross: WM x WF x VIENNA 8 1260 M) were evaluated, in order to better understand their implications.

Both types of crosses demonstrate that wild females actively remate. In Figure 2, when substracting the fluorescent percentage from the total egg:pupae conversion, for the A-type cross, we obtained 20.85 % nonfluorescent flies (against 16.15 % fluorescent) and for the B-type cross, 21.37% (against 9.63 % fluorescent). The approximately 21% non fluorescent offspring represent the contribution from the WM, while the 9.63 or 16.15% fluorescent offspring represents the

contribution of the VIENNA 8 1260 males. From these data, it is noted that the order of mating with VIENNA 8 1260 males can affect the level of egg:pupa conversion, as shown by fluorescence expression, with a greater expression coming when those males mate first. By dividing the non fluorescent/fluorescent egg:pupae values a 1.29 ratio is obtained for the A-type cross and 2.21 for the B-type cross. Both results suggest that the WM have higher mating ability than the VIENNA 8 1260 males (expressed in their offspring phenotype) wich is consistant with a lower mating competitiveness documented for mass reared strains (McInnis et al. 1996). The results shown in Fig. 3 for the 50 Gy dose, where the B-type cross (WM x WF x VIENNA 8 1260 M) has a higher expression of fluorescence emphasize the risk of using substerilizing doses. All other irradiation doses show lower fluorescence expression for B-type cross where mated females remate with VIENNA 8 1260 males as compared to the A-type cross where VIENNA 8 1260 males mated first. Apparently the effect of remating (2^{nd} mating) with VIENNA 8 1260 males on the expression of fluorescence increased at the lowest substerilizing dose, 50 Gy, while not at 80 Gy or any other higher doses. From here it can be concluded that it is very important which male mates first and that remating should be avoided in SIT. When there are multiple matings or remating, there is not an absolute advantage by precedence: the results support the sperm mixing process documented by Scolari et al (2011) and other authors who have discussed this issue. This subject should be implied in the mating tests by the FAO/IAEA/USDA (2003) but before that time there was not documented evidence of the mixing of sperm. These results emphasize the risk of remating in wild females if the VIENNA 8 1260 males do not have an adequate sexual performance, perhaps due to the origin of the strain or from using high irradiation doses in an attempt to prevent the vertical transmission of the transgene. Again, considering that the differences in sexual performance of mass reared, sterile males and wild males might be compensated for readjusting the S:W ratio. Despite these

differences, it can be concluded that the VIENNA 8 1260 males are capable of inducing adequate levels of sterility into wild females.

In the mating and re-mating experiments, results suggest that females exhibit a relative precedence in the use of the sperm: though there is mixing of sperms (both fluorescent and non-fluorescent pupae are obtained from each egg collection, from day 1 to day 10), sperm from the 2nd mating (re-mating) is spent first (this effect is more obvious in the A-type cross), a result that is consistent with the findings from the studies published by Lee, McCombs and Saul (2003) and Scolari et al (2014). The asymmetry in the results from the A-type and B-type crosses suggest a difference in the fitness of fluorescent (VIENNA 8 1260) and non-fluorescent sperm in favor of the later, either because a higher quality or quantity of non-fluorescent sperm and seminal fluid was transferred, a finding that is consistent with the lower reproductive performance of the VIENNA 8 1260 strain documented by Ramírez-Santos (2016), when compared with non-fluorescent strains. More pupae is produced in the A-type cross, when females mate with non-fluorescent males first, a finding that suggests a higher fitness for the non-fluorescent sperm. The relative amounts of fluorescent (f) and non-fluorescent (f-) pupae produced in each cross type also support the conclusion that VIENNA 8 1260 sperm showed a lower fitness. It also emphasizes the risk that remating represents and the need to increase either the quality of the VIENNA 8 1260 strain or the sterile to wild (S:W) male ratios in the field if the VIENNA 8 1260 strain is to be used in an AW-SIT program. Both types of crosses suggest that less sperm is transferred from the remating: in A-type cross only 38% pupae are non fluorescent coming from the re-mating sperm and in the B-type cross only 11% pupae are fluorescent, from the remating-sperm.

Reviewing the results already discussed in Fig. 3, data seem to show the effect of sperm precedence at substerilizing doses (i. e. 50 Gy) when results from the A-type and B-type cross are compared. For sterilizing doses (i. e. >80 Gy) which successfully eliminated the viability of the F1 eggs there

is no practical way of measuring sperm precedence by analyzing numbers of pupae because none was produced. From these results it can be concluded that the effect of sperm precedence would become more relevant at substerilizing doses.

In conclusion, irradiation dose affects the capacity of the VIENNA 8 1260 males to induce sterility and also its biological safety. Higher doses prevent the vertical transmission of the fluorescence transgene from the GMS into the wild population. Doses below 110 Gy may be considered substerilizing with a risk of transmitting the fluorescence gene to the wild population, with a subsequent loss of the ability of discriminating between fluorescent (f) VIENNA 8 1260 males and nonfluorescent (f-) wild males. With this strain it is necessary to consider the selection of an optimal dose (high enough to induce sterility, but without reaching levels that compromise the quality, especially the sexual performance, of the irradiated males), while at the same time establish the maximum level of biosafety control in mass rearing laboratories, as well as in the field. Under no circumstances should substerilizing doses be used with VIENNA 8 1260 males, in an attempt to increase their mating performance in the field, as has been suggested for other, nontransgenic strains (Parker and Mehta 2007). Based in our results, we recommend an irradiation dose of 140 or 145 Gy. The remating experiments suggest: a) a relative precedence in the use of sperm by the Medfly females (though f and f- pupae are mixed in each daily collection, sperm from the 2^{nd} cross is spent first) and b) a lower fitness of the VIENNA 8 1260 sperm after mating, as compared with the non-fluorescent sperm.

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Figures

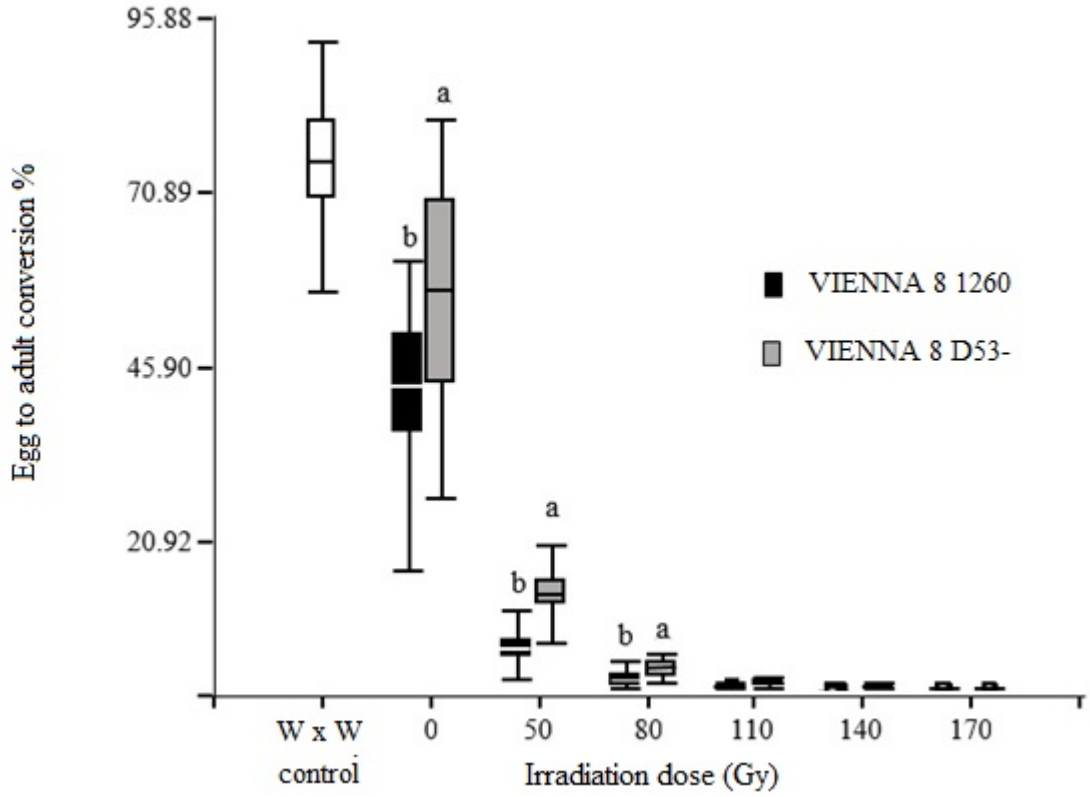

Fig. 1. Effect of irradiation dose on egg to adult conversión (e:a %) in the VIENNA 8 1260 and VIENNA 8 D53- strains. The white bar at the left shows the e:a % for the wild x wild fertile control. Each boxplot shows rectangles including the arithmetic means + 1 std deviation and the lines include maximum and minimum values.

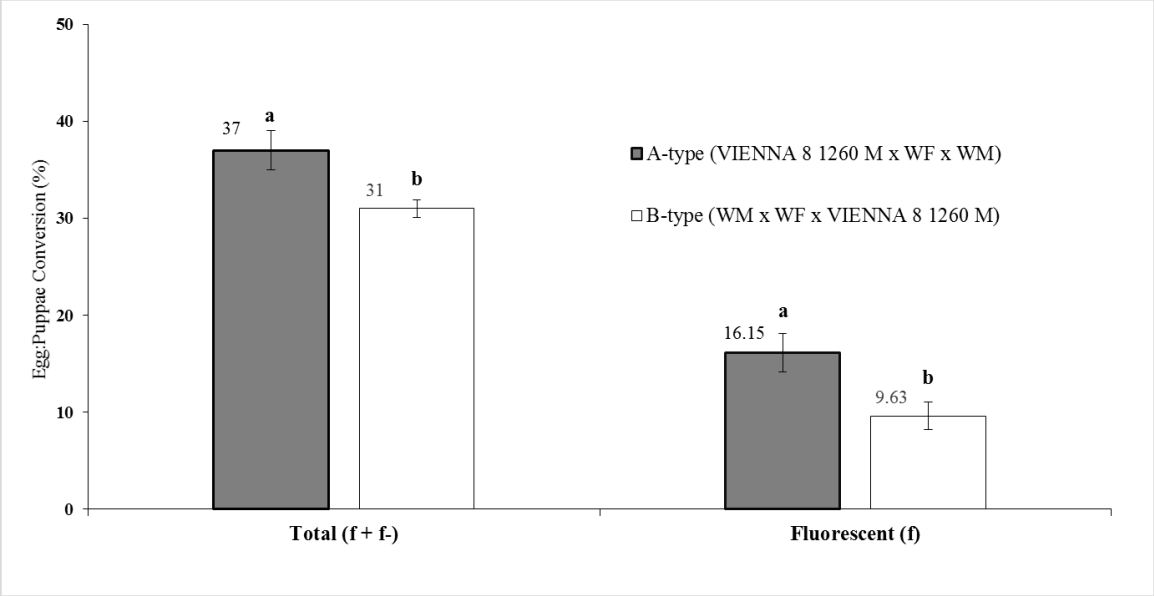

Fig. 2. Average egg:pupae conversion for fertile A-type (VIENNA 8 1260 M x WF x WM) and B-type (WM x WF x VIENNA 8 1260 M) crosses. The total includes fluorescent (f) and non-fluorescent (f-) pupae. The standard errors are included for each mean. Bars with different letters indicate significant differences ($P < 0.05$).

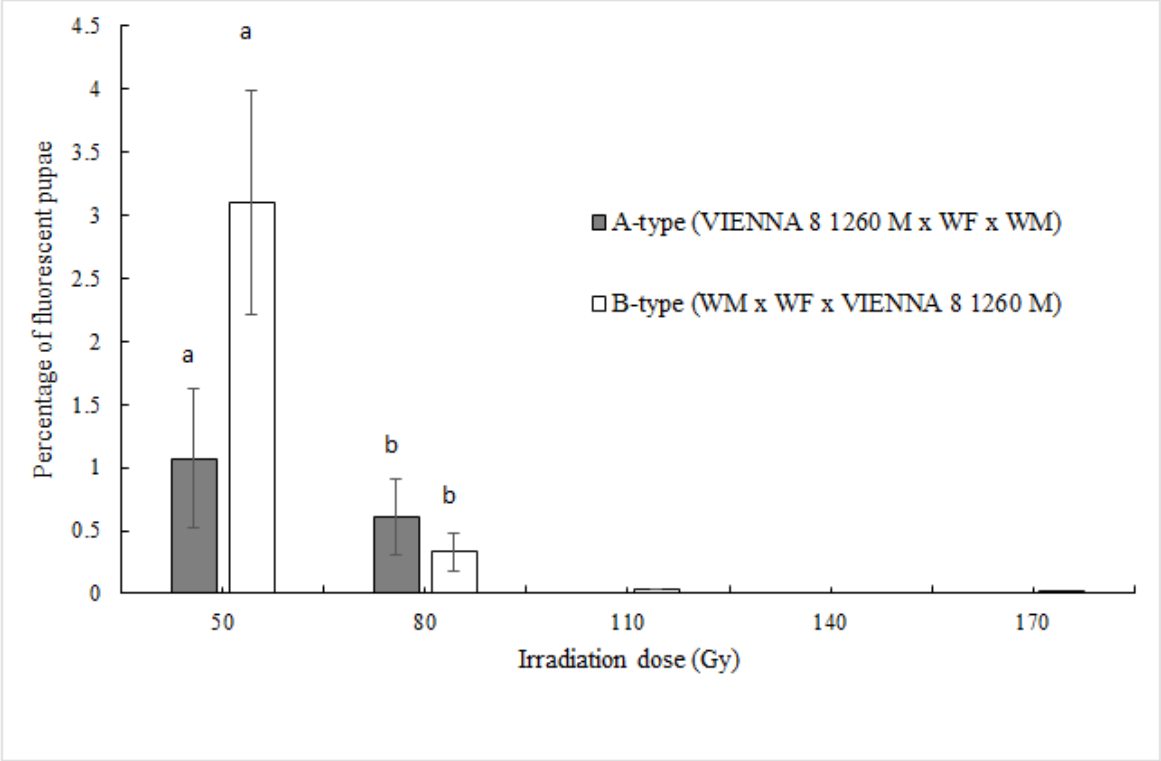

Fig. 3. Egg:pupae % expression of fluorescence in two types of crosses of VIENNA 8 1260 males irradiated at five different doses. Bars with different letters (within each cross type) are significantly different ($P<0.05$).

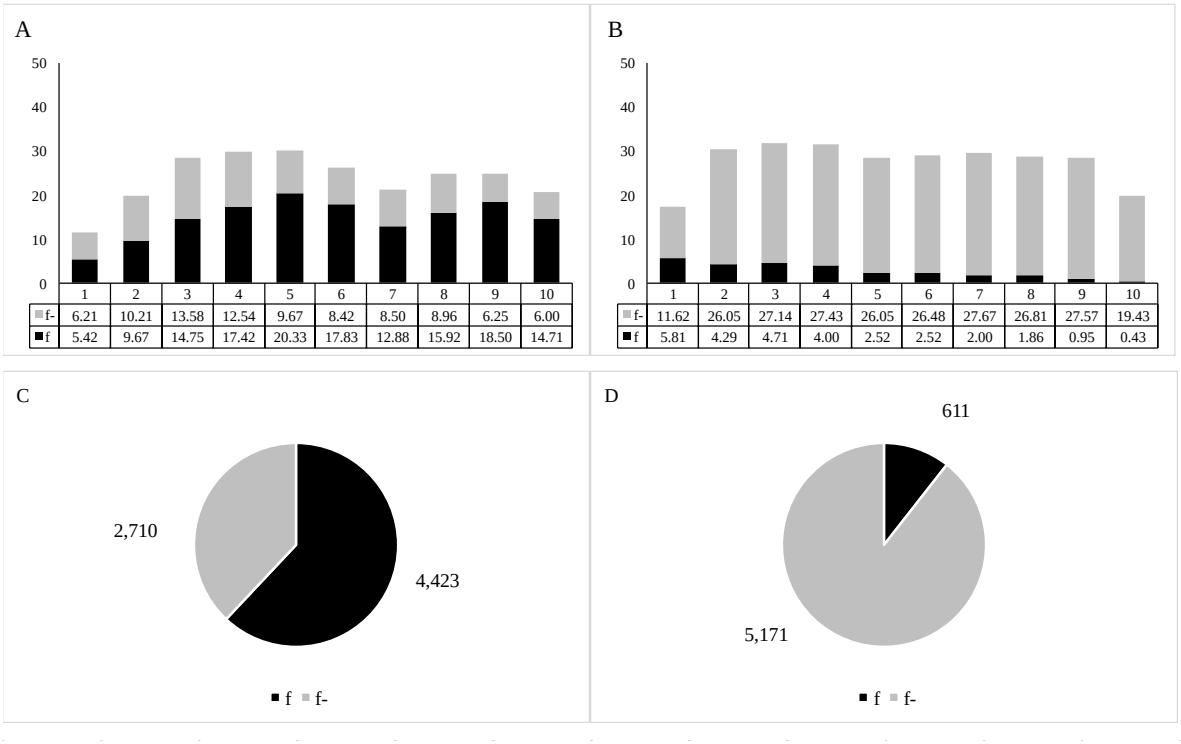

Fig. 4. Precedence of use of sperm by Medfly females. A-type (VIENNA 8 1260 M x WF x WM, N=21) and B-type (WM x WF x VIENNA 8 1260 M, N=24) crosses.
Average number of pupae produced from each daily egg collection from: A) A-type cross and B) B-type cross. Total pupae collected from: C) A-type cross and D) B-type cross.
f- = non fluorescent, f = fluorescent

Tables

Table 1. Fertility and sterility induction in wild females after mating (1:1 fly proportion) with VIENNA 8 1260 or VIENNA 8 D53- males irradiated at different doses. A WM x WF control (W) is included.

	Fertility (%)						Sterility (%)	
	W		V8 1260		V8 D53-		V8 1260	V8 D53-
Doses	**n**		**n**		**n**			
0 Gy	42696	90.51 ± 0.49	44285	71.10 ± 0.49 [a]	13102	81.08 ± 0.85 [a]		
50 Gy			44343	10.62 ± 0.49 [b]	13718	19.89 ±0.85 [b]	89.38	80.11
80 Gy			41267	6.73 ± 0.49 [c]	13850	5.42 ± 0.85 [c]	93.27	94.58
110 Gy			45625	1.22 ± 0.49 [d]	14671	2.07 ± 0.85 [d]	98.78	97.93
140 Gy			39973	0.27 ± 0.49 [d]	15388	0.62 ± 0.85 [d]	99.73	99.38
170 Gy			46557	0.26 ± 0.49 [d]	14854	0.12 ± 0.85 [d]	99.74	99.88

W = Wild, **n =** number of observed eggs
V8 1260 = VIENNA 8 1260, **V8 D53-** = VIENNA 8 without D53 inversion
Arithmetic means (±SE) with different letters in the same column denote statistically significant differences (P<0.05)

Table 2. Fertility and sterility induction n wild females after mating (1:1 fly proportion) with VIENNA 8 1260 or VIENNA 8 D53- males irradiated at a standard 145 Gy dose. A 0 Gy control is included.

		Fertility (%)		Sterility (%)
Strain	**N**	**0 Gy**	**145 Gy**	**145 Gy**
V8 D53-	30000	85 ± 0.30 a	0.20 ± 0.30a	99.80
V8 1260	33000	68 ± 0.29 b	0.17 ± 0.27a	99.83

n = number of observed eggs
V8 1260 = VIENNA 8 1260, **V8 D53-** = VIENNA 8 without D53 inversion
Arithmetic means (±SE) with different letters in the same column denote statistically significant differences (P<0.05)

Table 3. Percentage of fluorescence expression per generation of VIENNA 8 1260 vs wild (W) crosses.

	Fluorescence Expression (%)					
Generación	**V8 1260 M x WF**					
P	100 ± 0a					
	Cross 1		**Cross 2**		**Cross 3**	
	V8 1260* M x WF		**WM x V8 1260* F**		**V8 1260* M x V8 1260* F**	
F1	49 ± 2b		51 ± 1b		77 ± 2b	
	Mf x Ff	**M-f x F-f**	**Mf x Ff**	**Mf- x Ff-**	**Mf x Ff**	**Mf- x Ff-**
F2	82 ± 2c	0	79 ± 1c	0	88 ± 2c	0
F3	92 ± 2d	0	94 ± 2d	0	95± 3d	0
F4	94 ± 3d	0	94 ± 2d	0	96± 3d	0

M = male, **F =** female, **W =** wild
f = adult with fluorescence, **f- =** adult without fluorescence
V8 1260 = VIENNA 8 1260, **V8 D53-** = VIENNA 8 without D53 inversion, **V8 1260*** = fluorescent offspring of the P cross
Arithmetic means (+SE) with different letters in the same column denote statistically significant differences ($P<0.05$)

CAPÍTULO IV

El uso de la PCR como una herramienta de evaluación de la transferencia horizontal de marcadores de ADN entre una cepa genéticamente modificada de la mosca mediterránea de la fruta, *Ceratitis capitata* (Wied.) y su parasitoide específico *Fopius ceratitivorus* Wharton (Braconidae).

Los contenidos de esta sección se encuentran en el siguiente artículo:

Ramírez-Santos et al. 2016. Use of the PCR as a risk-assessment tool for the horizontal transfer of DNA markers between a genetically modified strain of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.) and its specific parasitoid *Fopius ceratitivorus* Wharton (Braconidae). Pendiente de enviar a revista para su publicación.

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Use of the PCR as a risk-assessment tool for the horizontal transfer of DNA markers between a genetically modified strain of the Mediterranean fruit fly (Diptera: Tephritidae) and its specific parasitoid *Fopius ceratitivorus* Wharton (Braconidae)

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Abstract

The genetically modified strain of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.) #1260_F-3_m-1 (VIENNA 8 1260), was developed from the genetic sexing strain VIENNA 8. It has two molecular markers that exhibit red fluorescence in the body and green fluorescence in testicles and sperm. These traits offer a precise tool to discriminate between the mass-reared sterile males and the non-fluorescent, wild and fertile males, thus increasing the effectiveness of the control programs for this pest. To asses the horizontal transfer risk of genetic sequences of the fluorescence transgene from the VIENNA 8 1260 strain to its parasitoid *Fopius ceratitivorus* (Braconidae), a DNA extraction and PCR amplification was performed using specific primers for the fluorescence markers at four independent laboratories. The results showed that the DNA extraction provided genetic material in optimal quantity and quality for the PCR amplification. The positive controls demonstrated that the technique is accurate for detecting the genetic markers and correlate with the phenotypic expression of fluorescence. No horizontal transfer of the transgene was detected from the VIENNA 8 1260 to its parasitoid for 16 generations. The conclusions are consistent when comparing the PCR results from four different laboratories.

Key words: Fluorescence transgene, VIENNA 8 1260.

Introduction

Within the order Diptera can be found several insect species that have a negative impact on agriculture and health (Franz and Robinson 2011). The mosquitoes and fluit flies are two important groups due to their high invasive capacity, favoured by factors such as the global climate change (Trumble and Butler 2009), the growing international trade (Pysek and Richardson 2010) and increasingly vulnerable zones that promote the pest establishment (Worner y Gevrey 2006, Johansson et al. 2009, Simberloff 2010). Due to the implications of the presence of these pests, the countries and regional organizations establish control programs (CP) to eradicate them or prevent their establishment (Hendrichs et al. 2002). A challenge for these programs is to find highly effective control tools with low cost and environmental impact. Such is the case of the Sterile Insect Technique (SIT) that has been essential for the control of several species of fuit flies under different programs of Area-Wide Integrated Pest Management (AW-IPM) around the world.

One of the main advances in the SIT has been the use of genetically improved strains of insects (Franz and Robinson 2011) created first by means of classic genetic techniques and more recently by genetic engineering. The new recombinant DNA biotechnologies have allowed the design and development of Genetically Modified Strains (GMS) of fruit flies, with desirable traits for mass rearing and field operations, enhancing their ability to control the target pests (Wimmer 2005). An example is the GMS of *Ceratitis capitata* (Wied.) #1260_F-3_m-1 (VIENNA 8 1260) that offers monitoring advantages to the control programs by the expression of a red fluorescent protein on the pupae and body of the adults, and a green fluorescent protein in the males testicles and sperm (Handler and Harrell 2001, Scolari et al. 2008), thus increasing the efficiency of the SIT by the accurate discrimination of the fluorescent, mass reared sterile males, and the non-fluorescent wild males (Enkerlin et al. 1996). What we are learning today about the control of an agricultural pest

by a GM insect may be safely applied tomorrow for the control of mosquitoes, one of the main groups of insects that act as vectors of human diseases (WHO, 2014).

The viability of using genetically modified insects, such as VIENNA 8 1260, could be determined by the need of new alternatives of control, either because the traditional methods have failed or they have lower efficiency. However, the use of these new technologies may be hindered by concerns about their viability or risks related to their use. These concerns may be either ecological or environmental, closely related to the social perception or the economic interests of global market groups that don´t have access to the generation of GMOs through biotechnology. As a consequence, the notion or risk associated with the use of these organisms, product of modern technologies, turns complex and polysemic (Pellegrini 2007). On the other side, the discussion on the potential ecological impact of these GMOs is complex, due to the dynamics of each species in the local population or metapopulations (Cornell and Lawton 1992, Lei and Hanski 1998, Hilbeck, et al 2015) as well as the technical and biological warranties that these GMOs should offer to the environment (Irvin et al. 2004).

There is a latent concern due to the risk of vertical (within the same species) or horizontal (between species or even kingdoms) transmission of transgenes. However, some authors agree that the SIT profiles as an opportunity for the safe use of these GM insects (Morrison et al. 2010, Scolari 2011). Regarding the use of the VIENNA 8 1260 strain in a CP, the reproductive sterility of the males achieved through irradiation may act as a biosafety mechanism that prevents the vertical transfer of transgenes by producing a 100% males strain, without females. But there is also the potential risk of horizontal transfer, also called lateral transfer, when genetically modified insects with high reproductive and colonizing capacities are introducing in an ecosystem new traits that don´t exist in the original species (Handler 2002). The horizontal transfer of genes is more common in bacteria

and plants, but it has been also documented in several species of other organisms, including insects (Robertson and Lampe 1995, Silva and Kidwell 2000, Hartl 2001, Lampe et al. 2003, Sormacheva et al. 2012). While most of these studies document the transfer of partial genomic sequences that don´t neccessarily have a phenotypic expression, they are indicators of the risk of transfer of fully functional genes. In a GMS, it can be a product of the unstability of the vector in the genome where it was integrated, so a way to limit this unstability is through rearrangements of the transgene sequence (i. e. inverted 3´ - 5´ terminals) thus blocking the recombination at the site of insertion (FAO/IAEA 2006). In the case of the *C. capitata* VIENNA 8 1260 strain the probability of horizontal transfer has been minimized by a system of three mechanisms of integration and stabilization (Schetelig et al. 2009).

Fopius ceratitivorus Wharton (Hymenoptera: Braconidae) is a promising egg-pupae parasitoid for the control of the Mediterranean fruit fly. This parasitoid was originally reported from Medfly collected in coffee berries from Kenya (Wharton, et al. 2000). In Guatemala, coffee (*Coffea arabica* L.) is the main host plant for *C. capitata* (Wied.) and its specific parasitoid *F. ceratitivorus* has been obtained from Kenyan coffee plantations through USDA-APHIS following proper quarantine and biosafety protocols (López et al. 2003). For both reasons it was important to include *F. ceratitivorus* in this study, as it is already being tested in Guatemalan Medfly-infested areas where VIENNA 8 1260 might be also released in the future. There are efforts to mass-rear *F. ceratitivorus* at several places, including Guatemala (López et al. 2003) and Hawaii (Bokonon-Ganta et al. 2007). In this research, the parasitoid *Fopius ceratitivorus* Wharton (Hymenoptera: Braconidae) was reared in the VIENNA 8 1260 GM flies for 16 generations, with the purpose of applying the PCR as a tool to assess the risk of horizontal transfer of genes from the fly host to its parasitoid. DNA was extracted from the parasitoids reared on fluorescent flies and PCR with primers specific

for the fluorescence sequences was applied to detect the transgenes as molecular markers, as well as their potential horizontal transfer from the fly host to its specific parasitoid. The DNA extraction is one of the techniques commonly used for the isolation and concentration of genomic material. It is also the first step in PCR and many recombinant DNA techniques. A review of the several DNA extraction techniques from insects has been recently published by Ashgar et al. (2015). The Polymerase Chain Reaction (PCR) is an *in vitro* biochemical process that allows amplifying DNA sequences with the aid of small known sequences (oligonucleotides or *primers*) of a given gene. In studies with insects, the PCR has been applied to several topics including: a) their origin and distribution (Kourti et al. 1992, Kourti 1997, Davies et al. 1999, Gasperi et al. 2002), b) the genetic structure of the insect populations (Baruffi et al. 1995, Basso et al. 2009, Alaoui et al. 2010, Beroiz et al. 2012), c) monitoring of sterile males sperm in wild populations (San Andrés et al. 2007) and d) genetic diversity in mass rearing (Liedo et al. n.d.). The interlaboratory approach applied in this study test the reliability of the PCR as a tool to detect the horizontal transfer of even partial sequences of the fluorescence transgene between the VIENNA 8 1260 host and its parasitoid (*F. ceratitivorus*).

Materials and Methods

The parasitoid *Fopius ceratitivorus* Wharton was reared on the fluorescent *Ceratitis capitata* (Wied.) VIENNA 8 1260 for 16 generations. In each generation, VIENNA 8 1260 females laid eggs in apple (*Malus domestica*) fruits. These fruits were then exposed to the parasitoid *F. ceratitivorus*, whose adults were recovered afterwards and a new cycle started. The VIENNA 8 1260 females came from a small colony (generation 36) following the FRS rearing system (Fisher

and Cáceres 2000), reared at the quarantine area of the San Miguel Petapa (SMP) laboratory, placed 15 km to the southeast of the Guatemala capital city (N 14º 29´ 2", W 90º 36´ 53").

The parasitoids used to initiate this evaluation (renamed as generation 0 = G0) were obtained from a colony that uses as a host a bisexual strain of *C. capitata* -Tolimán/99, following the methodology suggested by López et al. (2003). All the other parasitoids for subsequent generations (G1 to G16) come from the VIENNA 8 1260 generational rearing, as previously described.

Infestation of apple fruits with VIENNA 8 1260 eggs

Under controlled environmental conditions, 3000 males and 3000 females of the VIENNA 8 1260 strain were kept inside 31 x 31 cm square cages (SC) with 0.5 mesh plastic on their four sides. The flies were fed *ad libitum* with a mixture of 3:1 sucrose:hydrolized protein. To facilitate the fruit oviposition by the Medfly and later its parasitoids, the apples were prepared by puncturing them with a stainless steel pin, thus creating approximately 1500 punctures by fruit. After a mating period of 3 days, the apples (4-6 per cage) were exposed to the VIENNA 8 1260 females as an oviposition substrate. The exposition was repeated 3 times per cage and, in each exposition, 2 apples were left inside the cage for 24 hrs. All the infested apples were then kept for 24 hrs in a room at 24 ºC and 65 % relative humidity in order to promote the embrionary development of the VIENNA 8 1260 eggs. This procedure was repeated in each generation.

Parasitization of the VIENNA 8 1260 eggs by *F. ceratitivorus*

A total of 2000 males and 2000 females of the *F. ceratitivorus* parasitoids were kept inside 30 x 30 cm plexiglass cages with the same artificial diet provided to the VIENNA 8 1260 flies. The parasitoids were reared for a time period of 7 days, until they reached sexual maturity and mated.

Then, the apples infested with VIENNA 8 1260 eggs were introduced for 24 hrs in the cages with the parasitoids, thus allowing the colonization of the VIENNA 8 1260 eggs by *F. ceratitivorus*. After the parasitization time period ended, each apple was cut into four parts and put on an artificial diet prepared to feed for 6 days the parasitized VIENNA 8 1260 larvae. This diet is a modification of the standard Seibersdorf diet described by Braga et al. (2006). The VIENNA 8 1260 larvae were collected and pupated. The VIENNA 8 1260 adults (males and females) emerged at the 12th day after pupation, and the adult parasitoids (males and females) 3 days later.

The adult parasitoids recovered in each generation were introduced again in plexiglass cages and the parasitation procedure of the VIENNA 8 1260 eggs was repeated for the 16 generations that this study lasted. In each generation, a sample of 7 adult parasitoids of each sex was preserved in absolute ethanol, inside 1.5 ml Eppendorf vials.

PCR analysis of the parasitoid samples generationally reared on VIENNA 8 1260

In order to evaluate the potential horizontal transfer of DNA sequences of the fluorescence transgene from the VIENNA 8 1260 flies to its parasitoid, the DNA from the *F. ceratitivorus* samples was extracted and amplified by the PCR with primers specific for the fluorescence markers present in VIENNA 8 1260. The PCR analysis of parasitoids reared on VIENNA 8 1260 from the generations 0, 11, 12 and 14 was conducted at the Environmental Biotechnology and Agroecology Laboratory (LaBTAA) from the Colegio de la Frontera Sur (ECOSUR), Tapachula (Mexico). Samples of parasitoids from the generations 0 and 16 were analyzed at three additional laboratories: a) Institutional Laboratory of Genetics ECOSUR at San Cristóbal (Mexico), b) FAO/IAEA Laboratory of Agriculture and Biotechnology at Seibersdorf (Austria); and c) the Zoology and

Anthropology Laboratory of the Johann Friedrich Blumenbach Intitute, George August University, at Göttingen (Germany).

The total extraction of DNA from each individual parasitoid was performed for each sex and generation. A subsample of 5µl from every 30µl extraction sample was subject to electrophoresis in 1 % agarose gels to visualize the quality of the DNA extraction. For the PCR amplification, both a pure 1.0 µl subsample of extracted DNA and a 2.0 µl subsample of a 1:10 dilution were analyzed. The primers used were 1260DsRed-F and 1260DsRed-R, supplied by the Macrogen Inc company (Rockville, MD) and further detailed by Scolari et al. (2008). The PCR cycle was set with the following parameters: initial denaturalization 95ºC/5 min, 30 cycles of denaturalization 95ºC/30 sec, aligning 56.8ºC/30 sec, extension 72ºC/1 min and final extension 72ºC/5 min.

The DNA fragments resulting from the PCR amplification were analyzed in 1 % agarose gels loaded with a 10 µl subsample from each 20 µl PCR product. The size of the DNA bands was estimated by observing them under UV light and comparing their migration distances with those of the molecular weight markers coming from a 1 Kbp DNA ladder loaded in the first well of each agarose gel. As a positive control was used an amplicon of 700pb, corresponding to a sample from a VIENNA 8 1260 fluorescent adult, identified as #117. As a negative control was used a *F. ceratitivorus* adult parasitoid reared in the normal (bisexual) strain of *C. capitata*, with no contact with the VIENNA 8 1260 fluorescent strain.

To verify the quality of the DNA extraction and the reliability of the PCR results (that is depending upon the quality of the DNA extraction) another PCR was performed with a 1.0 µl subsample of the DNA previously extracted from the parasitoids samples and 2.0 µl of their 1:10 dilution, using the NS1-GCFung primers (Hoshino and Morimoto 2008) directed at the 18S eukaryotic ribosomal

unit marker, and the following PCR conditions: initial denaturalizatión 95ºC/5 min, 35 cycles of 95ºC/30 sec denaturalization, aligning 50ºC/40 sec, extension 72ºC/1 min and a final extension 72ºC/5 min. A 10 µl subsample from the 20 µl PCR product was analyzed with 1 % agarose gels electrophoresis. The DNA bands were compared under UV light against the molecular weight markers of a 1 Kbp DNA ladder. As a positive control was used an amplicon of 500 bp from sample #117 as template. As a negative control a blank was run with all the reagents and solvents used in the DNA processing (without DNA).

Complementary to the PCR analysis, an examination of the parasitoids reared on VIENNA 8 1260 fluorescent flies was conducted from G0 to G16, in order to verify their ability to express (or not) fluorescence under UV light and specific filters (GFP Plus for green fluorescence and Texas Red for red fluorescence) with the aid of a Leica® MZ-FL III stereoscope. This is a common procedure to verify the phenotypic expression of the fluorescence transgene in the VIENNA 8 1260 insects. This examination further reduces the uncertainty of the potential horizontal transfer of the transgene from VIENNA 8 1260 to its parasitoid. The results are sumarized in Table 1.

Results

The results of the DNA extraction of the parasitoids from generations 0, 11, 12 and 14 performed at the Environmental Biotechnology and Agroecology laboratory (LaBTAA) of the Colegio de la Frontera Sur (ECOSUR), Unidad Tapachula (Mexico) are presented in Fig. 1 and show DNA in quality and quantity optimal for the PCR, confirmed by the amplification of the direct 1µl subsample of the DNA extractions and the 2µl subsamples of the 1:10 dilutions for the PCR that amplified the DNA from the 18S eukaryotic ribosomal subunit (Fig. 1A).

When analyzing the PCR resulting from VIENNA 8 1260 parasitoid samples with the same amount of DNA, together with the specific fluorescence primers (1260DsREd-F and 1260DsRed-R), none showed products of amplification of the transgene sequence (Fig. 1B). The high quality of the DNA extraction (Fig. 1C) and the negative findings in PCR amplification of the transgene sequences measured in the VIENNA 8 1260 reared parasitoids, were consistent through all generations and no differences were detected due to the DNA dilution or the sex of the parasitoids. The positive controls demonstrated that the DNA extraction and PCR amplification with specific primers were able to produce bands in the agarose gels that confirmed the presence of the fluorescence transgene sequences in the VIENNA 8 1260 host flies.

The results reported from the other three laboratorios testing the same G0 and G16 samples are summarized in Table 1: a) Laboratory of Genetics/ECOSUR at San Cristóbal (Mexico), b) FAO/IAEA Laboratory of Agriculture and Biotechnology at Seibersdorf (Austria); and c) the Zoology and Anthropology Laboratory of the Johann Friedrich Blumenbach Intitute, George August University, at Göttingen (Germany). These laboratories confirm the quality of the DNA extraction and the negative results for the PCR amplification that measures the risk of vertical transfer of the fluorescent transgene from the VIENNA 8 1260 flies to their parasitoid for 16 generations. These findings are consistent with those previously reported by the LaBTAA/ECOSUR (Tapachula, Mexico) PCR analysis of the parasitoids at generations 0, 11, 12 and 14 (Fig. 1).

Discussion

The recent introduction of genetically modified organisms (GMO) in Health and Agriculture has generated an intense debate between those groups that oppose to the use of GMOs, perceiving in their use the *possibility* of an uncontrolled horizontal transfer (HT) of genes with unforeseen ecological consequences, and those groups in favor of the GMOs that balance their benefits against the *probability* that such HT may indeed occur and consider it an event so improbable, that it is virtually *impossible* that it should occur under controlled conditions. Thomas and Nielsen (2005) summarize the main mechanisms of, and barriers to horizontal gene transfer between bacteria, mechanisms and barriers that have been further discussed in other groups of organisms, including insects (Robertson and Lampe 1995, Silva and Kidwell 2000, Hartl 2001, Lampe et al. 2003, Sormacheva et al. 2012). Such natural mechanisms include, but are not limited to: cell transformation (the stable uptake, integration and functional expression of exogenous DNA), plasmids recombination and additive integration, conjugative transfer, and DNA or RNA sequences transfer mediated by transposons and other vectors (i.e. viruses).

This study starts with the first premise: that there is a posibility that the HT occurs, as the results of several published studies confirm with the horizontal transfer of partial sequences of foreign genetic sequences to insects. However, the results of this study support the second premise: the probability of occurrence of the HT is at least very low, measured by the PCR analysis negative results and the phenotypic examination of the VIENNA 8 1260 parasitoids under UV light and specific filters: no transfer of the transgene or their sequences was detected for 16 generations. This time period represents over a year of repeated contacts between the *F. ceratitivorus* parasitoids and the VIENNA 8 1260 host populations, a scenario that is very likely to occur if the GMOs are released in the field as part of an AW-IPM program.

The negative results of the examen of specimens under UV light and filters specific for fluorescence is a first indicator that the transgene was not transferred from the VIENNA 8 1260 host to its parasitoid. However, it could be proposed that the horizontal transfer was just partial or subject to mutations (resulting in a non functional transgene), even after the HT occurred. Both scenarios would be consistent with the negative results for the phenotypic analysys of the VIENNA 8 1260 reared parasitoids.

To absolutely rule out the occurrence of a HT was necessary to analyze the PCR results, that would be positive (in the form of DNA bands produced with the specific primers for the transgene sequences) even in the event of a partial horizontal transfer of the transgene sequences that could not be phenotypically expressed by the parasitoids. The results of the PCR amplification of the DNA that codifies for the 18S ribosomal unit (an example is shown in Figs. 1A and 1C) show that extracted DNA is reliable to obtain genetic material in quantity and quality suitable for the PCR amplification. The sharpness of the DNA bands confirms the reliability of the DNA amplification by PCR and the electrophoresis system. The comparative results of the positive and negative controls show that the PCR is able to detect the transgene sequences (positive control) without generating false positives (negative control).

The negative results of the PCR analysis for the fluorescence transgene in the VIENNA 8 1260 reared *F. ceratitivorus,* using the specific primers 1260DsRed-F and 1260DsRed-R show that: a) the transgene was not transferred from the VIENNA 8 1260 host to its parasitoids in none of the generations analyzed, b) the results are consistent in both dilutions (pure and 1:10 dilution), c) no differences were found betwen both sexes (the DNA bands that would indicate the presence of the transgene are absent in both sexes). Combining these results with the examination under UV light and filters of the VIENNA 8 1260 reared parasitoids, it can be concluded that no HT occurred

involving the fluorescence transgene, from the VIENNA 8 1260 to its parasitoids, in none of the generations analyzed. It is confirmed that the PCR is a reliable tool to assess the risk of the horizontal transfer of DNA markers between a genetically modified strain of the Mediterranean fruit fly (Diptera: Tephritidae) and its specific parasitoid, and that its use complement the traditional techniques used to detect these transfers, such as the verification under UV light and filters with the aid of an stereoscope to confirm the phenotypic expression of fluorescene.

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Figures

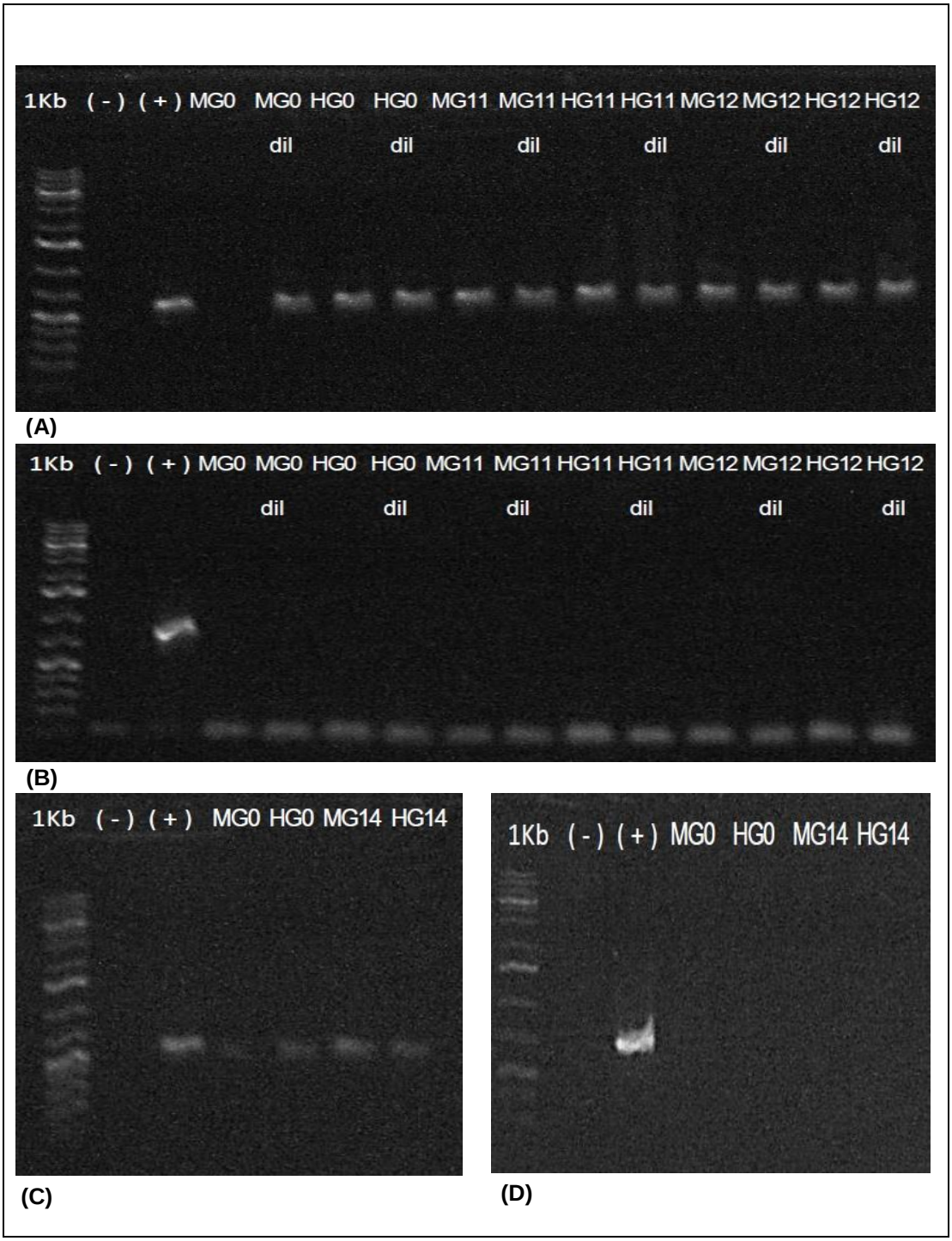

Fig 1. Picture of 1% agarose gel under UV light; (A) and (C) PCR amplification of the 18S ribosomal DNA, using the NS1-GCFung primer. 1 Kbp = DNA ladder markers, (+) = positive control, sample #117 amplicon of 500 bp; (-) = negative control; dil = 1:10 dilution.
(B) and (D) PCR amplification using the 1260DsRed-F and 1260DsRd-R primers. 1 kb = DNA ladder markers, (+) = positive control, sample #117 amplicon of 700 bp, (-) = negative control, dil = 1:10 dilution. M = male. H = female. G = generation.

Tables

Table 1. Detection of the fluorescence transgene in *F. ceratitivorus* parasitizing *C.capitata* VIENNA 8 1260 GMS.

		18S gene		PCR pure		PCR 1:10 dilution		
Sample	Filters	*Fopius* ♂	*Fopius* ♀	*Fopius* ♂	*Fopius* ♀	*Fopius* ♂	*Fopius* ♀	Laboratory
G0	-	+	+	-	-	-	-	(1) (2) (3) (4)
.								
.								
.								
G11	-	+	+	-	-	-	-	(1)
G12	-	+	+	-	-	-	-	
G13	-							
G14	-	+	+	-	-	-	-	(1)
G15	-							
G16	-	+	+	-	-	-	-	(1) (2) (3) (4)
Control +	+	+	+	+		+		
Control -	-	-	-	-		-		

Sample = generation 0 to 16. Filters = examination under stereoscope with UV light and filters specific for green and red fluorescence. 18S gene = PCR amplification of the 18S DNA. PCR = electroforesis of the PCR amplification products in *F. ceratitivorus* using the 1260DsRed-F and 1260DsRed-R primers. Laboratories: (1) LaBTAA, Tapachula (Mex), (2) Laboratory of Genetics San Cristobal (Mex), (3) FAO/IAEA Seibersdorf (Austria), y (4) Johann Friedrich Blumenbach Institute of the George August University, Göttingen (Alemania). Control+ = VIENNA 8 1260. Control- = *F. ceratitivorus* reared on normal strain (no contact with VIENNA 8 1260).

CAPÍTULO V

Conclusiones generales

La utilización de machos estériles genéticamente modificados puede mejorar la eficiencia de la TIE en el Manejo Integrado de Plagas en Areas Extensas (MIP-AE) y se pueden tomar medidas para garantizar su uso seguro en relación al ambiente, particularmente aplicando protocolos de bioseguridad en las plantas de cría masiva y dosis de irradiación que garanticen su esterilidad. La cepa transgénica VIENNA 8 1260 ofrece varias ventajas sobre los métodos tradicionales de marcaje e incluso puede combinarse con éstos para incrementar la certidumbre en la identificación de machos estériles provenientes de la cría masiva. Esta cepa, conserva una gran estabilidad en el mecanismo de sexado genético, evidenciado en una efectiva separación de machos y una tasa muy baja de recombinantes. También resultó altamente estable en la expresión de fluorescencia (100 %) en cuerpo y esperma, a lo largo de 16 generaciones.

A pesar de que se documentaron diferencias en el desempeño de la cepa VIENNA 8 1260 *vs* VIENNA 8 ó VIENNA 8 D53-, estos mismos resultados sugieren que tanto la calidad como el rendimiento de la cepa VIENNA 8 1260 pueden ser mejorados, lo cual en conjunto con la estabilidad en el sexado genético y la expresión de fluorescencia en el 100 % de la descendencia la hacen un candidato con potencial para ser incluido en un Programa de MIP-AE que utiliza la TIE para el control de la mosca del Mediterráneo. Otras cepas en cría masiva (p. ej. VIENNA 8 D53-) han demostrado que los costos operacionales iniciales pueden optimizarse con el tiempo.

Los resultados de este estudio indican que la cepa VIENNA 8 1260 puede ser criada masivamente en laboratorio y alcanzar niveles aceptables de rendimiento (MM/Ton), calidad y eficiencia (tasas de conversión huevo:pupa y huevo:adulto). En campo, los machos de la cepa VIENNA 8 1260 mostraron capacidad para sobrevivir y competir por hembras silvestres, induciendo en ellas esterilidad. Es importante reconocer que las diferencias en rendimiento y competitividad sexual documentadas para esta cepa podrían tener un mayor costo al requerir una mayor dosis de irradiación que elimine la TV, y un mayor número de machos estériles para incrementar la proporción esteril:silvestre en el campo. Sin embargo, con el uso de esta cepa podría haber una reducción en los costos por la eliminación del marcaje de pupas (con colorante Day Glo) y por una mayor certeza en la discriminación de machos estériles (f) y silvestres (f-) en los laboratorios de

identificación de adultos. Ademas de la reducción de costos directamente relacionados al monitoreo, los costos en los Programas de Control se podrían reducir sustancialmente sí se minimiza la probabilidad de dictaminar “falsos positivos”, principalmente en áreas libres de la plaga.

El solo hecho que el marcaje por Day Glo se limite a la superficie del cuerpo y al *ptilinum* (cabeza) de los machos estériles marcados, mientras la fluorescencia roja se manifiesta en los tejidos de todo el cuerpo de los machos VIENNA 8 1260, implica una mayor certidumbre en el marcaje y puede simplificar los actuales protocolos de identificación (que como primer paso requieren la disección de la cabeza y si hay duda, se continúa con la disección de órganos reproductivos). La presencia de fluorescencia verde en el esperma del macho VIENNA 8 1260 puede emplearse para obtener información adicional sobre el uso de esperma por las hembras silvestres (por ejemplo, si éstas han copulado con machos estériles). No se descarta el uso del gen fluorescente como un mecanismo secundario de marcaje que, en conjunto con el colorante Day Glo, brinde una mayor certeza y eficiencia en la identificación de machos estériles en campo.

Los resultados de este estudio muestran que los efectos generales de la irradiación sobre la fertilidad, fecundidad e inducción de esterilidad en la cepa VIENNA 8 1260 siguieron el mismo patrón que los documentados para la cepa VIENNA 8 D53-: a mayor dosis, menor fertilidad y fecundidad, así como mayor inducción de esterilidad por los machos irradiados sobre las hembras fértiles. Aunque en la cepa VIENNA 8 1260 la dosis de irradiación tuvo un efecto mayor (menor fertilidad y fecundidad) que en la cepa VIENNA 8 D53-, las dosis subesterilizantes tienen una interpretación más crítica en la cepa VIENNA 8 1260, debido a que es crucial evitar la transferencia vertical del transgen de fluorescencia a la población de moscas silvestres, con lo que se perdería la posibilidad de utilizar la fluorescencia como mecanismo de discriminación entre moscas estériles (f) y silvestres (f-).

Con el uso de la cepa VIENNA 8 1260 es primordial considerar una selección de la dosis correcta (suficiente para alcanzar un alto grado de esterilidad sin provocar efectos detrimentales en la calidad o competitividad sexual) y máximos controles de bioseguridad en los laboratorios de cría. Se recomienda una dosis mínima de 140 Gy, o una dosis

intermedia entre 140 y 170 Gy. Las diferencias documentadas en la competitividad de VIENNA 8 1260 no excluyen su uso en la TIE pudiendo alcanzar un nivel adecuado de inducción de esterilidad en las hembras silvestres, especialmente sí en la cría masiva se puede incrementar su desempeño a través de un proceso de selección de líneas con mayor fecundidad, calidad u otros parámetros deseables. Diversos estudios muestran que es posible compensar la menor competitividad sexual de los machos estériles incrementando la relación estéril:fértil, una práctica común en los Programas de Control de mosca del Mediterráneo.

La comparación entre VIENNA 8 1260, VIENNA 8 (ambas con inversión D53) y VIENNA 8 D53- confirma que las diferencias documentadas para la cepa VIENNA 8 1260 son parcialmente explicadas por la inversión D53 y no necesariamente son debidas en su totalidad al transgen fluorescente.

La disponibilidad de esperma f y f- permitió estudiar a mayor detalle la conducta de cópula y la precedencia en el uso de esperma en *C. capitata*. En los cruces tipo A (M VIENNA 8 1260 X HS X MS) y tipo B (MS X HS X M VIENNA 8 1260) se evidenció recópula en las hembras (mezcla de pupa f y f- en la F1). Dicha mezcla de pupa f y f- apoya el mecanismo de precedencia relativa en el uso de esperma: las hembras utilizan primero el esperma del macho con el que recopulan. La asimetría de los resultados al comparar los cruces tipo A y B sugieren una diferencia en el desempeño (fitness) del esperma, a favor de los machos no fluorescentes (silvestres). Estos resultados confirman la importancia de asegurar la competitividad sexual de los machos estériles de VIENNA 8 1260, quienes deben copular primero con las hembras silvestres, transferir una cantidad y calidad suficiente de esperma y fluido accesorio, que minimicen el riesgo de recópula y aseguren la inducción de esterilidad en la población silvestres.

Se confirmó que la técnica de PCR puede emplearse para evaluar el riesgo de transmisión horizontal del transgen fluorescente de la Moscamed hacia su parasitoide. Los resultados negativos de PCR, empleando los primers 1260DsRed-F y 1260DsRed-R, para la detección del transgen fluorescente en los parasitoides de *F. ceratitivorus* que se desarrollaron en individuos de la cepa VIENNA 8 1260, muestran que:

a) el transgen no fue transferido de los hospederos hacia los parasitoides en ningún caso a lo largo de las 16 generaciones analizadas, lo que se verificó por los cuatro laboratorios donde se hicieron los análisis;

b) los resultados son consistentes en las dos diluciones evaluadas (concentrada y dilución 1:10);

c) no se detectaron diferencias entre sexos (las bandas indicadores de la presencia del transgen están ausentes en ambos sexos).

Combinando estos resultados con el examen de los especímenes bajo el estereoscopio, se concluye que no ocurrió trasmisión horizontal del transgen responsable de la fluorescencia, de la cepa VIENNA 8 1260 hacia los parasitoides. Los resultados de la PCR correlacionan con los de técnicas tradicionales para detectar transferencias no controladas de material genético, tales como la verificación bajo estereoscopio utilizando filtros UV específicos que detectan la expresión fenotípica de fluorescencia en la cepa VIENNA 8 1260. Adicionalmente, este resultado sugiere que los Programas de Control pueden utilizar estereoscopios con filtros apropiados en los laboratorios de identificación de adultos para discriminar de manera segura entre insectos f y f-, sin necesidad de adquirir equipo sofisticado para otros análisis (como la PCR).

Se confirma que la presencia del gen exógeno responsable de la fluorescencia en la cepa VIENNA 8 1260 representa una carga detrimental mínima y que no se evidenció riesgo de una transferencia no controlada del transgen por el mecanismo de transferencia horizontal o transmisión vertical (particularmente si la dosis de irradiación se selecciona adecuadamente).

Se concluye que la presente tesis cumplió con el objetivo general de la investigación, que fue el de comparar el desempeño, en laboratorio y campo, de la cepa VIENNA 8 1260 con la cepa VIENNA 8 D53-, así como los objetivos específicos que fueron planteados: 1) evaluar el desempeño de machos y hembras de la cepa VIENNA 8 1260 en cría a escalas pequeña, media y masiva, y la competencia de los machos en condiciones de jaulas de campo (Capítulo II); 2) evaluar el efecto de diferentes dosis de irradiación en la

esterilidad y seguridad biológica, determinando la dosis óptima que evite la TV del transgen (Capítulo III) y 3) determinar la probalidad de la TH, utilizando para este caso la cercana relación huésped-parasitoide de *Ceratitis capitata* (Wied) con *Fopius ceratitivorus* Wharton (Capítulo IV).

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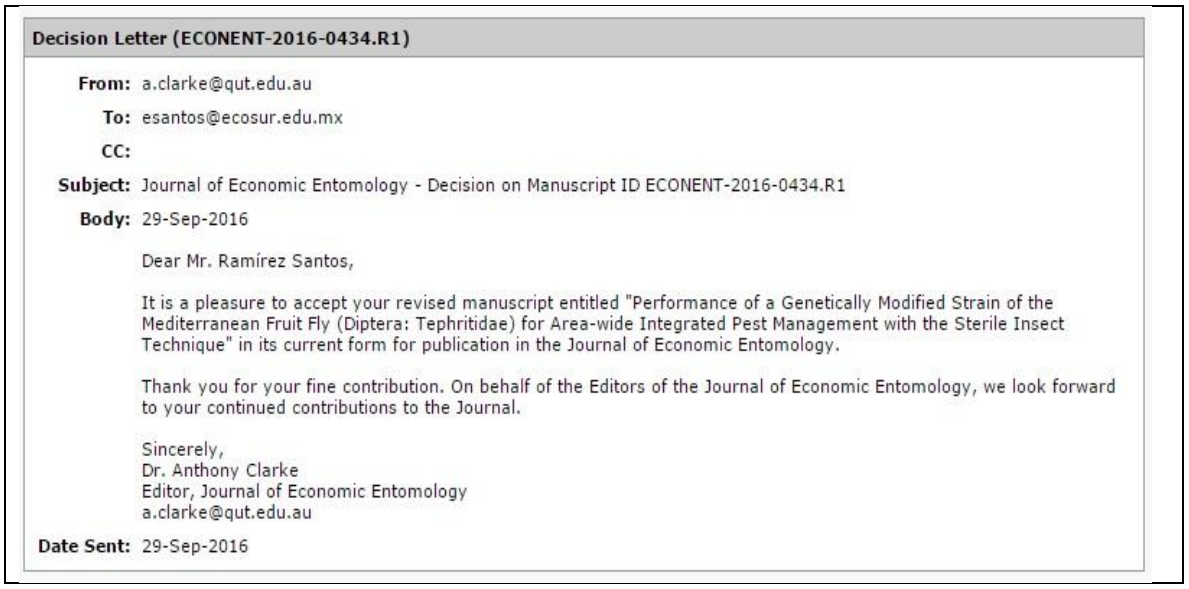
ANEXOS



Decision Letter (ECONENT-2016-0434.R1)

From: a.clarke@qut.edu.au

To: esantos@ecosur.edu.mx

CC:

Subject: Journal of Economic Entomology - Decision on Manuscript ID ECONENT-2016-0434.R1

Body: 29-Sep-2016

Dear Mr. Ramírez Santos,

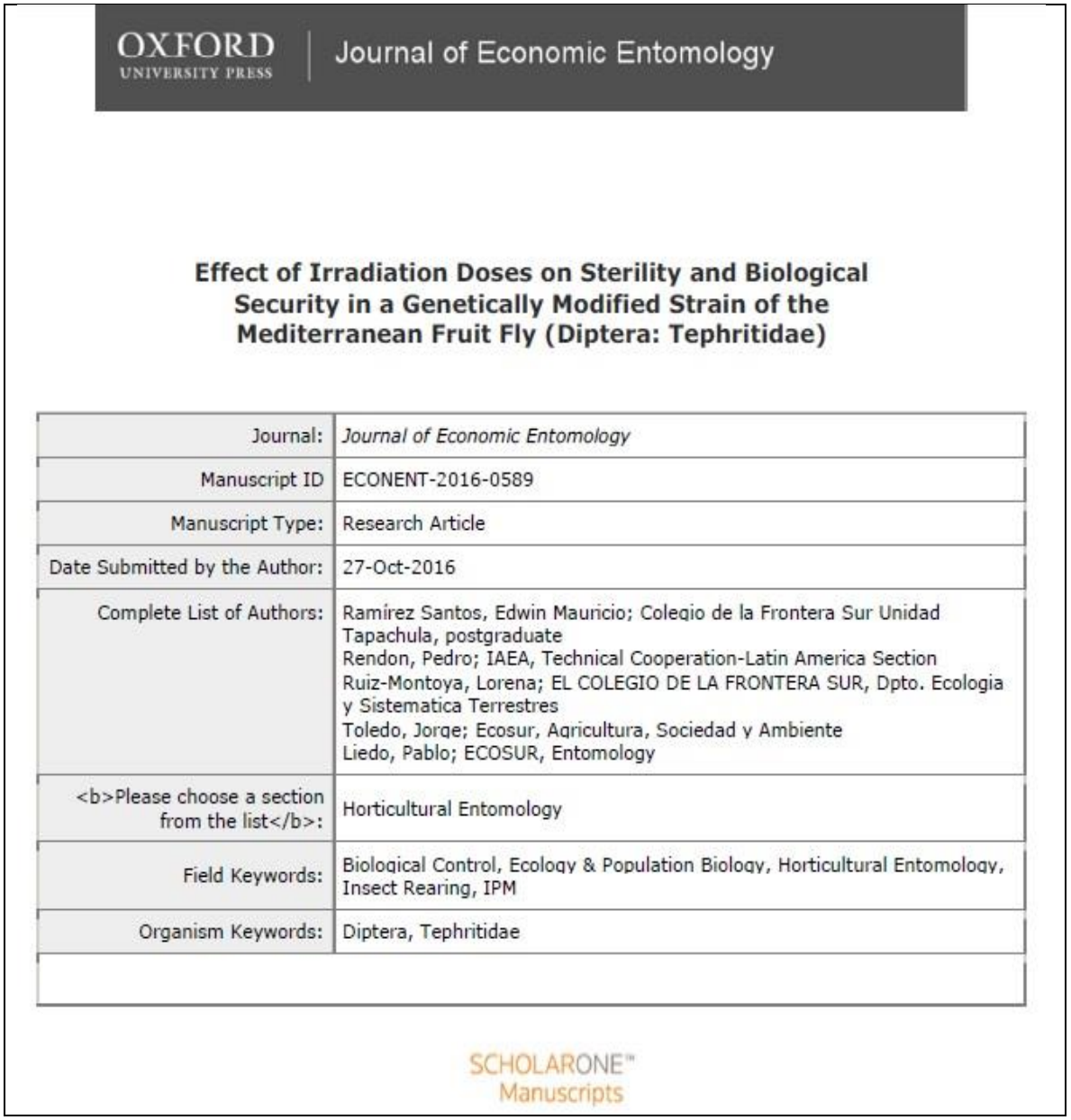
It is a pleasure to accept your revised manuscript entitled "Performance of a Genetically Modified Strain of the Mediterranean Fruit Fly (Diptera: Tephritidae) for Area-wide Integrated Pest Management with the Sterile Insect Technique" in its current form for publication in the Journal of Economic Entomology.

Thank you for your fine contribution. On behalf of the Editors of the Journal of Economic Entomology, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Anthony Clarke
Editor, Journal of Economic Entomology
a.clarke@qut.edu.au

Date Sent: 29-Sep-2016

Comprobantes de aceptación de artículo del Capítulo II en el “Journal of Economic Entomology (JEE)”.



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