Preventative releases of self-limiting *Ceratitis capitata* provide pest suppression and protect fruit quality in outdoor netted cages

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\textbf{ABSTRACT}

The Mediterranean fruit fly, *Ceratitis capitata* (Medfly) is considered a key pest of citrus fruit in many countries. Integrated pest management (IPM) approaches are frequently highly dependent on the use of insecticides. Oxitec’s self-limiting (prevents the insects’ offspring from surviving to adulthood) Medfly technology (OX3864A) offers an approach to manage Medfly outbreaks and may offer improved sustainability and economics for area-wide programmes. Netted cages were used to evaluate OX3864A deployed for the first time outdoors as a preventative treatment to suppress wild Medfly. Comparative assessments examined sexual competitiveness, the relative performance of OX3864A releases at both adult and pupal life stages, and the ability of OX3864A to protect against fruit damage. OX3864A males are as competitive as wild males. Deployment of both adult and pupal life stages of OX3864A effectively reduce Medfly abundance, resulting in elimination of the target pest populations. OX3864A deployment as adults also demonstrated the beneficial attribute of protecting fruit quality to preserve marketable yield. Data for adult and pupal deployment strategies are encouraging for self-limiting technologies targeting Medfly populations and support a broader evaluation of OX3864A Medfly or further improved self-limiting Oxitec strains of Medfly in open settings.

\textbf{ARTICLE HISTORY}

Received 12 March 2018
Accepted 25 March 2019

\textbf{KEYWORDS}

Medfly; Fruit fly; SIT; OX3864A; TSL; genetic-sexing

1. Introduction

Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (hereafter termed Medfly) is a highly polyphagous species, known to infest over 260 host plants including citrus fruits, stone fruits, deciduous fruits and some vegetables (Malacrida et al. 2007; Zeki et al. 2008; Diamantidis et al. 2011). It is native to Equatorial Africa, but has spread and established in tropical and temperate regions throughout the world (Bonizzoni et al. 2002; Gasperi et al. 2002; Malacrida et al. 2007; EPPO 2014). Yield reductions are mainly attributable to the damage incurred when larvae feed directly on the pulp as they tunnel through the fruit. This can rapidly spoil produce and induce premature fruit drop. In some cases, secondary damage due to bacterial or fungal infections that follow oviposition can reduce yields further still. Losses of produce are compounded by the elevated production costs, in some cases leading to significant economic impact across large regions (Lysandrou 2009).

Insect pest population control holds significant benefits to agriculture in terms of food yield. One of the most common methods of pest control is the use of pesticides. Use of pesticide in moderation protects crops against pests such as insects. However, the heavy use of pesticides could have important economic, environmental, and human health consequences (Aktar et al. 2009).

Control measures for Medfly typically rely on the use of chemical insecticides, sometimes in combination with protein-based baits or mass trapping with specific female attractants (Karsten et al. 2013; Siciliano et al. 2014). Use of these parasitoids showed some success as biological control against the Medfly and as such, they have been considered as part of an integrated pest management (IPM) approach. Use of parasitoids has also been part of IPM in Guatemala. Parasitoids have been, and are still, employed for the control of Medfly (Sime et al. 2007). Several species of entomopathogenic fungi are used as biological control agents for Medfly. For example, *Beauveria bassiana* (Balsamo) and *Metarhizium anisopliae* (Metschnikoff) were used to control populations of the Medfly and the olive fly in laboratory (Maria 2010). *B. thuringiensis* (Bt) is one of the environmentally friendly pesticides; is a bacterium that exists in the gut of maggots of several types of moth. It has been used as biological...
insecticide to control several pest insect families such as Lepidoptera, Diptera, Coleoptera, Hymenoptera and nematodes since 1920 (EPA 2001). A number of strains of Bt from Marrakech and south Morocco argan forest have been isolated and tested against Medfly (Aboussaid et al. 2010). The results demonstrated that some of the collected Bt strains can be used as part of integrated pest management against Medfly (Aboussaid et al. 2010).

None of the alternatives to insecticides such as parasitoids, protein-based baits or mass trapping have proved sufficiently effective to be used in a control programme. The Sterile Insect Technique (SIT) is an alternative pest management method which is an environmentally friendly and species-specific method. It uses biological techniques to eliminate or suppress pest insects without using pesticides (Klassen and Curtis 2005). In SIT, insects are mass reared and sterilised by radiation. Sterilized insects are released into the field where they search for a mate; hence they compete with wild insects for mating. Any mating between wild females and sterile males will result in females producing non-viable eggs, thus the targeted insect populations will be suppressed (Klassen and Curtis 2005).

SIT was reported to have successfully prevented the establishment of Medfly in areas of the USA (Dowell et al. 2000; Barry et al. 2004), Mexico (Hendrichs et al. 1983), and Chile (Espanza Duque 1999; Gonzalez and Troncoso 2007). The first major SIT programme against Medfly started in Guatemala and southern Mexico in 1977 as Medfly occupied a range across Central America and northwards into southern Mexico. The programme resulted in the pest being eliminated from Mexico and parts of Guatemala by 1982 (Klassen and Curtis 2005). SIT is still being used as a barrier in Guatemala to prevent the pest from becoming re-established in Mexico and the USA for over thirty years (Enkerlin et al. 2015). Effective Medfly programmes have allowed for the increase of agricultural exports, for instance earnings from Mexican agriculture exports between 1994 and 2004 tripled (Enkerlin et al. 2015).

Traditionally sterility in SIT is achieved through exposure to ionizing radiation. Nevertheless, this has well-documented adverse effects on longevity and mating performance of males (Robinson 2002; Barry et al. 2003; Kraaijeveld and Chapman 2004).

The release of insects carrying ‘self-limiting’ genetic elements provides an alternative that removes the need to subject insects to ionizing radiation. ‘self-limiting’ insects pre-dispose some (females) or all progeny to die at an immature life-stage, has been demonstrated as an effective management tool for specific insect pests across both agricultural and public health sectors (Ant et al. 2012; Gorman et al. 2015; Harvey-Samuel et al. 2015). Typically, the release of male-only cohorts provides the optimal approach, as this helps maximise the number of successful mating events with wild females. The two main advantages for male-only releases in the case of agricultural pests are: no oviposition of the females and no assortative mating of these females with the released males.

The development of a transgenic strain of Medfly (insects carrying dominant lethal transgene), which exhibited full functionality of a conditional female-specific self-limiting trait (Fu et al. 2007). Mating events between OX3864A males and wild females produce no female progeny that survive to functional adulthood, and therefore in practise, are anticipated to lead to population suppression of wild insects in the following generation (Leftwich et al. 2014). By providing a repressor (tetracycline (100 ng/ml) to the self-limiting gene product in the diet of developing larvae, the expression of the OX3864A self-limiting gene is prevented so that the strain can be reared normally. The strain, termed OX3864A, showed equivalency to its wild-type counterpart for life-history characteristics and sexual competitiveness in glasshouse studies (Leftwich et al. 2014). Repeated releases of analogous strains of other agricultural species have been shown to account for an increasing share of mating events and indoor (greenhouse) pest populations suppressed to the point of elimination (Ant et al. 2012; Harvey-Samuel et al. 2015). In addition, a dominantly inherited marker gene encoding a fluorescent protein is incorporated to provide a convenient means of identification (Fu et al. 2007).

Here we present new data for OX3864A Medfly strain, a conditional female-specific, self-limiting Medfly for the first time confirming its utility outdoors as a preventative treatment to suppress wild Medfly populations. Comparative assessments were used to examine male mating competitiveness, the relative performance of OX3864A releases at both adult and pupal life-stages, and the ability of OX3864A to protect against fruit damage.

2. Materials and methods

2.1. Insects

The maintenance, preparation and storage of OX3864A and wild insects took place at either of two insect-rearing facilities; the Omnium Agricole du Souss Auxiliary Production Site, Chtouka, Morocco and the Hassan II Institute of Agronomy and Veterinary Sciences, Agadir, Morocco. All flies were maintained in a controlled environment (25 ± 2 °C, RH 60–65%, 12:12 h light:dark cycle). Due to the established different developmental rates
between laboratory and wild caught Medfly (Gomulski et al. 2012; Leftwich et al. 2014), the ages of adults used for all studies were chosen to ensure a similar level of sexual maturity (8–10 days for wild and 3–5 days for OX3864A).

2.2. Wild insects

Wild Medfly were collected directly from the fruit of infested argan trees, *Argania spinosa* (L.) Skeels, in and around the Hassan II Institute of Agronomy and Veterinary Sciences, Agadir, Morocco. Dropped argan fruits were collected from the ground surrounding infested trees and were placed in perforated trays, allowing emerging larvae to crawl through the holes and pupate in a container of sand below. Pupae were sieved from the sand daily, and then maintained in sealed Petri-dishes until adult eclosion. Newly emerged adults were separated by sex and placed until sexual maturation (8–10 days post-eclosion) in either Bugdorm insect rearing cages (Watkins and Doncaster, UK; 27 L) for culturing and mating competition studies, or release devices (plastic boxes with a mesh lid to allow for air-circulation, 45 cm³/fly) for population suppression and crop protection studies. Adult wild flies were fed *ad libitum* on a diet of 3:1 sucrose and yeast extract (Fisher Scientific, UK).

2.3. OX3864A insects

OX3864A is a self-limiting strain of Medfly, whose initial development and characterisation has been detailed previously (Leftwich et al. 2014). In the absence of a dietary repressor, tetracycline, the sex-linked self-limiting phenotype that induces mortality prior to adulthood is expressed in all female larvae only. An OX3864A colony was established and larvae cultured on an artificial diet containing tetracycline (100 ng/ml) to prevent phenotypic expression of the self-limiting gene and enable the production of females. Larval diet contained: bran (25 g), sucrose (48 g), brewer’s yeast (65 g), sodium benzoate (2.6 g), and HCl 1% (100 ml) diluted with tap water to a final volume of 1 L (Tanaka et al. 1969). Pupae were collected and maintained in sealed Petri-dishes until adult eclosion. Newly emerged adults were separated by sex and placed until sexual maturation (8–10 days post-eclosion) in either Bugdorm insect rearing cages (Watkins and Doncaster, UK; 27 L) for culturing and mating competition studies, or release devices (plastic boxes with a mesh lid to allow for air-circulation, 27 cm³/fly) for population suppression and crop protection studies. Adult OX3864A were fed *ad libitum* on a diet of 3:1 sucrose and yeast extract (Fisher Scientific, UK). During population suppression studies, OX3864A were also deployed as pupae (8-days post pupation) from the same release devices as adults (plastic boxes with a mesh lid to allow for air-circulation, 27 cm³/fly). Recaptured adults and progeny fathered by OX3864A males were identified by visual inspection using a fluorescent microscope (Olympus SZX12 with Olympus U-RFLT fluorescent burner, Olympus, Japan) and excitation filter (530-550 nm, emission 575 nm) for the presence of the fluorescent protein encoded by the inserted DsRed2 genetic marker (Clontech Inc., USA).

2.4. Insecticide

Insecticide treatment plots were used as a positive control for OX3864A treated plots. Spinosad (Dow AgroSciences, USA), which is based on chemical compounds found in the bacterial species *Saccharopolyspora spinosa*, was chosen to treat Mandarin orange tree, *Citrus reticulata*, without fruit (1.8 m in height). Here, it was obtained as a commercially available formulation (Success 0.24 g/L) and applied at the recommended label rate for control of Medfly (1 L/ha diluted into a total volume of 30 L). In contrast to standard practice, a non-pressurised hand-held sprayer (as opposed to an airblast sprayer) was used to prevent overspray onto adjacent experimental plots (see discussion).

2.5. Mating competition study

Mating competition studies followed standard guidelines (FAO/IAEA/USDA 2014) and took place from September 2012 until December 2012 at the Hassan II Institute of Agronomy and Veterinary Sciences in Agadir. For each mating test, Medfly adults were released into an outdoor netted cage (1.7 m × 1.7 m × 1.7 m) that contained a single potted Mandarin orange tree, *Citrus reticulata* (1.4 m height). Immediately following dawn, at approximately 5:00 am, 50 × OX3864A males (5 days post-eclosion) and 50 × wild males (10 days post-eclosion) were released into each experimental netted cage and allowed to acclimatise for a minimum of 60 minutes prior to the introduction of 50 × wild females (10 days post-eclosion). From the introduction of females into the cages, courtship and mating behaviours were observed so that upon establishment of each mating pair, both individuals were collected and isolated in a 50 ml plastic tube with a cotton wool bung (BTP Drewitt Limited, UK). Male courtship behaviour has previously been comprehensively studied and described (Lux et al. 2002). Collection of mating pairs continued until no further pairs had been observed for a period of 60 minutes, which
typically occurred around midday (12.00 pm) (Mcinnis et al. 1996). Once collected, tubes containing individual mating pairs were placed in a shaded area within the cage. Copulation durations (time from formation to separation of a mating pair) were recorded and the identity of each male was confirmed by fluorescence microscopy (Olympus SZX12 with Olympus U-RFLT fluorescent burner, Olympus, Japan) and recorded as either wild (non-fluorescent) or OX3864A (fluorescent) (Figure 1). This experimental set up shown in Figure 2. This test was performed with seven replicates, copulation events with a duration of less than 30 minutes were discarded from the analysis, as they often fail to transfer sperm (Seo et al. 1990). The Relative Sterility Index (RSI) was used as a measure of male sexual competitiveness, enabling a comparison between OX3864A males and wild males (Mcinnis et al. 1996). An RSI value of 0 represents 100% of females mating with wild males, a value of 1 represents 100% of females mating with OX3864A males, and a value of 0.5 indicates equal competitiveness between the two types of males.

2.6. Population suppression study

Field-cage experiments to assess the suppression of wild Medfly by OX3864A treatments were carried out in an orchard at Ouled Dahou, Morocco from December 2013 till August 2014. Six netted plots, each measuring 4 m × 4 m × 2 m, utilised a double netted entrance and contained one Mandarin orange tree, Citrus reticulate, without fruit (1.8 m in height). Wild Medflies were not present in the netted plots prior to the initiation of the study. All six plots were inoculated with 800 male and 800 female wild adult Medflies (8–10 days post-eclosion); four treatment plots also received inoculations of 8,000 OX3864A male adults (3–5 days post-eclosion), and two treatment plots each received weekly inoculations of 8,000 OX3864A male pupae (8 days post-pupation). Daily recordings of temperature and humidity in each plot were taken via a USB data logger (EasyLog EL-USB-1, Lascar Electronics, UK). Water and a diet of 3:1 sucrose and yeast extract (Fisher Scientific, UK) were provided ad libitum during the experiment. This experimental set up shown in Figure 3.

To facilitate monitoring, four ripe mango fruits were placed in each plot twice weekly. Upon removal, mangoes from each plot were placed in separate sealed buckets with cardboard trays below, and then checked daily for larval emergence. Rolled-up filter paper (Fisherbrand QL135, Fisher Scientific, UK) was pushed into the flesh of each mango to absorb excess juice produced during mango senescence and decay. Upon pupation, pupae were screened for the presence of the DsRed2 fluorescent marker under a fluorescence microscope (Olympus SZX12 with Olympus U-RFL-T fluorescent burner, Olympus, Japan). Mangoes were kept until they had not produced larvae for three consecutive days. Pupae collected from each plot were maintained in individual, labelled Petri-dishes and following eclosion, the numbers of males and females were confirmed and recorded. Finally, all progeny were returned as adults to their original plots.

Adult numbers within the plots were assessed throughout the experiment using yellow sticky traps (Maxiboard, Russell IPM, UK). Monitoring was conducted on a weekly basis, and consisted of a single sticky trap per plot, placed for a continuous 24-hour period. Trapped insects were identified, sexed, and examined for the presence or absence of the genetic marker using a fluorescence microscope (Olympus

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**Figure 1.** Photographs showing side-by-side visual comparisons between OX3864A and wild-type Ceratitis capitata under A. white light and B. excitation filter (530–550 nm, emission 575 nm).
SZX12 with Olympus U-RFL-T fluorescent burner, Olympus, Japan).

2.7. Crop protection study

Assessments of the ability of OX3864A to protect fruit quality were carried out from December 2013 till August 2014 in an orchard at Ouled Dahou, Morocco. Six netted plots, each 4 m × 4 m × 2 m, utilised a double netted entrance and contained one Mandarin orange tree, *Citrus reticulate*, without fruit (1.8 m in height). Wild Medflies were not present in the netted plots prior to the initiation of the study.

From week 1 to week 6, two treatment plots received weekly inoculations of 3000 OX3864A adult males (3–5 days post-eclosion) to simulate a preventative release period. From week 3 to week 6, all plots received weekly inoculations of 200 male and 200 female wild Medfly (8–10 days post-eclosion). From week 7 onwards, the two control plots received no further treatment. From week 7 onwards, the two treatment plots that had been receiving preventative OX3864A inoculations were inoculated with 3000 OX3864A adult males (3–5 days post-eclosion) to simulate an operationally realistic application rate (15:1 OX3864A male:wild
male). The application rate for OX3864A males released in this study was greater than in the Population Suppression study as the intention was to achieve a significant effect in a shorter period. For comparative purposes, from week 7 and in accordance with recommended application intervals, the two remaining treatment plots received three applications at two-week intervals (i.e. on weeks 7, 9, and 11) of the insecticide, spinosad. Repeat treatments spaced at 14-day intervals target all individuals in the crop as they develop through the different life-stages. However, it must be noted that whilst label recommended rates and volumes were used, spinosad was applied with a non-pressurised handheld sprayer to protect adjacent plots. Typically, growers employ a pressurised airblast applicator to achieve a thorough penetration of the canopy.

Mango fruit suspended under the Mandarin trees in each plot were used to assess rates of Medfly infestation and their impact upon fruit quality. Five whole mango fruits were suspended in each plot, they were replaced with fresh fruits every 3 or 4 days alternately (i.e. 10 mangoes per week per plot). Medflies were able to freely oviposit on the mango fruit, and progeny allowed to develop. The numbers of oviposition punctures and pupae within each fruit were recorded. Using a fluorescence microscope (Olympus SZX12 with Olympus U-RFL-T fluorescent burner, Olympus, Japan), all pupae were identified as either OX3864A or wild, based on the presence or absence of the fluorescent genetic marker. Pupae collected from each plot were identified as either OX3864A or wild, based on the presence or absence of the fluorescent genetic marker. Pupae collected from each plot were maintained in individual, labelled Petri-dishes and progeny allowed to develop. The numbers of oviposition punctures and pupae within each fruit were recorded. Using a fluorescence microscope (Olympus SZX12 with Olympus U-RFL-T fluorescent burner, Olympus, Japan), all pupae were identified as either OX3864A or wild, based on the presence or absence of the fluorescent genetic marker. Pupae collected from each plot were maintained in individual, labelled Petri-dishes and following eclosion, the numbers of males and females were confirmed and recorded. Finally, all progeny were returned as adults to their original plots. This experimental set up shown in Figure 4.

To monitor infestation levels within plots, both the number of oviposition punctures in the introduced fruit and the subsequent pupal emergence from that fruit was recorded on a weekly basis. Damage to fruit was assessed three times, during the period of inoculation with wild insects (weeks 1–7), the period during which insecticide treatments took place (weeks 8–14), and the subsequent period (weeks 15–22).

Adult numbers within the plots were assessed throughout the experiment using yellow sticky traps (Maxiboard, Russell IPM, UK). Monitoring was conducted on a weekly basis and consisted of a single sticky trap per plot placed for a continuous 24-hour period. Trapped insects were identified, sexed, and examined for the presence or absence of the genetic marker using a fluorescence microscope (Olympus SZX12 with Olympus U-RFL-T fluorescent burner, Olympus, Japan). Daily recordings of temperature and humidity in each plot were taken via a USB data logger (EasyLog EL-USB-1, Lascar Electronics, UK).

2.8. Statistical analysis

For all three studies (mating competition, population suppression and crop protection) a Shapiro-Wilk test was used to determine if data were normally distributed or not. The RSI in mating competition study was the proportion of wild male and OX3864A male mating with wild female obtained from the 187 mating pairs and was analysed with a Generalized Linear Mixed Model (GLMM) with a binomial error family including mating groups (wild male to wild female or OX3864A male and wild female), replicates and their interactions as their random effect. a two-way ANOVA was performed on mating duration values, with mating type (wild or OX3864A male) and Cage (7 separate cages tested) as factors as well as their interactions. Count data in both population suppression and crop protection study (the number of ovipuncture, larvae or pupae in fruit) was analysed with a general mixed linear model (GLMM) with a poisson error family including treatments (OX3864A male releases, insecticide or no treatment) and replicates as a random effect. The number of pupae analysed in the population suppression study was analysed with a general linear mixed model (GLMM) with a quasipoisson error family (Poisson distributed errors corrected for overdispersion) including treatments (OX3864A male releases, insecticide or no treatment) and replicates as a random effect. The number of pupae analysed in the population suppression study was analysed with a general linear mixed model (GLMM) with a quasipoisson error family (Poisson distributed errors corrected for overdispersion) including treatments (OX3864A male releases, insecticide or no treatment) and week (from week 1 to week 31) as well as their interactions. Finally, for the climatic study, linear regression models were used to analyse the relationship between ambient temperature and pupae production during the crop protection study. Confidence Intervals (CIs) are given at the 95% level, and values presented to a minimum of two significant figures. All statistical analyses were performed using R software, v3.5.2 and RStudio v1.1.463 (R Development Core Team, 2018).

3. Results

3.1. Mating competition study

Across all seven replicates, 187 mating couples were recovered. The mean number of mating couples assessed per replicate was 27 ± 5 out of a possible 50. For all replicates, mating events involved at least 20% of each mating group (OX3864A male, wild male, and wild female). The mean RSI value observed for OX3864A males was 0.42 with the standard deviation (sd) of ±0.12 (P = 0.029, degrees of freedom (d.f.) = 6, n = 187, GLMM
(binomial distribution)); demonstrating significantly better mating rate from wild Medfly males. The data is normally distributed (Shapiro–Wilks’ test: \( W = 0.94, P = 0.64 \)).

Copulation duration data between Medfly females and OX3864A males, on average 126.6 minutes ± 4.8 (sd), were not significantly different from Medfly females and wild Medfly males, on average 124.2 minutes ± 4.0 (sd) \( (F = 0.49, \text{df} = 1, P = 0.49) \). The data was normally distributed (Shapiro–Wilks’ test: \( W = 0.99, P = 0.14 \)).

### 3.2. Population suppression study

Total pupal production (Figure 5A) in plots treated with OX3864A began to diverge from those of the untreated plots at week 17, declining rapidly thereafter with no pupal production from week 21 in the plots treated with OX3864A adults and from week 25 in the OX3864A pupae-treated plots. OX3864A adult and pupae-treated plots suppressed wild Medflies at equivalent rates across the weeks \( (P = 0.32, \text{Null deviance} = 25494 \text{ on df} = 186, \text{Residual deviance} = \) \( ) \)
The data was not normally distributed (Shapiro–Wilks’ test: $W = 0.89$, $P = 2.5e-10$). Adult female emergence from the weekly fruit introductions was monitored (Figure 5B). The number of fluorescent pupae originating from the weekly fruit introductions provided an indirect measurement of the mating outcomes within the treatment plots (Figure 5C). Fluorescent pupae were observed in treated plots two weeks following the introductions.
first inoculation with OX3864A. The proportion of fluorescent pupae reached 100% by week 21 for both treatments (OX3864A adult and OX3864A pupal treatment). Wild female emergence had ceased in both OX3864A male adult and both pupal treatment plots by week 19 and 22 respectively. As any developing insects may take two weeks to come through, elimination of the target population in both plots treated with OX3864A adults was confirmed by week 24, and in both plots treated with OX3864A pupae by week 27. The number of wild females declined post-release in treatment plots, it reached to zero at week 17 for adult treatment plots and week 19 for pupal treatment plots (Figure 5D).

### 3.3. Crop protection study

Mean percentages of fruit with ovipuncture and/or larval fruit damage in untreated plots was consistently the highest and ranged between 86 and 99% at the three assessment points (Table 1). Mean fruit damage in both treatments declined significantly between assessment points and no fruit damage was observed in the OX3864A-treated plots during the final treatment period as the population had been eliminated.

Comparisons of infestation levels, measured by the number of ovipuncture in fruit, between OX3864A and insecticide treated plots were not significantly different for weeks 4-7 ($P = 0.2$, Null deviance: 264.2 on df = 16, Residual deviance: 94.5 on df = 12 GLMM (poisson distribution)) (Figure 6A). The data was not normally distributed (Shapiro–Wilks’ test: $W = 0.87634$, $P < 2.2e-16$). Medfly control from the sustained release of OX3864A males manifested as a gradual decline in the wild female population, female emergence from the introduced fruit was also measured on a weekly basis (Figure 6C). There was no wild female emergence in the OX3864A-treated plots from week 13. In contrast, female emergence in the insecticide-treated plots reduced sharply following the initial spinosad application and was evident at relatively low levels until the end of the experiment.

During weeks 8-14, fruit damage (ovipuncture damage and larval presence) in the insecticide treated plots dropped markedly following the first spinosad application (Figure 6A and 6B). The fruit infestation levels (ovipuncture damage) in OX3864A and insecticide treated plots were comparable during that period with reductions in fruit infestation (oviposition puncture) of 83% and 85% respectively (Table 1), the mean values of infestation levels (ovipuncture damage) between OX3864A and insecticide-treated plots were not significantly different for weeks 8-14 ($P = 0.07$, Null deviance: 667.2 on df = 28, Residual deviance: 164.5 on df = 19, GLMM (poisson distribution)) (Figure 6A).

After spinosad treatments had ceased, fruit infestation levels (ovipuncture and larval fruit damage) increased slightly in the insecticide-treated plots. Infestation levels (ovipuncture damage) between

### Table 1. Mean percentages of fruit with damage shown across three time periods for OX3864A-treated, insecticide-treated, and untreated plots.

| Period (weeks) | OX3864A-treated | Insecticide-treated | Untreated |
|---------------|-----------------|---------------------|-----------|
| Oviposition punctures |                  |                     |           |
| 4–7           | 94.0 ± 3.7      | 96.9 ± 11.9         | 98.5 ± 11.6 |
| 8–14          | 83.0 ± 7.7      | 85.0 ± 3.24         | 94.7 ± 7.6 |
| 15–22         | 1.0 ± 0         | 69.0 ± 12.2         | 99.3 ± 7.0 |
| Larval presence |                 |                     |           |
| 4–7           | 97.7 ± 6.4      | 94.8 ± 6.3          | 98.4 ± 14.0 |
| 8–14          | 74.5 ± 5.7      | 65.5 ± 13.7         | 86.3 ± 14.5 |
| 15–22         | 1.0 ± 0         | 32.6 ± 19.7         | 99.0 ± 6.9 |

Figure 6. Fluctuations in metrics for Medfly abundance and fruit damage from weeks 4-22. The arrows indicate the weeks that insecticide treatment occurred (weeks 7, 9, and 11). A: total number of oviposition punctures B: Total number of larvae found in fruit. C: Female emergence from introduced fruit. D: Number of wild females trapped.
OX3864A and insecticide-treated plots were highly significantly different for weeks 8-14 ($P = 2.7e-16$, Null deviance: 1374.3 on df = 42, Residual deviance: 871 on df = 24 GLMM (poisson distribution)) (Figure 6A).

Although there was still a 62.6% reduction in fruit damage compared to the control plots (Table 1). These results correlate with the numbers of females emerging in insecticide-treated and untreated plots (Figure 6C).

### 3.4. Climatic monitoring

Climatic monitoring in the untreated plots highlighted spikes in ambient temperatures during weeks 7, 15, 16, and 17, resulting in temperatures above 40 °C on a total of 14 separate days (Figure 7). The mean temperature across the study period was 23.3 °C. There was no obvious effect of temperature on pupal production during the trial (adjusted $r^2 = 0.02722$ and $r^2 = 0.1157$, $t = -1.144$, df = 1, $P = 0.2794$) (Figure 7).

### 4. Discussion and conclusion

This study has demonstrated that in a caged field environment, OX3864A males were comparable with wild males in terms of their ability to compete for and mate with wild female Medflies. The obtained RSI for OX3864A (0.42 +/− 0.12) was not significantly different from 0.5, in accordance with previously published data from a contained laboratory setting (Leftwich et al. 2014). In comparison, the RSI for the genetic sexing Temperature Sensitive Lethal (tsl) Vienna 8 strain of Medfly, mass-reared for SIT programmes worldwide, was previously documented as 0.22 (Paranhos et al. 2006). Given that the tsl strain is in operational use, the data provided here suggest that mating competitiveness is unlikely to be a barrier to area-wide deployment of OX3864A.

Ant et al. (2012) presented data from a study conducted in Israel (Rempoulakis and Nestel, personal communication) regarding olive fruit fly (Bactrocera oleae) mating competitiveness. They calculated an RSI value of 0.4 for wild-type olive fruit fly males that were manually-sexed and tested for mating competitiveness against wild olive fruit flies collected in Israel. However, they also demonstrated that when the wild-type males were irradiated (exposed to 100 Gy of gamma rays) the RSI value fell to less than 0.2 (Ant et al., 2012).

For some insect species, pupal releases would not be viable due to operational constraints, for example due to pupae that cannot easily be separated from the larval rearing medium, or rapid eclosion rates giving insufficient time for distribution. Prior to this study, pupal releases of OX3864A had not been attempted. This side-by-side comparison of pupal and adult releases demonstrated that pupal releases for this strain were generally favourable. It is possible that the time necessary for eclosion of pupae and sexual maturation of adult males resulted in a delay in the control observed in the plots where pupae were released as opposed to ones where mature adults were released. Additionally, losses during the pupal eclosion stage may have been higher in the outdoor plots (as opposed to the laboratory), contributing to the observed lower numbers of OX3864A being recovered as progeny.
in pupal release plots when compared to the adult release plots. This results in the typically larger numbers of wild Medfly progeny being recovered from the pupal-treated plots. It also appears this may have contributed to a slower pest suppression in pupal release plots. Overall, despite these general trends, an operational system must also account for an array of other economic inputs and the high efficacy and proximity of these two datasets warrant a more detailed cost-benefit assessment.

The level of control observed with Spinosad was high, although in these experiments spinosad did not achieve elimination and the population showed evidence of resurgence. If the experimental constraints of the adjacent plots had permitted the recommended airblast applications, we consider it likely that spinosad would have performed better than observed both in terms of pest management and the protection of fruit quality.

In plots preventatively treated with OX3864A, rapid suppression of the wild Medfly populations was observed. Indeed, from week 15 onwards there was no Medfly presence in any of the OX3864A-treated plots. These observations were reflected by the absence of any fruit damage. In contrast to chemical treatments that have requisite harvest intervals during which applications must cease, releases of OX3864A males can in theory be maintained to ensure there is no re-infestation during critical harvest periods.

Several spikes in climatic temperature were observed during the course of the studies. Although there was no consistent relationship between ambient temperature and the level of pupal emergence from fruit (Figure 7). Large fluctuations in temperature are likely to have direct effects on Medfly reproductive rates, although in this case, any effects would have been similar across all plots and are therefore unlikely to have affected comparisons between treatments.

Overall, the data offered a valuable first assessment of how OX3864A self-limiting genetic technology translates from contained settings to field use and can provide an efficient tool for preventing damage to fruit by reducing pest abundance. It should be noted that these experiments were undertaken in closed (netted) outdoor environments. The successful deployment of OX3864A as pupae demonstrated potential for flexibility in terms of application method, which may offer cost reductions and/or operational advantages. Although assessed in this instance as a stand-alone tool, it is envisaged that operational use would be in the context of integrated pest management that may provide further technical and cost synergies.

Acknowledgements

The authors would like to thank colleagues and collaborators for valued discussion throughout the planning and execution phases. Kevin Gorman for editing the manuscript, Said Akhzam for technical assistance and Mohammad Reza Tabrizi for his advice on statistical analysis.

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