Development of genetic control technology for Tephritid pests

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Abbreviation	Description
3'-UTR	3' untranslated region
5'-UTR	5' untranslated region
β2tubulin	Member of tubulin family, the proteins that make up microtubules.
cDNA	Complementary DNA that is synthesized from a messenger RNA.
DNA	Deoxyribonucleic acid
DsRed2	Variant of *Discosoma* sp. red fluorescent protein with faster maturation and lower non-specific aggregation.
EcoRI	Endonuclease enzyme that is isolated from strains of E. coli.
Fok1	Flavobacterium okeanokoites, is a bacterial type IIS restriction endonuclease.
FSL	Female-specific lethality
GMO definition	Genetically Modifies Organism
hsp70	Heat-shock protein 70
Hsp83	Heat-shock protein 83
I-ppo1	from the slime mould *Physarumpolycephalum,* restriction endonuclease.
Lab reared	A strain of insect that is reared in a Laboratory conditions
mCherry	Variant of *Discosoma* sp. red fluorescent protein, preferred to other fluorophores due to its colour, as well as its photostability.
Medfly	Mediterranean fruit fly
NT	Reared off-tetracycline
Off-tet	Diet with out tetracycline
On-tet	Diet containing tetracycline
Olive fly	Olive fruit fly
PCR	Polymerase Chain Reaction
PEL	Paternal Effect Lethal
Q-PCR	Quantitative PCR-Real time PCR
RIDL	Release of Insects carrying a Dominant Lethal
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SIT	Sterile Insect Technology
T	Reared on tetracycline
Tet	Tetracycline
tetO	Tet-Operator
Tet-off system	A form of Tetracycline-controlled transcriptional activation, for controlling expression of genes of interest
tetr	Tet-Repressor
topi	*matotopetli*
tTAV	*tetracycline transactivator variant*
Znf	*3Zincfinger*
ZsGreen	Zoanthus sp. green fluorescent protein

Declaration of Authenticity

This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) studentship and Oxitec Ltd. I hereby declare that this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. None of this work has been submitted previously for a qualification at the Cardiff University or another institute of higher education.

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Abstract

The olive fly, *Bactrocera oleae*, is the single most important pest in olive plantations. Currently, control of olive fly relies on the heavy use of chemical pesticides. The sterile insect technique (SIT) is a highly effective, species-specific and environmentally non-polluting method of pest control that involves the mass-release of sterilised insects. SIT is considered a potentially valuable method for the control of olive fly. Previous olive fly SIT attempts failed due to an inability to produce large numbers of flies, low egg production rates and lack of a method to separate the sexes.

RIDL (Release of Insects carrying a Dominant Lethal) is a biotechnology-based variant of SIT. This could potentially overcome several problems of classical SIT, including the radiation damage to insects. To develop fly male sterility, we have identified and tested several different germline specific promoters and several potential effector genes. These have been linked to the 'tet-off' expression system, which is suppressed by dietary tetracycline, and were initially tested in the Mediterranean fruit fly (*Ceratitis capitata)* for practicality. In the absence of tetracycline, tTAV binds to its target sequence, tetO, and activates expression of downstream genes. Flies carrying a promoter construct (*topi-tTAV* or *β2-tubulin-tTAV*) in medfly were crossed to flies carrying effector constructs (*tetO-I-ppoI*, *tetO-3zincfinger* or *tetO-ProtamineFokI*). A combination of *β2-tubulin-tTAV* and *tetO-ProtamineFokI* gave the best male sterility in medfly.

A construct containing both elements was designed, and transposon-based germline transformation was used to generate and test ten olive fly strains. Progeny assessment off tetracycline indicates high penetrance of the male-sterile phenotype in all strains, with only 0.0-2.4% viable progeny; this sterile phenotype appears to be completely suppressed by provision of dietary tetracycline.

Outline

Fruit flies belonging to the Tephritidae family are the pests that have the most significant economic impact on fruits and vegetables. Although Tephritidae males do not have any economic impact, the female flies damage fruit of their host species by piercing them with their ovipositor where females deposit their eggs. Larvae feed on the flesh of fruits and vegetables hence further damage their hosts.

Olive fly (Olive fruit fly; *Bactrocera oleae* (Gmelin)) and medfly (Mediterranean fruit fly; *Ceratitis capitata* (Wiedemann)) are at the centre of this study as both have a major economic impact in the Mediterranean region. Although different methods of pest management have been developed to control the population of these pests, their control mostly relies on chemical pesticide. In this thesis, I study the development of a new technology which is based on a Sterile Insect Technology (SIT) methodology to replace the use of chemical pesticides. SIT is based on mass rearing of targeted species, their steriliation by irradiation and their mass release. Sterile males mate with their wild female counterparts but no progeny are produced, leading to a gradual decline in the insect pest population.

Radiation is destructive to insects; it impairs their fitness and mating competitiveness. Oxitec has developed a genetic variant of SIT, called RIDL (Release of Insect carrying Dominant Lethal). RIDL could potentially overcome the disadvantage of radiation while offering the benefit of genetic sexing. Genetic sexing is an additional operational improvement for controlling fruit fly populations using SIT as it eliminates the number of assortative matings happening between the co-released insects. Oxitec has developed a set of conditional female-lethal RIDL olive fly and medfly strains providing highly penetrant female-specific lethality and dominant fluorescent marking. We are aiming to improve our current strains by rendering

the released males sterile. Additional potential improvements include development of a robust genetic marker for field use and a sex marking system for a cost-effective mass rearing system.

To develop fly male sterility we have identified and tested different germline specific promoters and 'lethal' effector genes. These have been linked to the tet-off system (a binary gene regulation system based on the tetracycline-responsive transcriptional factors under which the effect of transgene will be suppressed by dietary tetracycline) and were initially tested in medfly. I looked at various testis specific promoters crossed to a variety of nuclease effectors in chapter three. A selected promoter will control the transcription of the tTAV protein, which then induces expression of an effector gene downstream of *tetO*. Expression of the effector in the male germline damages sperm and thus renders the males sterile.

topi and *β2-tubulin* were used in promoter constructs while *I-ppoI*, *3zincfinger* (Znf) nuclease and *ProtamineFokI* sequences downstream of *tetO* were used in the effector constructs. After obtaining transgenic lines from each individual construct, promoter lines were crossed to effector lines. While our results demonstrate statistical significance (60-1% viable progeny) with all promoter and effector combinations tested in this study, we decided to proceed with *β2-tubulin* and *ProtamineFokI* which in combination, reduced embryonic hatch rates to 1% of control levels.

Once the best combination of promoter and effector sequences had been identified, the promoter and effector modules were incorporated into a single construct, aiming to generate tet-repressible male sterility. These results are described in chapter 4.

I tested 5 individual insertion lines. All gave fertile females; the fertility of the males ranged from 1-10% of control levels. To test whether the expression of the construct is sex-specific (male), and tissue-specific (testis), RT-PCR and Q-PCR were performed. This revealed an

enrichment of expression in testes, but some expression also in somatic tissue. This correlates with the relatively poor viability of the adult flies in the absence of tetracycline.

To examine how the paternal effect lethality (pel) technology interacts with the female specific lethal (fsl) technology and the possible effects on the performance of a final product containing both transgenes, females of pel lines were crossed to males of fsl lines. Both pel and fsl strains are based on the tet-off system hence in the absence of dietary tetracycline both transgenes should be effective. No female progeny were produced in the absence of tetracycline whereas a normal 50:50 male to female ratio was obtained when larvae were grown on food containing tetracycline (permissive conditions). Males containing both insertions had dramatically reduced fertility, as expected.

An "all in one" construct conferring sperm marking, the pel, enhanced transformation marker, and fsl was tested in medfly. The single transgenic line showed fully penetrant female-lethality and male sterility in the absence of tet. However, more detailed examination revealed that this line did not produce sperm. A re-mating test was performed on this strain. The results showed that the proportion of re-mating in wild-type females mated with males of this strain was the same as that of wild-type females mated to wild-type males.

Once the medfly construct (containing both promoter and effector components) showed significant male sterility, we optimised the tet-off based *β2-tubulin* and *ProtamineFokI* construct and redesigned it with a new transformation marker. A mexfly (Mexican fruit fly; *Anastrepha ludens* (Loew)) muscle actin promoter sequence was used to design the new transformation marker. An mCherry fluorescence protein sequence was placed in between the Protamine and *FokI* in order to mark the sperm. This construct was tested in olive fly and these results are described in chapter 5. When raised in the absence of tet, males with this

construct had fertility of 0-2.4% of wild type levels. They were fully fertile on tet. Female fertility was not affected in any of the transgenic strains tested.

In chapter 6 the *hsp83* gene sequence from medfly was used to design an alternative testis-specific promoter construct. The transcription start site of *hsp83* in the germline is different from that in the somatic tissue. Three versions of *hsp83* promoter driving *DsRed* were designed and transgenic flies generated. Although hsp83-DsRed mRNA was detected in the male germline by RT-PCR, the males did not have red fluorescence in their germline (spermatocytes, spermatids or sperm).

Sex-specific marking in olive fly is shown in chapter 7. Here the *tra* (*transformer*; sexual determination gene) gene was used to design a construct to generate males and females expressing different fluorescent markers. The transgenic males and females were marked successfully with different fluorescence markers, and could be sorted as pupae on this basis.

PCR was used to develop a monitoring method to evaluate the mating success of transgenics flies in the field. Chapter 8 describes how we used Y chromosome and RIDL-specific primers in PCR assays to differentiate the type of sperm DNA present in recently inseminated medfly and olive fly females.

Chapter 1-Introduction

1.1. Agriculture today

Agriculture is an important industry, over seven billion people live in the world today and that number is growing. It is important to be able to supply safe, nutritious food to the world population. Use of modern technology to advance various aspects of current agricultural systems is important for this industry.

Reduction of pest populations 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).

Insecticides increase air pollution; wind carries the suspended particles of them in the air to other regions, potentially contaminating a larger area (Newton 2004). Insecticides contribute to water pollution, impacting the aquatic environment; their residues have also been detected in groundwater and rain (Gilliom et al. 2007). Another environmental impact of insecticides is soil contamination; many insecticides are persistent and can reduce the biodiversity in the soil, with negative effects on the soil quality (Kellogg et al. 2000).

Pesticides influence non-target species, and not all economically important insects are pests. Insects such as pollinators, natural enemies of pests, producers of silk, honey, etc are good examples of beneficial insects (Aktar et al. 2009). Beneficial insects have been the centre of focus for modern agricultural practices which aim to target insect pests while leaving beneficial insects unaffected.

Excessive use of insecticides can result in evolution of pest insects through genetic mutations and selection to become resistant to insecticides. After resistance has evolved the targeted pest insect populations can increase (Kakani et al. 2014).

Human health issues are also very important factors to consider, and the effect depends on the toxicity of the pesticides and the length of exposure (Carson 1962). Children are still developing and are typically more susceptible to health risks from pesticides than adults (Lorenz 2009). The health effects on humans can be from mild skin irritation, to cancer, genetic mutation, birth defects, or even death (Carson 1962). Employing biotechnology and genetic engineering in insect pest control has the capacity to advance agriculture and crop production, with potentially considerable benefits in the efficiency and cost effectiveness of the methods employed. Further this can be done in an environmentally-friendly way.

1.2. Tephritid fruit flies and their economical impacts on agriculture

The order Diptera is one of the largest orders of insects with over 6,200 species in the UK alone (Panagiotakopulu 2004). This group of insects has a huge economic and medical influence on human life as many of the species in this order are livestock and agricultural pests, or vectors of disease. Diptera also have some benefits such as pollination of plants (Keasrns 2001), use in forensics (examination of insects found around a body) (Panagiotakopulu 2004) or medical research (Gonzalez 2013).

The order of Diptera consists of two suborders; the Brachycera which have thick bodies with short stylate antennae and the Nematocera which have long and thin bodies with multi-segmented antennae (Panagiotakopulu 2004). The families Tephritidae and Drosophilidae belong to the Brachycera suborder. All Dipteran insects have a holometabolous life cycle which comprises an egg, larva, pupa and adult, with complete metamorphosis. The duration of each life stage varies within and between species depending on species-specific factors, and environmental factors including temperature.

The Tephritidae family consists of four genera; Ceratitis, Anastrepha, Bactrocera and Rhagoletis and consists of approximately 450 species (Holt et al. 2002), many of them are significant agricultural pests. From the genus Ceratitis, 10 species are considered to be economically significant agricultural pests, however one of the species of this genus is classified to be the most destructive agricultural pest in the world; *Ceratitis capitata*. *Ceratitis capitata*, the Mediterranean fruit fly (medfly) is the most recorded damaging fruit fly pest, and it is the key target for many pest control programs (Diamantidis et al. 2008). Medfly originated in Equatorial Africa and has spread to tropical and temperate regions all around the world (Bonizzoni et al. 2002; Malacrida et al. 2007). Another economically important pest species of this genus is *Ceratitis rosa*, the Natal fruit fly which is native to

many African countries and found predominantly in southern and eastern Africa (Virgilio et al. 2013). *Ceratitis savastani* (the caper fruit fly), native to Afro-tropical regions, is now widespread throughout the Mediterranean region and has also been found in Pakistan (Mazzon & Martinez-sañudo 2014).

The *Bactrocera* genus has around 40 species that are classified to be significant agricultural pests (White and Elson-Harris 1992). The most damaging ones are *Bactrocera cucurbidae* (the melon fruit fly), *Bactrocera dorsalis* (the Oriental fruit fly), *Bactrocera oleae* (the olive fruit fly), *Bactrocera tryoni* (the Queensland fruit fly), and *Bactrocera zonata* (the peach fruit fly). The melon fruit fly is native to the most of southern Asia and it can be found throughout several countries in Africa, and in some Pacific island groups (Dhillon et al. 2005). The Oriental fruit fly occurs in the northern part of Asia (Drew & Hancock 1994; EPO). The olive fruit fly is native throughout the Mediterranean and South Africa, and established in California in the 1990s (Economopoulos 2002). The Queensland fruit fly is native to Australia (White & Elson-Harris 1992). The peach fruit fly is native to India and is present in numerous tropical countries of Asia. This pest also established in Egypt in the late 1990s and is now distributed throughout that country (Delrio & Cocco 2010).

The *Anastrepha* genus contains 15 species that are considered economically significant pests (White & Elson-Harris 1992). They are native to tropical and subtropical America (Aluja 1994). The important ones are *Anastrepha fraterculus* (the South American fruit fly), *Anastrepha ludens* (the Mexican fruit fly), *Anastrepha obliqua* (the West Indian fruit fly) and *Anastrepha suspensa* (the Caribbean fruit fly).

The *Rhagoletis* genus of Tephritidae comprises a small number of economically significant fruit fly pests such as *Rhagoletis pomonella* (the apple maggot), *Rhagoletis indifferens* (the cherry fruit fly) and *Rhagoletis mendax* (the blueberry maggot) (Foote et al. 1993).

Although size varies between different Tephritids, they all have similar morphology during the larval and pupal stages (Headrick and Goeden 1998). Fruit flies cause damage to crops when a) an adult female penetrates the flesh of the fruit with her ovipositor as she lays her embryos, thus causing 'sting damage' on the surface of fruit often resulting in secondary infestations by neighbouring fungi and bacteria; b) the hatching larvae tunnel and feed in the fruit mesocarp resulting in extensive fruit damage and premature drop of the fruit. Depending on species, sexually mature adult female flies can lay up to eight hundred eggs during their reproductive life time; even in the absence of mating. Moreover, female fruit flies deposit pheromones on the fruit surface as they oviposit their embryos, deterring other females from laying in the same fruit (Stoffolano 1987). This behavioural investment in the prospective progeny results in extensive crop damage. In most Tephritids, larvae feed inside the fruit from 1st instar through to the final instar (the 3rdinstar). When they are ready to pupate, they emerge from the fruit and drop into the soil.

1.2.1. Mediterranean fruit fly

Ceratitis capitata commonly known as the Mediterranean fruit fly (medfly) is the most economically significant pest of all Tephritids (Diamantidis et al. 2011) (Figure 1.1). Medfly is native to Equatorial Africa, but, as it can easily adapt to different environmental conditions it has spread to tropical and temperate regions across the world such as Australia, the Mediterranean and North and South America (Bonizzoni et al. 2002; Malacrida et al. 2007). Microsatellite analysis in wild medfly from different geographical locations demonstrated that current medfly populations originated from a single ancestral population from eastern and southern Africa. Differences between various geographical populations are well-matched with a structure of migration to different regions (Bohonak et al. 2001). The new world medfly has genetically diverged from the ancient medfly population, in this case establishment of medfly from an old habitat into a new region, has led to a new genetically diverse populations. For instance the population found in most of California is genetically diverse from that in the Los Angeles area, as they established from different populations, via at least two immigration events (Gasperi et al. 2002).

Medfly's adaptive behaviour both in terms of adult fly tolerance to temperature and humidity, as well as its ability to oviposit in a wide range of host plant species, render this pest as one of the most economically important insect pest species worldwide; the Mediterranean fruit fly can infest over 260 host plants including citrus fruits, stone fruits, deciduous fruits and some vegetables (Malacrida et al. 2007). Adult medfly feed on nectar or fruit juice for sugars and bird droppings for protein (Manrakhan & Lux 2007). Medfly is readily reared in laboratory conditions, with larvae fed on standard Drosophila diet, and has a circa 25-day life cycle under optimum temperature (25±1 ºC) (Figure 1.1). In the wild, medfly can live for up to two

or three months depending on temperature, where they over-winter, they can survive as adults, eggs and larvae (in fruit), or as pupae in the ground.

Medfly adults are present all through the year in areas with mild winter and mild summer as ripe fruit is available whole year around (Maria 2010). In regions with extreme weather conditions, a small percentage of individuals will survive each winter and reproduce the whole population from them in spring and early summer. This imposes a strong selection pressure towards the evolution of insects that withstand the cold (Papadopoulos et al. 2001).

Medfly larvae are damaging crops by feeding on the flesh of the fruit or vegetable so they extensively injure them. As well as the economic expenses caused by loss of production of crops as a result of the damage by larvae, other expenses include efforts for population control of medfly, protection of crops and also limited exportation to medfly free zones (due to quarantine regulations) (Diamantidis et al. 2011). Medfly can use over 260 plants as host which causes considerable damage to many varieties of economically significant crop plants (Zeki et al. 2008).

Medfly has 5 pairs of autosomal chromosomes and a pair of sex chromosome (XX: female, XY: male). The genome of medfly has been sequenced (www.hgsc.bcm.edu/ftp-archive/I5K-pilot/Mediterranean_fruit_fly/) and is currently under characterisation.

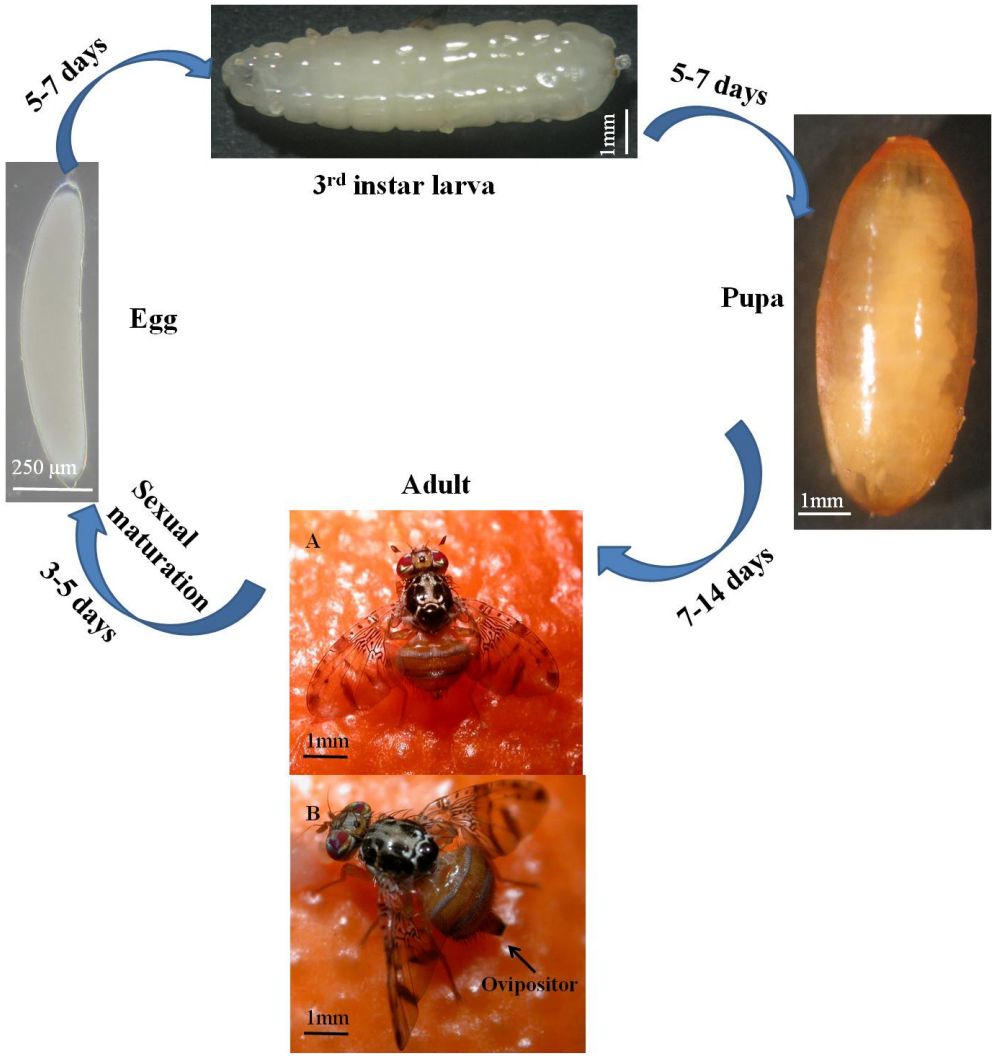


Figure 1. 1 The life cycle of medfly; 20-25 days, in optimum temperature (25±1 ºC).

A: Male medfly; B: Female medfly, arrow indicates the female oviposition structure –characteristic of all fruit flies; the ovipositor. The medfly egg is 1 mm long, curved, smooth and shiny white. A medfly embryo would take up to three days to hatch in optimum temperature. There are three larval instars in medfly. Larvae are active feeders in all stages. The third instar larva is between seven to nine millimetres in length, with eight ventral fusiform areas. The 3 larval stages last from five to seven days at optimal conditions (Ricalde et al. 2012). Larvae feed on fruit and vegetable, causing fruit damage. After two moulting periods, the 3rd instar larva which is highly active will jump out of fruit into the ground to pupate in the soil. Metamorphosis, which involves a dramatic remodelling from the larval structure to make the adult, starts at this point. Medfly pupae are cylindrical, four to five millimetres long, with a dark reddish-brown hue. The pupal duration is one to two weeks depending on temperature. The adult fly is three and a half to five millimetres long. It is yellowish with a brown tinge, especially on the abdomen, with black and white thorax. Photographs A and B obtained from Kirsty Stainton.

1.2.1.1. Medfly mating behaviour

Medfly males and females sexually mature within two to three days after emergence (in laboratory conditions; 25 degrees Celsius) and 8-10 days in the wild. In the wild, mating occurs under the surface of sunlit leaves during early morning and lasts nearly three hours. Males initiate mating in medfly by forming a lek, which is a gathering of three to six males in an attempt to compete for mating (Bateson et al. 2001). Medfly male calling behaviour provides potential visual, acoustic and pheromonal cues to females, and starts by them curling their abdomen upward and emitting pheromones followed by rapid wing-fanning on arrival of females to the site. Females will sample several males within the lek before choosing a mate. The mating selection behaviour of female exerts competition between males, thus males must be sexually competitive to have a successful courtship and mating. This behaviour often will cause a small number of males to achieve the majority of successful mating, and most males will remain unmated (Robinson et al. 2002).

Female medfly have a complex system for sperm storage, with two spermathecae (Figure 1.2 A&B) for long-term storage and a fertilization chamber for storage of sperm prior to use in egg fertilization (Figure 1. 3A) (Twig & Yuval 2005). Sperm survive for up to eighteen days after copulation in both storage organs (Perez-Staples & Aluja 2006). The male medfly intermittent organ possesses three ejaculatory openings, one for each of the female's storage compartments. As a result, sperm is simultaneously fed into both the spermathecae and the fertilization chamber (Twig & Yuval 2005). The complicated sperm storage system and multiple mating (if the mating was incomplete) in female medflies, render them capable of adapting key traits associated with sperm use (Shelly & Whittier 1994).

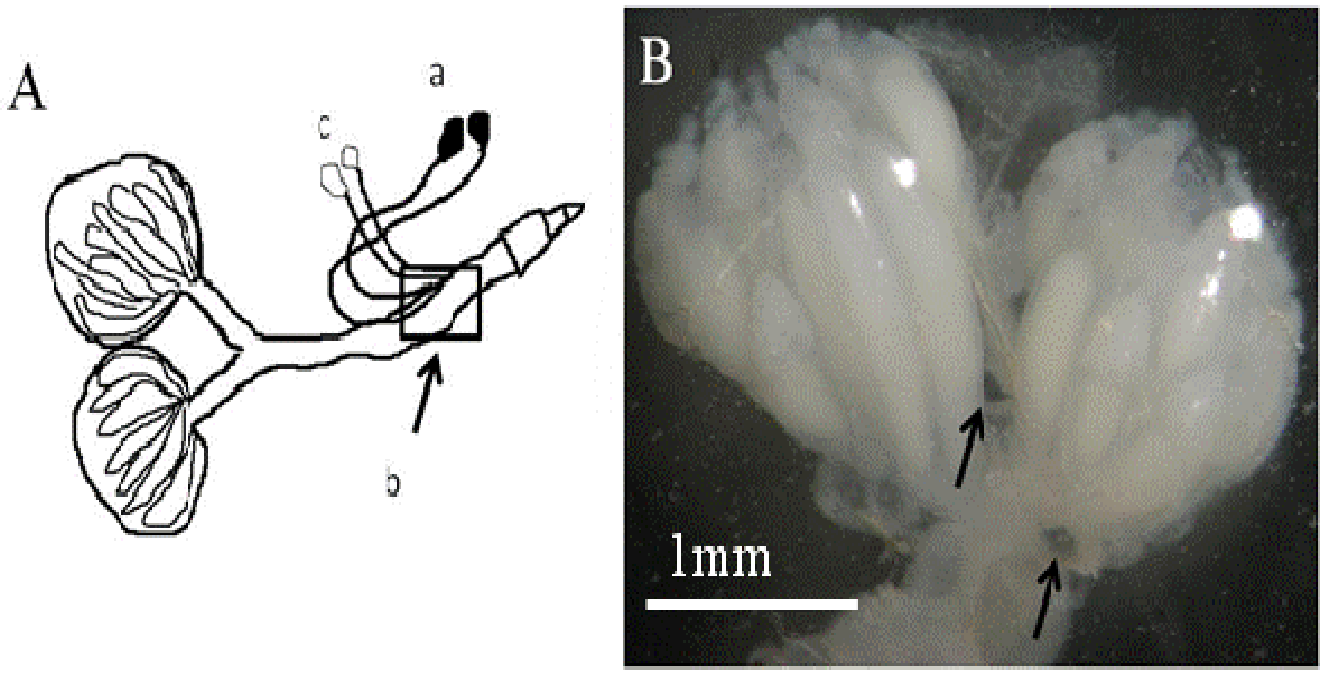


Figure 1. 2 The reproductive apparatus of the medfly female.

A: Schematic diagram of the insect ovaries. a: spermathecae; b: fertilisation chamber; c: accessory gland. B: Micrograph of medfly ovaries, arrows indicate spermathecae.

1.2.2. Olive fruit fly

Bactrocera oleae known as olive fruit fly (olive fly) is a monophagous species and the major insect pest of olives (Tsiropoulos 1977; Tsiropoulos 1980) (Figure 1.3). Olive fly females oviposit a single egg on each olive fruit. Hatching larvae feed on the fruit of olive trees causing direct damage and reducing both crop yield and quality. As in other fruit flies, indirect damage occurs through secondary infestations in the "oviposition opening", which causes black spots on the olives and makes them unmarketable. Premature drop of the crop and high acidity of olive oil are other factors that are detrimentally affected by the presence of olive fly. The olive fly originated in Africa, established throughout the Mediterranean, Middle East and South Africa, and has been found in California since the late 1990s (Economopoulos 2002). In the United States, the first olive fly was captured in Los Angles on 1998 and had spread all through olive cultivation in California by 1999.

An important feature of olive fly is that females lay one egg per olive fruit (Gutierrez et al. 2009). Considering that a single female lives up to 25 days in temperate climatic conditions, and can lay 200-500 eggs during her adult life time (Daane et al. 2005), one understands the economic impact of this pest to olive growers around the globe.

Olive is a seasonal fruit whose development starts in spring and whose maturation occurs in summer, with harvest in autumn. Successive infestation will be from the start of summer till the early days of winter (Economopoulos 2002). In winter with mild temperature and rather cool summer, the development of the population of olive fly starts in spring and continues for few generation till the beginning of autumn (Gutierrez et al. 2009). The duration of the life cycle is directly related to environmental conditions, specifically the climate and the state of the olives. In mild winters and relatively cool summers, the olive fly populations increase. The olive fly has two to three generations per year in cooler regions and four to five

generations per year in warmer climates (Al-Zaghal & Mustafa 1986).The olive fly's life cycle is similar to that of medfly (see section 1.2) (Figure 1.3).

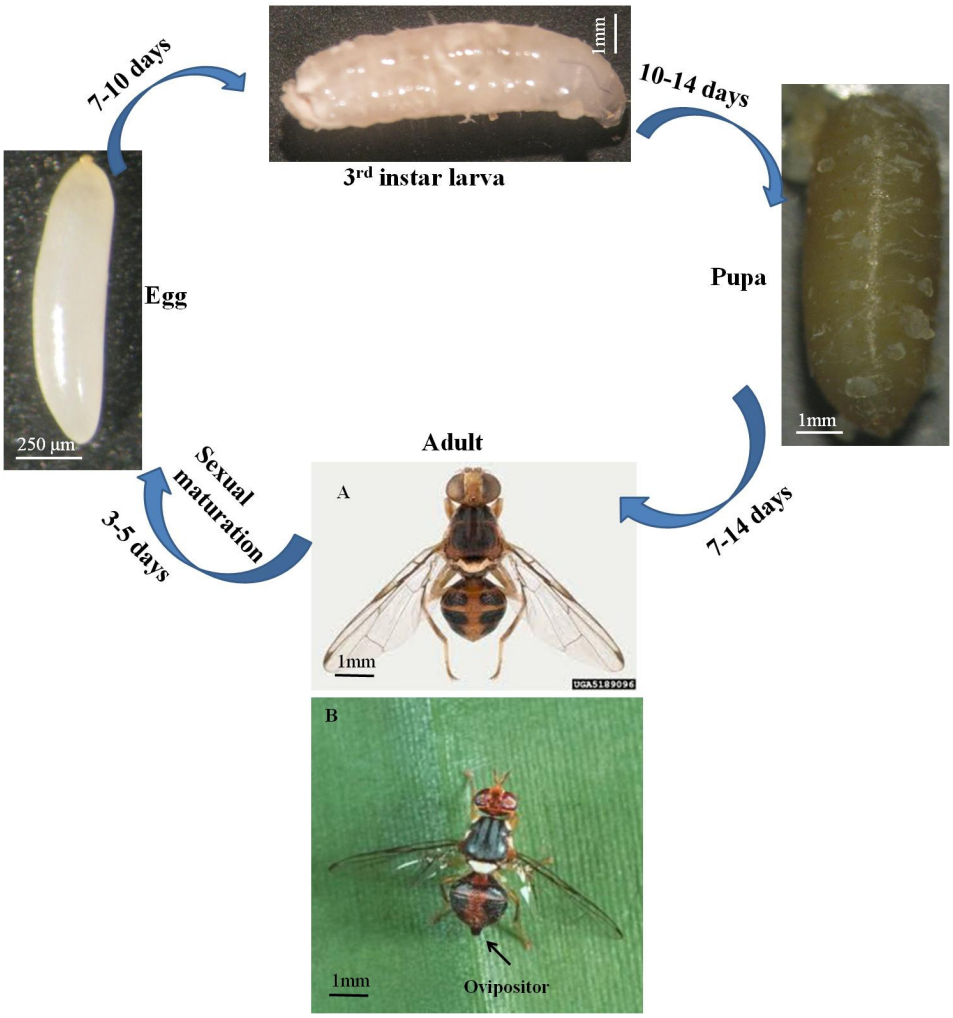


Figure 1. 3 The life cycle of olive fly (up to 30 days).

A: The olive fly male. B: The olive fly female with an ovipositor. The egg is around 0.8-1.2 mm long, elongated and slightly flattened, with a small and white microfleece nodule on the anterior end which is important for the respiration of the embryo. Larvae feed on ripening olive fruit till its last stage. There are three instars; and the larva will eventually be six to seven millimetres long and it is morphologically similar to the larva of the medfly. Third instar larvae crawl out of the olives and pupate on the ground. Upon exit from the olive fruit, larvae become shorter, broader and non-motile. Pupae are 3.5 to 4.5 millimetres long, varying in colour from creamy white to yellow-brown, when environmental conditions are dry. The change in the colour can also indicate the age of the pupae (Tzanakakis 2003). The adult olive fly is four to five millimetres long with a brown head, the female has two circular spots under the antennae close to the eyes (A & B). It has a light-brown abdomen with a pair of black spots. The female olive fly has a distinct ovipositor which is always black (B). The duration of egg to sexually adult development, is about 35 days at 25ºC (Sime et al. 2006). Photograph A by Natasha Wright, Florida Department of Agriculture and Consumer Services. Photograph B obtained from Rafael Estevez Rodriguez (Oxitec).

Very limited information is available on genetics of the olive fly. The olive fly has 5 pairs of autosomal chromosomes and a pair of sex chromosome (XX: female, XY: male). Considerable genetic variation between wild and laboratory populations has been found and this variation seems to be greater than that caused by different geographical locations (Tsakas & Krimbas 1975; Zygouridis et al. 2014). Selection for survival and reproduction on in animals reared on the artificial larval medium appears to have skewed the population genetics in the laboratory population (Yokoyama et al. 2006). In addition, temperature and light (which is stable at laboratory conditions) could also contribute to the observed variation between laboratory and wild olive fly populations.

Genetic resistance to different types of insecticides has been detected among wild populations of olive fly. Repetitive use of the same pesticide compounds that have been traditionally employed for the control of this pest have led to selection of the resistance alleles (Kakani& Mathiopoulos 2008). Laboratory research on olive fly showed that they have potential to evolve resistance to dimethoate (Tsakas & Krimbas 1975), organophosphates (Skouras et al. 2007), pyrethroids (Kakani et al. 2008) and spinosad (Kakani et al. 2014). The development of insecticide resistance in olive fly increases the risk of a new olive fly invasion to areas that olive fly has not been detected yet (Gutierrez et al. 2009).

1.2.2.1. Olive fruit fly mating behaviour

Male and female olive flies are sexually mature two to three days after emergence from the pupal stage (in the laboratory conditions) and 8-10 days in the wild. The virgin females start producing sex pheromone three days after emergence, which attracts males. The cycle peak of sex pheromone production in females occurs at ten-day intervals, and each peak of production lasts two to three days. Although females produce pheromones throughout their life, as the female ages the quantity of the sex pheromone decreases. This quality reduction is also present in mated females for the first ten days after mating. With a single successful mating the male olive fly transfers sufficient sperm into the female's spermathecae to fertilize all the eggs she will lay (Tzanakakis 1967). The sperm storage organ in the female olive fly is similar to that of the medfly with the presence of two spermathecae for long-term storage and a fertilization chamber for storing sperm prior to use in egg fertilization soon after mating (Twig & Yuval 2005) (Figure 1.4).

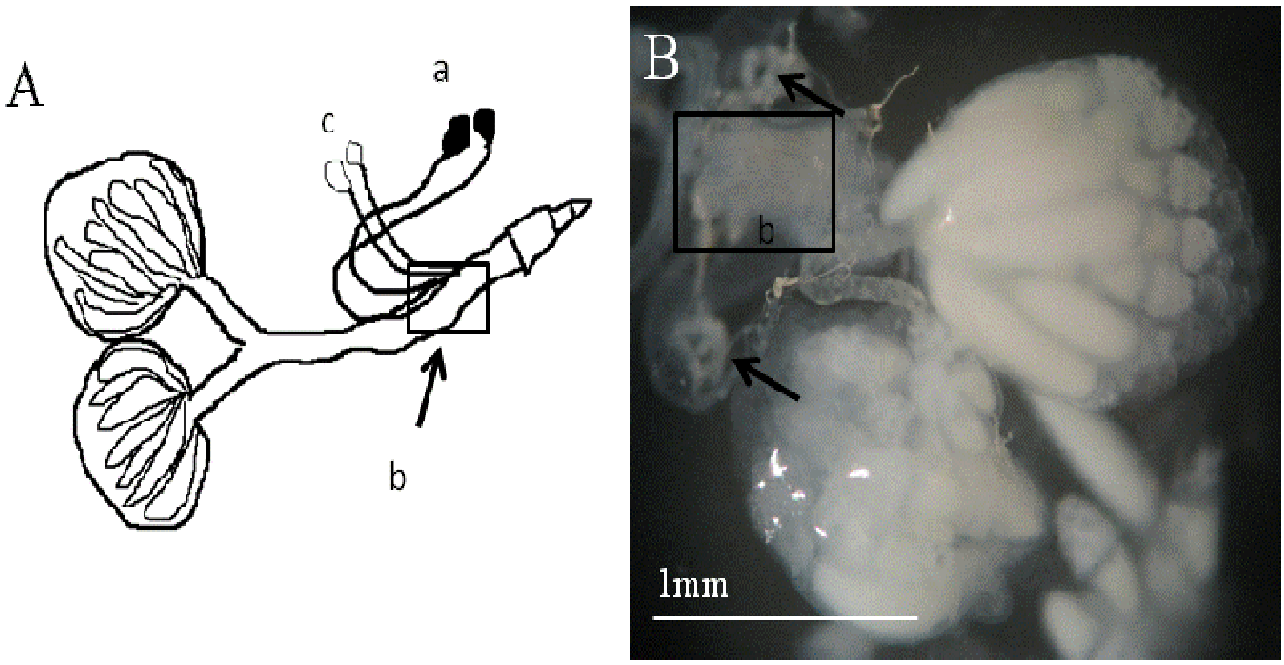


Figure 1. 4 The reproductive apparatus of the olive fly female.

A: Schematic diagram of the insect's ovaries. a: spermathecae; b: fertilisation chamber; c: accessory gland.
B: Micrograph of olive fly ovaries; b: fertilisation chamber; arrows indicate spermathecae.

1.3. Insect pest control of Tephritid fruit flies (medfly and olive fly)

1.3.1. Chemical control

Chemical agents are used frequently to control medfly and olive fly populations. Extended research over many years has been carried out to develop protocols to replace these chemicals with more effective and environmentally friendly methods. Chemical pest control dates back 4,500 years, to the birth of agriculture (Chauhan 2012). Synthetic chemical pesticides became popular after World War II when the manufacturing of hundreds of new synthetic compounds, mainly organophosphates, made their broad usage possible (Chauhan 2012). Extensive use of pesticide over the years has driven the evolution of pesticide resistance in many species. In addition, when chemicals are applied to a region, they can also influence non-target species (Aktar et al. 2009).

In recent years, organophosphate compounds have been the most common insecticides used against the medfly and the olive fly, mostly in the form of bait spraying. The most widely used are dimethoate, fenthion, formothion, malathion and phosphamidon (Navarro-Llopis et al. 2013). These compounds have the ability to penetrate the fruit mesocarp, as a result the deposited larvae and eggs will die. However a pesticide residue remains on the fruit.

Malathion inhibits cholinesterase which is an enzyme required for the breakdown of the acetyl-choline; a neurotransmitter (Walker et al. 1997). The absence of this enzyme leads to an accumulation of acetyl-choline in the nervous system causing muscle contractions and finally death in insects. In humans, low levels of exposure cause symptoms including nausea, eye irritation, dizziness (Gupta et al. 1980); high-level exposure can cause death. The aerial application of malathion is required in order to control medfly as they have good dispersal capabilities (Bergsten et al. 1999). Aerial treatment will suppress the population of adult

medfly by over 90% (Bergsten et al. 1999) so to achieve closer to 100% suppression; multiple treatments are obligatory.

A substitute for malathion in medfly and olive fly population management is spinosad, which is a combination of two metabolites isolated from the soil bacterium, *Saccharopolyspora spinosa* (Fussenegger et al. 2000). Spinosad affects the insects through their digestive system and hence it is used in traps with hydrolysed protein to attract medfly (Fussenegger et al. 2000). The interaction of spinosad with acetyl-choline receptors causes the excitation of the nervous system and finally paralyses the insect. Although it has low toxicity in mammals and birds and its toxicity profile is better than malathion, it is moderately toxic to aquatic life (both invertebrate and vertebrate) and extremely toxic to honeybees (Fussenegger et al. 2000).

1.3.2. Biological control

Chemical pesticides may leave residues on fruit and also have adverse effects in the environment and ecosystems of targeted areas, therefore a number of alternative control methods to insecticides have been studied and tested. These methods include the use of parasitoids, fungi, *B. thuringiensis*is, pheromones, food and visual lures, mass trapping with or without pheromones, and transgenic crops.

Parasitoids reduce the host population by a variety of methods; some are predators of larvae while others attach to or within a host organism and in due course sterilise, kill, and sometimes consume, the host (Rousse et al. 2005). Introduced parasitoids to control the targeted insect are typically not native to that habitat therefore their effect on an ecosystem is unpredictable. An additional setback in using the biological approach is the fact that some of these organisms have become pests themselves (Rousse et al. 2005).

Braconid parasitic wasps have been investigated as biological control agents for Tephritids such as medfly and olive fly (Bautista et al. 1999). Some examples are the *Fopius arisanus* (Sonan) females that lay their eggs into host embryos, *Diachasmimorpha longicaudata* (Ashmead) and *Opius concolor* (Szepl) which attack second and third instar larvae of the host. All three species have many different Tephritid hosts (Rousse et al. 2005)**.** In all cases the parasitoid grows inside the host larva waiting for pupation of the host, at which time it emerges from the host body. During this emergence, the host individual will die (Rousse et al. 2005; Bautista et al. 1999).

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; their effectiveness is under investigation for olive fly (Sime et al. 2007). Due to an overlap in pupal sizes between these parasitoids and the medfly or the olive fly's pupae, the parasitised pupae are often discarded (in mass rearing facilities) hence their rearing and sorting for release still needs to be optimised (Bautista et al. 1999). Further, although parasitoids can suppress small populations of medfly or olive fly, they are in effective on large infestations especially in regards to olive fly which is a seasonal insect and builds large populations in the autumn months prior to harvest (Yokoyama & Rendo 2008).

Several species of entomopathogenic fungi are used as biological control agents for medfly or olive fly. 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). The studies showed the potential of fungi species in controlling the fruit fly population is feasible when applied to the base of trees (Maria 2010). One common method of applying these species is spraying them on plants or onto the soil where infesting pests are present. Another method is to set up a bait station to which adult flies will be attracted and thus infected. The benefit of the bait station over spraying is that it spreads infection among flies via escaped flies which stay alive for a few days. In addition, these fungi are found not to be pathogenic to humans as confirmed by the Environmental Protection Agency (USA). They mainly affect insects and they are not known to affect birds, mammals or plants but can affect honeybees (EPA 2001).

*B. thuringiensis*is (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 insects families such as Lepidoptera, Diptera, Coleptera, 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).

Recent research has demonstrated that there are a few negative issues regarding the use of Bt insecticides, mainly due to resistance. Bt has been used intensively to control pink bollworm, as a result this insect has become resistant to Bt cotton (Bagla 2010). Another issue could be the persistence of Bt; some stains of viable Bt may remain inactive in laboratories or nature for years (Van Cuyk et al. 2011) although this needs to be confirmed in field conditions.

1.3.3. Mass trapping and attractant

A recent and innovative method in controlling fruit fly populations is mass trapping. In some species mass trapping can target specifically either the male or female population of fruit flies. The traps that are used for male mass trapping are pheromone-based while the traps for female mass trapping are based on protein bait (natural protein baits such as Torula Yeast) (Heath et al. 2007)

The mass trapping strategy is a more environmentally friendly approach than broad spectrum insecticides as it targets mostly insects of interest (IAEA 1999). Mass trapping can be quite expensive however, particularly in targeting large fruit fly populations (Leza et al. 2008). Due to cost limitations, mass trapping works best as part of an integrated pest management approach including SIT (Heath et al. 2007).

Trap attractant baits usually include food, visual lures, chemical attractants and pheromones (Heath et al. 1995). The attractant which is used for medfly contains three synthetic food attractants: Ammonium Acetate, Putrescine and Trimethylamine (IAEA 1999). These traps are being widely used in areas that have problem with medfly. This kind of trapping which is based on female can detect immigrant, or increasing, medfly populations early therefore it can improve the efficiency of an IPM programme. Another advantage of female trapping in IPM programme is the beneficial insects won't be trapped (Heath et al. 2007).

1.3.4. The Sterile Insect Technique

The Sterile Insect Technique (SIT) is an alternative pest management method which uses biological techniques to eliminate or suppress pest insects without using pesticides (Klassen & Curtis 2005). SIT can also be used to prevent the current populations of pest insects from infesting a new area. In the 1950s, SIT was introduced by Raymond Bushland and Edward Knipling in an attempt to eradicate the livestock pest New World Screwworm in the USA (Knipling 1985). 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 (Figure 1.5). Any mating between wild females and sterile males will result in females producing non-viable eggs, thus the targeted insect populations will be suppressed (Dyck et al. 2005).

New World Screwworm was eliminated from the USA, Mexico, Central America and Panama using SIT (Del Valle 2003; Knipling 1985). SIT has also been applied effectively to control other pests such as tsetse fly (*Glossina* sp.), a vector of trypanosomiasis in Zanzibar (Feldmann et al. 2005). It has been also applied in Canada and western USA to control population of Codling moth, *Cydia pomonella* (Linnaeus) which is pest of apples and pears (Bloem et al. 2005). An SIT program is used to prevent the population of pink bollworm, *Pectinophora gossypiella* (Saunders) from spreading in within the USA (currently there is a stabilised population in Arizona) (Bloem et al. 2005). Although SIT can be effective when sterile adults of both sexes are released, it becomes more successful in many species of insects if only sterile males are released. One study, undertaken in south-western Guatemala, found that a male-only release of medfly gave three to five-fold higher efficiency compared to mixed sex releases (Rendón et al. 2004). The lower efficiency of mixed sex release might be caused by the presence of sterile females; sterile males court these sterile females, rather

than dispersing and mating with the wild population. Finally, release of females can be directly detrimental as it is the females (even sterile ones) that cause damage to various crops (Rendón et al. 2004) either by ovipositing in fruit.

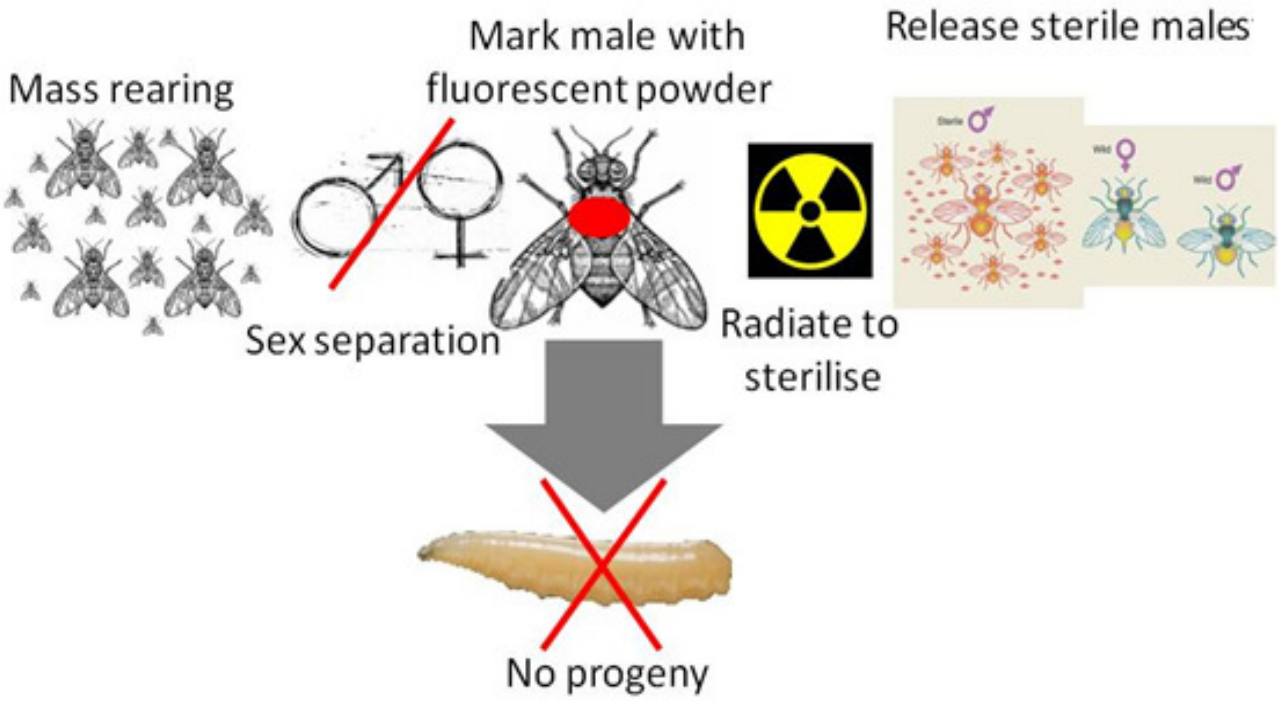


Figure 1. 5 Sterile insect technique (SIT) stages.

SIT relies on the mass production and release of sterile insects. Where sex separation is possible, only sterile males are released. Traditionally, sterility is induced by-rays or gamma irradiation. Sterilised males are marked with a fluorescent powder for monitoring purposes. When a sterile male mates with a wild female no progeny survive, leading to population suppression.

1.4.The Medfly and Olive fly control using SIT

1.4.1. SIT Success stories

Eradication successes using irradiated bi-sex releases include the New World screwworm, *Cochliomyia Hominivorax*, from North and Central America (reviewed in Klassen and Curtis, 2005), and from Libya (FAO 1992; Lindquist et al. 1992); the tsetse fly, *Glossina austeni*, from Unguja Island in Zanzibar, Tanzania (Vreysen et al. 2000); the melon fly, *Bactrocera cucurbitae*, from Japan (Koyama et al. 2004; Kuba et al. 1996); the Mexican fruit fly, *Anastrepha ludens,* from north-western Mexico (Reyes et al. 2000); and the Queensland fruit fly, *Bactrocera tryoni*, from western Australia (Sproule et al. 1992). SIT has prevented establishment of the Mediterranean fruit fly (medfly, *Ceratitis capitata*) in California and Florida, USA (Barry et al. 2004; Dowell et al. 2000), Mexico (Hendrichs et al. 1983) and Chile (Esparza Duque 1999; Gonzalez and Troncoso 2007). Similarly, regular sterile releases of pink bollworm (*Pectinophora gossypiella*) inhibited the long-term establishment of this pest in the San Joaquin Valley, California (Staten et al. 1999) and now provide a major component of an eradication programme (Grefenstette et al. 2009).

1.4.2. Medfly SIT

Medfly is a major pest of several key crops worldwide causing significant damage to crops and vegetables. It is a major quarantine insect for Japan and the USA. Due to its adaptive nature medfly has spread throughout the subtropical, tropical, Mediterranean, and temperate regions. Because of this, medfly is the most-studied fruit fly pest today, both at the genetic and molecular level. It is also the primary target for many pest control programmes (chemical and biological) (Mumford & Enkerlin 2001; Malacrida et al. 2007). Medfly infestation has been detected in the southern parts of the USA, particularly California (from 1975 to 2008) and Florida (from 1929 to 2010) (Bergsten et al. 1999; Diamantidis et al. 2011).

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 & 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. SIT in Australia was introduced to stop existing populations of medfly in Western Australia infesting southern Australia. A medfly SIT programme was set up in Los Angeles in 1994 leading to great reductions in medfly infestations (Papadopoulos 2008). Effective medfly programmes have allowed for the increase of agricultural exports, for instance earnings from Mexican agriculture exports between 1994 and 2004 tripled (Klassen & Curtis 2005).

1.4.3. Olive fly SIT

The population management of olive fly relies on chemical insecticides which have high economic and environmental costs and supply the selection pressure to allow resistance to become established into a population (Başkurt et al. 2011). None of the alternatives to insecticides such as parasitoids, pheromones, food-lure and visual lures, and mass trapping, have proved sufficiently effective to be used in a control programme. Organophosphorous insecticides are most frequently used (Başkurt et al. 2011). *Bactrocera oleae* was among the first insects to be considered for a sterile insect technique programme following the success of the screwworm programmes. Despite decades of research using irradiated flies, these sterile flies performed poorly (Economopoulos 2002). This was primarily due to altered diurnal mating rhythms between the released irradiated insect and the wild insects leading to mating isolation between the two populations (Economopoulos 2002). The irradiated males and females mated among themselves instead of mating with the wild population, leading to suppression failure after a three-year pilot study in Greece. The other limitations of developing SIT for olive fly were low quality of the irradiated flies, inability to produce adequate numbers of sterilised flies at a reasonable cost (mass production of olive fly), low egg production rates and lack of methods to separate the sexes to ensure only males are released (Ant et al. 2011). Male-only release of olive fly will partially overcome the problem of preferred mating times between the irradiated and the wild population, since the release males will not have any co-released females with which to mate.

1.4.4. Irradiation as a method of sterilisation

The traditional method of sterilising insects in SIT is exposure of late pupae to a source of ionising radiation. One of the disadvantages of using irradiation in SIT is the fitness cost that it confers on the released male cohort. Irradiated males have reduced mating competitiveness, reduced capability for successful sperm transfer and a shorter lifespan (Barry et al. 2003). All these factors result in a 4- to 10-fold decrease in fitness of irradiated males compared to wild males (Alphey 2002); it is an inevitable cost of the sterilisation processes. The adverse effects on the insect increase with increasing radiation doses. In irradiating flies, it is important to keep a balance between sterilising the released flies and the costs in terms of insect fitness.

1.4.5. Drawbacks

Radiation to a level sufficient to cause full sterility is not always compatible with sufficient viability and vitality for release programmes therefore partially sterile insects have been considered for SIT. In Drosophila, it was shown that radiation-induced mutations included dominant lethal alleles which could be transmitted to offspring. This caused partial sterility in irradiated Drosophila (Blaylock & Shugart 1972).

The level of sterility is an important factor prior to any SIT release. High level of sterility is compatible with a low release ratio for population suppression, while if the level of sterility is lower the release ratio will need to be higher (Klassen & Creech 1971). The degree of sterility of partially sterile insects depends on the dose of radiation which varies between species. Diptera, Hymenoptera, and Coleoptera orders are classed as radiation-sensitive, while Lepidoptera, Homoptera and Acari orders are classed as radiation-resistant. The difference in molecular repair mechanisms of these orders are responsible for the high radio-resistance in Lepidoptera (LaChance & Graham 1984).

The first mathematical modelling of partial sterility has been evaluated by Knipling on Lepidoptera (1970). Partially sterile Lepidoptera have the ability to transmit dominant lethal genes to offspring so that -at least theoretically-higher suppression levels can be achieved. Lepidopteran males treated at radiation level of 250Gy, were over 90% sterile. The same dose resulted in 100% sterility of their F1 offspring. The study suggested that the use of partially sterile insects, increases the over flooding ratio of the sterile-to-wild by ¼ of what is generally required for fully sterile insects (Knipling 1970). Sterility of several other pest insects was studied using mathematical models which demonstrated very similar results to Knipling's findings (Bloem & Carpenter 2001). A field study of partially sterile lepidopteran further supported the feasibility of this concept (Bloem & Carpenter 2001).

Phuc et al. 2007 studied the penetrance and effectiveness of few RIDL strains in *A. aegypti*, for how much lethality needed to get good population suppression with the late acting lethal. These strains of A. *aegypti* at best gave only 95–97% penetrant lethality. The initial modelling was based on assumption of 100% penetrant lethality, however the modelling was extended to investigate the effect of incomplete penetrance. The modelling proved that moderate levels of non-lethality for example up to 8%, would have little undesirable effect. The levels of non-sterility were also investigated on the effect of incomplete sterility in a conventional SIT program (Barclay 2001), which showed similar results. The comparison of both modelling on both RIDL penetrance and sterility on irradiated *A. aegypti* showed consistent results. After modelling showed that it didn't matter that the RIDL strain in *A. aegypti* wasn't fully penetrant and the adverse effect would be little, the best strain was selected and was being used in the various trials in Malaysia (Lacroix et al. 2012), Cayman (Harris et al. 2011) and Brazil (Thompson 2013). These trials proved that suppression was achievable with this strain.

1.4.6. Sexing for male only release

A sexing mechanism is an advantage for an SIT insect-pest management programme; single-sex releases mean that the males seek wild female mates more eagerly in the absence of sterile females (Rendón et al. 2004; Morrison et al. 2010). Sexing also makes it possible to remove the sex that will damage the crop (female cohort) making it more acceptable to the farmers, and reduces the cost of a programme by not rearing and releasing the unwanted females.

Classic genetic sexing strains (here-after referred to as GSS) for medfly are based on a selectable recessive marker that is used for sorting or killing. A translocation on Y chromosome which carries a dominant wild-type allele of this marker. The combination of these two factors means that females express the recessive trait, while males do not, and this creates a mechanism that makes the males and females to be different from each other hence they can be automatically sorted on a large scale.

The first GSS was based on the white pupae mutation (*wp*, located on chromosome 5) (Robinson 2002). In large scale mass-rearing of this strain, significant levels of genetic instability were observed on *wp* females and it was difficult to be distinguished from males. In medfly SIT, a male-only release was achieved by using a GSS (TSL: Temperature-sensitive lethal mutation strain of medfly) (Franz et al. 1996). TSL is a recessive mutation based on a naturally occuring mutation that is used as a selectable marker for separation of the sexes and Y-autosome translocations to link the inheritance of the wild-type allele to sex (Willhoeft & Franz 1996; Morrison et al. 2010). In this strain, when a higher temperature (34°C) is applied during the embryonic stage the females die because they are homozygous for a recessive temperature-sensitive lethal mutation, while males survive because they have a wild type copy attached to the Y-chromosome by a (Y:5) translocation. The *temperature*

sensitive lethal gene (*tsl*) is located on chromosome 5 (Franz 2002).

In a comparison between male only release and bisexual sterile medfly release, a male-only release resulted in improvement in the success of trials and gave a 3-7 fold performance improvement of the irradiated medflies (Rendón et al. 2004).

1.5.Genetic enhancements to SIT

To improve the efficiency of SIT (significant operational and cost-effectiveness improvements), classical genetics or genetic modification through transgenesis have been employed (Morrison et al. 2010). Classical genetics was used to develop a method to automatically separate sexes (see section 1.4.3. for more details). Furthermore there is genetic marking which will be a good way of distinguishing the released sterile insects from the target wild population. Visible mutations have been used as transformation markers for medfly, Sergeant (Sr2) is a dominant mutation was used as a transformation marker which marks the third stripe on the abdomen of the fly (Niyazi et al. 2005).

Genetic sterility can be an alternative method to radiation for insect sterilisation. RIDL™ (Release of Insects carrying a Dominant Lethal) uses modern genetics and current molecular biology approaches to develop strains to use in SIT programmes (Dyck et al. 2005). RIDL technology can also confer genetic sexing, which facilitates generation of male-only populations for single sex release programmes (Fu et al. 2007). It also offers the benefit of genetic marking using fluorescent protein genes. It is likely that the inclusion of a genetic marker will compromise the fitness of the released males but the fitness penalties are likely to be less detrimental than the effects of the fluorescent powder (Catteruccia et al. 2005; Handler et al. 2001). Dominant lethality causes death in relatively early stages of life, before adulthood (Gong et al. 2005). RIDL can replace irradiation, which is harmful to most insects, so that the released males are more potent for mating than the irradiated insects, therefore potentially fewer males will need to be released to control the target insect populations (Thomas et al. 2000).

1.5.1 Transposable elements in insect transformation

Transposable elements (TEs) are a class of genetic elements which have the ability to mobilise themselves from one position in a genome to another (Kidwell & Lisch 1997). Three classes of transposable elements are identified: class I TEs use a reverse transcription mechanism to transpose; class II TEs relocate themselves from one position into a new position without copying themselves meaning they transpose directly between DNA sites (Pimpinelli et al. 1995). Class III TEs or MITES are miniature inverted-repeat transposable elements; they are small fragments of DNA that do not encode any protein but are capable of non-replicative relocation into a new site in the genome. Class II TEs have been used to transform insects by mobilizing the transgene into the insect's genome (Handler, 2002). Intact class II TEs have remobilisation ability as they have short inverted repeats at their terminal and a transposase gene (Pimpinelli et al. 1995). The class II TEs need their terminal sequences for transposition and excision. Other non-transposon DNA sequences, e.g. a gene construct, if flanked by these terminal sequences, are able to mobilise into host genomes, facilitated by the relevant transposase (O'Brochta 1996). Most elements have limited target site specificity, e.g. 2bp or 4bp recognition sites; so the insertion sites within the host genome are broadly distributed. For some elements the insertion site distributions are close to random (*P-element* family, (Craig 1991)), while for the other elements, there is quite a strong site bias (Tn7, (Craig 1991)).

There are different types of class II transposable elements that are used in insect transformation. These include *Minos* (Loukeris et al. 1995), *Hermes* (Jasinskiene et al. 1998) and *piggyback* (Handler & McCombs 2000). There are five families of class II TEs which are all short inverted repeat type elements; the *P*-element family is extensively used to transform *Drosophila melanogaster* however they only work in a very limited range of species (Craig

1991). The hobo or hat family was first isolated from *D. melanogaster* (Kidwell & Lisch 1997). The mariner family was first isolated from the mutant of *D. mauritiana* (Thornburg et al. 2006). The Tcl family was initially isolated from the nematode *Caenorhabditis elegans* (O'Brochta 1996). The TTAA-specific transposable element was identified in baculoviruses that infect Lepidoptera (Handler 2002).

The *piggyBac* transposable element has been successfully used for the transformation of several economically important species, making *piggyBac* the most widely used transposable element for pest insect transformation. *piggyBac* was originally defined in Lepidoptera but it has also been found in plants, fungi and across the animal kingdom (Keith et al. 2008). Insect species that have been transformed with *piggyBac* include *Drosophila melanogaster* (Handler & McCombs 2000); *Aedes aegypti* (Kokoza et al. 2001); *Anopheles gambiae* (Grossman et al. 2001); *C. Capitata* (Handler & McCombs 2000); and *C. hominivorax* (Allen et al. 2004). The summary list of insect species that have been transformed with class II TEs are listed in table 1.1. Furthermore, *piggyBac* is one of the few transposable elements which exclusively inserts and excises precisely. This, together with the high rate of transformation across different insect taxa, made it the preferred TE for use in the genetic transformation of insects in Oxitec.

Table 1. 1 Summary of transposable element-mediated stable germline transformation of non-drosophila insect species (Table is updated from Morrison et al. 2010).

Family	Species name(s)	TEs	Reference
Mosquitoes			
Culicidae	Yellow fever mosquito, *Aedes aegypti*	*Mariner*	(Coates et al., 1998)
		Hermes	(Jasinskiene et al., 1998)
		piggyBac	(Kokoza et al., 2001)
	Asian tiger mosquito, *Aedes albopictus*	*piggyBac*	(Labb et al., 2010)
	Aedes fluviatilis	*piggyBac*	(Rodrigues et al., 2006)
	New World malaria mosquito, *Anopheles albimanus*	*piggyBac*	(Perera et al., 2002)
	African malaria mosquito, *Anopheles gambiae*	*piggyBac*	(Grossman et al., 2001)
	Indo-Pakistan malaria mosquito, *Anopheles stephensi*	*Minos*	(Catteruccia et al., 2000)
		piggyBac	(Ito et al., 2002; Nolan et al., 2002)
	Southern house mosquito, *Culex quinquefasciatus*	*Hermes*	(Allen et al., 2001)
Fruit flies			
Drosophila	Spotted wing drosophilid, Drosophila suzukii	*piggyBac*	(Schetelig and Handler, 2013)
Tephritidae	Mexican fruit fly, *Anastrepha ludens*	*piggyBac*	(Condon et al., 2007b)
	Caribbean fruit fly, *Anastrepha suspensa*	*piggyBac*	(Handler and Harrell, 2001b)
	Oriental fruit fly, *Bactrocera dorsalis*	*piggyBac*	(Handler and McCombs, 2000)
	Olive fly, *Bactrocera oleae*	*Minos*	(Koukidou et al., 2006)
	Queensland fruit fly, *Bactrocera tryoni*	*piggyBac*	(Raphael et al., 2010)
	Mediterranean fruit fly, *Ceratitis capitata*	*piggyBac*	(Handler et al., 1998)
		Hermes	(Michel et al., 2001)
		Minos	(Loukeris et al., 1995)
Other Diptera (pest, myiasis, biting flies)			
Muscidae	Housefly, *Musca domestica*	*piggyBac*	(Hediger et al., 2001)
		Mariner	(Yoshiyama et al., 2000)
	Stable fly, *Stomoxys cacitrans*	*Hermes*	(O□Brochta et al., 2000)
	Horn fly, *Haematobia irritans*	piggyBac	(Xu et al.,, 2015)
Calliphoridae	Australian sheep blowfly, *Lucilia cuprina*	*piggyBac*	(Heinrich et al., 2002)
	New World screwworm, *Cochliomyia hominivorax*	*piggyBac*	(Allen et al., 2004)
Wasps, bees and ants			
Hymenoptera	Sawfly, *Athalia rosae*	*piggyBac*	(Sumitani et al., 2003)
	Honeybees , *Apis mellifera*	*piggyBac*	(Schulte et al., 2014)
Beetles			
Coccinellidae		*piggyBac*	(Kuwayama et al., 2006)
Tenebrionidae	Red flour beetle, *Tribolium castaneum*	*piggyBac & Hermes*	(Berghammer et al., 1999)
		Minos	(Pavlopoulos et al., 2004)
Butterflies and moths			
Nymphalidae	Squinting bush brown butterfly, *Bicyclus anynana*	*piggyBac & Hermes*	(Marcus et al., 2004)
	buckeye butterfly, *Junonia coenia*	*piggyBac*	(Beaudette et al., 2014)

1.5.2. Conditional expression systems

The primary aim of this research is to develop a male-sterile strain of olive fly for use in a RIDL SIT approach to control this pest. RIDL™ technology uses a binary gene regulation system based on the tetracycline-responsive transcriptional factors (tet-off system) (Gossen &Bujard 1992) (Figure 1.6). In this system, transposable elements are used to introduce the desired gene sequences into the genome of pest insects (Gong et al. 2005).

An ideal regulated transgene system is the one that is highly induced under stimulating conditions and is inactive, or nearly inactive, under basal conditions (Markstein et al. 2008). In addition it is practical to be able to be able to dictate in which tissue the expression is active (Tzou et al. 2000). In RIDL the presence of tetracycline or analogues to tetracycline in the larval diet represses the lethal system which allows mass-rearing of the strain (Schetelig & Handler 2012). Several conditional gene expression systems have been generated that have ability of offering both restricted gene expression and be regulated in time. Examples of these systems are the FLP recombinase gene and FRT sites (from yeast), steroid hormone responsive transcription factors (GeneSwitch and ER-GAL4), temperature-sensitive repressors of the GAL4-UAS system (TARGET), and tetracycline-responsive transcriptional system (tet-On and tet-Off) (McGuire et al. 2004).

The tet-off system was successfully used to efficiently regulate a dominant repressible lethality system in *D. melanogaster* (a repressible transcription factor that its expression is driven by sex-specific or non–sex-specific promoter , which in turn controls the expression of a selectively lethal gene product) (Thomas et al. 2000). Furthermore it can be used as the lethal mechanism itself, and not only as a means of regulating a lethal system (Gong et al. 2005). tTA is a transcriptional transactivator that is composed of the tet-responsive tetR DNA binding protein fused to the VP16 transcriptional activator. In the absence of tetracycline it

binds to its target sequence, tetO, and activates expression of downstream genes. In the presence of tetracycline the binding of tTA to tetO is abrogated, and there is no activation of the downstream gene (Gossen & Bujard 1992). Expression of tTA will be controlled by a selected promoter in a tissue- and time-specific manner (Gong et al. 2005).

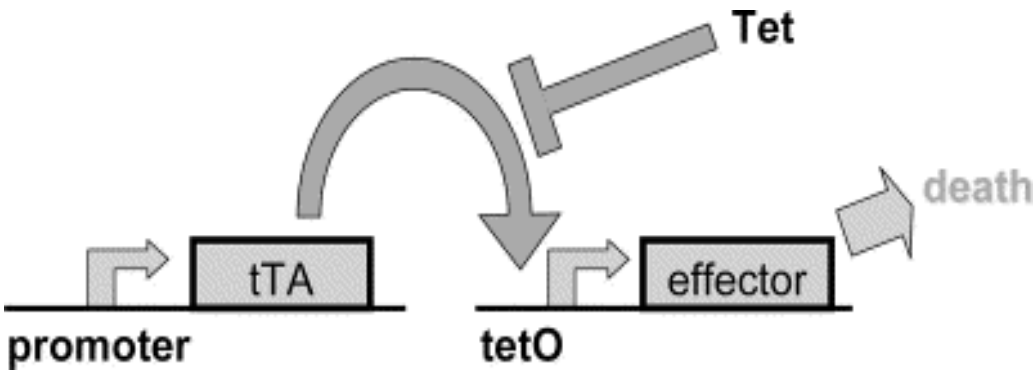


Figure 1. 6 The basic bipartite tTA system.

A selected promoter controls the transcription of (tTA) protein, introducing developmental specificity to the system. In the absence of tetracycline tTA protein binds to the tetO sequence, which is upstream of a minimal promoter and an effector gene coding sequence. tTA binding drives expression of the effector gene. In the presence of tetracycline, tetracycline binds to tetR domain, and prevents tTA from binding tetO, hence the effector is not transcribed (Gong et al. 2005).

1.5.2.1. Spermatogenesis

The study of reproductive biology of male insects can play an important role in controlling their population in SIT programmes. The germ cells only exist in reproductive organs; these are different from somatic cells which can be found in all tissues, in many ways. The germ cells are toti-potent, that is they retain the ability to differentiate to any tissue via contributing to the next generation and to pass on genetic information (Reinke et al. 2000). Differentiation of the male germline (spermatogenesis) produces mature male gametes and is broadly conserved among species. *D. melanogaster* has been used as a model organism to study gene expression in the male germline, which gives a clear view on the developmental stages of testis and the position of the germ cell in each developmental stage (White-Cooper 2009).

The various phases of male germline cell stages in spermatogenesis are: germline stem cell; spermatogonium; spermatocyte; spermatid; spermatozoan (Figure 1.7). All stages of spermatogenesis in insects occur in individual cysts; depending on the species and what stage the cysts are, the number of cells in each cyst is different (Gönczy et al. 1992). During spermatogenesis two types of cell divisions occur: mitosis, when gonial cells are being multiplied and meiosis when haploid spermatids are being formed. The stem cell niche is located at the tip of the testis (hub) where there is a small group of somatic cells. Stem cells are self-renewing cells and are capable of dividing throughout the reproductive life of the organism. They both maintain the stem cell population in testis and also produce spermatogonial cells (Cox et al 2000). The spermatogonial cells undergo a series of mitotic multiplications before exiting mitosis and differentiating into primary spermatocytes.

The level of transcription and gene expression increases extensively throughout the primary spermatocyte stage, then decreases dramatically prior to the meiotic divisions in the germ

cells (Reinke et al. 2000). The transcription of many genes exclusively occurs in primary spermatocytes. These genes include the ones that encode proteins that are required only for spermatogenesis such as Protamines (they replace histones in sperm) (Braun 2001) or *topi* (DNA binding protein) (Perezgasga et al. 2004) and also genes that are encode male germline-specific isoforms of proteins that are functional elsewhere in the body such as *fzo* (protein mediator of mitochodrial fusion) (Hales & Fuller 1997). In addition, many genes are transcribed elsewhere in body but use different promoters to drive their expression in primary spermatocytes, like cytoplasmic dynein (motor activity of cytoplasmic transport) (White-Cooper 2010).

There are sixteen primary spermatocytes per cyst in *D. melanogaster*, which undergo two meiotic divisions (meiosis I and meiosis II) in metasynchrony at the end of the primary spermatocyte growth phase. The result of these meiotic divisions is a cyst of 64 interconnected haploid spermatids; at this stage most of the transcription activity is shut down. However, transcripts from many genes are stable and present for days after meiosis where they play an important role in regulating the timing of protein production, especially during the post-meiotic stages (Eddy 1998).

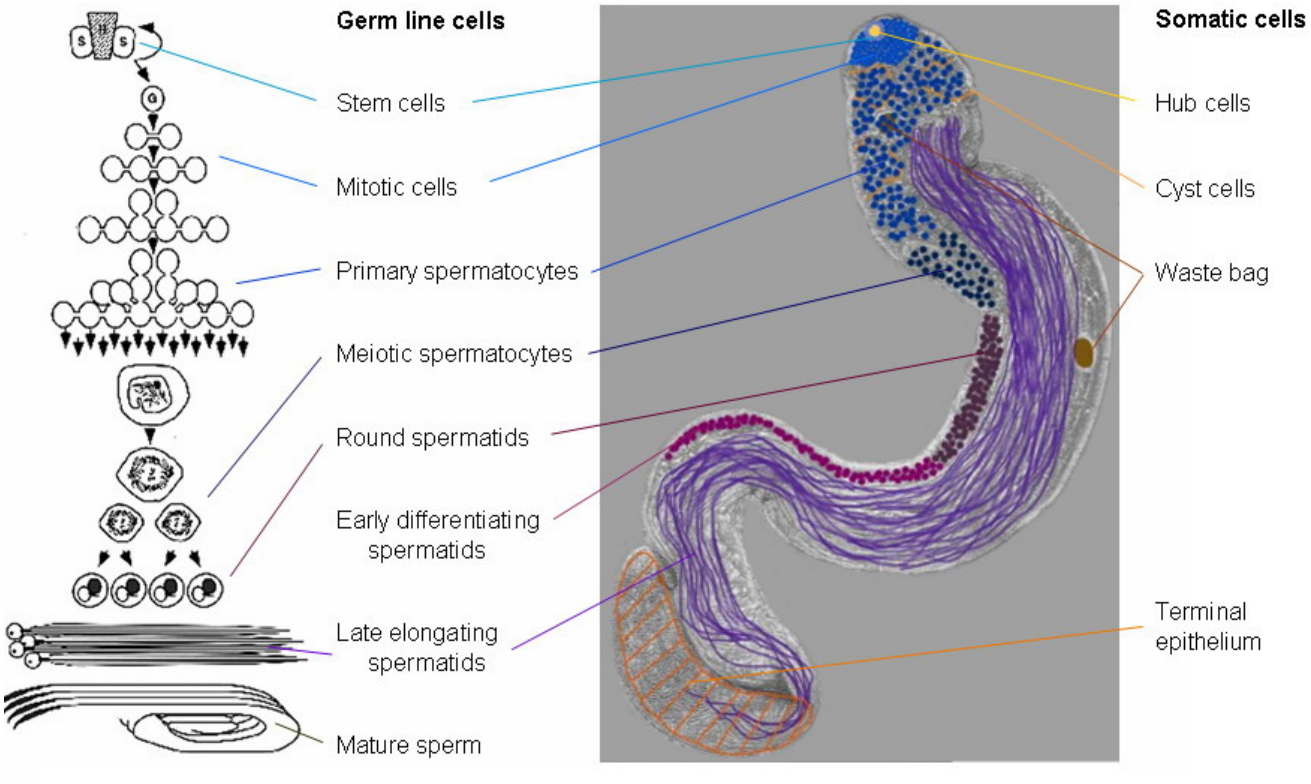


Figure 1. 7 The spermatogenesis cells and stages in *D. melanogaster*.

A schematic diagram of the *Drosophila* testis where all stages of spermatogenesis in *D. melanogaster* are depicted. The photograph obtained from **http://flyted.zoo.ox.ac.uk/**.

1.5.2.2. Spermatogenesis in fruit flies

Spermatogenesis in medfly and olive fly is very similar to *D. melanogaster* (White-Cooper 2010). The olive fly genome has not yet been sequenced but medfly and *D. melanogaster*'s sequencing have been completed (www.hgsc.bcm.edu/ftp-archive/I5K-pilot/Mediterranean_fruit_fly/; http://flybase.org). The construction of EST libraries (expressed sequence tag) from medfly has generated a vast amount of sequence and associated expression data that will permit the identification and study of homologous medfly genes involved in numerous biological processes (Gomulski et al. 2008). Like *D. melanogaster*, the testes of medfly are divided into a number of cysts (Figure 1.8), each with a different group of germ cells developing in synchrony within a somatic capsule, all derived from an original stem cell. The division sequences of spermatogenesis are one stem cell division, six spermatogonial mitotic amplification divisions. resulting in 64 germline cells in each spermatocyte cyst (Figure 1.8C). This is followed by meiosis to produce 256 germline cells in each spermatid cyst (Owusu-Daaku et al. 2007).

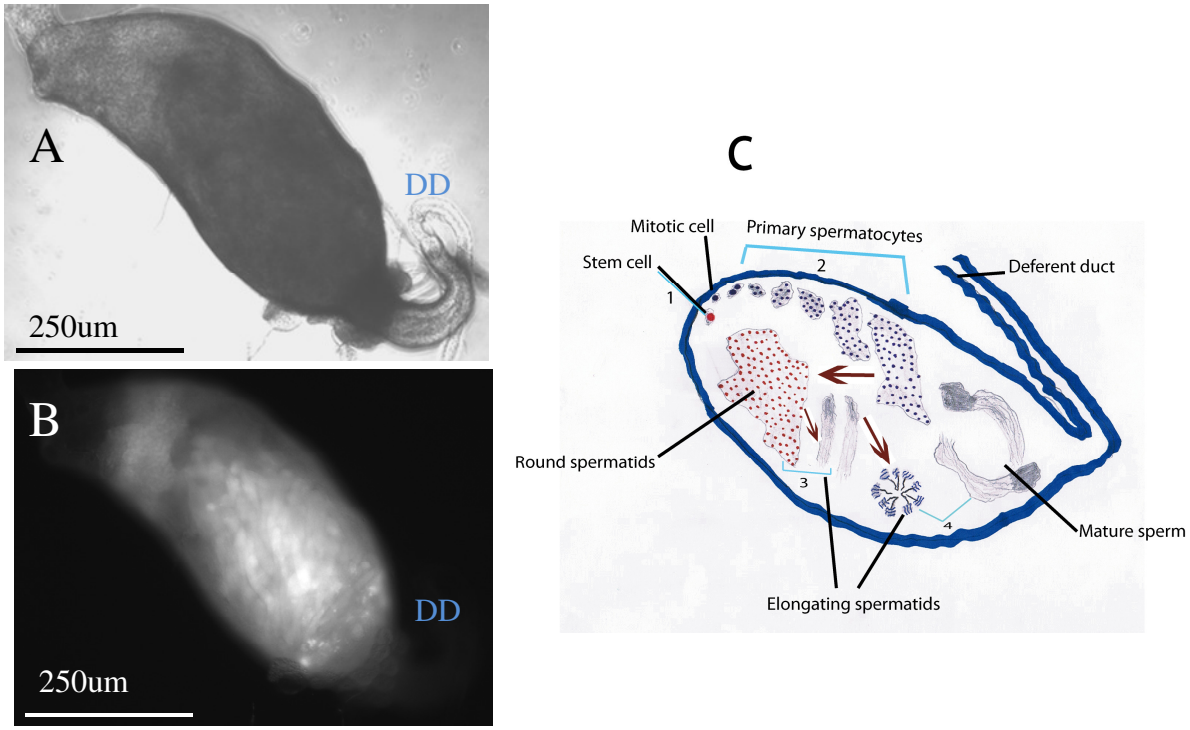


Figure 1. 8 Spermatogenesis in medfly and olive fly.

A: Olive fly testis (bright field), B: Olive fly testis stained with Hoechst 33342 which reveals the overall internal organisation of different cell types in the testis. DD: Deferent duct. C: Schematic diagram of Tephritidae testis and all stages of spermatogenesis.

1.5.3. Selection of promoter and effector to generate sterile male

Male sterility in insects can be the result of aspermia (inability to produce spermatozoa), sperm malfunction (impairment in function of sperm, such as immotile sperm), and dominant lethal mutations in the sperm genome. The desirable sterility in SIT technology is dominant lethal mutations. Males are able to produce viable and motile sperm to be transferred to females and these sperm have the ability to fertilize eggs. As the genetic material of the sperm is damaged, the resultant zygote will not develop past the early embryonic stages. Radiation in SIT generates dominant lethal mutations by introduction of double-stranded breaks in the chromosomes. Sperm doesn't have the ability to repair such breakage in chromosomes therefore the broken ends of chromosomes stay unattached until fertilization. This results in zygotic death as these chromosome fragments are unable to participate in the early mitotic divisions because they lack centromeres, leading to aneuploidy of the embryo (Smith & Borstel 1972).

The same ideology is employed in developing genetic sterility using the RIDL technology. Dominant lethal mutations occur by introduction of multiple double strand breaks into DNA. To achieve this in a genetic sterilisation system, endonucleases may be used to introduce double strand breaks into the insects' germline DNA, which will mimic the mechanism of the traditional SIT method; irradiation.

Windbichler *et al.* generated a genetically sterile of strain of *Anopheles gambiae* that carry a DNA nuclease under the control of a male germline-specific promoter. Specifically they expressed *I-PpoI* under control of the *β2-tubulin* promoter, which expresses in primary spermatocytes. *I-PpoI* targets a sequence found only in the X-linked *Anopheles gambiae* 28S ribosomal genes. They were able to generate early embryonic lethality in the progeny, i.e.

paternal effect lethality, however this system is constitutively ON, and not repressible (Windbichler et al. 2007).

My aim is to generate a repressible paternal effect lethality strain; the combination of two different testis specific promoters (*β2-tubulin* and *topi*) and four varieties of effectors (*3zincfinger*, *EcoRI*, *I-PpoI* and *ProtamineFokI*) in conjunction with the tet-off system for conditional expression will be tested. The final desired strain will ideally also contain a sexing mechanism (for male-only release) and fluorescent sperm (for monitoring).

1.5.4. Gene expression in spermatogenesis and promoter selection

An ideal male sterile strain will make sperm that females cannot distinguish from normal sperm, but that will not support embryonic development. In an attempt to generate such transgenic male sterile strains we explored the following structural hypothesis: tTA expression under the control of a male germ line promoter would drive expression of an effector gene downstream of the tetO operator. The aforementioned configuration would drive adequate expression of an effector gene in male germ cells conditionally. The effector will have the ability to damage the male germ cells and defective sperm would be produced. This would only occur when the insects are reared in the absence of tetracycline. Ideally the effector would mimic the mechanism of radiation-induced sterility, i.e. because DNA double strand breaks. The initial aim of this project is to evaluate a series of different promoters and different nucleases to select the most potent combination.

Expression of some genes is specific to one cell type while a number of genes are expressed in all tissues. The promoter of a gene that is expressed in a specific tissue will control the gene expression in a tissue-dependent manner and possibly to a certain developmental stage. Any gene driven by such promoters will ideally only be expressed in tissues where the transgene product is desired, while the transgene would remain silent in all other cells in the individual. Tissue-specific promoters may additionally be induced by endogenous or exogenous factors, in which case they can also be classified as inducible promoters. An example of such a promoter is the *diptericin* promoter from *D. melanogaster*, it is induced in the fat body and the digestive tract in response to bacterial infection (Tzou et al. 2000). It is preferable to use promoters from the species of interest or from a closely related species to achieve efficient and reliable expression of transgenes in particular tissues, since the promoters have been optimised for the species by evolution. As stated in section 1.5.2.1,

although transcription is extremely high in primary spermatocytes, it drops considerably with the onset of the first meiotic division. This developmental transition in spermatogenesis has a very important role in relation to engineering male sterility using the 'tet-off' system.

The timing of transcription and translation of both promoter-*tTA* and *tetO*-effector cassettes is very important. Ideally, a system in testes would comprise a male germline specific promoter acting in early-mid primary spermatocytes, driving expression of tTA in these cells by using UTRs that promote efficient translation in primary spermatocytes. In absence of tetracycline in the diet, the tTA activates expression of an effector gene under the control of tetO in male germ cells. The effector coding sequence could be flanked by UTRs incorporating translational delay signals, so that the effector protein is not translated until the spermatid stages. Expression of a nuclease inducing double strand breaks in the DNA in pre-meiotic cells is likely to lead to defects in chromosome segregation in meiosis. Moreover the DNA repair machinery is active in these cells, so there would be opportunities for the cells to repair any induced damage (Fuller 1998).

The promoter of a gene expressed in early primary spermatocytes is an ideal candidate to drive transcription of a *tetO*-effector in slightly more advanced primary spermatocytes. Translation of the *tetO*-effector in spematids should cause sufficient DNA damage to sterilise sperm. While a few genes are known to be transcribed post-meiotically (Barreau et al. 2008) the promoter of a post-meiotically expressed is not considered suitable for use. There is a limited time window available for post-meiotic transcription, and the expression of the *tTA* would be insuffient to drive expression of the *tetO*-effector. An activation of the effector driven by the *tetO* sequence should not occur too early in the spermatogenesis either as this will most likely result in a failure to produce sperm (Figure 1.9). As stated in section 1.5.2.1,

the female has a complicated sperm storage system and has to receive a 'normal' amount of sperm during copulation otherwise she will search for a mate again (Shelly & Whittier 1994).

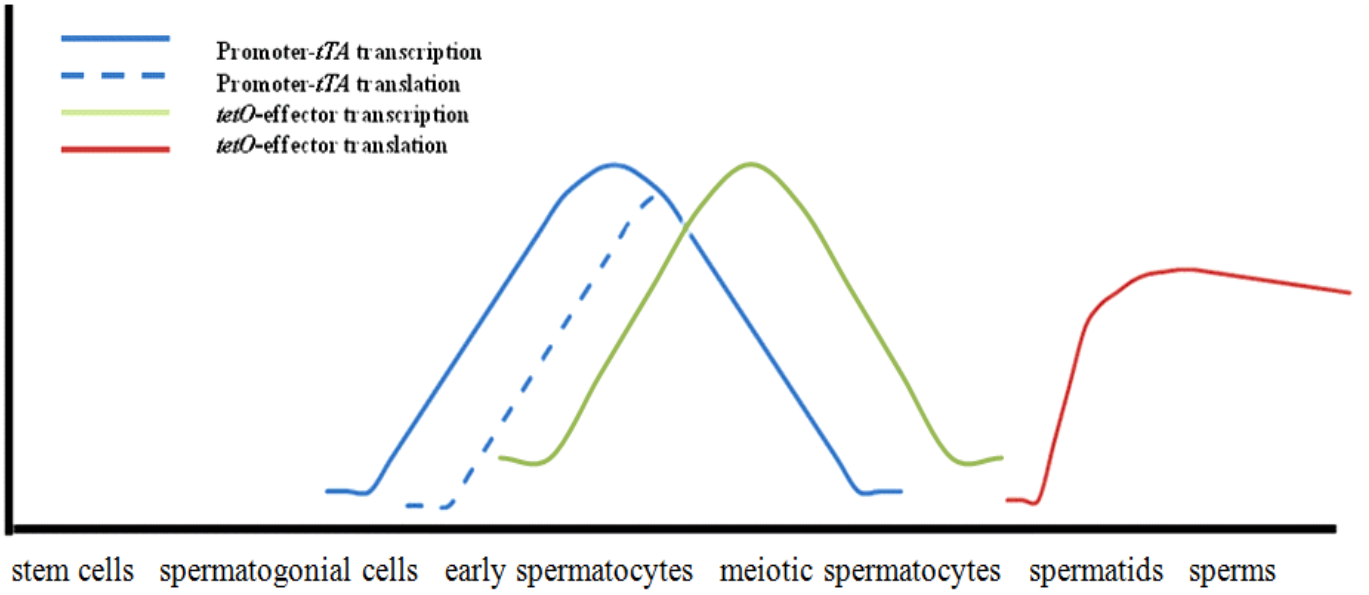


Figure 1. 9 Ideal timing for transcription and translation of promoter and effector molecules for Tephritidae male sterility.

A promoter acting in the early-mid primary spermatocytes drives expression of tTA using UTRs that promote efficient translation of the mRNA in the primary spermatocytes. Then tTA activates the transcription of the *tetO*-effector in late spermatocytes and translation in spematids so that enough double stranded DNA breaks are inflicted to sperm to cause sterility, but sperm retains normal morphology and motility.

The *topi* gene encodes a testis-specific predicted Zn-finger protein (Perezgasga et al. 2004). *topi* is an essential gene in primary spermatocytes for the transcriptional activation of many downstream genes. These genes are later required for progression into the meiotic divisions and spermatogenesis. It is transcribed exclusively in primary spermatocytes, and has a peak expression in early-mid primary spermatocytes. In a previous study, the *topi* promoter from *D. melanogaster* driving tTA in *Ceratitis capitata* and *Aedes aegypti* was assessed. When *topi*-tTA strains were crossed to strains containing a tetO-DsRed construct, expression of DsRed in testis and sperm was found to be very weak and not fully penetrant (Jin 2011). Although the results were encouraging, the expression of *tetO* and subsequently that of the fluorescent protein was inadequate for the purposes of this study.

β2-tubulin is another testis-specifically expressed gene (Kemphues et al. 1982) and it is essential for the formation of normal microtubules in spermatocytes and spermatids (Hoyle et al. 1995). The *β2-tubulin* gene was discovered in *D. melanogaster*, its function starts during early spermatogenesis, it is activated at the larval stage and expresses throughout male adulthood in the post-mitotic male germ cells (Fackenthal et al. 1993). *β2-tubulin* gene transcription initiates in early spermatocytes; at this time there is a switch from *β1-tubulin* isoform production (Buttgereit & Renkawitz-Pohl 1993). The *β2-tubulin* promoter has been used in this study as a driver of *tTA*. The mRNA produced from constructs with the wild type *β2-tubulin* promoter and 5'UTR is translated too late, thus it does not drive *tTA* protein early enough to drive expression of the effector and produce infertile sperm. Therefore, we used an altered form of *β2-tubulin* upstream region (alteration of 5'UTR) so that its translation starts earlier in spermatogenesis. The 5′ UTR of a gene is accountable for translation initiation and affects its translation rate (Wilkie et al. 2003). The 5′UTR region of *β2-tubulin* was replaced by the 5'UTR of *hsp83* from medfly. *hsp83* gene in medfly encodes a heat shock protein 83

in which expresses in all stages of the medfly development. This gene is particularly well expressed in the male germline, even in the absence of heat shock (Jin 2011).

1.6. Current RIDL strains for Medfly and olive fly

Oxitec has developed a system called RIDL® (Release of Insects carrying a Dominant Lethal gene) as a genetic alternative to classical SIT. According to RIDL, lethal (such as female specific lethal or bi-sex lethal) genes could replace the radiation-induced dominant lethal mutations of classical SIT. The lethality needs to be conditional, so that the strain can be propagated under controlled, permissive conditions; that is in the presence of tetracycline as a dietary 'antidote' which prevents expression of the lethal system. These lethal genes could be designed to selectively kill females which would allow integrated genetic sexing simply by rearing the flies intended for release under restrictive conditions (Fu et al. 2007).

Previously, researchers used female specific promoters in *Drosophila melanogaster* to generate female only protein expression (Heinrich & Scott 2000; Thomas et al. 2000), which resulted in male only flies. In another attempt to create female lethal strain; sex specific splicing has been used to generate female only gene expression (e.g. Sex-lethal (*Sxl*) (Keyes et al. 1992)), which can initiate the splicing of sex-specific intronic sequences in females and can therefore be used in the regulation of lethality in transgenic insect strains. The *transformer* gene in medfly (*Cctra*) is used to regulate sex-specific alternative splicing which creates a system for inducing sex specific expression (Fu et al. 2007) (Figure 1.10). In this system, F1 males are fully viable while females are eliminated at an early larval stage.

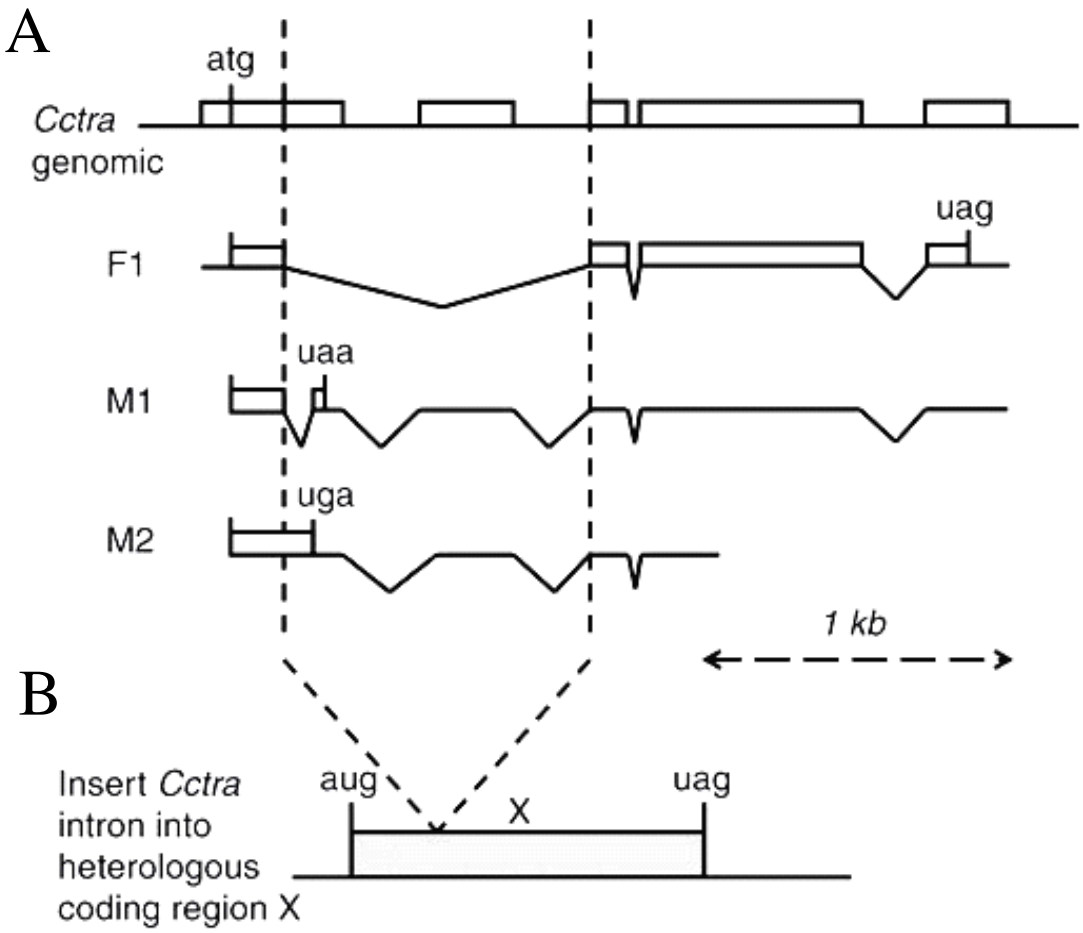


Figure 1. 10 The transformer gene of medfly (*Cctra*) and the alternative splicing that was used in designing female-specific lethal strains for use in SIT.

A number of extant female-specific lethal strains in Oxitec are based on the *tra* intron splicing in fruit flies. A: the native DNA sequence of *tra* with the three splice variants (F1, M1 and M2) illustrating the sequence for the female-specific intron. Males only produce M1 and M2 variants. The F1 variant is a complete splicing of the intron. Exon two has stop codons, and therefore the Tra protein in males is truncated. B: *cctra* intron was inserted into the tTAV coding region to generate sex-specific gene regulation (Diagram from Fu et al. 2007).

Schetelig et al. 2009 developed conditional embryonic-lethal strains in medfly which can also be used in combination with female specificity. In this system a promoter/ enhancer (P/Es) element from cellularisation-specifically- expressed genes drive the expression of tTA. tTA activates the expression of the lethal gene *hidAla5* and leads to embryonic lethality in medfly.

1.7. Research aim and objectives

The primary aim of this study is to use RIDL technology to produce repressible male-specific sterility (use a lethal gene that generates sterility in males) in Tephritidae (olive fly and medfly) that could be used in SIT programmes to replace radiation. The process of developing (finding the best combination of promoter and effector) such strains is reported in Chapter Three. The optimised outcome product from Chapter Three was further investigated in Chapter Four. The final product of these strains could potentially be used for large scale releases of sterilised males. Medfly has been used as the model organism for the development and improvement of male-specific sterility technology due to its relatively short life-span, the simplicity of transformation, rearing, handling and its significance as a key pest insect. However, the main aim was to establish that these technologies could be functional in olive fly. Chapter Five reports on the achievement of applying this work to olive fly.

It was crucial to test and develop other promoters that can be used as an alternative if needed; therefore Chapter Six provides information regarding *hsp83*.

Development of sex specific markers in olive fly maybe used for the sex separation of olive fly in an SIT programme (Chapter Seven). Males and females which are carrying sex specific markers will express different fluorescent markers. This allows for mechanical sex separation at pupal stages. It is crucial to develop a technique to detect our product in the field therefore PCR was used to develop a protocol to identify whether trapped females in the field have mated and whether the mating choice was with a RIDL or wild male (Chapter Eight). By optimizing a PCR protocol and using Y chromosome primers or transgene primers it is possible to determine whether a specific female has mated, and if so, whether the male was wild type or RIDL.

Chapter 2-Materials and Methods

2.1. Rearing of the laboratory strain medfly

The lab strain medfly at Oxitec were kept in a sealed room at a temperature of 26±1ºC, 35±5% RH (relative humidity) and a photoperiod of 14:10 (L:D) at all stages of the life cycle. The medfly larvae were fed on a standard *D. melanogaster* diet. This diet contains 133g maize meal, 146g sugar and 93g yeast, 20g of Agar, 50ml of the Nipagen/Ethanol solution (50g Nipagen to 500ml Ethanol) and 1700ml de-ionised water. To make the diet that contains tetracycline, 0.25g of tetracycline was dissolved in 20ml of water and added to the diet mixture. The adult medfly were fed on 1:4 ratio of yeast powder to sucrose (fed to flies as a solid mix). The adult medfly were kept in a cage with a fine mesh on one side to provide an oviposition surface for adult females; females oviposit through the mesh. Eggs were collected into a pot of water placed below the mesh. Eggs that are in the pot of water can be kept for up to a week. The collected eggs were placed into a bottle containing standard *D. melanogaster* diet after filtering them through a filter paper (Fisher brand; Range: QL125, Size: 125mm). The culture bottles containing third instar larvae were placed on their sides, and without lids, in a Tupperware box containing a layer of sand. Several pieces of folded filter paper (Fisher brand; Range: QL135, Size: 125mm) were placed into the bottle to reduce the moisture level and also provide a surface for larvae to crawl out of the bottle. The third instar larvae make their way to the top of the bottle, jump out of the bottle and pupate on the sand (Figure 2.1).

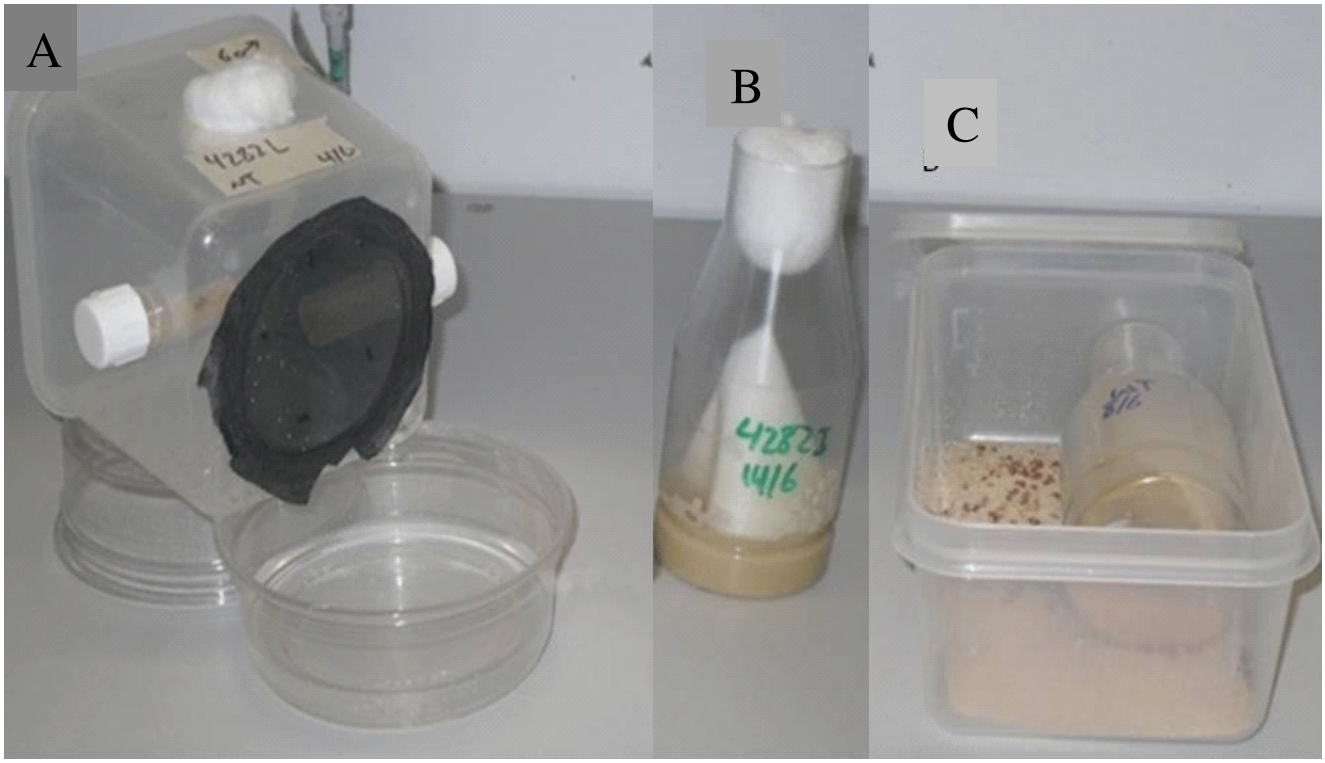


Figure 2. 1 The process of rearing the lab strain medfly.

A: The adult medfly are kept in a cage (10 x 10 x10cm) with water and diet containing yeast and sugar. The adult females lay their eggs through a fine mesh which are then collected into a pot of water. B: The collected eggs are filtered and placed into a bottle of *D. melanogaster* standard diet. C: The bottle is placed into a Tupperware box (20 x 14 x 8.5cm), the third instar larvae crawl out to pupate.

2.2. Rearing of the laboratory strain olive fly

The lab strain adult olive fly and eggs were kept in a sealed room at a temperature of 24±1ºC, 35±5% RH and a photoperiod of 14:10 (L:D). The lab strain larvae were pupae are kept at a temperature of 26±1ºC, 35±5% RH. Olive fly larvae were fed on olive fly diet. The olive fly diet was made as follows: 20ml de-ionised water was added to a small beaker with a stirrer containing 0.5g potassium sorbate, and then the heater was turned on. 20ml olive oil, 7.5ml Tween 80 and 2g of Nipagen was added to the beaker and the beaker was heated up gently until the Nipagen had dissolved. 30g of soy hydrolyzed, 20g sucrose, and 75g yeast and 580ml de-ionised water was added to a mixing bowl, and mixed thoroughly for 10 minutes. Hydrochloric acid (4.5ml of 10M) and the olive oil mix was added and mixing continued for another few minutes. Finally 250g cellulose powder was added slowly whilst the mixer was still running.

The adult olive fly was fed on 10g yeast hydrolyzed, 40g icing sugar and 3g of egg yolk powder (fed to flies as a solid mix). The adult olive fly were kept in a cage with a cone covered in wax (bee wax and petroleum jelly) inside it, to provide an oviposition surface. Eggs that are laid into the cone can be kept for up to a day. The eggs that were collected inside the cone were washed into a beaker and placed in a petri dish containing wet filter paper (Whatman; Diameter 90mm). The filter paper had enough water to avoid it drying out over two days. The collected eggs were placed into a petri dish of food after 2 days. Petri dishes containing 3rd instar larvae are placed into a Tupperware box containing a layer of sand where they make their way to the top of the petri dish jump out and pupate on the sand (Figure 2.2)

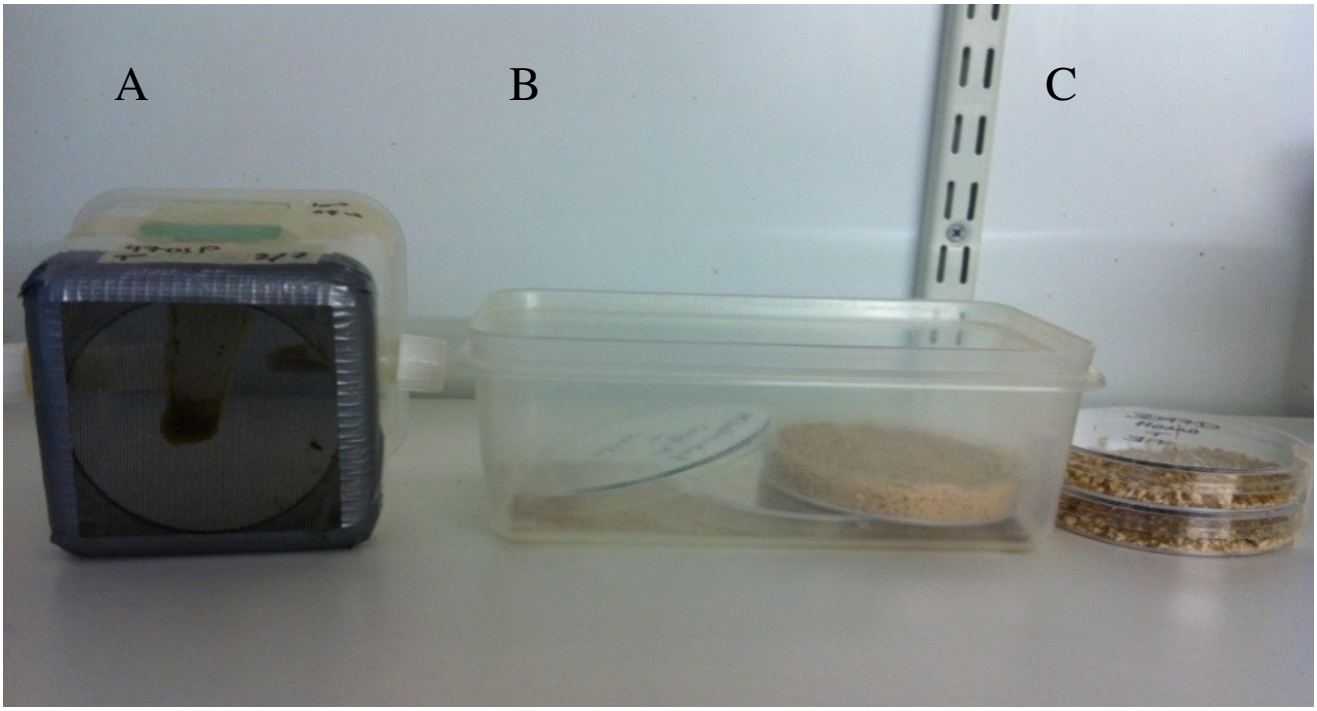


Figure 2. 2 The process of rearing the lab strain olive flies.

A: The adult olive fly are kept in a cage (10 x 10 x 10cm) with water and diet containing yeast hydrolyzed, icing sugar and egg yolk powder. The adult olive fly females lay their eggs through a waxed cone which are collected inside the cone. B: The laid eggs are collected and placed into a petri dish of olive fly larval diet. The petri dish containing third instar larvae is placed into a Tupperware box with a layer of sand (20 x 14 x 8.5cm). C: Pupae are collected into a petri dish.

2.3. Genetic modification of insects

For genetic modification of insects, specifically Diptera, it is important to understand the processes of fertilisation, cellularisation and embryogenesis. Shortly after fertilisation of medfly or olive fly egg, the fusion of male and female pronuclei is followed by rapid mitotic divisions, in the absence of cell division so a syncytium is formed (many nuclei sharing common cytoplasm). Following three to six divisions nuclei move to the periphery of the embryo to form the syncytial blastoderm (Edgar and O'Farrell 1989). At the end of this stage the plasma membranes extend between nuclei, to isolate individual nuclei into individual cells (Edgar and O'Farrell 1989). The eggs of insect of interest can be injected before the nuclear migration and formation of blastoderm, with a mixture of plasmid DNA (construct) and transposase (helper) DNA (or mRNA) as at this stage all nuclei (including those that will contribute to the germline) are located within the common cytoplasm. When nuclei migrate to the cell periphery the nuclei which first reach the posterior pole of the embryo become encapsulated by cell membranes and develop into pole cells. These will become adult germ-line cells. In *D. melanogaster*, the formation of pole cells happens at about 1.5-2 hours after females oviposit (depending on the temperature). In some Tephritids including medfly and olive fly pole cell formation does not occur until three hours or even longer (depending on the temperature) after females oviposit (Gabrieli et al. 2011). It is crucial to inject the DNA mix before pole cell formation, to ensure that it is incorporated in these cells on cellularisation, since only the pole cells have the developmental capacity to differentiate into germline cells of the adult. Engineered DNA with the help of transposable elements will integrate into host genomes through a recombination process (Thomas et al. 2000). The transgene is marked with genes encoding fluorescent proteins which allow for identification of transgenic insects (Handler & Harrell II 2001).

The process of micro-injection is stressful for embryos leading to a lower survival rate than non-injected embryos. The G_0 adults that survived this process were back crossed to wild-type insects of the opposite sex and the G_1 progeny are screened for transgenic individuals. For *D. melanogaster* crosses of G0 adults is typically done individually, for Tephritid species we typically cross the survived adults in batches since the transformation efficiency is very low.

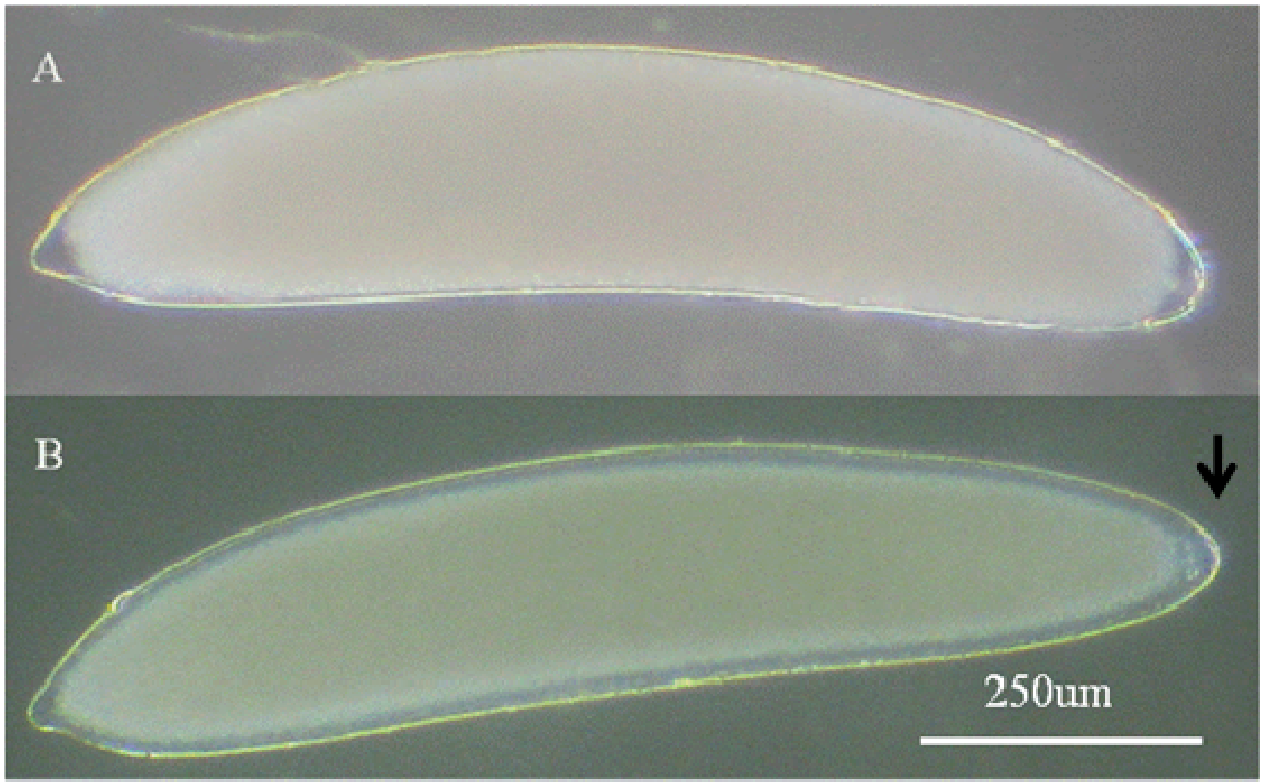


Figure 2. 3 The comparison between pre-blastoderm and blastoderm stage of a medfly egg.

A: A medfly egg in a pre-blastoderm stage. B: A medfly egg in a blastoderm stage; cells are starting to form. The arrow shows formation of pole cells at the posterior end of the egg.

2.2.1. The micro-injection of medfly and olive fly

Freshly laid eggs were collected from wild-type cages within an hour of oviposition. Eggs were treated with 10% bleach (Sodium hypochlorite solution from Sigma-Aldrich; reagent grade, 10-15% chlorine available) for 1.5 minutes for medfly eggs and 5% bleach for 1 minute for olive fly eggs. Glue was made by dissolving the glue from double-sided sticky tape in heptane (Sigma-Aldrich; absolute grade), and a thin strip was painted on a cover slip and left to dry. This glue strip was used to hold the eggs in place. The de-chorionated eggs were lined up in parallel, with their posterior poles all facing in the same direction. The lined up eggs were desiccated in a heater at a temperature of 37ºC for 1 to 6 minutes. Halocarbon oil (700 and 27 mixed 10:1 (v:v) (Sigma-Aldrich)) was placed on top of the desiccated eggs to prevent further desiccation. If the egg was too turgid, it would burst and die after being pierced with the needle during injection. Embryos which were too flaccid also died. For injection, Eppendorf Femtotip II needles were used. The needle was back-loaded with 4 µl of vector/helper mix (600ng/µl and 300ng/µl respectively). For each egg, the needle was inserted into the posterior, and a small amount (the precise volume was not controlled) of DNA solution was dispensed. After injection, cover slips containing the injected eggs were placed on an apple juice agar plates (15g of agar, 12.5g of sucrose, 20ml of 10% Nipagen/Ethanol, 250ml of apple and 750ml of de-ionised water), and incubated at 25±1ºC temp for 3 days. Once the larvae emerged, they were transferred into a petri dishes containing mexfly (Mexican fruit fly) larval diet for medfly and olive fly larval diet for olive fly. Mexfly larval diet is made up as follows; 158g corn grits, 84g sugar, 64g torula yeast, 1g sodium benzoate, 2.3g nipagen (methyl-4-hydroxybenzoate), 1g guar gum, 6g citric acid, 84g wheat germ, 1g vitamin premix, 65g cellulose and 550ml of de-ionised water and 50ml of tetracycline stock solution (2mg/ml) (if appropriate).

The surviving G0 adults were crossed to wild-type, 6 males G0 to 20 wild-type females or 12 G0 females to 6 wild-type males).

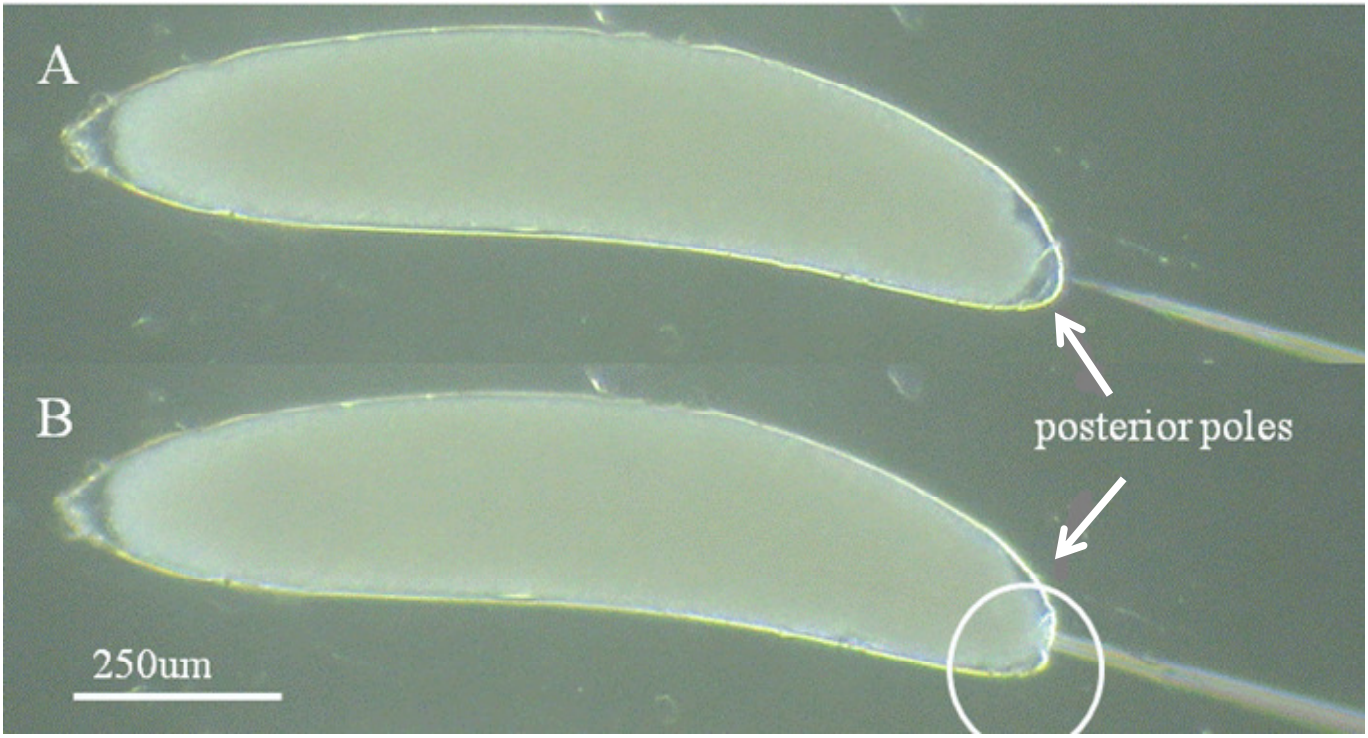


Figure 2.4 Micro-injection of a medfly egg.

A: A medfly egg which is about to be injected. B: The same medfly egg is being injected with the DNA plasmid. The white circle shows where the injection needle is penetrating the egg. The arrows show the posterior end of the egg.

2.2.2. Experimental design of fertility assays

The medfly promoter strains were made with *topi-tTAV* and *β2-tubulin-tTAV* based constructs. The medfly effector strains were made with tetO-*Ippo1,* tetO-*protamine-FokI* and tetO-*ZnF* based constructs (see section 2.7). To check for paternal effect lethality phenotypes, transgenic lines that contained *topi* or *β2-tubulin* promoters driving tTAV were crossed to each of the tetO-effector lines. Cages were set up with 6 medfly males from the effector lines and 12 virgin medfly females of promoter lines. The eggs from these crosses were separated into two batches, and hatched onto tetracycline-containing or normal diet (without tetracycline). Individuals carrying both promoter and effector insertions were selected from the progeny of these crosses on the basis of their fluorescence phenotypes.

Double-heterozygous male progeny of the above crosses were crossed with wild-type virgin females at a ratio of three females to one male. The reciprocal cross was also performed. The adults of each cross were maintained on or off tetracycline. Males reared off tetracycline were expected to be infertile, while female fertility should be unaffected. Two control cages with wild-type flies were also made (wild-type male and female, one fed on tetracycline and one off tetracycline). 5-7 days after setting up the cages, females were allowed to lay eggs into a freshly changed pot for 24 hours. Around 100 eggs from each cross were allowed to develop on wet Whatman filter paper in a petri dish for 48 hours, and the number of hatched and unhatched eggs was counted. Experiments were performed in duplicate or triplicate.

2.3. Statistical analysis of sterility assay

For statistical analysis of the experiments, a Chi Squared test (X^2) was performed. The statistical tests were done using the R statistical package. For the male and female paternal effect lethality assay, the three replicates were pooled together rather than taking the average, as it gives bigger sample sizes (Hanley & Lippman-Hand 1983).

2.4. Micro Dissection

The adult males and females of medfly or olive fly were dissected in testis buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris pH 6.9) with fine forceps to isolate testes, ovaries or spermatheacae. If the samples were to be examined by microscopy, they were placed on a clean slide in testis buffer, testes were cut open, and a cover slip was placed on top. If the samples were to be used for molecular biology (e.g. RT-PCR), they were transferred after dissection into a small drop of buffer in the lid of a 1.5ml eppendorf tube, and stored at -80°C until needed.

Transgenic pupae were screened using a fluorescence microscope (Olympus SZX12 with Olympus U-RFLT fluorescent burner) with filters for RFP and GFP. Testes were checked with an Olympus BX50 with Olympus U-RFLT fluorescent burner and filters for RFP and GFP. The same field of view was also examined by phase contrast microscopy.

2.5. DNA extraction using squishing buffer

To extract DNA using squishing buffer, 2µl Proteinase K (10mg per ml) was added to 100µl squishing buffer (10mM Tris HCl (pH 8.0), 1mM EDTA (pH 8.0), 25mM NaCl). The sample (e.g. insect abdomen) was homogenized with 20µl (15µl if it is smaller e.g. spermatheacae) of enzyme + buffer using a sterile pestle. The sample was incubated at 37°C 30 minutes followed by 95°C 10 minutes, and then stored at 4°C (typically these incubations were conducted in a thermal cycler). Samples were spun at full speed in a micro-centrifuge for 5 minutes. Extracted DNA was used for PCR with the following conditions.

Steps	Process	Temperature	Time	No. of repeats
1	Initial denature	94°C	2 minute	x1
2	Denature	95°C	10 seconds	x5
3	Anneal	60°C	45 seconds	
4	Extend	72°C	1 minute	
5	Denature	95°C	10 seconds	x5
6	Anneal	57°C	30 seconds	
7	Extend	72°C	1 minute	
8	Denature	95°C	10 seconds	x25
9	Anneal	55°C	30 seconds	
10	Extend	72°C	1 minute	
11	Extend	72°C	6 minute	x1
12	Pause	4°C	-	-

2.5.1. Standard genomic DNA extraction

The GeneJET™ Genomic DNA Purification Kit from Fermentas was used to extract DNA from insects. One medfly or olive fly was placed into a 1.5 ml eppendorf tube and 200µl cell lysis solution was added; the sample was homogenised thoroughly with a pestle. The homogenized sample was incubated at 57°C for one to three hours. 20µl Proteinase K (200 µg/ml) was added to the cell lysate and incubated for 10 min at 57°C. 20µl RNase A (100mM) (provided in the kit) was added and the sample thoroughly mixed, then incubated a further 10 min at room temperature. 400µl of 50% ethanol was added and the mixture was applied to the column (Nucleospin tissue), and placed in a collection tube. The column was centrifuged for 1 minute at 6000g. The flow through and collection tube was discarded and the column placed in a new collection tube. 500 µl of Wash Buffer 1 (with ethanol added) was added to the column and the assemblage was centrifuged for 1 minute at 8000g. The flow through was discarded and the column placed back in the collecting tube. 500µl Wash buffer 2 (with ethanol added) was added to the column and this was centrifuged in the micro-centrifuge for 3 minutes at maximum speed. After discarding the flow through and replacing the column in the same collection tube, the assemblage was centrifuged for 1 minute at maximum speed. Finally the Nucleospin tissue column was placed into a 1.5ml microcentrifuge tube, the required volume (up to 100µl) of elution buffer was added, and the samples were centrifuged at maximum speed 1 one minute to elute the DNA.

2.6. RT-PCR for RNA samples

2.6.1. RNA extraction

200µl of Trizol (from Life Technology) was added to samples, which were then homogenised thoroughly either using a micro-centrifuge pestle or using a syringe and fine gauge needle. The homogenate sample was incubated for 5 minutes at room temperature the centrifuged at 12,000g for 10 minutes at 4°C. 40µl of chloroform was added and the sample shaken vigorously. The sample was incubated for 10 minutes at room temperature followed by centrifuging at 12,000g for 15 minutes at 4°C. After centrifugation the upper, aqueous, phase (containing RNA) was transferred to a fresh tube. 100µl isopropanol was added and the sample was vortexed for 5-10 seconds. A 10 minute incubation at room temperature was followed by centrifuging at 12,000g for 8 minutes at 4-25°C. The supernatant was removed and the pellet washed with 500µl of 75% ethanol. The RNA was re-suspended in RNAse free water. RNA was treated with DNAse1 kit (from Sigma-Aldrich) to remove any potential DNA contamination from the samples. The quantity and quality of RNA was crudely assessed by electrophoresis in a 1% agarose gel in TAE buffer (for this it is essential to use a freshly poured gel and fresh buffer). For more accurate assessment the RNA was quantified using a NanoPhotometer P 300.

Alternatively, RNA extraction was carried from single flies, 10 testes or 5 ovaries using Qiagen RNeasy Mini kit from Qiagen, UK according to manufacturer's instructions. For RNAse treatment Qiagen RNase-Free DNase from Qiagen, UK was used.

β-mercaptoethanol (β-ME) was added to RLT buffer (10µl β-ME per 1ml RLT). 350µl β-ME/RLT was added to each sample and this was homogenised using a micro-pestle or syringe with fine gauge needle. Samples were centrifuged at 3500rpm for 1 min to pellet the

chitin and the supernatant was transferred to a Qiashredder column. This was centrifuged for 2 min at maximum speed. The column was discarded and the collection tube was further centrifuged for 3 min at max speed. The supernatant was transferred to a clean micro-centrifuge tube, 1 volume (350µl) of 70% ethanol was added to the supernatant, and the sample was mixed immediately by pipetting. The sample was loaded onto an RNeasy spin column and was centrifuged for 15s at max speed. Columns were washed using wash buffer (350 µl of RW1). 10µl of DNase stock and 70µl Buffer RDD was added to the column and incubated at room temperature for 15 minutes. 350µl Buffer RW1 was added to the RNeasy spin column, after centrifuging for 15s at max speed the flow-through was discarded. 500µl of Buffer RPE (with ethanol added) was added to the RNeasy spin column and was centrifuged for 15s at max speed. The flow-through was discarded. The RNeasy column was placed in a new 1.5ml micro-centrifuge tube (supplied in the kit). 30-50µl RNase-free water was added directly to the spin column membrane and was centrifuges for 1 min at max speed to elute the RNA. RNA was stored at -80ºC.

2.6.2. cDNA synthesis

cDNA Synthesis was performed using the RevertAid First Strand kit from Thermo Scientific, UK. Before cDNA synthesis the quantity of RNA was determined using a NanoPhotometer P 300. For each reaction 1µg of RNA was used, and the total volume made up to 11.5µl using RNAse free water. 11.5µl of RNAse free water was used as a template for the no-template negative control reactions.

To each reaction 1µl Oligo-dT (10µM) was added to the 11.5µl RNA/water and the mix incubated at 65ºC for 5 minutes then incubated at 4ºC for at least 1 min. Afterward 4µl of 5x Reaction Buffer, 0.5µl Ribolock, 2µl of 10mM dNTP mix and 1µl RevertAid were added to each reaction (No RevertAid enzyme was added to “No RT” control reactions). The reactions were incubated at 42ºC for 60 minutes, then 70ºC for 5 minutes, before being chilled to 4ºC. These incubation steps were typically carried out in a thermal cycler.

2.6.3. General PCR Method

In all PCRs controls were included: RNAse free water as the template to check for contamination of reagents, wild-type insect as a negative control to check for primer specificity, and diluted pure plasmid as a positive control. In RT-PCR, a no RT control was included to check for genomic DNA contamination of reagents. For all PCRs the BIOTAQ™ PCR Kit (Bioline) was used. Reagents in PCR generally were:

Components	**Volume/Reaction**
Template	0.5-4µl
5 x Reaction buffer	5µl
10mM dNTP	0.5µl
F primer (10mM)	0.75µl
R primer (10mM)	0.75µl
Taq polymerase	0.3µl
RNAase free H_2O	Make it to 20µl

PCR mix were placed in a PCR machine followed by standard genotyping programme as stated in section 2.4

2.7. Mating competition and re-mating assay

Mating competition and re-mating experiments were designed according to guidelines for mating competition tests and re-mating tests (IAEA/USDA/FAO 2003). These tests were designed to be carried out in medium cages (30x30x30cm) in lab conditions. Wild-type and heterozygous OX4751 larvae were reared at equal densities to obtain adult flies. To have only infertile adult males from OX4751, larvae were reared off-tet. Wild-type flies were separated by sex immediately after eclosion, and the genders were kept in separate cages. Test cages were set up of 200 wild-type males, 200 OX4751 males, and 200 wild-type females. Cages were provided with adult diet and water, and were kept at 26ºC (50% relative humidity) for 5-6 days to reach sexual maturity. Mating tests were performed in contained room facilities at Oxitec. Mating pairs were removed from the cages using a 1.5ml tube. The time when the mating started for each individual mating pair was recorded on the tube. Every 10 minutes, they were checked to find out whether the couples were still mating. After all mating pairs had finished mating, they were cooled on ice and males were checked for the absence or presence of the DsRed2 fluorescence using a fluorescence stereo microscope.

For re-mating experiments two separate cages were set up: the first cage contained 100 mature wild-type females (four days old) along with 100 mature wild-type males (four days old). The second cage contained 100 mature wild-type females (four days old) along with 100 mature OX4751D males (four days old). Mating pairs from both cages were removed; Mated females were sorted according to the genotype of their first mate (OX4751, n =98 or wild-type, n =97) and relocated the following day to new cages with adequate number of fresh wild and OX4751 males to give a 1:1:1 ratio of mated females, wild males, and OX4751 males. The cages were monitored daily for re-mating pairs over the next 15 days. Re-mating couples were isolated, and the genotype of the second male was assessed by fluorescence

microscopy (the absence or presence of the DsRed2 fluorescent protein). After removing a male of a specific genotype from a cage, it was replaced by a fresh one from the same genotype to sustain equal ratios.

2.8. Construct design, amplification and their schematic diagrams

All constructs used in this report were designed and built by the molecular team at Oxitec Ltd. To design each individual construct, a meeting was held. My findings from previous experiments were considered and new ideas from all team members were taken into consideration. New construct designs involved both agreeing on the next experimental step (e.g. incorporation of a new genetic module), and improvements to designs previously tested modules. The details of their composition are included here for reference. In all of the construct schematics below, the plasmid backbone is omitted, and the region that will insert into the insect genome is shown as a linear fragment.

2.8.1. Amplification of a plasmid construct

To build a construct the first step was to amplify the target gene using PCR (section 2.5 for standard PCR). The PCR products were prepared for ligation into a desired plasmid backbone using the T4 DNA ligase from New England Biolab®, UK. The initial reaction contains the following:

COMPONENT	20 µl REACTION
10X T4 DNA Ligase buffer	2 µl
Vector DNA	50 ng (0.020 pmol)
Insert DNA	37.5 ng (0.060 pmol)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

The mixture was mixed gently and incubated for 5 min at room temperature. The reaction tube is placed on ice and followed by the transformation step. One vial of E. coli cells (TOP10 Chemically Competent, 60 µl) was defrosted on ice. 2 µl of the T4 DNA ligase reaction was added to the vial of defrosted E. coli cells and mixed gently. The mixture was incubated on ice for 30 min, heat-shocked for 30 s at 42°C in a water bath then replaced on ice. SOC medium (250 µl) was added to the reaction at the room-temperature. The transformation mixture was placed in a shaking incubator at 37°C for 1 h. 50 µl of transformation was spread on pre-warmed LB plates containing 50 µg/ml ampicillin. The plate was incubated overnight at 37°C.

SOC medium contains: 0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4 and 20 mM glucose. Glucose was added after autoclaving and the final solution was sterilised by passing it through a 0.2 µm filter.

96 well round bottom plates were set up with 100µl/well of LB media with ampicillin (concentration 100 µg/ml). 96 well PCR plates were set up with PCR reaction components for colony PCR. Colonies from the transformation plate were picked and sequentially inoculated into the PCR reaction well, and into the cell culture well. The culture plate was incubated in a shaking incubator at 37°C for 1-2 h.

The PCR reactions for screening were set up using standard PCR reaction (see section 2.6). PCR was run with followed programme:

Steps	Process	Temperature	Time	No. of repeats
1	Initial denature	94°C	2 minute	x1
2	Denature	94°C	10-20 seconds	x3
3	Anneal	60°C	30-60 seconds	
4	Extend	68°C	30s-2minute	
5	Denature	94°C	10-20 seconds	x3
6	Anneal	57°C	30-60 seconds	
7	Extend	68°C	30s-2minute	
8	Denature	94°C	10-20 seconds	x24
9	Anneal	54°C	30-60 seconds	
10	Extend	68°C	30s-2minute	
11	Extend	68°C	6 minute	x1
12	Pause	4°C	-	-

The PCR product was checked on a 0.8% agarose gel and colonies containing the desired fragment were identified. The colonies of interest were recovered by inoculating 2 ml of LB

+ ampicillin medium with 20 µl inoculated LB media from the appropriate well of the cell culture plate, and incubated at 37°C overnight. DNA plasmid was extracted from cultures using the Qiagen Qiaquick® Mini-prep kit (Qiagen). 50 µl of purified DNA was sent to GATC Ltd for sequencing.

2.8.2. Schematic diagrams of constructs of this thesis

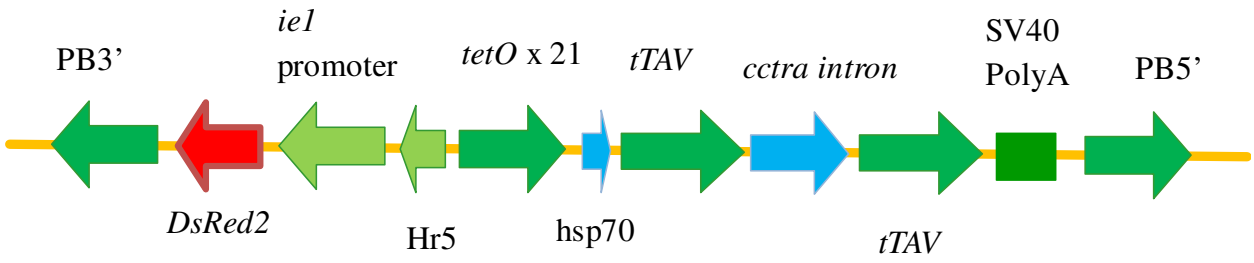


Figure 2. 5 Schematic diagram of OX3097 and OX3864.

PB-*Hr5IE*-DsRed-SV40-*tetO*21-*hsp70-tTAV-Cctra intron-tTAV*-SV40.

The OX3097 and OX3864 constructs are PiggyBac vector (PB) containing a dominant selectable tranformation marker (DsRed, a fluorescent protein, driven by the *Hr5-IE1* promoter). A *tetO* sequence present in a 21 mer array (*tetO*21) drives expression of tranformation marker, with an SV40 3' UTR (Simian vacuolating virus 40). tTAV with an *hsp70* 5'UTR (heat shock protein 70) drives the expression of *tTAV,* interrupted by the *C. capitata tra* intron (*cctra*), with the 3'UTR from SV40. OX3864 has four-ended PB which was injected into medfly, transgenics lines with this construct were used in sections 4.3.3 and 8.3.1. OX3097 is a single-ended PB which was injected into olive fly, transgenics lines with this construct were used in section 8.3.2.

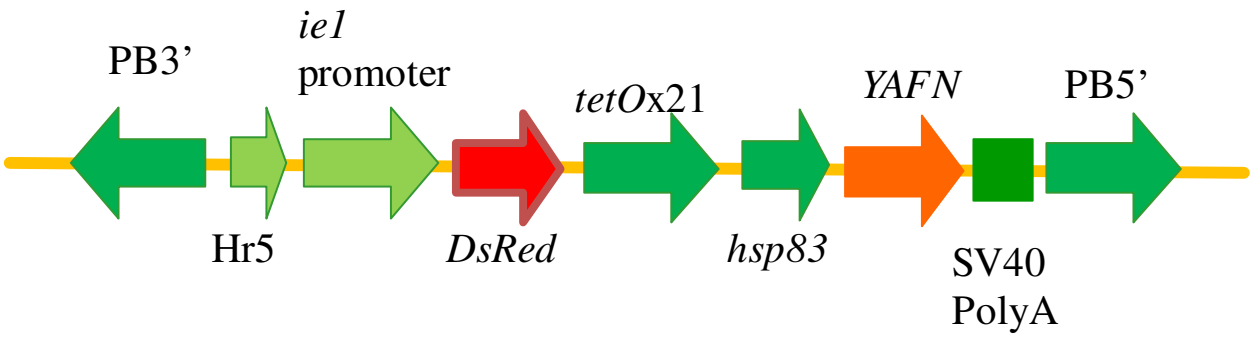


Figure 2. 6 Schematic diagram of OX4104.

PB-YAFN-hsp83-tetO21-Hr5-IE1-Red.

The OX4104 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (DsRed, a fluorescent protein, driven by the *Hr5-IE1* promoter). A *tetO* sequence present in a 21mer array (*tetO*21) drives expression of *YAFN* (*ZnF* protein from *D. melanogaster*). This construct has an *hsp83* 5'UTR (heat shock protein 83) and an SV40 (Simian vacuolating virus 40) 3'UTR. Medfly transgenics with this construct were used in section 3.3.4, 3.3.5, 3.3.6, 3.3.12 and 3.3.13.

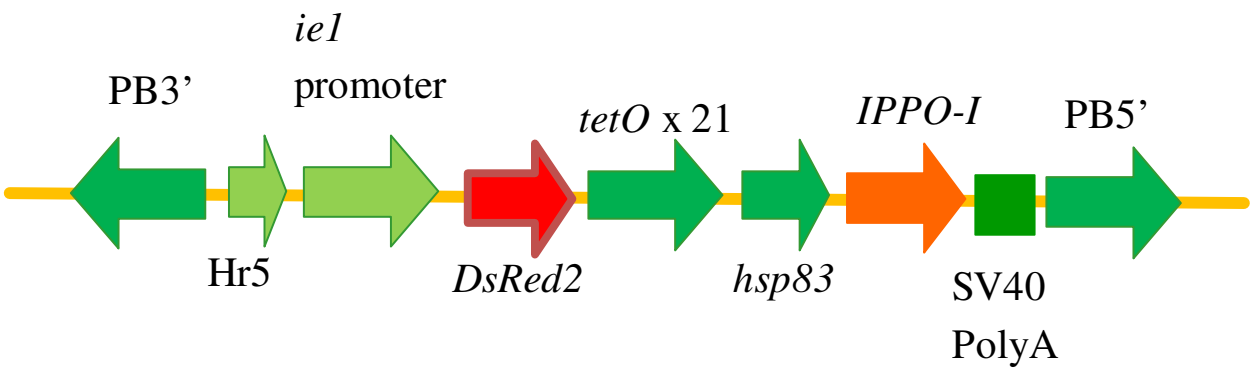


Figure 2. 7 Schematic diagram of OX4112.

PB-*IPPO-1*-hsp83-tetO21- Hr5-IE1-Red.

The OX4112 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (DsRed, a fluorescent protein, driven by the *Hr5-IE1* promoter). A *tetO* sequence present in a 21mer array (*tetO*21) drives expression of *IPPO-1* (homing endonuclease from slime mould), with an *hsp83* 5'UTR and an SV40 3'UTR. Medfly transgenics with this construct were used in section 3.3.1, 3.3.2, 3.3.3, 3.3.10 and 3.3.11.

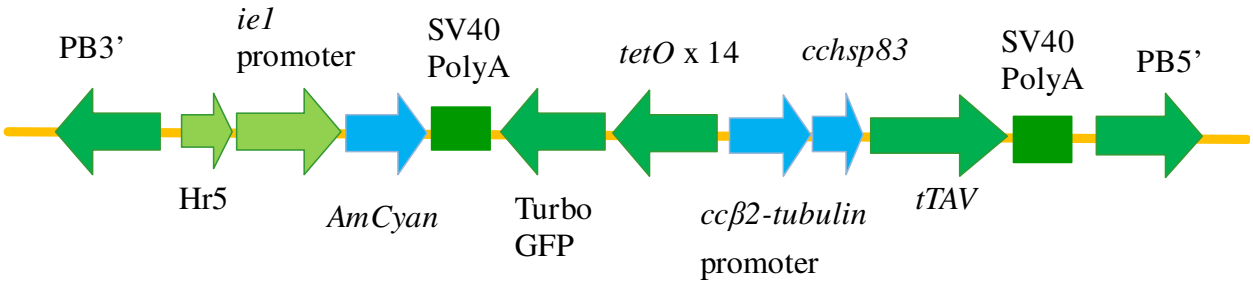


Figure 2. 8 Schematic diagram of OX4282.

PB-*Hr5IE*-AmCyan-SV40-*TurboGFP-tetO*14-*ccTubulin-hsp83-tTAV*-SV40.

The OX4282 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter /enhancer sequence (from baculovirus)). The *C. capitata β2tubulin* promoter *(ccβ2tubulin)* drives expression of *tTAV* with an *hsp83* 5'UTR and an SV40 3'UTR. A *tetO* sequence present in a 14mer array (*tetO*14) drives expression of the Turbo GFP with an *hsp83* 5'UTR, and an SV40 3'UTR. Medfly transgenics with this construct were used in section 3.3.1, 3.3.4 and 3.3.7.

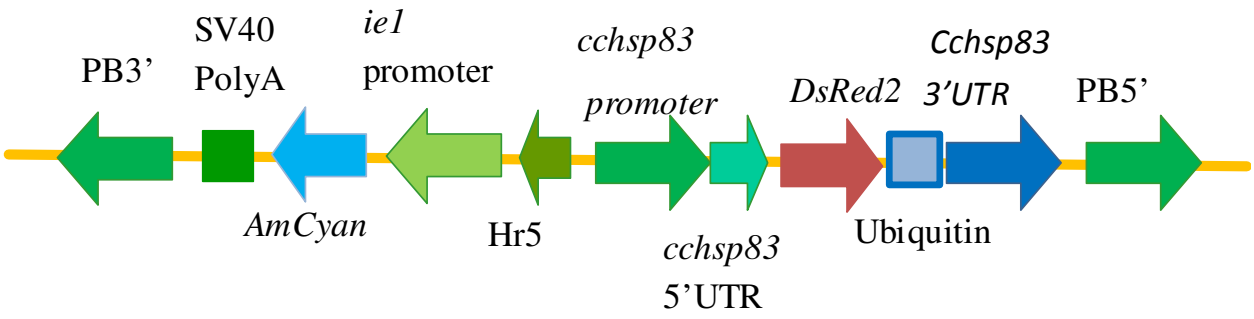


Figure 2. 9 Schematic diagram of OX4324.

PB-cchsp83-DsRed –cchsp83-3'UTR- Hr5-IE1-AmCyan

The OX4324 construct is a PiggyBac vector (PB) containing a dominant selectable transformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter). The *C. capitata hsp83* promoter combined with *cchsp83* 5'UTR drives expression of DsRed with a *C. capitata hsp83* 3'UTR. Medfly transgenics with this construct were used in section 6.3.1, 6.3.2 and 6.3.3

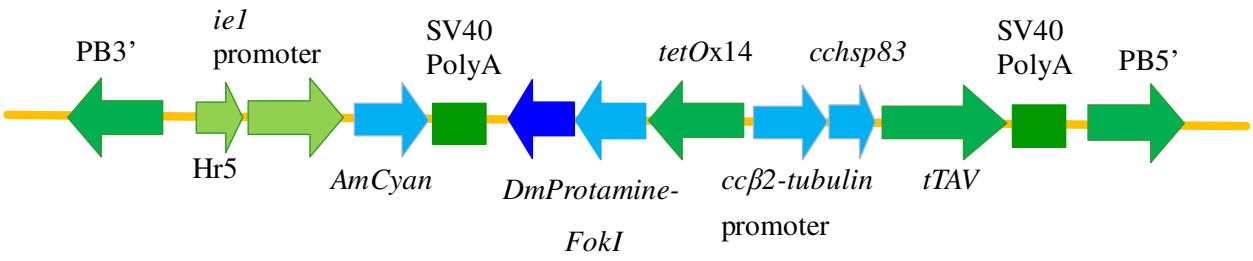


Figure 2. 10 Schematic diagram of OX4353.

PB-Hr5-IE1-AmCyan-SV40-teto14-DmProtamine-nuclease-ccTubulin-hsp83-tTAV-SV40.

The OX4353 construct is a PiggyBac vector (PB) containing a dominant selectable transformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter). A *tetO* sequence present in a 14mer array (*tetOx14)* drives expression of DmProtamine-nuclease (dProtB chain from *D. melanogaster)*, with an *hsp70* 5' UTR and SV40 3' UTR. The *cc-β2tubulin* drives expression of tTAV with an *hsp83* 5'UTR and an SV40 3'UTR. Medfly transgenics with this construct were used in section 4.3.1, 4.3.2, 4.3.3, 4.3.4, 4.3.5 and 4.3.6.

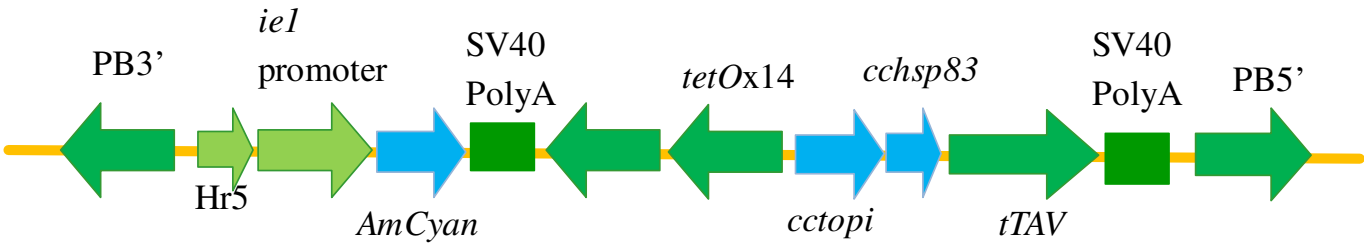


Figure 2. 11 Schematic diagram of OX4371.

PB- Hr5-IE1-AmCyan-SV40-*TurboGFP*-teto14-*cctopi-tTAV*-SV40.

The OX4371 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter). A *tetO* sequence present in a 14mer array (*tetOx*14) drives expression of *turboGFP*, with an *hsp83* 5'UTR and an SV40 3'UTR .The *C. capitata topi* promoter (*cctopi*) drives expression of the *tTAV* with an *hsp83* 5'UTR and an SV40 3'UTR. Medfly transgenics with this construct were used in section 3.3.2 and 3.3.5.

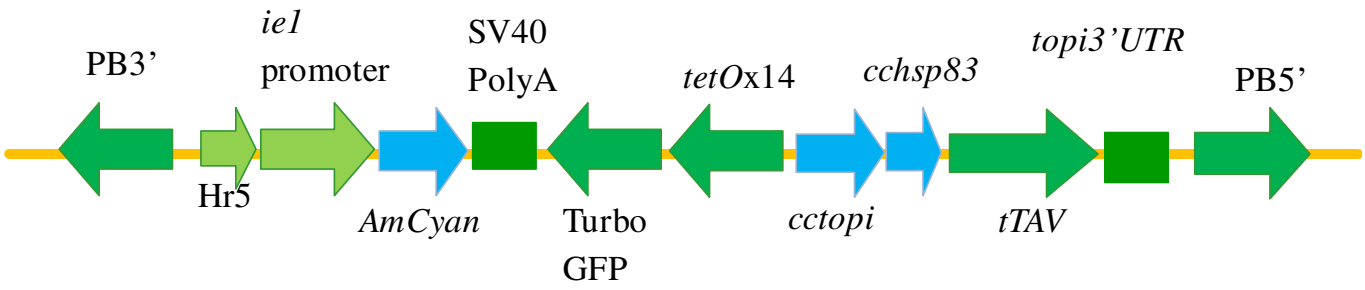


Figure 2. 12 Schematic diagram of OX4391.

PB- Hr5-IE1-AmCyan-SV40-*TurboGFP*-*tetO*14-*topi*-ubi-*tTAV*-*topi*3'UTR.

The OX4391 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter). A *tetO* sequence present in a 14mer array (*tetOx*14) drives expression of *turboGFP*, with an *hsp83* 5'UTR and an SV40 3'UTR. *C. capitata topi* drives expression of *tTAV* with an *hsp83* 5'UTR (heat shock protein 83) and a *topi* 3'UTR. Medfly transgenics with this construct were used in section 3.3.3, 3.3.6 and 3.3.8.

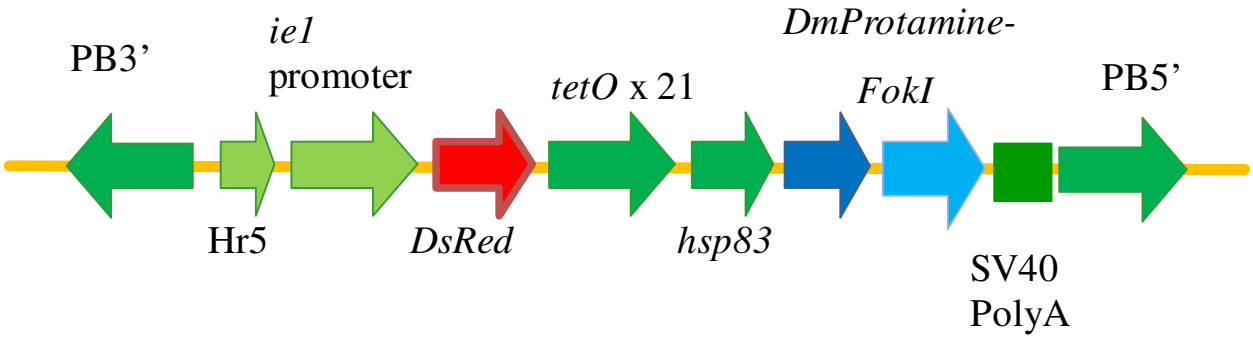


Figure 2. 13 Schematic diagram of OX4458.

PB- *DmProtamine-FokI-nuclease* -hsp83-tetO21-Hr5-IE1-Red.

The OX4458 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (DsRed, a fluorescent protein, driven by the *Hr5-IE1* promoter). A *tetO* sequence present in a 21mer array (*tetO*21) drives expression of *Dm-ProtamineFokI* (*FokI* is from *Flavobacterium okeanokoites* fused to *D. melanogaster's Protamine B* (dProtB chain)), with an *hsp83* 5'UTR and an SV40 3'UTR. Medfly transgenics with this construct were discussed in section 3.4.

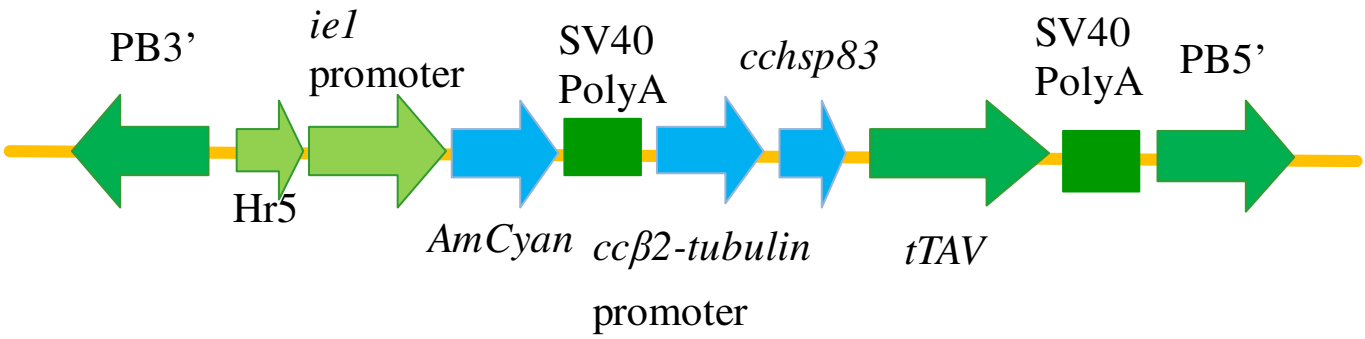


Figure 2. 14 Schematic diagram of OX4483.

PB-*Hr51E1*-AmCyan-SV40-*ccTubulin-hsp83*-tTAV-SV40

The OX4483 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter). The *C. capitataβ2-tubulin* promoter drives expression of *tTAV* with an *hsp83* 5'UTR (heat shock protein 83) and an SV40 3'UTR (Simian vacuolating virus). Medfly transgenics with this construct were used in section 3.3.9, 3.3.10, 3.3.12 and 3.3.14.

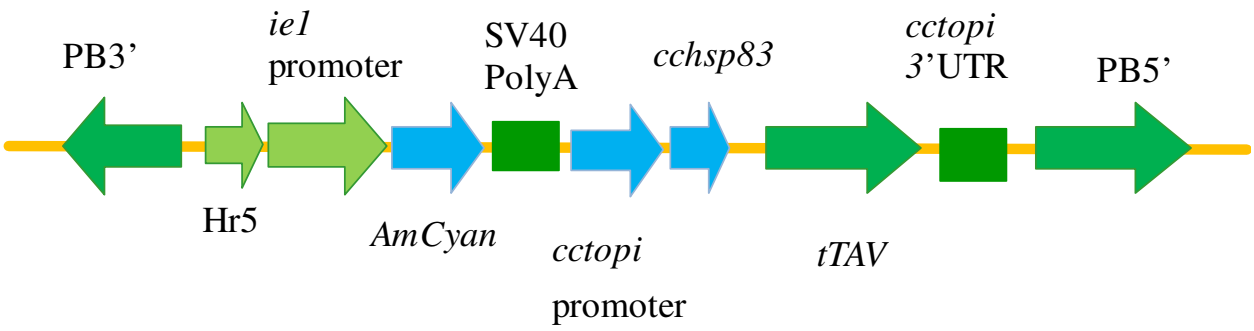


Figure 2. 15 Schematic diagram of OX4485.

PB-*Hr5-IE1*-AmCyan-SV40-*topi*-ubi-tTAV-*topi*:3'UTR

The OX4485 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter). *C. capitata topi* promoter drives expression of *tTAV* with an *hsp83* 5'UTR (heat shock protein 83) and a *topi* 3'UTR. Medfly transgenics with this construct were used in section 3.3.9, 3.3.11, 3.3.13 and 3.3.15.

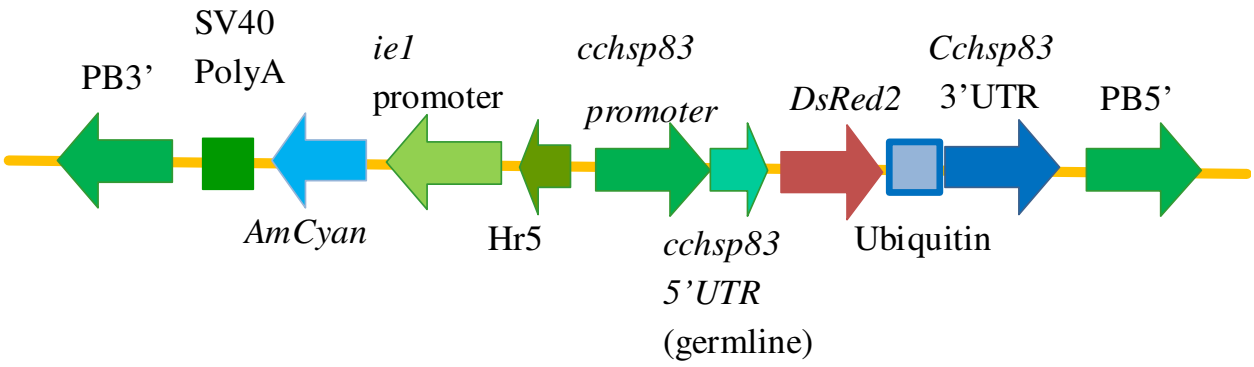


Figure 2. 16 Schematic diagram of OX4642.

PB-mutated cchsp83-DsRed –cchsp83-3'UTR- Hr5-IE1-AmCyan

The OX4642 construct is a PiggyBac vector (PB) containing a dominant selectable transformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter). The *C. capitata hsp83* promoter combined with *cchsp83* 5'UTR from germline drives expression of DsRed with a *C. capitata hsp83* 3'UTR. The 5'UTR was mutated to delete the somatic TATA-box (promoter = 2599 bp, germline 5'UTR 554 + intron = 281 bp). Presence of ubiquitin here should result in post-translational separation of two protein domains which will be separated by ubiquitin protease, leading the translation of red fluorescence protein only in germline cells. Medfly transgenics with this construct were used in section 6.3.1, 6.3.2 and 6.3.3.

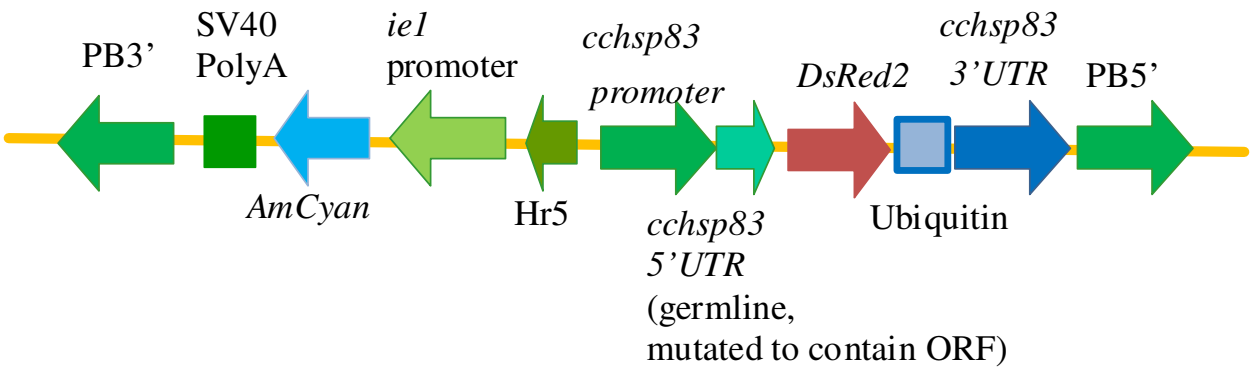


Figure 2. 17 Schematic diagram of OX4656.

PB- cchsp83-promoter-cchsp83 germline 5'UTR (mutated to contain ORF) DsRed –cchsp833'UTR-Hr5-IE1-AmCyan

The OX4656 construct is a PiggyBac vector (PB) containing a dominant selectable transformation marker (AmCyan, a fluorescent protein, driven by the *Hr5-IE1* promoter). The *C. capitata hsp83* containing *hsp83* germline 5'UTR drives expression of DsRed with a *C. capitata hsp83* 3'UTR. To delete the somatic transcript without deleting its 5'UTR (which is part of the germline 5'UTR), a couple of point mutations to open the reading frame even before the transcription start for the somatic transcript was made and a KOZAK start was inserted to enable the translation from upstream to the somatic 5'UTR (promoter = 1153 bp, 5'UTR = 552 bp, intron = 281 bp and part of exon 2 =134 bp). The new ORF is different from the original OFR for the endogenous Cchsp83-protein. The start codon for the endogenous Cchsp83 is immediately after the intron in the 5'UTR, deleting or mutating it might affect the splicing of that intron. ATG-start untouched but is out of frame compared to the new ORF i.e. is not going to translate DsRed. Presence of ubiquitin here should result in post-translational separation of two protein domains which will be separated by ubiquitin protease, leading the translation of red fluorescence protein only in germline cells. Medfly transgenics with this construct were used in section 6.3.1, 6.3.2 and 6.3.3.

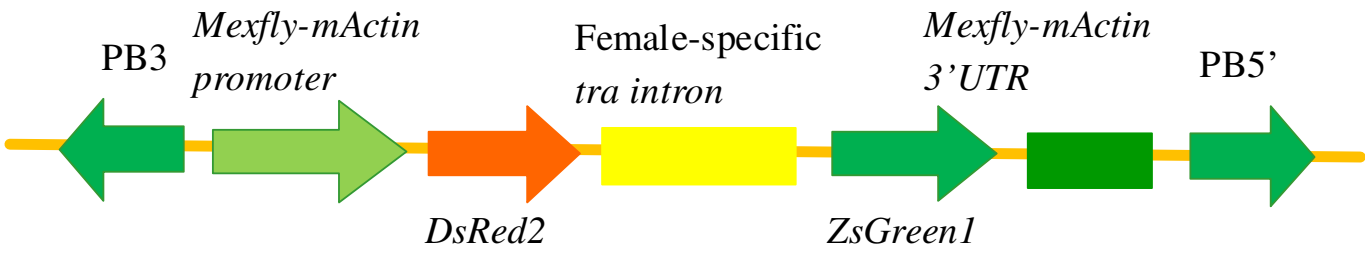


Figure 2. 18 Schematic diagram of OX4676.

PB-mActin-Dsred2-tra intron-ZsGreen-mActin3'UTR-PB

The OX4676 construct is a PiggyBac vector (PB) containing a dominant selectable transformation marker (DsRed2, a fluorescent protein, driven by the Mexfly muscle Actin). mActin drives expression of a transcript including coding regions for Dsred2 and Zs green. The first intron of *tra* gene from olive fly is included in this construct between the two fluorescent protein coding regions. In females the first intron will be spliced out, producing an mRNA that encodes a fusion protein of DsRed2 and ZsGreen.

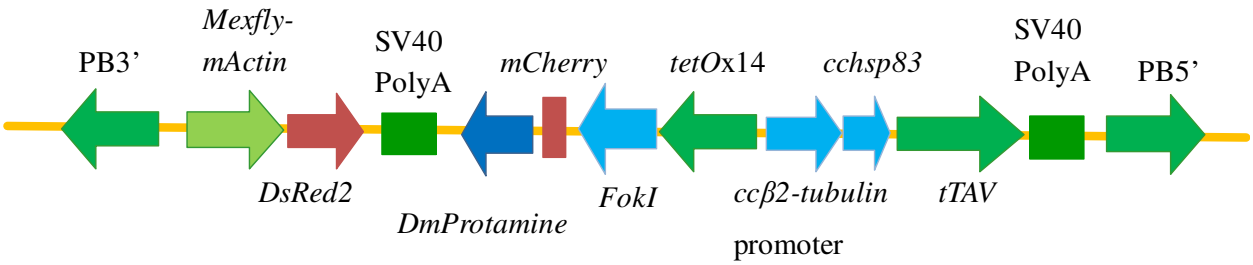


Figure 2. 19 Schematic diagram of OX4705.

PB-Mexfly-mActin-DsRed-SV40-DmProtamine-mCherry-nuclease-teto14-ccTubulin-hsp83-tTAV-SV40PB.

The OX4705 construct is a PiggyBac vector (PB) containing a dominant selectable transformation marker (DsRed, a fluorescent protein, driven by the muscle actin promoter (from the Mexican fruit fly)). A *tetO* sequence present in a 14mer array (*teto14)* drives the expression of DmProtamine-mCherry-FokI (dProtB chain from *D. melanogaster*'s Protamine B*)*, with an *hsp83* 5'UTR and an SV40 3' UTR. The *cc-β2tubulin* promoter drives expression of tTAV with an *hsp83* 5'UTR and an SV40 3'UTR. Olive fly transgenics with this construct were used in section 5.3.1, 5.3.2, 5.3.3 and 5.3.4.

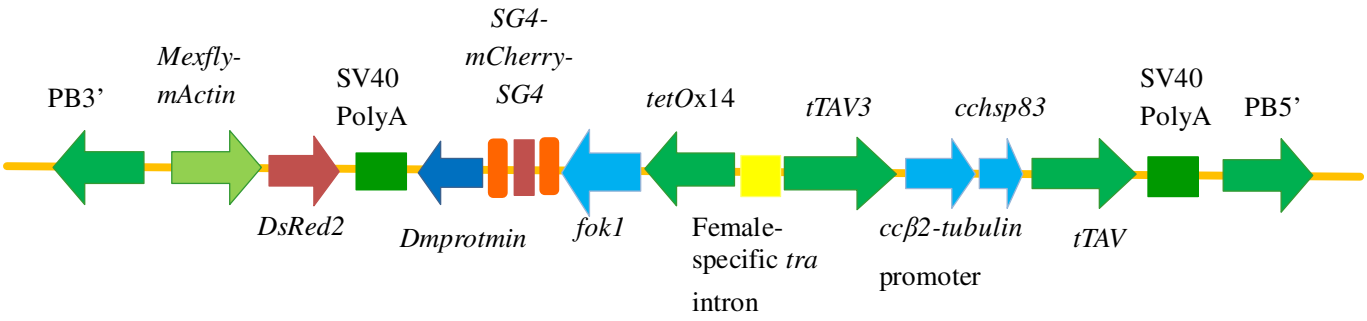


Figure 2. 20 Schematic diagram of OX4751.

PB-Mexfly-mActin-DsRed-SV40- DmProtamine-mcherry-nuclease- teto14-ccTubulin-hsp83-tTAV-SV40PB.

The OX4751 construct is a PiggyBac vector (PB) containing a dominant selectable transformation marker (DsRed, a fluorescent protein, driven by the muscle actin promoter (from the Mexican fruit fly)). A *tetO* sequence present in a 14mer array (*teto14)* drives the expression of DmProtamine-mCherry-FokI (dProtB chain from *D. melanogaster*'s Protamine B*)*, with an *hsp83* 5'UTR and an SV40 3' UTR. The *cc-β2tubulin* promoter drives expression of tTAV with an *hsp83* 5'UTR and an SV40 3'UTR. It also has the inclusion of female specific *tra* intron and extra sequence of *tTAV*. Medfly transgenics with this construct were used in section 4.3.7, 4.3.8, 4.3.9, and 4.3.10.

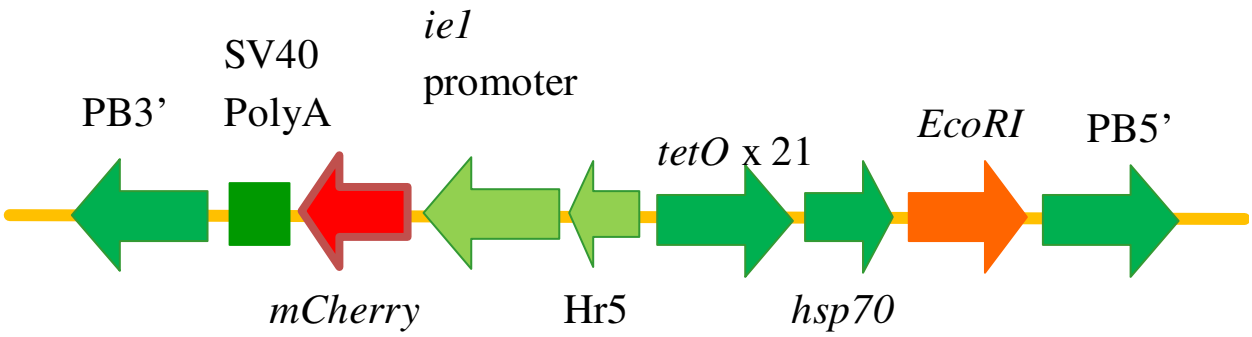


Figure 2. 21 Schematic diagram of OX4763.

PB-*EcoRI*-mCherry-hsp83-tetO21-Hr5-IE1-Red.

The OX4763 construct is a PiggyBac vector (PB) containing a dominant selectable tranformation marker (mCherry, a fluorescent protein, driven by the *Hr5-IE1* promoter). A *tetO* sequence present in a 21mer array (*tetO*21) drives expression of *Eco*R*I* (from *Escherichia coli*), with an *hsp70* 5'UTR and an SV40 3'UTR. Medfly transgenics with this construct were used in section 3.3.7, 3.3.8, 3.3.14 and 3.3.15.

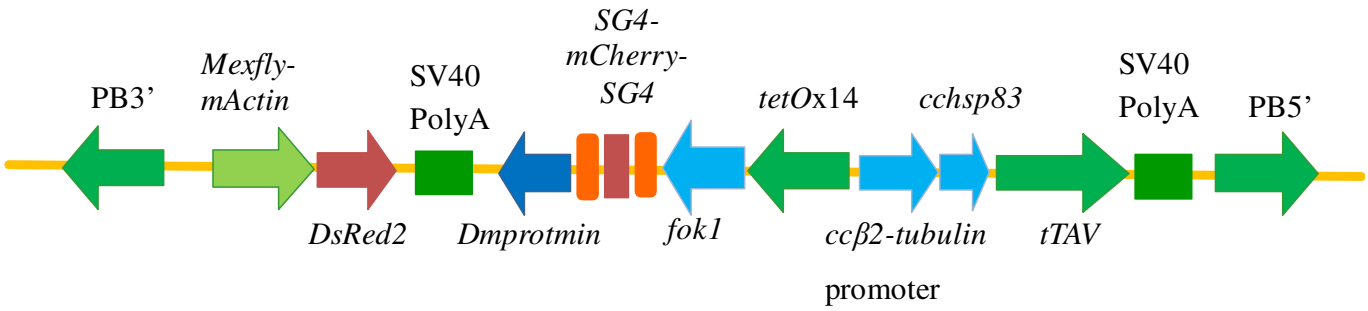


Figure 2. 22 Schematic diagram of OX4801.

PB-Mexfly-mActin-DsRed-SV40-DmProtamine-SG4-mCherry-SG4-nuclease-teto14-ccTubulin-hsp83-tTAV-SV40PB.

The OX4801 construct is a PiggyBac vector (PB) containing a dominant selectable transformation marker (DsRed, a fluorescent protein, driven by the muscle actin promoter (from the Mexican fruit fly)). A *tetO* sequence present in a 14mer array (*teto14)* drives the expression of DmProtamine-mCherry-FokI (dProtB chain from *D. melanogaster*'s Protamine B*)*, with an *hsp83* 5'UTR and an SV40 3' UTR. The *cc-β2tubulin* promoter drives expression of tTAV with an *hsp83* 5'UTR and an SV40 3'UTR. It also has the inclusion of two short peptide linkers (SG4) flanking the mCherry coding sequence. Olive fly transgenics with this construct were used in section 5.3.5, 5.3.6 and 5.3.7.

2.9. Primer sequences

Table 2.1 below shows primer sets and their sequences that have been used in this thesis.

Table 2. 1 List of primer sequences.

Primer name			Primer sequence
OX4353	cctra2	Forward	GGCCGATCTCGTGGCTTTTGC
		Reverse	GTGGCGAATAGGAACGACTACGG
	tTAV	Forward	ACGGTGCCAAAGTCCATCTC
		Reverse	CGATGGTGCTGCCGTAGTTG
	Protamine-fok1	Forward	GAGTCAGAAGTGCAGCAAGCAG
		Reverse	CTCCAGGATGCGGTCCTGGGTG
OX4705	ADH	Forward	CGTTTGGGCTTCATCGGTTACG
		Reverse	GCGTCTTGGTGTTGTTGAGCTTATC
	tTAV	Forward	ACGGTGCCAAAGTCCATCTC
		Reverse	GGCATCGAATCGGTTGTTGGGGTC
	Protamine-mCherry	Forward	GAGTCAGAAGTGCAGCAAGCAG
		Reverse	ACGCGGTCTCTGTGTCCAAGGGCGAAGAGGA
OX4324	hsp83 5'UTR-DsRed2	Forward	CACACTCATCGGGTAGTCATGAC
		Reverse	ACACTTTGTGTCATCATGTAGGAGG
OX4642	hsp83 5'UTR-DsRed2	Forward	CTCGATCTCGAACTCGTGGC
		Reverse	TCCTACATGATGACACAAAGTGTCAC
OX4656	hsp83 5'UTR-DsRed2	Forward	CGGAGCAACAAAGAAAGTGTTC
		Reverse	GGATGCCCTCCTTGTCCTGG
OX4676	mActin-DsRed2	Forward	CCATGGTCTTCTTCTGCATCAC
		Reverse	TGTGTGACGATGAGGTTGCTG
Y-chromosome	medfly	Forward	GTTGATACTAGTGATTGTAAATAGTAAAGTGC
		Reverse	GCGTCAGTGTAGCGTGAAGACG
OX3864	flaFRT	Forward	GAACTTCTTCGAACGGGAGTAG
		Reverse	GCCAGTTCGGTTATGAGCCGTGTAC
Y-chromosome	Olive fly	Forward	CACAAAGAGCTGGTGCAGTC
		Reverse	CTGCCAGCTGAGGTAGTACG
OX3097	K10	Forward	CACTTAAGCGACAAGTTTGGCCAAC
		Reverse	CCAGAGCGATACAGAAGAAGC

Chapter 3: Identifying a suitable promoter and effector combination to engineer paternal effect lethality in Tephritidae

3.1. Introduction

In principle the current strategy of RIDL provides a considerable advantage for SIT implementation. I aim to replace radiation, which is at present by far the most widely used method of sterilization. By reducing or eliminating the production of viable larvae in the field, inclusion of genetic sterilization may help gain the regulatory and public acceptance of transgene-enhanced derivatives of SIT, relative to Oxitec's current female specific lethal strains.

While the primary damage to crops by Tephritids fruit flies is caused by oviposition, larval feeding causes additional and highly visible damage. A dominant lethal system leading to death of offspring prior to larval hatching would eliminate damage from larval feeding, though not oviposition damage. The specific route chosen here to achieve this benefit was to develop a system for conditional damaging of sperm DNA, thereby producing infertile males, and ultimately to produce paternal effect lethality strains for potential field use.

In this version of RIDL, a gene expressed in the male germline will result in males producing non-functional sperm. Due to the early acting paternal effect lethality, fully-penetrant paternal effect lethality strains will produce no viable offspring. In this new system, it is desirable that certain normal sperm functions are retained (such as motility) in order to allow them to reach the spermatheca. The frequency of re-mating of females with fewer sperm in their spermathecae is greater than those with full spermathecae (Taylor et al. 2001). Efficient mating reduces female interests to re-mating in many insects (Chapman et al. 1998). Irradiated male medfly have an impaired ability to decrease the interest of a female in further

mating, compared to non-irradiated male medfly (Kraaijeveld & Chapman 2004). A female mated to an irradiated male will additionally favour a wild male rather than another irradiated male for re-mating. Both of these aspects can diminish the effectiveness of SIT.

Increased re-mating rates in females mated to irradiated males, further compounds the reduced competitiveness of the males by increasing sperm competition. Tephritid females can mate several times, storing and simultaneously preserving viable ejaculates from multiple males. SIT success depends on the transfer and fertilisation of eggs by sterile sperm, thus reduced sperm competitivity may be a key issue concerning sterile strain effectiveness.

In an ideal system the males would be able to mate with and inseminate the females, but eggs fertilised by such sperm should not be capable of completing embryonic development and hatching as larvae. This system should not affect the male's mating ability. If sterile males are not able to reduce receptivity of female to re-mating (i.e. induce appropriate levels of refractoriness to re-mating), the females may look for another mate (Lux et al. 2002). Although sperm of such a fly should be capable of delivering its genetic material to the eggs, it will consist of damaged DNA which will not be capable of fertilisation or alternatively it will cause lethality in the earliest embryonic stage.

One key factor in SIT studies is to evaluate the level of sterility required to get population suppression. A high level of sterility facilitates a low ratio of release of sterile insects to achieve suppression and vice versa (Klassen & Creech 1971).

Partially sterile insects can be used for SIT. As radiation is generally harmful to insects - it impairs their fitness, mating competitiveness and fecundity, radiation to a level sufficient to cause full sterility is not always compatible with sufficient viability and vitality for release programmes (see section 1.3.). In previous studies a lower dose radiation was considered to reduce the adverse effect of radiation. A radiation-induced mutation was observed in

irradiated Drosophila using a lower dose of radiation. That was a dominant lethal gene which would be passed on to offspring was in partially sterile Drosophila (irradiated Drosophila) (Curtis 1968). This is result in reduction in number of eggs that hatched and the offspring are highly sterile.

The partial sterility achieves by treating insects with different doses of radiation (Knipling 1970). Study of application of partially sterile insects indicated that the number of release reduces as the over flooding ratio of the sterile-to-wild decreases by ¼ of what is normally essential for completely sterile insects (Knipling 1970). Several other mathematical models studies on partial sterility of pest insects confirmed very similar results to Knipling's findings (Bloem & Carpenter 2001). The field study of partially sterile insects, also presented that the idea of using partially sterile insects is feasible (Carpenter et al. 2005). A lower dose of radiation will inducing sterility of offspring and improve the fitness and competitiveness of the released insects such as dispersal, increased mating ability, and sperm competition. The degree of sterility of partially sterile insects depends on the dose of radiation; the dose response varies between species. Different orders of insects show variety of response to radiation. Diptera, Hymenoptera, and Coleoptera orders are classed as radiation-sensitive, while Lepidoptera, Homoptera and Acari orders are classed as radiation-resistant (Klassen 2005). The difference in molecular repair mechanisms of these orders are responsible for the high radio-resistance in Lepidoptera (LaChance & Graham 1984). While studies on incompletely sterile insects demonstrated that population suppression is achievable even with partial sterility, I aim to develop a strain in an insect of interest that is 100% sterile. This has several practical advantages. Firstly it should suppress the targeted population with fewer releases, and thus make the project cost efficient. Secondly, it will avoid the problem from partial sterility of larvae developing inside fruit and making the fruit unsellable. Thirdly,

acceptance from regulatory bodies and the public is likely to be easier to obtain if there is no residual fertility of the released males.

To make an insect paternal effect lethal strain, a sperm-damaging protein (effector) is needed to be expressed in the male germline of the insect of interest. Ideally the expression will be specific to the male germline, to avoid damage to other tissues of the targeted species. I have chosen to generate sperm damage by directly damaging sperm DNA. DNA damage in other tissues of the insect of interest is likely to impair the fitness and mating capability. The effect on fitness and mating capability depends both on the level of expression of the effector, and on the location(s) of its expression. Therefore, the aim of the first part of this project is to identify the best combination between a promoter and an effector that confers male germline specificity without compromising the mating capability of the males carrying both the transgene insertions. In this chapter, medfly was used as a model organism.

3.2. Experimental design

3.2.1. Promoters

In this project flies carrying two different promoter sequences (derived from medfly homologues of the *D. melanogaster* genes *topi* and *β2-tubulin*) were used to drive expression of *tTAV*. Several constructs with *topi* or *β2-tubulin* promoters driving tTAV as their main component were designed. OX4282, OX4371 and OX4391 had *TurboGFP* (TurboGFP is an improved variant of the green fluorescent protein CopGFP cloned from copepod *Pontellina plumata* (text taken from Evrogen website)) under the direct control of tetOx14. In these constructs *tetO* sequence was cloned head to head to the *topi* and *β2-tubulin*-promoter-*tTAV* component. The aim was to allow for easy monitoring of the promoter activity in testis. In the absence of tetracycline in the larval diet, *tTAV* expressed in spermatocytes should bind to *tetO* and induce expression of the *TurboGFP* marker. The testes of males carrying this construct should show the *TurboGFP* expression when reared off tetracycline, and the *TurboGFP* expression should be repressed by addition of tetracycline in the larval diet. However, *tetO* acts as a bidirectional promoter, and so might enhance further expression of *tTAV* via a positive feedback loop. This effect has previously been reported in the RIDL female specific lethal strains (Gong et al. 2005). (See Figure 2.8 for the OX4282 (*β2-tubulin* based construct), 2.11 for the OX4371 (*topi* based construct) and 2.12 for the OX4391 (*topi* based construct)).

OX4483 (*β2-tubulin* based construct, Figure 2.14) and OX4485 (*topi* based construct, Figure 2.15), have only the *topi* or *β2-tubulin*-promoter-*tTAV* component, and lack the tetO binding site for tTAV. Therefore there is no possibility for enhancement of expression via positive feedback in these constructs.

3.2.2. Effectors

Nucleases are enzymes which are capable of cleaving the phosphodiester bond between DNA or RNA base pairs. Sequence-specific DNA endonucleases have a specific DNA recognition site which can be anything from 4-45 nucleotides long. Nucleases were first isolated in the 1960s by Stuart Linn and Werner Arber, who discovered the two enzymes responsible for bacteriophage growth restriction in Escherichia coli (Linn et al. 1968). One of the enzymes was a DNA methylase, while the other was a restriction nuclease that cleaved un-methylated DNA at various locations along the DNA. Nucleases can be of two types: endonucleases and exonucleases although some nucleases may have both properties. Endonucleases cut the bonds of the DNA from within the molecule. A free 3' or 5' hydroxyl group at the end of the polynucleotide chain is not needed for their activation, thus they break down a nucleotide chain into two or more shorter chains (oligonucleotides). Exonucleases cut at the ends of the DNA molecule and a free 3' or 5' hydroxyl group at the end of the polynucleotide chain is required in order for them to be activated, therefore shorter nucleotide chains are formed (nucleosides) (Beumer et al. 2006).

The preferred nucleases for the purpose of this project are endonucleases with short recognition sequences, and thus high frequency target sites, which damage DNA in multiple locations. Dimerisation is another important key feature in nuclease selection. A monomeric enzyme will generally work at very low concentrations as its activity depends linearly on concentration. On the other hand, a dimeric enzyme's function has a non-linear response to concentration; at low concentrations the probability of two molecules binding each other, or both at the same target site, is low, and so they essentially cannot form a dimer to cut DNA. At high concentrations the probability of dimer formation is much higher. The non-linear

response to concentration provides protection against the leaky expression of effectors in non-target tissues. Here we tested four different nuclease enzymes.

3.2.2.1. I-PpoI

Originating from the slime mould *Physarum polycephalum*, *I-PpoI* was originally found in the large-subunit of the ribosomal DNA. *I-PpoI* is a homing endonuclease that recognizes a 13- to 15-bp DNA sequence in a portion of the large-subunit rRNA gene. Homing endonucleases coding sequences are implanted in introns or intein and the DNA double-strand break initiates in a site-specific manner in the intronless or intein less alleles. This causes generation of recombinogenic ends and after a gene alteration procedure, the intron or intein will be duplicated. Homing endonucleases differ from other endonucleases in structure, recognition properties, and genomic location (Windbichler et al. 2007). *I-PpoI* cleaves 120bp double stranded rDNA repeats, which in *A. gambiae* are found on chromosome X (Lin & Vogt 1998) (Figure 3.1). Previously, *I-PpoI* was designed to be expressed during spermatogenesis in *A. gambiae* to target the X chromosome of spermatozoa. This was designed to result in generating an excess of spermatozoa carrying chromosome Y, therefore the sex ratio in the targeted population would become male biased (Windbichler et al. 2007).

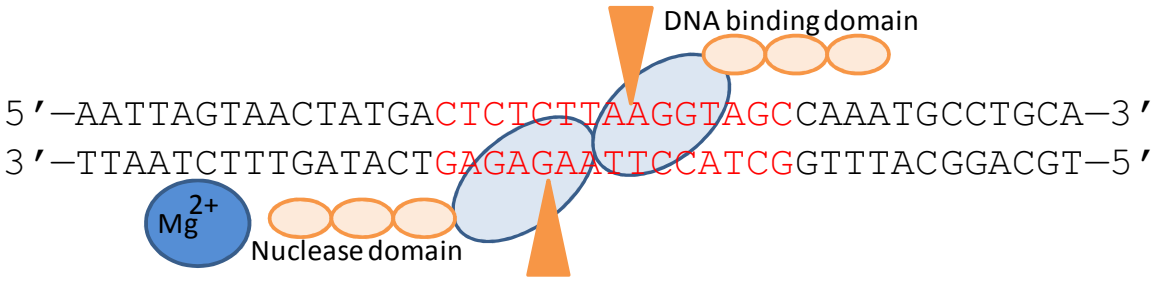


Figure 3. 1 *I-PpoI* catalyses the hydrolytic cleavage of the double stranded DNA in a specific site which results in a double strand break.

I-PpoI recognises a large and asymmetric sequence of rDNA and binds to the sequence of interest in the absence of Mg2+, but it requires Mg2+ cation as its cofactor for cleavage. *I-PpoI* consists of a DNA-binding domain which attaches to the DNA at the recognition site. The nuclease domain of *I-PpoI* cuts DNA at the recognition site. The recognition sequence (CTCTCTTAAGGTAGC) is shown in red, and the specific cleavage site of the two DNA strands is shown by orange arrowheads.

3.2.2.2. ProtamineFokI

*Fok*I endonuclease is naturally present in *Flavobacterium okeanokoites*. It contains a DNA binding domain towards the N-terminus and a DNA cleavage domain in the C-terminal region. *Fok*I cuts DNA non-specifically between nine and thirteen nucleotides downstream of the recognition site. One characteristic that makes the *Fok*I endonuclease desirable for our research is that it has activity only as a dimer. This means the cleavage does not start till the dimer forms. Another characteristic of *Fok*I endonuclease is that the DNA binding and the nuclease domains are separable, therefore the nuclease domain can be attached to heterologous DNA binding domains to make synthetic nucleases.

In this project, the nuclease domain of *Fok*I was fused to *Dm-ProtamineB*, in this design the Protamine serves as a DNA targeting domain (Figure 3.2). Protamines are small nuclear proteins which replace histones in the late haploid phase of spermatogenesis. Protamines are essential for the condensation of sperm DNA and DNA stabilization (Minczuk et al. 2008). In *D. melanogaster* there are two *protamine* genes –*protamineA* and *protamineB*, giving dProtA and dProtB proteins. The gene have evolved from a single ancestral gene by tandem duplication; their amino acid sequences are extremely similar and are functionally identical (Kanippayoor et al. 2013).

As Protamine binds to the sperm DNA in a non-sequence-specific manner at multiple locations and the *Fok*I cleavage domains have to form a dimer to become active, the proposed design provides the desired nuclease features (see section 3.2.2).

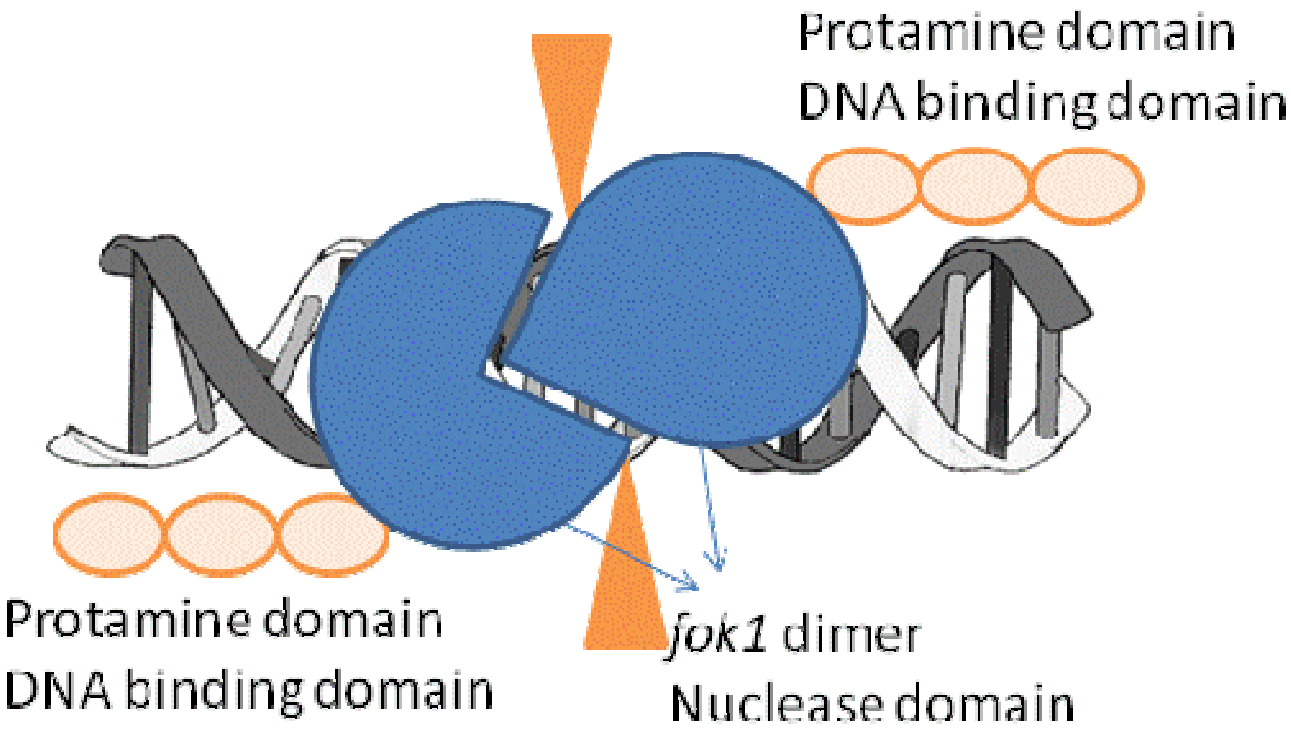


Figure 3. 2 The *Fok*I endonuclease dimerises and catalyses the cleavage of the double strand DNA where Protamine binds to DNA in a non-specific site.

Protamine binds to sperm DNA in a non-specific manner at multiple locations. *Fok*I endonuclease forms a dimer, is activated, and cleaves the DNA.

3.2.2.3. Zinc fingers nucleases

Zinc fingers (*ZnF*) are small protein motifs which chelate with one or more zinc ions in their structure to help stabilize their folds. They have 4 H (histidine) or C (cystine) resides that act to chelate a single Zn 2+ ion (per finger), in a tetrahedral arrangement to give a "finger" looping out that contacts DNA with site specificity (Beumer et al. 2006).

The 3-*ZnF* nuclease protein is an artificial restriction enzyme in which 3-*ZnF* DNA binding domains are fused to a DNA-cleavage domain (Figure 3.3). The nuclease domain used is from *Fok*I which cleaves DNA in a non-specific manner (3.2.2.2). The *ZnF* is a DNA-binding domain which directs the *Fok*I cleavage domain to a specific DNA target site (Smith et al. 2000). Different *ZnF* have different target sites, so the target site specificity can be engineered in principle by the combination of fingers used. The specific 3-*ZnF* module used here was found to have significant off-target cutting in *D. melanogaster*. I used it because it was less specific rather than more specific. The fusion protein also requires dimerisation to be able to cleave DNA since the *Fok*I cleavage domain is active only as a dimer (Smith et al. 2000).

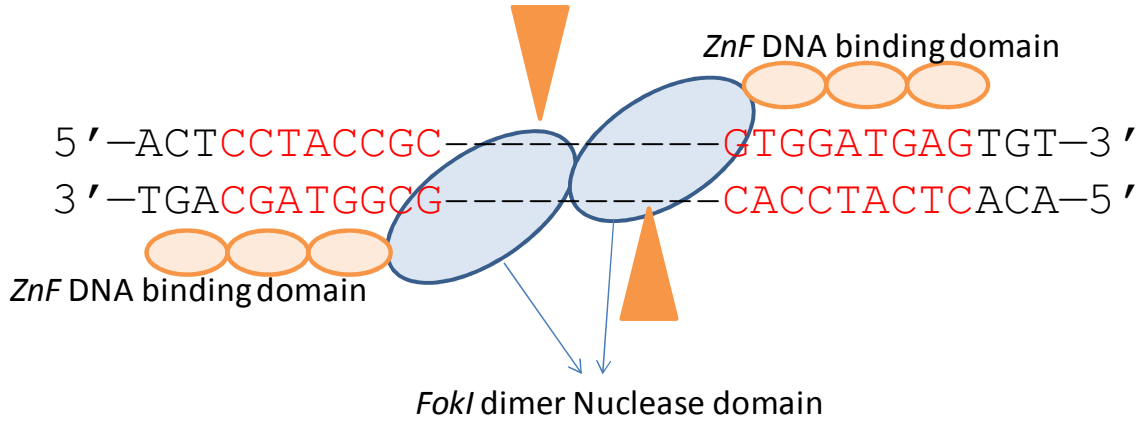


Figure 3. 3 The *ZnF* endonuclease dimerises and catalyses the cleavage of the double strand DNA at a specific site.

The middle finger of the *ZnF* DNA binding domain recognizes the DNA binding site (which can target a broad range of DNA sequences). The *ZnF* nuclease domain cleaves DNA. The recognition site of the *ZnF* nuclease is shown in red.

3.2.2.4. EcoRI

EcoRI is an endonuclease and is in the family of type II restriction enzymes. It is isolated from *E. coli* and it has a role in the restriction modification system in *E. coli*. *EcoRI* has a homodimer structure and it is highly selective in binding to specific nucleotide sequences in DNA (Figure 3.4). It has widely been used in molecular biology as a restriction enzyme for recombinant DNA technology.

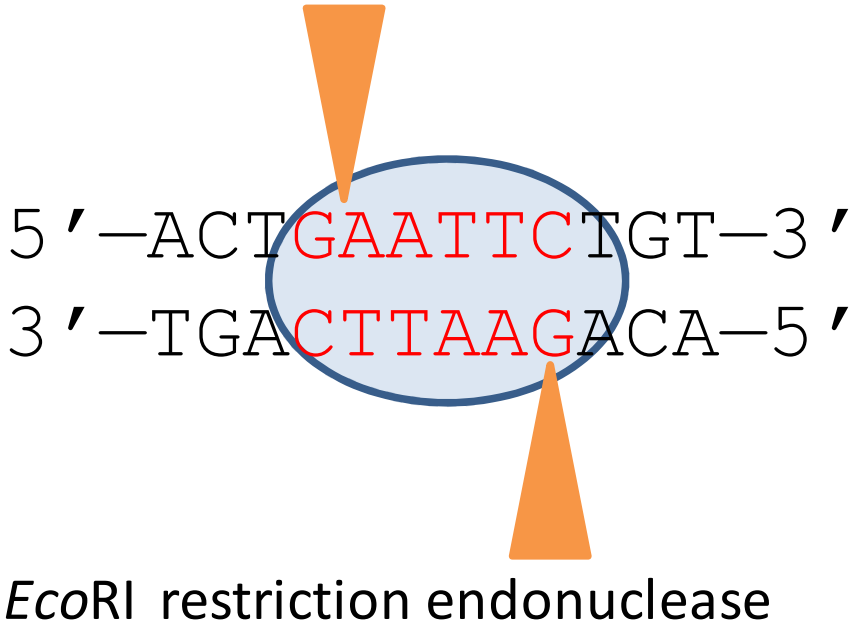


Figure 3. 4 The *EcoRI* nuclease catalyses the cleavage of the double strand DNA at a highly specific site.

It binds to DNA at the GAATTC site, cleaves DNA and produces 4 bp 3' overhangs. The recognition site of the *EcoRI* nuclease is shown in red.

Four different tetOx14-effector constructs were tested. Each of these constructs contains a different endonuclease as its active component; *I-ppoI* (Figure 2.7), *ZnF* (Figure 2.6), *ProtamineFokI* (Figure 2.13) and *Eco*R*I* (Figure 2.21).

OX4112I has the homing endonuclease, *I-ppoI*, under the control of *tetO*. In Anopheles gambiae *β2-tubulin* directly driving the expression of *I-ppoI* resulted in male sterility (Windbichler et al. 2007). *I-ppoI* selectively cleaves the ribosomal DNA (rDNA), which in *An. gambiae* is exclusively located on the X-chromosome. Cleavage of rDNA in X-chromosome bearing spermatozoa should disable these spermatozoa. The intended effect was to generate an excess of spermatozoa carrying the Y-chromosome. Progeny from these males should be all males, and therefore the sex ratio in the targeted population should become male biased. In the first attempt, the heterozygous male *An. gambiae* induced early dominant embryonic lethality in a cross with wild-type females (Windbichler et al. 2008), i.e. death of all embryos irrespective of sex. This was interpreted as that the paternal transmission of RNA or protein from *I-ppoI* attacks the maternally-derived X-chromosome in progeny which eventually cause lethality in all embryos. Galizi et al. 2014 showed that this strain of *An. gambiae* (demonstrated by Windbichler et al. 2007) is capable of suppressing caged wild-type *An. gambiae* populations.

To improve the chance of developing a strain with desired characteristics, two other effectors were tested as well with similar design. The OX4104F strain contains *tetO*-inducible *ZnF* (*3zingfinger* nuclease), an artificial restriction enzyme in which a ZnF DNA-binding domain (CCTACCGC) is fused to the nuclease domain of *FokI* as a DNA-cleavage domain. OX4763 construct has an *EcoRI* based effector under the control of *tetO*. *EcoRI* cleaves the DNA at a specific site (GAATTC), randomly distributed throughout the genome. These effectors do not

specifically target the X chromosome, so all spermatozoa should be equally affected. Figure 3.5 represents the experimental design for male fertility test.

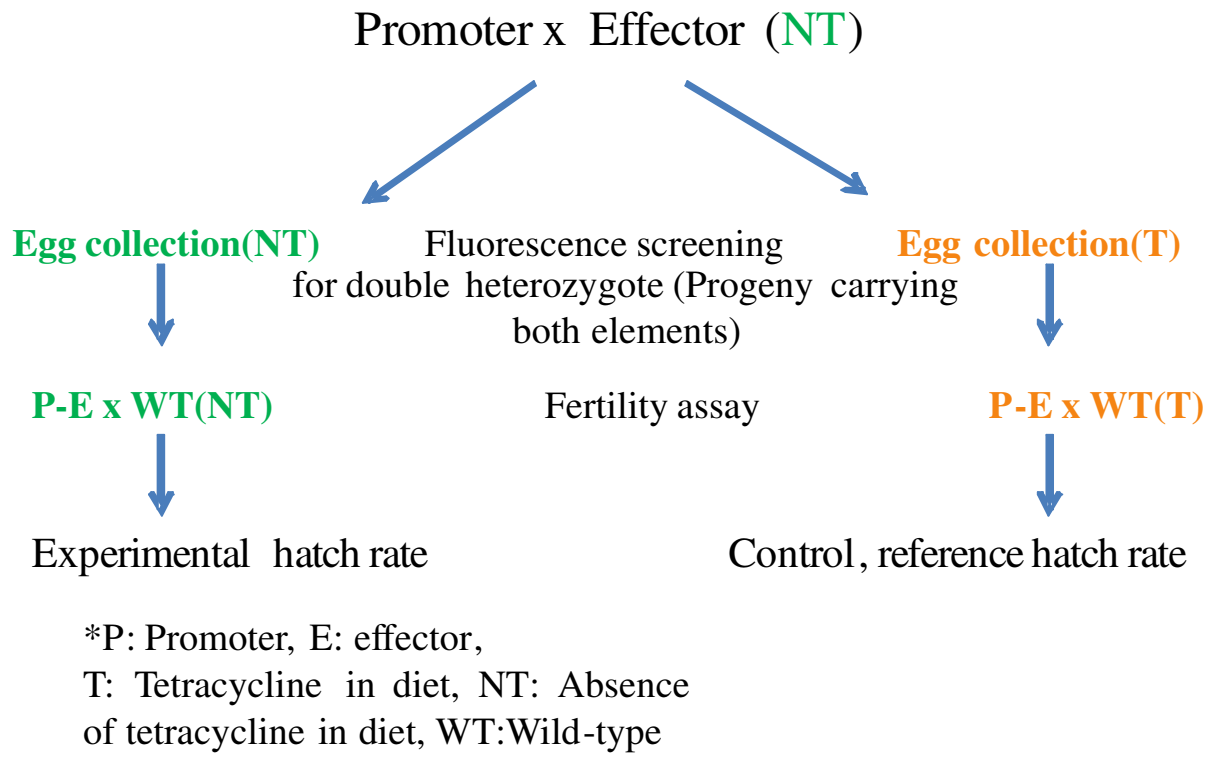


Figure 3. 5 The outline of experimental design for the "male fertility" test.

The individual strains of flies carrying either the effector or promoter constructs were reared on a diet without tetracycline (at larval stage) and then flies carrying the effector construct crossed to the flies carrying the promoter construct. Eggs from the crosses were collected and reared off and on tetracycline (in the larval diet). The male individuals that contained both transposons were crossed to wild-type females. Males with both construct, fed on a diet deficient in tetracycline (at larval stage), should be infertile. T: Flies reared on tetracycline. NT: Flies reared off-tetracycline.

In this chapter; eight different strains of medfly were generated and used. Five of these strains carry promoter constructs while four of them carry effector constructs (I tested 3 effectors and a colleague tested the other one). The prefix OX indicates that they were made at Oxitec. The construct numbers relate to the time when the constructs were generated, numbers are used sequentially, hence higher numbers represent newer constructs. The alphabetic letters were used to distinguish lines from one construct which were from different G0 pools, and thus certainly have different insertion sites. Where more than one transgene insertion was detected from a G0 cross, the two (or more) derived lines were distinguished by a further letter or number, and only maintained if they had obvious differences. The OX4282 (*β2-tubulin*) construct has three lines; G, I, L. G and L had a single autosomal insertion and I was a multiple insertion. The OX4373 (*topi*) construct has two single copy autosomal insertion lines (B and E). The OX4391 (*topi*) construct; there are 5 single autosomal insertions: B, C, D, G and H, the OX4483 (*β2-tubulin*) construct has one Y-chromosome insertion (line A) and four single autosomal insertions (lines D, E, I, Q). The OX4485 (*topi*) construct has four single copy autosomal insertion lines (Da, Db, Ja, Jb), while line E has a single copy inserted in the Y-chromosome. Only one (single copy autosomal) insertion of OX4112I (*I-ppoI)* was available. Similarly, OX4014 (*ZnF*) strain has only one line with a single autosomal copy. The OX4763 (*EcoRI*) construct has two lines; D3 and D4, both of which are single autosomal insertion. Some strains (OX4112 and OX4763) have deleterious effects on the fitness of the flies. Extra care was applied to rearing of these strains such as keeping them in a bigger population, collecting eggs more than once and also collecting more progeny from them. All the strains that were used here were maintained as heterozygous lines, through selection for transgenics at every generation.

To determine the number of insertions in each strain; the number of wild-type and transgenic progeny (at pupal stage) were counted. If there is only one copy of transgene then the number

of wild-type and transgenic will be equal. If there is more than one insertion the number of transgenics will be higher unless there are two closely linked insertions. If the number of transgenic progeny is lower than their non-transgenic siblings, it likely the transgene insertion or the insertion site that is the cause of the reduced fitness has a deleterious effect on the fitness of the flies.

3.3. Results and discussion

Each promoter line was crossed to each effector line. Offspring were reared either on a normal diet (“off-tetracycline” or “off-tet”) or on a diet supplemented with tetracycline (“on-tet”) to a final concentration of 100 ng/ µl. Heterozygous flies from the promoter lines and the effector lines were crossed. 25% of the progeny should have both inserts, and be sterile in the absence of tetracycline in the larval diet. The double- heterozygous transgenic progeny were identified based on the presence of the characteristic fluorescence marker of both constructs. Double- heterozygotes male transgenic progeny were crossed to wild-type females. The male fertility was assessed.

The expression of the effector should be repressed by the addition of tetracycline (Figure3.5). The male and female fertility tests which were performed in this chapter relied on crosses of multiple flies, with twice as many females as males in each cross. Furthermore, the cages were observed, to see whether the males attempted to mate.

3.3.1. The combination of *β2tubulin-tTAV* (OX4282) with the effectors (*tetO-I-ppoI* (OX4112I), *tetO-ZnF* (OX4104F) and *tetO-EcoRI* (OX4763)) has limited effects on male fertility

OX4282 has the medfly *β2-tubulin* driving expression of *tTAV* in the testes of the targeted fly. Eight female flies from each OX4282 strain (three independent insertion lines: of OX4282G, OX4282I, and OX4282L) were crossed to four OX4112I male flies. Similarly, they were crossed to OX4104F and to OX4763D3 and OX4763D4. The progeny of these crosses were screened at the pupal stage and those containing both constructs were isolated. The numbers of: a) wild-type; b) transgenic carrying the OX4282 construct (AmCyan marker); c) transgenic carrying the OX4112, OX4104 or OX4763 constructs (DsRed2 marker); and, d) transgenic carrying both constructs (AmCyan and DsRed2 markers) were counted. This would reveal if there were any observable detrimental effects of carrying any individual construct or both constructs on fly viability. Reduction in viability may be a result of endogenous coding sequence disruption at the genomic locus of transposition (insertional mutagenesis, although note that all the flies were heterozygous for the insertions and most insertional mutagenesis defects will be recessive), and/or through the expression of deleterious transgene products. Approximately 200 progeny were counted for each cross. The results are shown in Figure 3.6.

For all OX4282 x OX4112I, the number of individuals with both transgenes was significantly lower than expected (Chi Squared test, p values <0.006, Figure 3.6). The OX4282I and L both appeared to have single insertions of the transgene (~25% or fewer of the progeny were AmCyan). Conversely the OX4282G has more than one insertion of the transgene (>25% of the progeny was AmCyan). The OX4282I strain showed significantly reduced viability as a

heterozygote (12% of progeny). This effect was increased in combination with the OX4112I; only 3% of progeny apparently had both insertions.

The progeny carrying OX4112I or double-heterozygotes are much lower than expected. This could be due to errors in the scoring. The transformation maker (DsRed2) in OX4112I is expressed very weakly in flies (even in very young pupae - the developmental stage with the least opaque cuticle), and therefore screening can be difficult and may lead to human error. Therefore there may be some false negatives for this construct in the scoring – i.e. consequently it is possible that some of the progeny containing the OX4112I insertion may have been miss-identified as wild –type or single insertion OX4282 during fluorescence screening. Another possibility is that OX4112I has a detrimental effect on insect developmental viability, especially when it is combined with the OX4282 construct.

OX4282 x OX4112 crosses	No. of wild-type progeny (%)	No. of OX4112 progeny (%), *P* Value	No. of OX4282 progeny (%), *P* Value	No. Of OX4282 x OX4112 progeny (%), *P* Value
OX4282G x OX4112	81 (28.1)	63 (21.8), 0.37	112 (38.8), 0.18	32 (11.1), 0.006
OX4282I x OX4112	42 (42.8)	41 (41.8), 0.91	12 (12.2), 3.7 x 10^{-5}	3 (3.1), 4.2 x 10^{-9}
OX4282L x OX4112	42 (36.2)	25 (21.5), 0.05	29 (25), 0.15	20 (17.2), 0.009

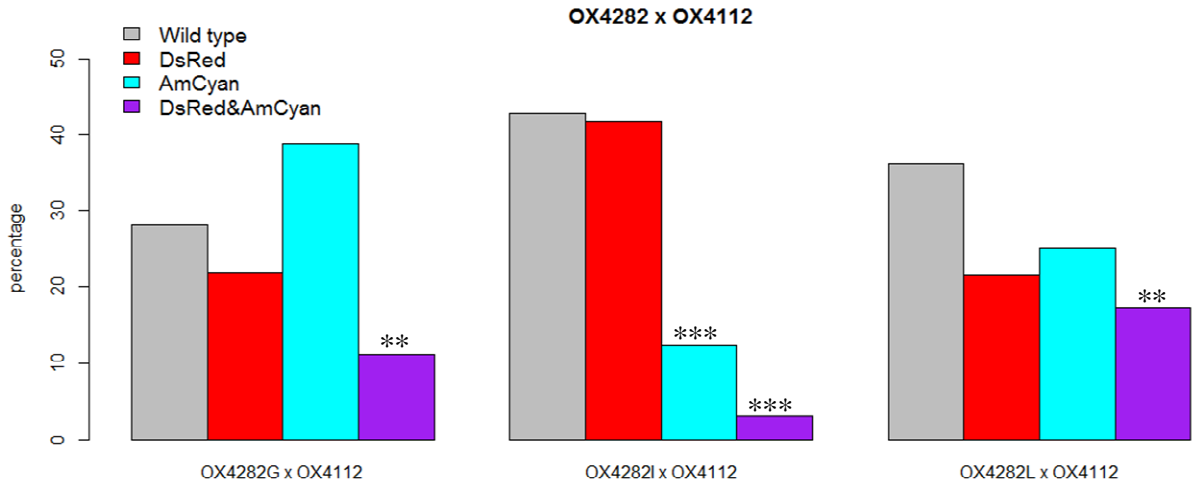


Figure 3. 6 The results from fluorescence screening of progeny from crosses of the OX4282 strain (*ß2-tubulin*) x the OX4112I strain (*I-ppoI*).

The table shows the actual number and percentage of wild-type and transgenic progeny from OX4282 (*AmCyan*) or OX4112I (*DsRed2*) and OX4282 x OX4112I (both markers for double-heterozygotes) resulting from the crosses of OX4282 x OX4112I (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4282 and OX4112I transgene. The bar chart illustrates percentage ratio of the observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion except OX4282G. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

In all OX4282 x OX4104F, the different fluorescent phenotypes showed 1:1:1:1 segregation patterns, consistent with single insertions and no detrimental effect of the presence of both transgenes (as the number of individuals containing both constructs is equal in comparison to the other phenotypes in all crosses) (Figure 3.7). The OX4282G line is the only line which shows adverse effect on flies carrying this construct as the p value is 0.03. Comparing the OX4282G x OX4104F cross to the OX4282G x OX4112I cross, there is much lower number of OX4282G progeny scored in the progeny pool of the OX4282G x OX4104F cross. This can be because of the expression OX4112I in flies is very weak and therefore identifying them is difficult which may lead to human error. There might be some false negatives for this construct in the scoring – i.e. consequently it is likely that some of the progeny with the OX4112I insertion may have been miss-identified as single insertion OX4282G during fluorescence screening.

OX4282 x OX4104 crosses	No. of wild-type progeny (%)	No. of OX4104 progeny (%), *P* Value	No. of OX4282 progeny (%), *P* Value	No. of OX4282 x OX4104 progeny (%), *P* Value
OX4282G x OX4104	60(30.6)	56(28.5), 0.79	32(16.3), 0.037	48(24.4), 0.40
OX4282I x OX4104	48(23.4)	59(28.7), 0.45	46(22.4), 0.88	52(25.3), 0.77
OX4282L x OX4104	63(31.9)	56(27.7), 0.65	40(19.8), 0.11	43(21.2), 0.17

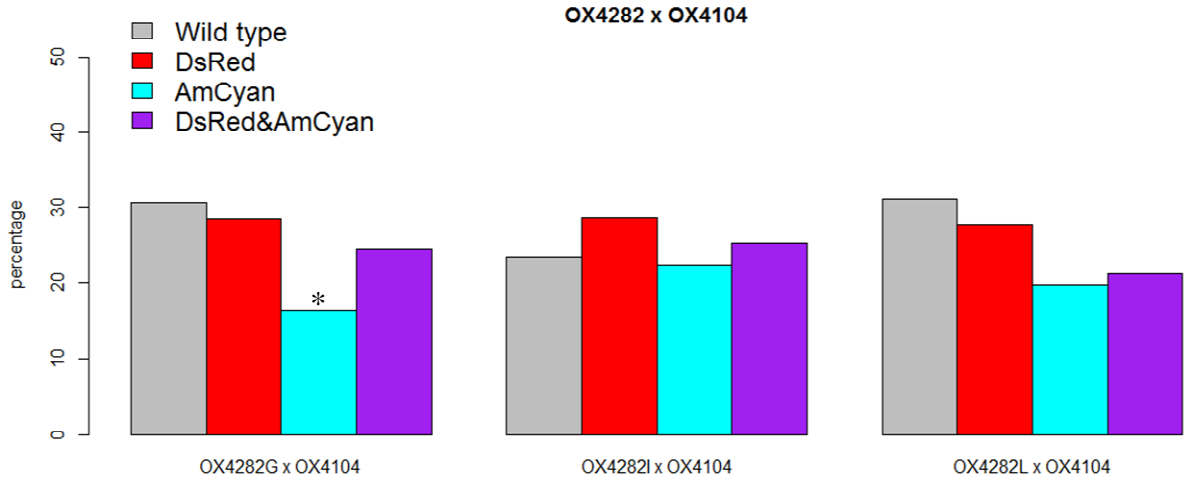


Figure 3. 7 The result of fluorescence screening of progeny from crosses of OX4282 strain (*ß2-tubulin*) x OX4104F strain (*ZnF*).

The table shows the actual number and percentage of wild-type and transgenic progeny from OX4282 (*AmCyan*), OX4104F (*DsRed2*) and OX4282 x OX4104F (both markers for double-heterozygotes) resulting from the crosses of OX4282 x OX4104F (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4282 and OX4104F transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have a single autosomal insertion. The Y axis shows the percentage of individuals in each phenotypic class. The asterisks on bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

The results for OX4282 x OX4763 are shown in Figure 3.8. If the number of transgene insertions is one in all of the lines of these crosses, the number of wild-type progeny, progeny carrying OX4763 construct, progeny carrying OX4282 construct and double-transgene progeny should be equal. Both OX4763 strains contain a single insertion. Although the DsRed2 marker expresses strongly in the OX4763D3, the DsRed2 maker in the OX4763D4 line is expressed very faintly. This means that screening can be hard and allows for human error in classification. The error in identifying DsRed2 maker during screening might be one of the reasons for low number of OX4763D4 progeny. There may also be a somatic expression of the transgene, with a consequent fitness penalty on progeny carrying this construct. The early death in the progeny results in observing a lower number of the progeny with red marker in this experiment.

OX4282 x OX4763 crosses	No. of wild-type progeny (%)	No. of OX4763 progeny (%), *P* Value	No. of OX4282 progeny (%), *P* Value	No. of OX4282 x OX4763 progeny (%), *P* Value
OX4282I x OX4763D3	52(25.4)	54(26.4), 0.89	48(23.5), 0.77	50(24.5), 0.88
OX4282L x OX4763D3	49(24.1)	53(26.1), 0.78	45(22.1), 0.77	56(27.5), 0.63
OX4282I x OX4763D4	80(47.3)	22(13), 9.9 x 10^{-6}	50(29.5), 0.05	17(10.1), 8.62 x 10^{-6}
OX4282L x OX4763D4	65(34.7)	33(17.6), 0.01	69(36.9), 0.80	20(10.7), 0.0003

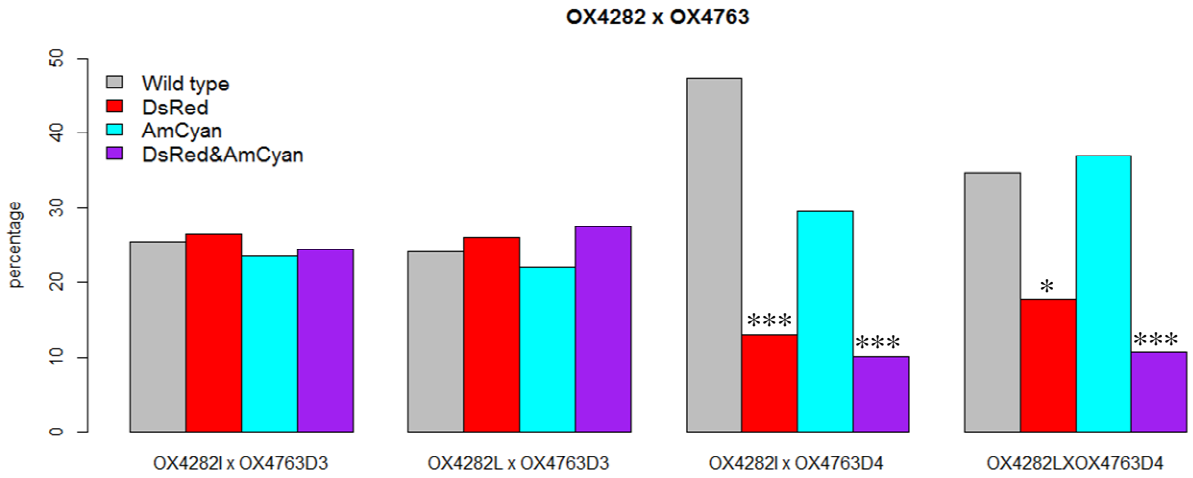


Figure 3. 8 The results from fluorescence screening of the progeny from crosses of the OX4282 strain (*β2-tubulin* based strain) with OX4763 strain (*EcoRI* based strain).

The table shows the actual number and percentage of wild-type and transgenic progeny from the OX4282 (*AmCyan*), OX4763 (*DsRed2*) and OX4282 x OX4763 (both markers for double-heterozygotes) resulting from the crosses of OX4282 x OX4763 (progeny were fed on tetracycline diet (100ng/ul) at larval stage).A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4282 and OX4763 transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

Six double-heterozygote males from each of the above crosses were crossed to twelve wild-type females and the fertility assay was performed in the absence and presence of tetracycline (100ng/µl) in the larval diet (according to section 3.2). To assess if any effects observed from these crosses were male specific, twelve double-heterozygotes female progeny were also crossed to six wild-type males.

There was no difference in the fertility of OX4282 (G or I) x OX4112I double heterozygote males reared off or on- tetracycline. The results are shown in Figure 3.9.

The OX4282L x OX4112I double heterozygotes, showed a significant reduction in fertility (50%, p=0.0003) when reared off-tetracycline relative to the on-tetracycline control. *I-ppoI* selectively cleaves rDNA, in A. gambiae this is X-linked, and expression of *I-ppoI* can generate an excess of spermatozoa carrying the Y-chromosome (Windbichler et al. 2007). Willhoeft, 1996 suggests that rDNA is found on in both the X and Y-chromosome in medfly, although it is possible that there are more copies of rDNA in the X-chromosome than the Y-chromosome.

Here at best, only 50% reduction in egg viability was observed which show reproductive capacity of males decreased by 50%. If *I-ppoI* is cutting rDNA in the X-chromosome more males than females will be present in the offspring. This was tested by growing the viable eggs on a standard diet. 80% of the viable eggs developed into males and 20% of them were females. Potentially, relatively low levels of *I-ppoI* cutting of rDNA could differentially affect the sex-chromosomes.

Females from all OX4282 x OX4112I cross were also tested for fertility. Females of the double heterozygote of the OX4282 x OX4112I had equal fertility in the absence or presence

of tetracycline in their diet at larval stage. Therefore the fertility defect that was seen with the OX4282L x OX4112I is male specific (Figure 3.9).

OX4282 x OX4112 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	364	347	95.5	0.82
	NT	447	418	93.2	
OX4282G x OX4112(m)	T	391	332	85.1	0.49
	NT	365	228	78.9	
OX4282I x OX4112(m)	T	336	258	76.7	0.84
	NT	299	235	78.5	
OX4282L x OX4112(m)	T	357	297	83.1	0.0003
	NT	400	277	52.9	
OX4282G x OX4112(f)	T	395	360	90.3	0.99
	NT	475	426	90.6	
OX4282I x OX4112(f)	T	350	316	90.3	0.12
	NT	455	349	76.0	
OX4282L x OX4112(f)	T	388	348	89.9	0.99
	NT	400	365	90.8	

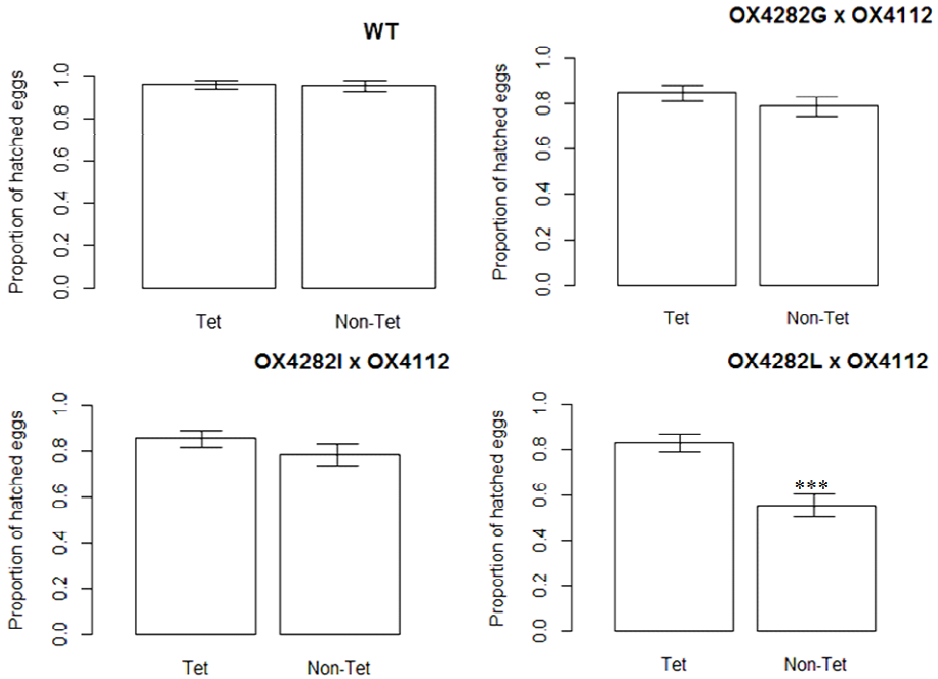


Figure 3. 9 The male and female fertility test for medfly lines carrying both *ß2-tubuli*n driven *tTAV* (OX4282) strain and *tetO-Ippo1* (OX4112I) strain.

The effect and repressibility of the transgene expression were tested by crossing double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4282 (G, I and L); OX4112I crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4282 (G, I and L); OX4112I crossed to wild-type males are presented with an (f). The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in males of OX4282 (G, I and L); OX4112I crossed to wild-type females. In OX4282 (G or I); OX4112I (males) crossed to wild-type females; the number of hatched eggs didn't differ significantly between Tet and Non-Tet (p-value>0.05). However, this difference is significant in OX4282L; OX4112I (males) crossed to wild-type females (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show 95%confidence intervals.

OX4283 x OX4104 showed a significant reduction in fertility of male flies carrying both construct ($P=2.2 \times 10^{-16}$, Chi Squared test) in all crosses, except for OX4282G x OX4104F. No effect was seen on the fertility of female flies double-heterozygous for OX4282 x OX4104, therefore the sterility is male-specific (Figure 3.10).

OX4282 x OX4104 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	394	380	96.3	0.94
	NT	355	340	95.8	
OX4282G x OX4104(m)	T	397	359	90.3	0.12
	NT	362	277	76.8	
OX4282I x OX4104(m)	T	380	299	78.6	2.2×10^{-16}
	NT	416	107	25.3	
OX4282L x OX4104(m)	T	412	343	82.9	2.2×10^{-16}
	NT	474	70	15.2	
OX4282G x OX4104(f)	T	381	346	91.0	0.77
	NT	319	299	93.7	
OX4282I x OX4104(f)	T	362	333	92	0.97
	NT	370	339	91.6	
OX4282L x OX4104(f)	T	396	340	86.1	0.34
	NT	492	384	77.7	

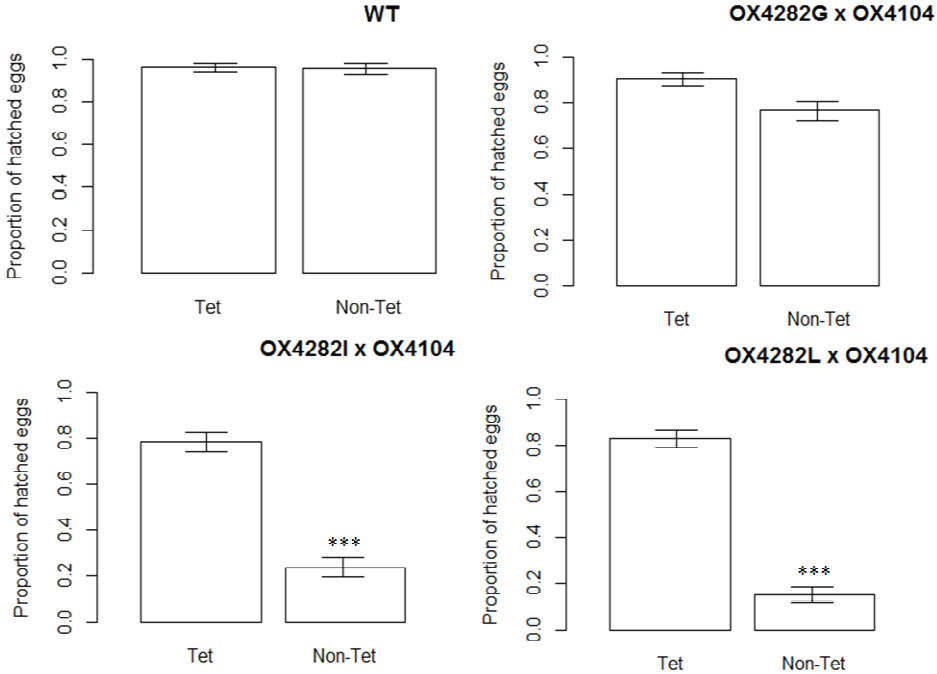


Figure 3. 10 The male and female fertility test for medfly lines carrying both the *ß2-tubulin* driven *tTAV* strain (OX4282) and *tetO-ZnF* strain (OX4104F).

The effect and repressibility of transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4282 (G, I and L); OX4104F crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4282 (G, I and L); OX4104F crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males ofOX4282 (G, I and L); OX4104F crossed to wild-type females. In OX4282 G, OX4104F (males) crossed to wild-type females; the number of hatched eggs didn't differ significantly between Tet and Non-Tet (p-value>0.05). However, this difference is significant in OX4282 (I and L); OX4104F (males) crossed to wild-type females (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

The results for OX4282 x OX4763 are shown in Figure 3.11. Although the reduction in egg viability was significant off tetracycline in comparison to on tetracycline in all cases, the egg viability was found to be relatively low (compared to wild-type controls) even in the presence of tetracycline. Females of the OX4282 (I or L) x OX4763 (D3 or D4) also showed a significant reduction in egg viability off-tet in most cases except OX4282I x OX4763D3. This indicates that the effector has impaired the fertility in females as well. This could be explained by leaky expression of the effector transgene in the female germline. The results are shown in Figure 3.11.

OX4282 x OX4763 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	385	359	93.2	0.50
	NT	392	344	87.7	
OX4282I x OX4763D3(m)	T	356	281	78.9	2.2×10^{-14}
	NT	392	72	18.36	
OX4282L x OX4763D3(m)	T	423	276	65.2	8.06×10^{-8}
	NT	408	103	25.2	
OX4282I x OX4763D4(m)	T	376	276	73.4	2.2×10^{-14}
	NT	413	89	21.5	
OX4282L x OX4763D4(m)	T	489	298	60.9	1.9×10^{-7}
	NT	443	129	29.1	
OX4282I x OX4763D3(f)	T	458	334	72.9	0.08
	NT	468	286	61.1	
OX4282L x OX4763D3(f)	T	482	345	71.5	0.006
	NT	559	266	47.5	
OX4282I x OX4763D4(f)	T	407	264	64.8	0.008
	NT	392	146	37.2	
OX4282L x OX4763D4(f)	T	446	286	64.1	0.02
	NT	374	183	48.9	

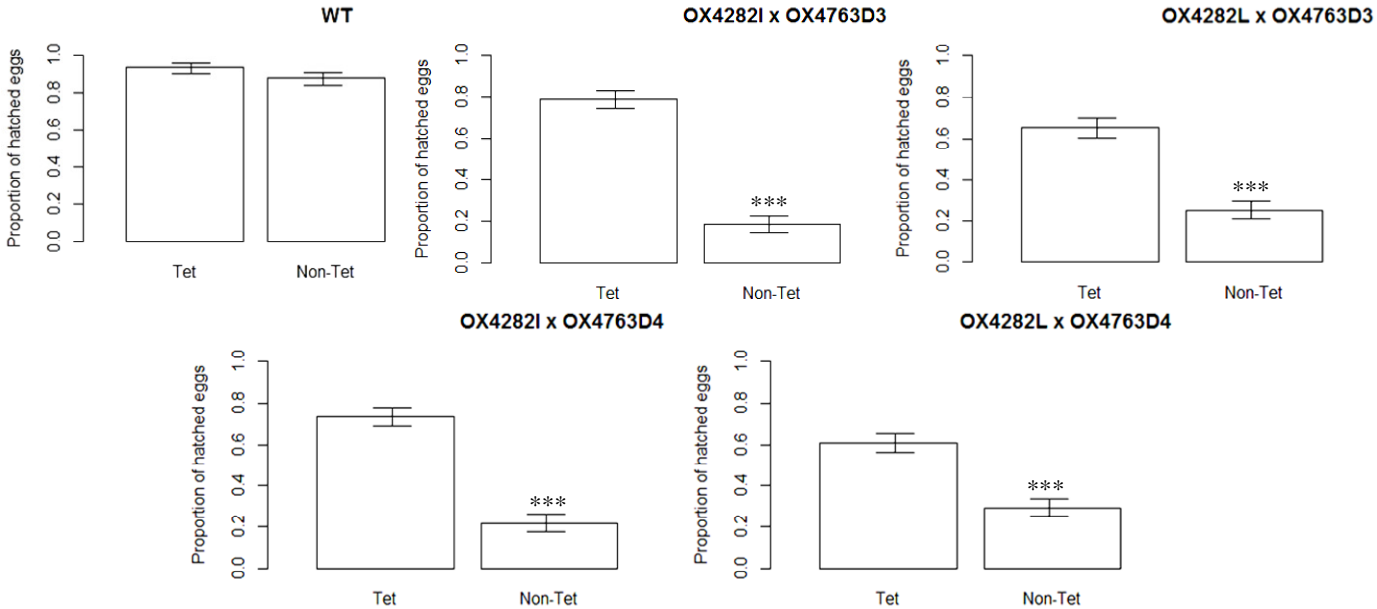


Figure 3. 11 The male and female fertility test for medfly lines carrying both *β2-tubulin* driven *tTAV* strain (OX4282) and *tetO-EcoRI* strain (OX4763).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4282 (I and L); OX4763 (D3 and D4) crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4282 (I and L); OX4763 (D3 and D4) crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of males of OX4282 (I and L); OX4763 (D3 and D4) crossed to wild-type females. In all of OX4282 (I and L), OX4763 (D3 and D4) (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched egg. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show 95% confidence intervals.

3.3.2. The combination of *topi-tTAV* construct (OX4371) with the effector strains (*tetO-I-ppoI* (OX4112I) or *tetO-ZnF* (OX4104F)) has limited effects on male fertility

In OX4371, *tTAV* is driven by the medfly *topi* promoter. The *topi* promoter based strains OX4371 (B, E) were crossed to the *I*-ppoI or *ZnF* effector based line (8 females of OX4371 with 4 males from OX4112I or OX4104F). As before, around 200 progeny were counted at pupal stage for each cross to check for detrimental effects on the flies carrying both individual constructs and constructs in combination. The number of wild-type and transgenic flies carrying the OX4112I or OX4104F (*DsRed2* marker), or the OX4371 constructs (*AmCyan* marker) and transgenic progeny which exhibit both marker (*AmCyan* and *DsRed2*) were screened and counted.

The results for OX4371 x OX4112 are shown in Figure 3.12. Both of lines of the OX4371 had single insertions of the transgene. However, for the line OX4371E x OX4112I the number of individuals carrying both constructs (*DsRed2* and *AmCyan*) is lower than the 25% expected from an innocuous dominant marker. As for previous crosses, the expression of the *DsRed2* marker in the OX4112I line is very weak and could be mis-scored.

OX4371 x OX4112 crosses	No. of wild-type progeny (%)	No. of OX4112 progeny (%), *P* Value	No. of OX4371 progeny (%), *P* Value	No. Of OX4371 x OX4112 progeny (%), *P* Value
OX4371B x OX4112	33(22.6)	42(28.7), 0.39	40(27.3), 0.49	31(21.2), 0.83
OX4371E x OX4112	34(31.1)	30(27.5), 0.63	26(23.8), 0.32	19(17.4), 0.04

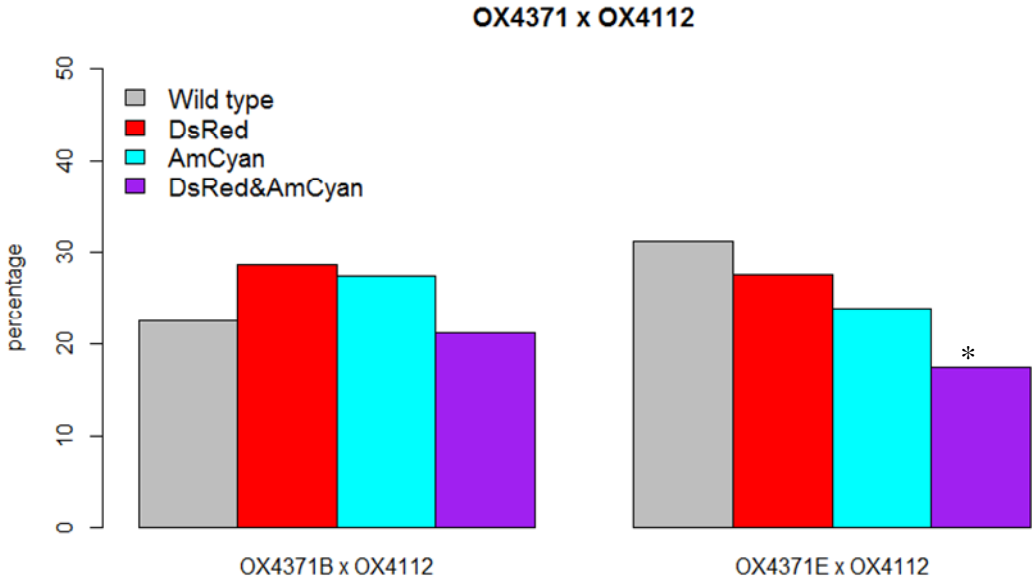


Figure 3. 12 The results from fluorescence screening of progeny from the OX4371 strain (*topi* based strain) x OX4112I strain (*I-ppoI* based strain).

The table shows the actual number and percentage of wild-type and transgenic progeny from the OX4371 (*AmCyan*), OX4112I (*DsRed2*) and OX4371 x OX4112I (both markers for double-heterozygotes) resulting from the crosses of OX4371 x OX4112I (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4371 and OX4112I transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have a single autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

No detrimental effects on viability of flies of OX4371 x OX4104F were detected and the observed segregation of different fluorescent phenotypes confirms previous results indicating a single insertion of each transgene, Figure 3.13.

OX4371 x OX4104 crosses	No. of wild-type progeny (%)	No. of OX4104 progeny (%), *P* Value	No. of OX4371 progeny (%), *P* Value	No. of OX4371 x OX4104 progeny (%), *P* Value
OX4371B x OX4104	56(26.7)	49(23.4), 0.63	53(25.3), 0.84	51(24.4), 0.73
OX4371E x OX4104	57(25.6)	61(27.4), 0.80	59(26.5), 0.90	45(20.2), 0.42

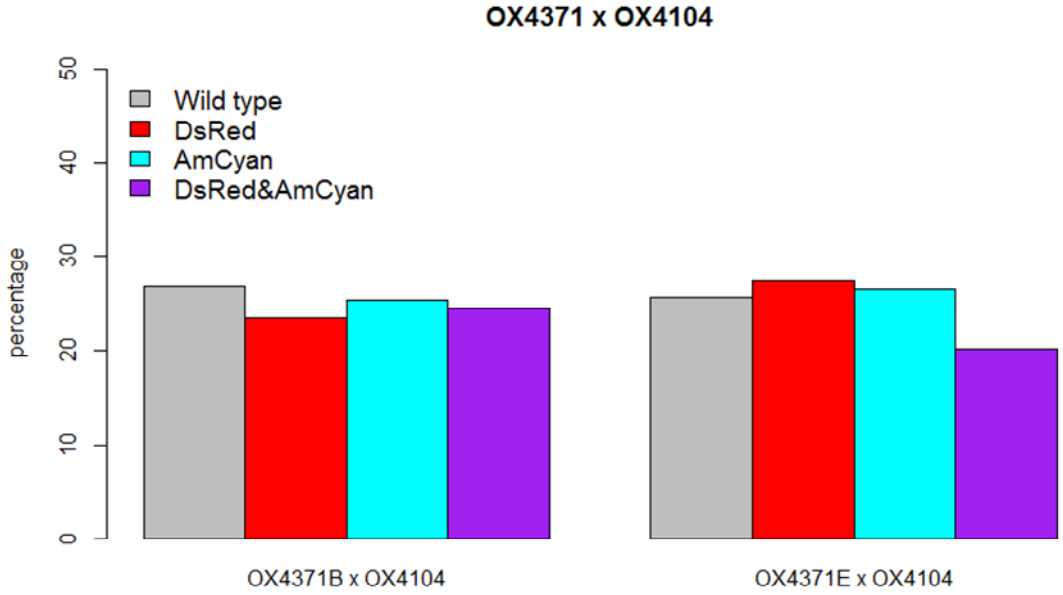


Figure 3. 13 The resulted from fluorescence screening of the progeny from crosses of the OX4371 strain (*topi*) with OX4104F strain (*ZnF*).

The table shows the actual number and percentage of wild-type and transgenic progeny of OX4371 (*AmCyan*), OX4104F (*DsRed2*) and OX4371 x OX4104F (both markers for double-heterozygotes) resulting from the crosses of OX4371 x OX4104F (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4371 and OX4104F transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar charts indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

Double-heterozygotes progeny from all OX4371 x OX4112I or OX4371 x OX4104F crosses were selected, and six males of each were crossed to twelve wild-type females to check for male fertility. Female progeny were also crossed to wild-type to check if any observed sterility is male specific. Since the expression of the effector should be repressed by the addition of tetracycline, the male fertility was studied in the absence and presence of dietary tetracycline in the larval diet (100ng/µl). As shown in Figure 3.10, the reproductive capacity of male flies carrying both OX4371E and OX4112I was reduced to 29% relative to controls (Chi Squared test, p=0.002). The females showed no reduction in fertility; therefore, the sterility in flies carrying both OX4371E and OX4112I insertions in their genome is male specific (Figure 3.14). No reduction in reproductive capacity of either males or females was found in the OX4371B x OX4112I double heterozygotes.

OX4371 x OX4112 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	364	347	95.5	0.82
	NT	447	418	93.2	
OX4371B x OX4112(m)	T	464	428	91.8	0.69
	NT	420	373	88.5	
OX4371E x OX4112(m)	T	479	448	93.2	0.002
	NT	425	274	64.9	
OX4371B x OX4112(f)	T	378	340	90.1	0.97
	NT	361	333	90.3	
OX4371E x OX4112(f)	T	438	402	91.8	0.99
	NT	361	331	91.7	

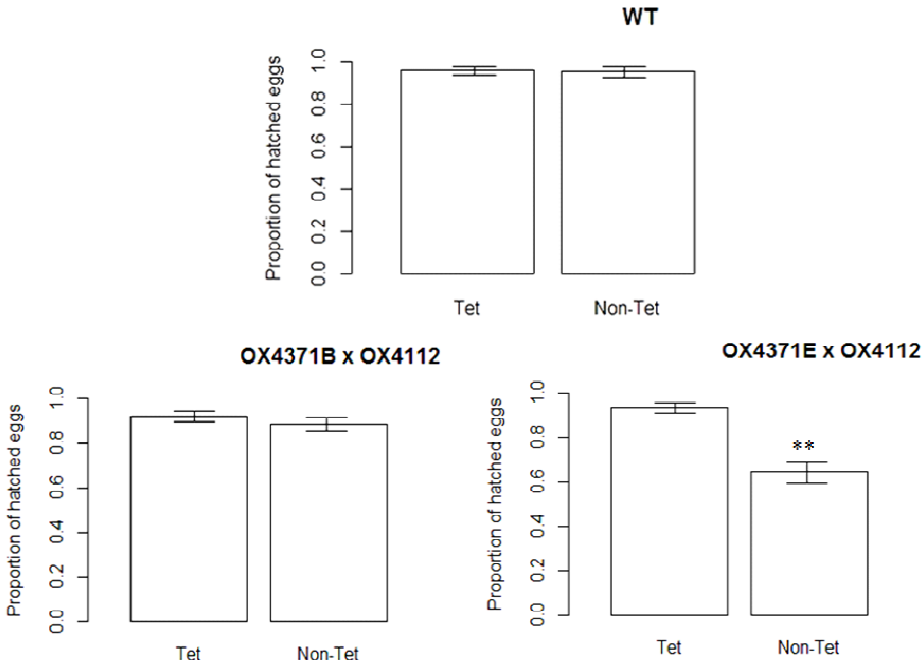


Figure 3. 14 The male and female fertility test for medfly lines carrying both *topi* driven *tTAV* (OX4371) strain and *tetO-IppoI* (OX4112I) strain.

The effect and repressibility of the transgene expression were tested by crossing double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sixth table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4371 (B and E); OX4112I crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4371 (B and E); OX4112I crossed to wild-type males are presented with an (f).The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in males of OX4371 (B and E); OX4112I crossed to wild-type females. In OX4371B; OX4112I (males) crossed to wild-type females; the number of hatched eggs didn't differ significantly between Tet and Non-Tet (p-value>0.05). However, this number is significant in OX4371E; OX4112I (males) crossed to wild-type females (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).The error bars show confidence intervals which was set at 95% confidence intervals.

Figure 3.11 presents results from the fertility test for OX4371 x OX4104F; the reduction in reproductive capacity in all cases was significantly different ($p<0.05$) when comparing the males that were reared on tetracycline diet vs. non tetracycline.

To check if the observed sterility is male specific, the female from the double-heterozygotes were crossed to wild-type males and egg viability was assessed (Figure 3.15). The results indicate that the observed sterility for this combination of constructs is male specific, as females of these crosses produced viable eggs.

OX4371 x OX4104 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	394	380	96.3	0.94
	NT	355	340	95.8	
OX4371B x OX4104(m)	T	338	262	77.6	3.1×10^{-6}
	NT	383	167	44.2	
OX4371E x OX4104(m)	T	384	287	74.7	1.3×10^{-6}
	NT	365	150	41.2	
OX4371B x OX4104(f)	T	356	329	92.4	0.91
	NT	383	350	91.5	
OX4371E x OX4104(f)	T	405	364	89.8	0.97
	NT	387	349	90.1	

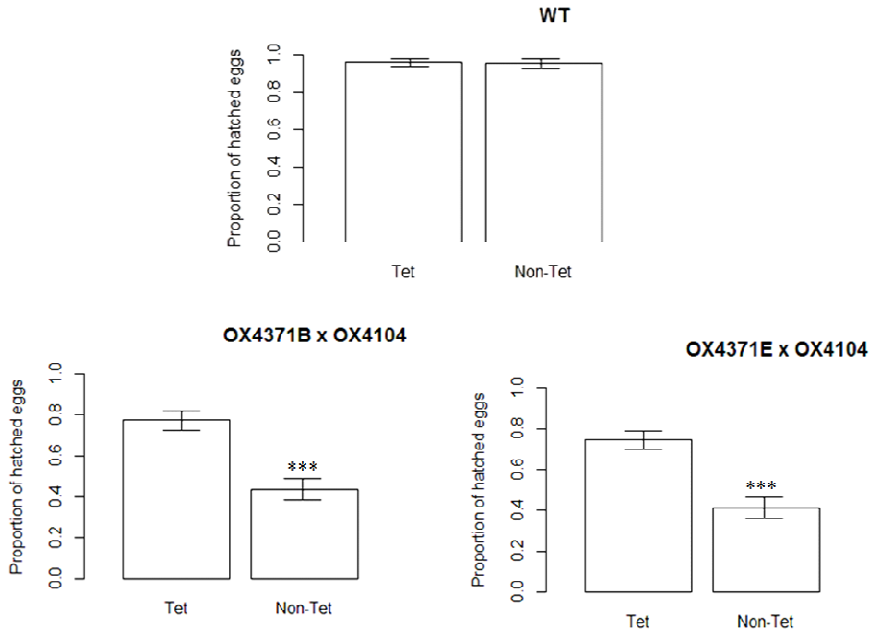


Figure 3. 15 The male and female fertility test for medfly lines carrying both *topi* driven *tTAV* strain (OX4371) and *tetO-ZnF* strain (OX4104F).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4371 (B and E); OX41104F crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4371 (B and E); OX4104F crossed to wild-type males are presented with an (f).The bar chart illustrates the proportion of hatched egg to non-hatches eggs in males of OX4371 (B and E); OX4104F crossed to wild-type females. In the OX4371 (B and E); OX4104F (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05).The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).The error bars show confidence intervals which was set at 95% confidence intervals.

3.3.3. The combination of the second *topi-tTAV* construct (OX4391) with the effector strains (*tetO-I-ppoI* (OX4112I), *tetO-ZnF* (OX4104F) and *tetO-EcoRI* strain (OX4763)) has limited effects on male fertility

The OX4391 construct (Figure 2.12) carries the *topi* promoter sequence (from medfly) as the main regulatory element controlling expression of *tTAV*. The difference between the OX4391 construct and OX4371 construct (see section 3.3.2) is in their 3' untranslated region (3'-UTR). The 3' UTR regulates the translation efficiency, mRNA stability and also polyadenylation position. In OX4371 the 3'UTR is *SV40* polyA, a common choice of 3'UTR used for transgene expression. In OX4391 the *topi* 3'UTR replaces the *SV40* polyA 3'UTR. The rational for this alteration is that the 3′ UTR from *topi* will potentially help provide the desired tTAV expression pattern (spermatocyte specificity with early translation). The 5'UTR in both constructs is *Cchsp83* (*C. capitata*).

To investigate the paternal effect lethality of OX4391 x OX4112I, OX4104F or OX4763D3 and D4; 8 females from all insertion lines of OX4391 (B, C, D, G, H) were crossed to 4 males of OX4112I, OX4104F or OX4763D3 and D4. Around 200 progeny from each crosses were counted (at pupal stage) to reveal any detrimental effect of each constructs. The numbers of the wild-type progeny and the transgenic progeny carrying OX4112I, OX4104F or OX4763D3 and D4 (*DsRed2* marker) or OX4391 construct (*AmCyan* marker) and also the progeny that have both constructs (expressing both *DsRed2* and *AmCyan* marker) were counted. The results for OX4391 x OX4112I are shown in Figure 3.16. The OX4391G, OX4391C and OX4391D lines have a single insertion of the transgene while the OX4391B and OX4391H lines appear to have two copies (proportion of fluorescent progeny>25%).

OX4112I has a single insertion therefore the number of progeny carrying the *DsRed2* marker should be 25% of the whole progeny. Here in some of the crosses, there is less progeny from OX4112I. As explained earlier, this could be due to the faint expression of *DsRed2* in this strain which results in their miss-identification during fluorescence screening.

OX4391 x OX4112 crosses	No. of wild-type progeny (%)	No. of OX4112 progeny (%), *P* Value	No. of OX4391 progeny (%), *P* Value	No. of OX4391 x OX4112 progeny (%), *P* Value
OX4391B x OX4112	37(22.8)	33(20.3), 0.70	77(47.5), 0.003	15(9.2), 0.01
OX4391C x OX4112	49(38.8)	18(14.2), 0.0007	45(35.7), 0.71	14(11.1), 8.54×10^{-5}
OX4391D x OX4112	68(30.2)	47(20.8), 0.19	72(32), 0.82	38(16.8), 0.05
OX4391G x OX4112	70(35.5)	67(34), 0.85	34(17.2), 0.01	26(13.1), 0.001
OX4391H x OX4112	46(25.5)	3(1.6), 4.6×10^{-6}	126(70), 5.4×10^{-6}	5(2.7), 1.8×10^{-1}

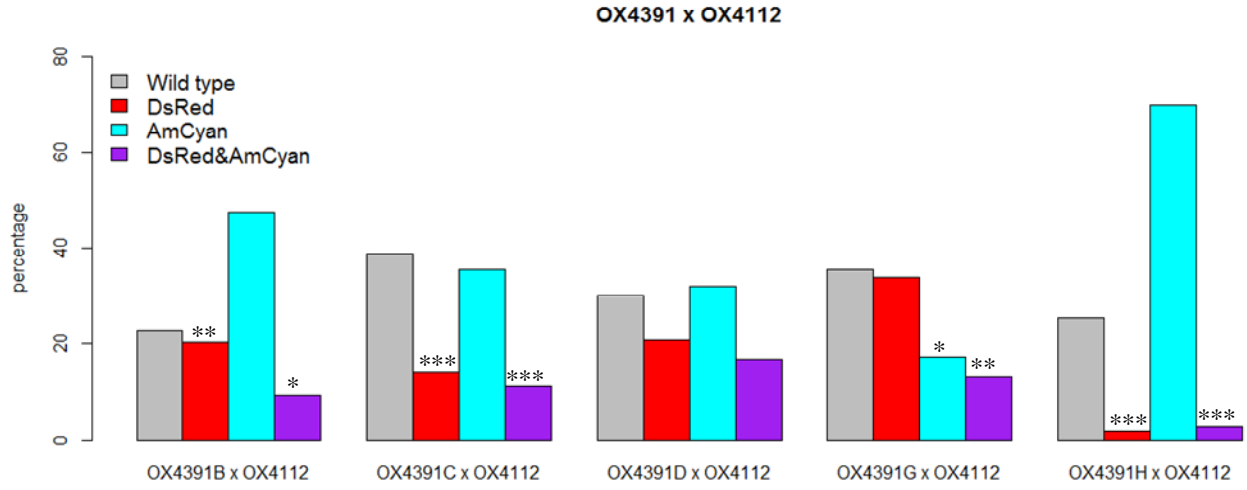


Figure 3. 16 The results from fluorescence screening of progeny from crosses of OX4391 strain (*topi* based strain) x OX4112I strain (*I-ppoI* based strain).

The table shows the actual number and percentage of wild-type and the transgenic progeny of the OX4391 (*AmCyan*), OX4112I (*DsRed2*) and OX4391 x OX4112I (both markers for double-heterozygotes) resulting from the crosses of OX4391 x OX4112I (progeny were fed on tetracycline diet (100ng/ul) at larval stage).A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4391 and OX4112I transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

Figure 3.17 shows single insertions for all of the lines carrying OX4391 and OX4104F.

OX4391 x OX4104 crosses	No. of wild-type progeny (%)	No. of OX4104 progeny (%), *P* Value	No. of OX4391 progeny (%), *P* Value	No. of OX4391 x OX4104 progeny (%), *P* Value
OX4391B x OX4104	56(27.7)	52(25.7), 0.78	48(23.7), 0.58	46(22.7), 0.48
OX4391C x OX4104	62(27.1)	55(24.1), 0.66	53(23.2), 0.57	58(25.4), 0.80
OX4391D x OX4104	49(23.6)	53(25.6), 0.78	51(24.6), 0.88	54(26.1), 0.73
OX4391G x OX4104	59(28.7)	50(24.3), 0.54	49(23.9), 0.50	47(22.9), 0.41
OX4391H x OX4104	61(30.5)	47(23.5), 0.34	44(22), 0.24	48(24), 0.37

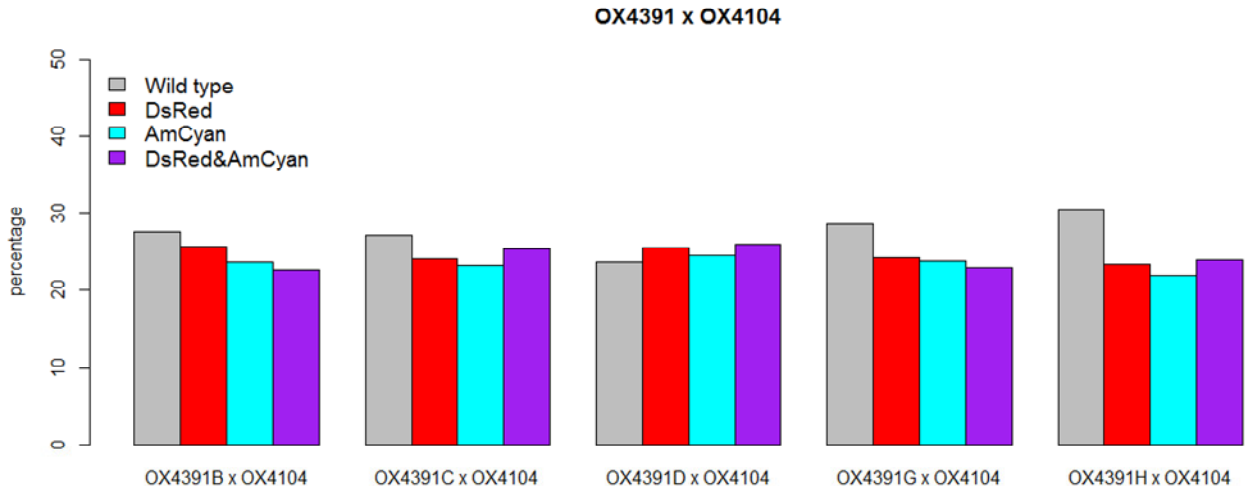


Figure 3. 17 The result from fluorescence screening of the progeny from crosses of the OX4391 strain (*topi*) with OX4104F strain (*ZnF*).

The table shows the actual number and percentage of wild-type and transgenic progeny of the OX4391 (*AmCyan*), OX4104F (*DsRed2*) and OX4391 x OX4104F (both markers for double-heterozygotes) resulting from the crosses of OX4391 x OX4104F (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4391 and OX4104F transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage pupae in each phenotypic class. The asterisks on the bar charts indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

The results for OX4391 x Ox4763 are shown in Figure 3.18. As explained in section 3.3.2 the number of progeny from the OX4763D4 appears to be lower in all crosses. This can be due to human error or adverse effects of the construct.

OX4391 x OX4763 Crosses	No. of wild-type progeny (%)	No. of OX4763 progeny (%), *P* Value	No. of OX4391 progeny (%), *P* Value	No. of OX4391 x OX4763 progeny (%), *P* Value
OX4391B x OX4763D3	45(21.8)	57(27.6), 0.40	61(29.6), 0.27	43(20.8), 0.88
OX4391H x OX4763D3	57(28.2)	60(29.7), 0.84	40(19.8), 0.22	45(22.2), 0.40
OX4391B x OX4763D4	71(38.3)	30(16.2), 0.002	65(35.1), 0.70	19(10.2), 5.5×10^{-5}
OX4391H x OX4763D4	68(40.4)	21(12.5), 0.0001	61(36.3), 0.63	18(10.7), 3.1×10^{-5}

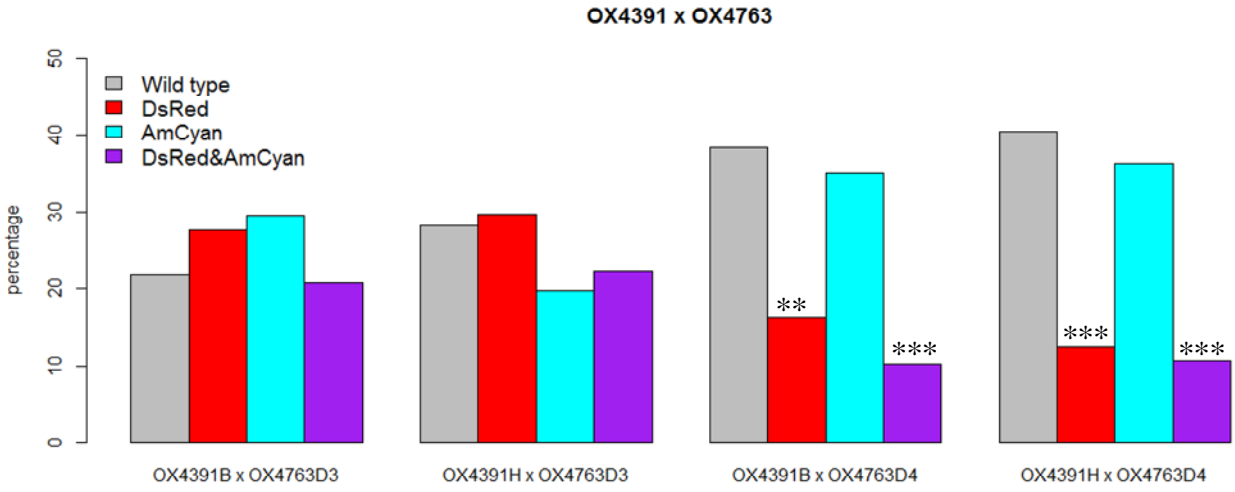


Figure 3. 18 The results from fluorescence screening of the progeny from crosses of the OX4391 strain (*topi*) with OX4763 strain (*EcoRI*).

The table shows the actual number and percentage of wild-type and transgenic progeny of OX4391 (*AmCyan*), OX4763 (*DsRed2*) and OX4391 x OX4763 (both markers for double-heterozygotes) resulting from the crosses of OX4391 x OX4763 (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4391 and OX4763 transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

To check fertility of male flies, 6 double-heterozygotes males from each line were crossed to wild-type females. Furthermore females containing both insertions were crossed to wild-type males to check if any fertility defect detected is male specific.

As is shown in Figure 3.19, three of the double-heterozygotes lines (OX4391(C, D, G) x OX4112I) showed no reduction in reproduction capacity of the males. However, the double-heterozygote males, OX4391 (B or H) and OX4112I showed reduction in reproduction capacity by approximately 50% compared to controls. The male fertility was compared in the absence and presence of tetracycline in the larval diet (100ng/µl). To investigate whether the *I-ppoI* effector biases the sex ratio of the offspring that do survive (see section 3.3.1), hatched larvae were reared on a standard diet. 71% of the eggs that hatched were males while only 29% of them were females. The results from the female crosses indicate that the reduction in reproduction capacity is male-specific as there female fertility was unaffected.

OX4391 x OX4112 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	364	347	95.5	0.82
	NT	447	418	93.2	
OX4391B x OX4112(m)	T	410	372	91.1	7.4×10^{-7}
	NT	377	159	42.1	
OX4391C x OX4112(m)	T	433	405	92.9	0.97
	NT	392	368	93.8	
OX4391D x OX4112(m)	T	408	373	91.2	0.83
	NT	338	301	89	
OX4391G x OX4112(m)	T	393	378	95.9	0.87
	NT	438	428	97.5	
OX4391H x OX4112(m)	T	379	351	92.6	5.5×10^{-7}
	NT	362	199	54.2	
OX4391B x OX4112(f)	T	469	405	86.7	0.52
	NT	390	359	92	
OX4391C x OX4112(f)	T	381	346	90.8	0.99
	NT	461	419	90.8	
OX4391D x OX4112(f)	T	504	469	93.1	0.90
	NT	375	345	91.8	
OX4391G x OX4112(f)	T	355	323	91	0.40
	NT	405	357	88.3	
OX4391H x OX4112(f)	T	393	348	88.8	0.76
	NT	353	316	89.5	

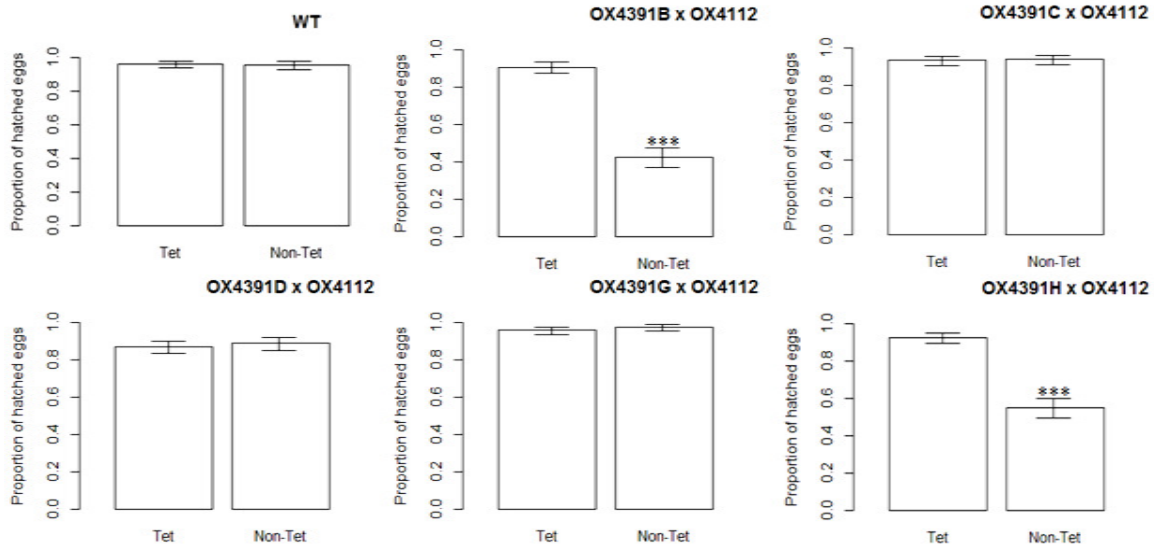


Figure 3. 19 The male and female fertility test for medfly lines carrying both *topi* driven *tTAV* (OX4391) strain and *tetO-IppoI* (OX4112I) strain.

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4391 (B, C, D, G and H); OX4112I crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4391 (B, C, D, G and H); OX4112I crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of OX4391 (B, C, D, G and H); OX4112I crossed to wild-type females. In OX4391 (C, D or G); OX4112I (males) crossed to wild-type females; the number of hatched eggs didn't differ significantly between Tet and Non-Tet (p-value>0.05). However, this number is significant in OX4391 (B or H); OX4112I (males) crossed to wild-type females (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

As shown in Figure 3.20, in majority of the lines from the combination of OX4391 x OX4104F, the reduction in egg viability was not significant in the absence of tetracycline in the larval diet compared to the control (presence of tetracycline in the larval diet). Only OX4391H crossed to OX4104F showed a 50% reduction. The sperm function of progeny is expected to be reduced which results in a fewer viable eggs. The expression of *ZnF* (the effector) should be repressed by the addition of tetracycline, therefore the male fertility was compared in the absence and presence of tetracycline in the larval diet (100ng/µl).

Females from individual lines were crossed to wild-type males to check if the observed sterility was male specific. The various combinations of OX4391 x OX4104F in medfly appeared to give male specific sterility, as results from female crosses did not show a significant reduction in the absence of tetracycline in the larval diet (Figure 3.20).

OX4391 x OX4104 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	394	380	96.3	0.94
	NT	355	340	95.8	
OX4391B x OX4104(m)	T	436	365	83.8	2.2×10^{-16}
	NT	425	125	29.4	
OX4391C x OX4104(m)	T	488	427	87.5	0.003
	NT	537	355	66.1	
OX4391D x OX4104(m)	T	482	446	92.4	0.18
	NT	495	405	81.8	
OX4391G x OX4104(m)	T	342	312	91.2	0.53
	NT	349	297	85.2	
OX4391H x OX4104(m)	T	411	335	81.1	3.6×10^{-16}
	NT	417	163	38.8	
OX4391B x OX4104(f)	T	387	358	92.6	0.90
	NT	381	357	94.1	
OX4391C x OX4104(f)	T	372	341	92.1	0.93
	NT	393	357	90.8	
OX4391D x OX4104(f)	T	379	350	95.5	0.78
	NT	390	350	89.8	
OX4391G x OX4104(f)	T	353	325	92.1	0.60
	NT	392	355	88.3	
OX4391H x OX4104(f)	T	394	359	90.6	0.96
	NT	367	336	91.6	

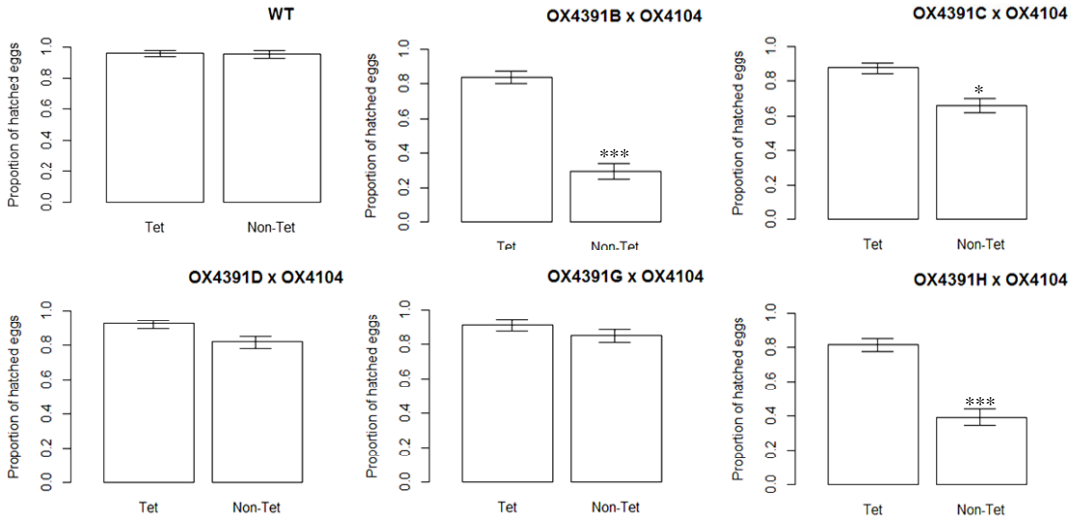


Figure 3. 20 The male and female fertility test for medfly lines carrying both *topi* driven *tTAV* strain (OX4391) and *tetO- ZnF* strain (OX4104F).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4391 (B, C, D, G and H); OX4104F crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4391 (B, C, D, G and H); OX4104F crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of OX4391 (B, C, D, G and H); OX4104F crossed to wild-type females. In all of OX4391 (B, C, D, G, H), OX4104F (males) crossed to wild-type females; number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

The results for OX4391 x Ox4763 are shown in Figure 3.21. As explained in section 3.3.2 the number of progeny from the OX4763D4 appears to be lower in all crosses. This can be due to human error or adverse effects of the construct.

OX4391 x OX4763 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	385	359	93.2	0.50
	NT	392	344	87.7	
OX4391B x OX4763D3	T	432	360	83.3	2.2×10^{-14}
	NT	403	112	27.7	
OX4391H x OX4763D3	T	406	314	77.3	2.2×10^{-14}
	NT	91	416	21.8	
OX4391B x OX4763D4	T	396	261	65.9	1.1×10^{-12}
	NT	392	88	22.4	
OX4391H x OX4763D4	T	438	319	72.8	1.3×10^{-7}
	NT	442	158	35.7	
OX4391B x OX4763D3(f)	T	480	342	71.2	0.20
	NT	549	260	47.3	
OX4391H x OX4763D3(f)	T	487	333	68.3	0.64
	NT	492	321	65.2	
OX4391B x OX4763D4(f)	T	438	319	72.8	0.06
	NT	442	158	35.7	
OX4391H x OX4763D4(f)	T	463	282	60.9	0.28
	NT	348	187	53.7	

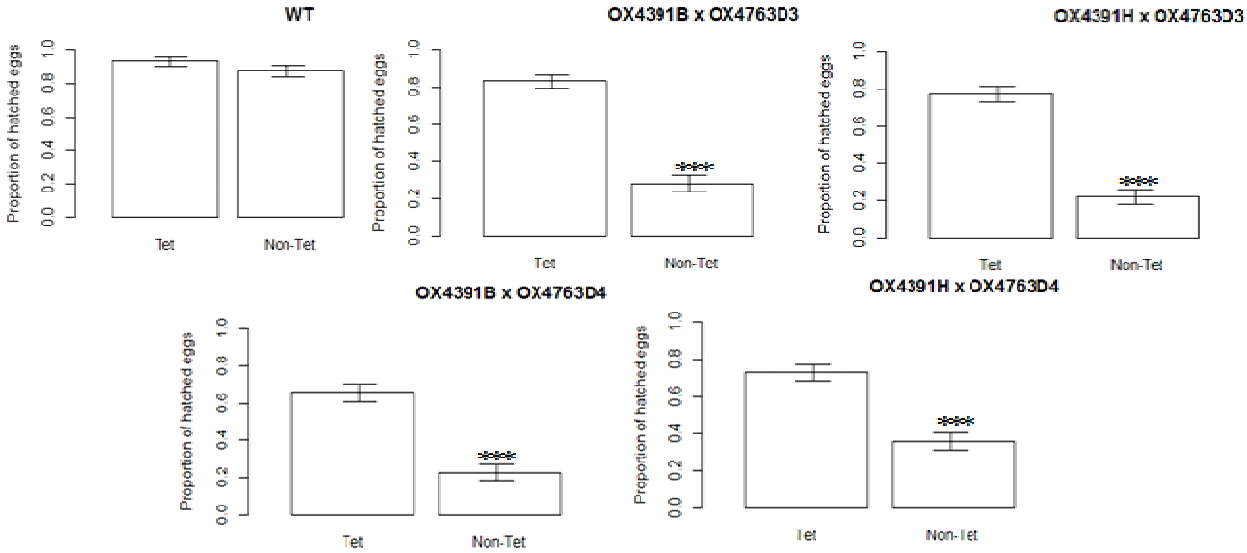


Figure 3. 21 The male and female fertility test for medfly lines carrying both *topi* driven *tTAV* strain (OX4391) and *tetO- EcoRI strain* (OX4763).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4391 (B and H); OX4763 (D3 and D4) crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4391 (B and H); OX4763 (D3 and D4) crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of OX4391 (B and H); OX4763 (D3 and D4) crossed to wild-type females. In OX4391 (B and H); OX4763 (D3 and D4) (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

These results demonstrate that *β2-tubulin-tTAV* (OX4282) and *topi-tTAV* (OX4391, OX4371) are both capable of driving an effector to reduce male fertility in medfly. A more dramatic effect was seen when the effector was *EcoRI* (OX4763), than when it was *I-ppoI* (OX4112) or *ZnF* (OX4104). However, even in the best combination, it is still not close to 100%. Furthermore, both *topi* and *β2-tubulin* drivers crossed to *EcoRI* had high effect on female fertility, indicating that the *EcoRI* based construct impairs fertility of female flies as well as male flies. Population suppression is feasible using SIT and only partially sterilized males, although, as discussed in section 1.3 and 3.1, there are some disadvantages to this. I therefore chose to continue to develop the system with the aim of achieving 100% sterility.

3.3.4. The generation OX4483 (β2-tubulin promoter based construct) and OX4485 (topi promoter based construct) in medfly

To avoid issues relating to positive feedback in the construct design (see Section 3.2) OX4483 and OX4485 constructs were designed. The experimental constructs are; (1). (OX4483 PB-Hr1E-AmCyan-SV40-ccTubulin-hsp83-tTAVNew-SV40) and (2) (OX4485 PB-Hr1E-AmCyan-SV40-topi-ubi-tTAV2-topi 3'UTR). In these *topi* or *β2-tubulin* promoters drive expression of tTAV, with no other elements included on the transgene. To generate transgenics for these construct, pre-blastoderm medfly embryos were micro-injected with the experimental constructs plasmid DNA together with a *piggyBac* DNA helper construct.

OX4483 was injected into 1000 medfly embryos and OX4485 was injected into 1100 medfly embryos. From those 260 OX4483-injected embryos and 202 OX4485-injected embryos survived to the L1 larval stage. 201 larvae from OX4483 injections and 112 larvae from OX4485 injections were survived to adulthood (20% egg to adult survival for OX4483 and 10% egg to adult survival for OX4485). Around 32% of these progeny displayed AmCyan transient expression, confirming the presence of plasmid in those individuals in both sets of injections. The results are shown in Table 3.1, and are consistent with previous results at Oxitec (Ant 2013; Bilski 2012; Jin 2011).

All surviving adult flies (with or without transient expression) from both injection sets were crossed to wild-type flies (10 G0 males from survivors crossed to 30 wild-type females and 20 females from G0 survivors crossed to 10 wild-type males). The cages were labelled alphabetically (A, B, C…). The G1 progeny from individual crosses were collected and screened for expression of the transformation marker (AmCyan fluorescent). Five

independent lines were obtained from injections with OX4483 and six independent lines were obtained from injections with OX4485.

Table 3. 1 The generation of OX4483 and OX4485 transgenic strains.

OX4483 plasmid (*β2-tubulin* based construct) and OX4485 plasmid (*topi* based construct) were injected into preblastoderm medfly embryos. 5 transgenic lines were obtained from the OX4483 (*β2-tubulin* based construct) injections and 6 transgenic lines were obtained from the OX4485 (*topi* based construct) injections.

Construct No.	No. of eggs injected	G0 Larvae	G0 Adults	No. of transgenic lines obtained
OX4483 *β2-tubulin -tTAV*	1000	260	201	5 lines (A, D, E, I, Q)
OX4485 *topi-tTAV*	1100	202	112	6 Lines (Da, Db, E, Ja, Jb)

To estimate the number of transgene copies inserted into each line; 6 males from each individual strain were crossed to 12 wild-type females. The number of wild-type versus transgenic was scored. The observed segregation of fluorescent phenotypes confirmed the presence of a single insertion of the transgene in all strains and similar ratios of males to females in eclosed adults confirmed their autosomal localization in most strains. This was an exception in the OX4483A and OX4485E lines where the presence of only male transgenics suggested that the transgenes are located on the Y-chromosome. OX4485Da and OX4485Db strains were generated from the same cage. They had a different fluorescence pattern so they were separated as two different lines. OX4485Da had fewer fluorescent nuclear dots resulting in overall dim fluorescence, while OX4485Db is showed more positive nuclear signals resulting in brighter fluorescence overall.

Both lines were observed for three generations, they continued to be distinct from each other in their fluorescence pattern but uniform in their own lines. The same was detected in OX4485Ja and OX4485Jb lines.

3.3.5. The combination of the *β2-tubulin-tTAV* construct (OX4483) with effector strains (*tetO-I-ppoI* (OX4112I), *tetO-ZnF* (OX4104F) and *tetO-EcoRI* strain (OX4763)) has limited effects on male fertility

To test whether these drivers would improve the sterility previously observed, the OX4483 strain of medfly which carries a *β2-tubulin* (latest version) based promoter that drives the expression of *tTAV* crossed to variety of effector strains (*tetO-I-ppoI* (OX4112I), *tetO-ZnF* (OX4104F) and *tetO-EcoRI* strain (OX4763)) of medfly. To examine male fertility, an experiment was set up as explained in section 3.2. From the progeny of such a cross, 25% of them should have the *β2-tubulin* promoter driving expression tTAV in the male germline, and consequent expression of the effector linked to *tetO*. Expression of the effector should be repressed by addition of tetracycline, therefore fertility of males of OX4483 x OX4112I, OX4104F or OX4763D3 and D4 was compared in the absence and presence of tetracycline in the larval diet (100ng/µl).

As previously shown, OX4483 x OX4112, OX4104F or OX4763D3 and D4 crosses might have fitness cost on the flies that are carrying them. To check if the combination of both transgene has any detrimental effects on the viability of flies, an experiment was set up, where number of wild-type and transgenic progeny were scored. Figure 3.18 illustrates the results from this experiment, where the OX4483 (A, D, E, I and Q) flies were crossed to the OX4112I flies. The observed segregation of different fluorescent phenotypes confirmed a single insertion of in each transgenic strain. The presence of both transgenes in a single organism appears to be detrimental as the number of the individuals containing both constructs was lower in all crosses (Figure 3.22).

OX4483 x OX4112 crosses	No. of wild-type progeny (%)	No. of OX4112 progeny (%), *P* Value	No. of OX4483 progeny (%), *P* Value	No. of OX4483 x OX4112 progeny (%), *P* Value
OX4483A x OX4112	98(40)	65(26.5), 0.09	40(16.3), 0.001	42(17.1), 0.002
OX4483D x OX4112	51(37.2)	26(18.9), 0.01	44(32.1), 0.53	16(11.6), 0.0002
OX4483E x OX4112	72(32.2)	47(21.1), 0.12	68(30.4), 0.82	36(16.1), 0.02
OX4483I x OX4112	54(32.2)	12(10.6), 1.1 x 10^{-2}	34(30.1), 0.81	13(11.5), 2.4 x 10^{-6}
OX4483Q x OX4112	86(29.1)	57(19.3), 0.15	95(32.2),0.69	57(19.3), 0.15

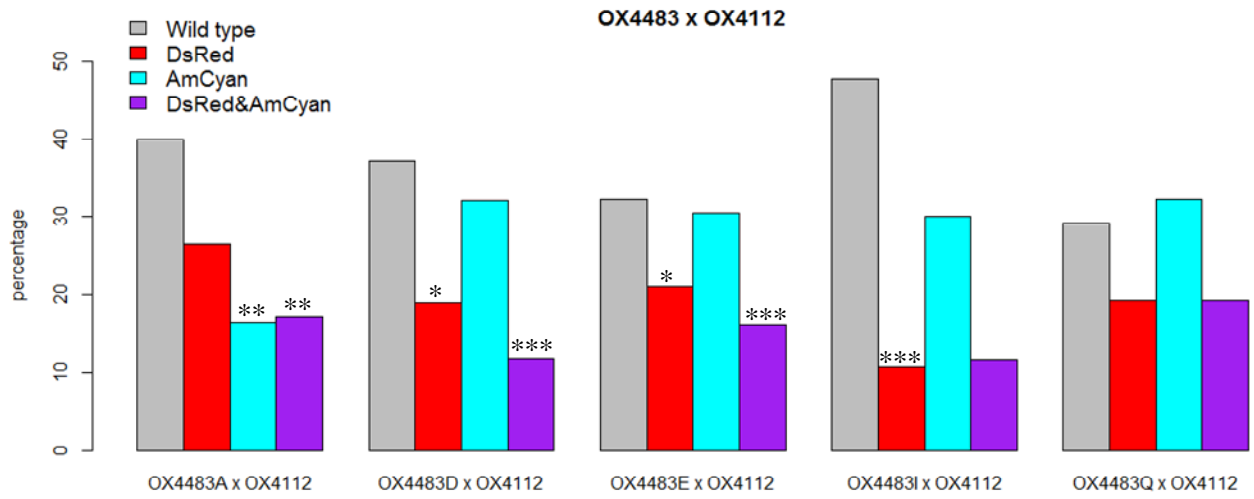


Figure 3. 22 The results from fluorescence screening of the progeny from the crosses of OX4483 strain (*ß2-tubulin*) with OX4112I strain (*IppoI*).

The table shows the actual number and percentage of wild-type and transgenic progeny of the OX4483 (*AmCyan*), OX4112I (*DsRed2*) and OX4483 x OX4112I (both markers for double-heterozygotes) resulting from the crosses of OX4483 x OX4112I (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4483 and OX4112I transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

Figure 3.23, illustrates the ratio of different type of pupae of the OX4483 strains crossed to OX4104F (see section 3.2). The presence of both transgenes in a single organism does not have any detrimental effect.

OX4483 x OX4104 crosses	**No. of wild-type progeny (%)**	**No. of OX4104 progeny (%), *P* Value**	**No. of OX4483 progeny (%), *P* Value**	**No. of OX4483 x OX4104 progeny (%), *P* Value**
OX4483A x OX4104	56(22.3)	63(25.1), 0.68	67(26.6), 0.53	65(25.9), 0.60
OX4483D x OX4104	65(30.5)	56(26.2), 0.57	48(22.5), 0.27	44(20.6), 0.16
OX4483E x OX4104	67(27)	55(22.1), 0.35	68(27.4), 0.81	58(23.3), 0.32
OX4483I x OX4104	66(30)	65(29.5), 0.95	44(20), 0.15	45(20.4), 0.17
OX4483Q x OX4104	63(23.9)	68(25.8), 0.78	70(26.6), 0.70	62(23.5), 0.95

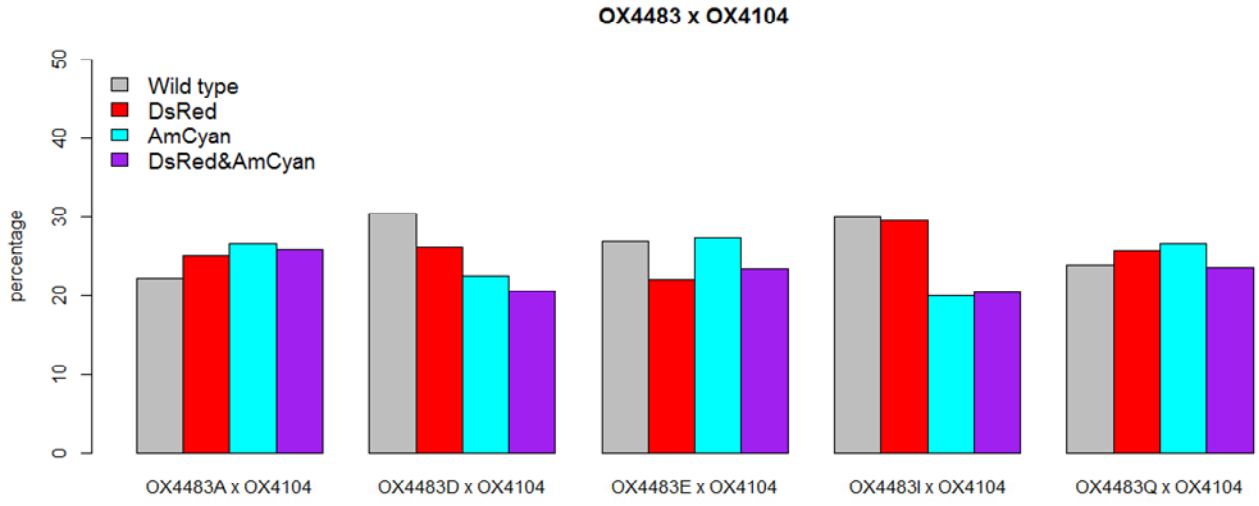


Figure 3. 23 The results from fluorescence screening results of the progeny from crosses of the OX4483 strain (*ß2-tubulin*) with OX4104F strain (*ZnF*).

The table shows the actual number and percentage of wild-type and transgenic progeny of the OX4483 (*AmCyan*), OX4104F (*DsRed2*) and OX4483 x OX4104F (both markers for double-heterozygotes) resulting from the crosses of OX4483 x OX4104F (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4483 and OX4104F transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class.

For OX4483 x OX4763D3 or D4 I again confirmed a single insertion of each transgene and found that the presence of both transgenes in a single organism was detrimental (Figure 3.24). Generally there are lower numbers showing the *DsRed2* fluorescent phenotype than the other fluorescent phenotypes and wild-type in all lines of OX4763D4. Although the *DsRed2* marker is expressing robustly in OX4763D3, the *DsRed2* maker expression in OX4763D4 is very dim, screening can therefore be hard which may result in human error. This is one potential explanation as to the presence of fewer red progeny observed in this experiment, although it cannot explain the OX4763D3 result.

OX4483 x OX4763 crosses	No. of wild-type progeny (%)	No. of OX4763 progeny (%), *P* Value	No. of OX4483 progeny (%), *P* Value	No. of OX4483 x OX4763 progeny (%), *P* Value
OX4483A x OX4763D3	69(28.7)	63(26.2), 0.73	53(22.1), 0.34	55(22.9), 0.41
OX4483D x OX4763D3	51(24.5)	57(27.4), 0.68	47(22.6), 0.77	53(25.4), 0.90
OX4483Q x OX4763D3	70(28.2)	63(25.4), 0.69	64(25.8), 0.74	51(20.5), 0.27
OX4483A x OX4763D4	63(33.8)	34(18.2), 0.03	59(31.7), 0.79	30(16.1), 0.01
OX4483D x OX4763D4	71(41.2)	31(18.0), 0.002	47(27.3), 0.09	23(13.3), 0.0001
OX4483Q x OX4763D4	56(32)	29(16.5), 0.02	61(34.8), 0.72	29(16.5), 0.02

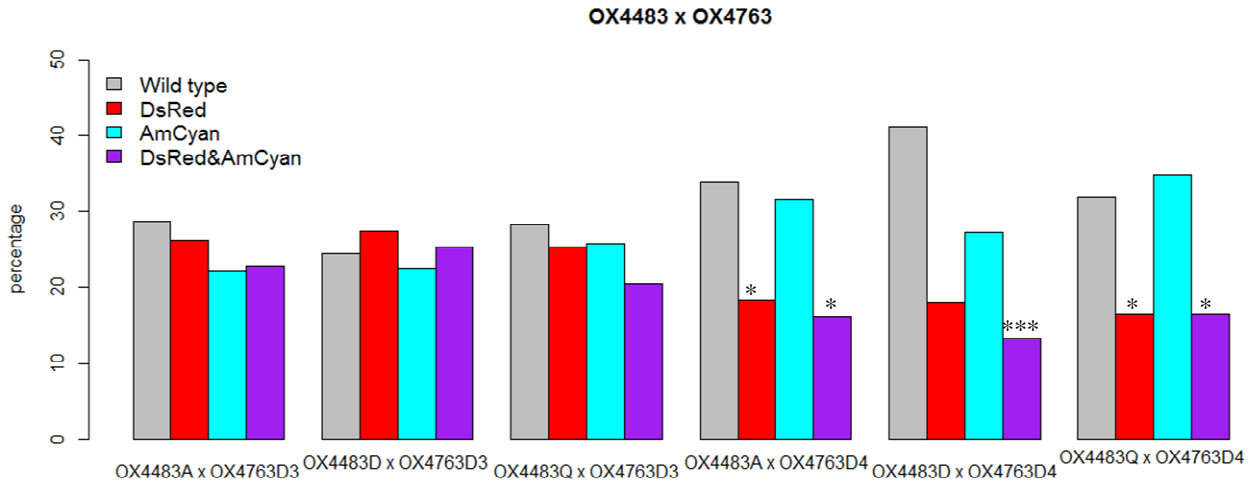


Figure 3. 24 The results from fluorescence screening of the progeny from crosses of the OX4483 strain (*ß2-tubulin*) with OX4763 strain (*EcoRI*).

The table shows the actual number and percentage of wild-type and transgenic progeny of the OX4483 (*AmCyan*), OX4763 (*DsRed2*) and OX4483 x OX4763 (both markers for double-heterozygotes) resulting from the crosses of OX4483 x OX4763 (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4483 and OX4763 transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

Male progeny containing both transgenes from the OX4483 x OX4112I, OX4104F or OX4763D3 and D4 crosses were crossed to wild-type females (6 males to 12 females) to test whether paternal effect lethality could be induced in these flies.

A significant reduction in egg hatching was found for OX4483D x OX4112I ($p<0.05$), furthermore there was a highly significant effect from the OX4483A, OX4483I and OX4483Q strains crossed to OX4112I ($p<0.005$). No significant effect was found for OX4483E x OX4112I strain ($p>0.05$) (Figure 3.25).

Females from individual crosses were also crossed to wild-type males to check if these crosses gave a male specific effect. Combinations of OX4483 x OX4112F in female medfly did not show a significant reduction in the egg viability in the absence of tetracycline in the larval diet (Figure 3.25).

OX4483 x OX4112 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	455	304	67.7	0.06
	NT	354	288	81.3	
OX4483A x OX4112(m)	T	407	280	68.7	0.003
	NT	371	181	48.7	
OX4483D x OX4112(m)	T	439	307	71.6	0.0003
	NT	423	184	43.1	
OX4483E x OX4112(m)	T	398	233	56.8	0.0004
	NT	562	232	43.6	
OX4483I x OX4112(m)	T	375	298	79.4	9.6×10^{-6}
	NT	410	172	19.3	
OX4483Q x OX4112(m)	T	549	410	74.9	2.2×10^{-16}
	NT	536	93	17.3	
OX4483D x OX4112(f)	T	337	305	80.9	0.72
	NT	353	297	81.1	
OX4483E x OX4112(f)	T	376	291	77.4	0.80
	NT	391	310	79.4	
OX4483I x OX4112(f)	T	591	483	82.4	0.92
	NT	372	299	80.3	
OX4483Q x OX4112(f)	T	414	320	77.6	0.85
	NT	396	312	79.9	

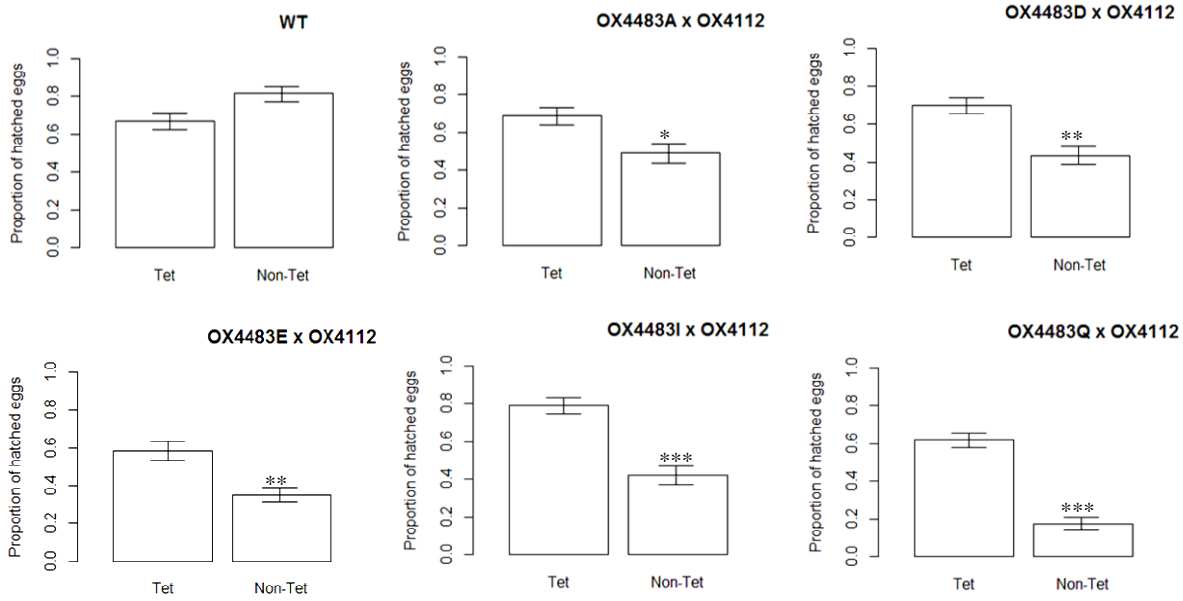


Figure 3. 25 The male and female fertility test for medfly lines carrying both *ß2-tubulin* driven *tTAV* strain (OX4483) and *tetO-IppoI* strain (OX4112I).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4483 (A, D, E, I and Q); OX4112I crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4483 (A, D, E, I and Q); OX4112I crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of OX4483 (A, D, E, I and Q); OX4112I crossed to wild-type females. In all cases of OX4483 (A, D, E, I and Q); OX4112I (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicates a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

As shown in Figure 3.26, the reduction in the egg viability is significant between flies reared on tetracycline or off tetracycline. OX4483Q crossed to OX4104F gave a highly significant difference between the number of viable eggs between on tetracycline and off tetracycline reared flies, ($p<0.0005$). OX4483D and OX4483I line crossed to OX4104F also generated a highly significant difference between the number of viable eggs between on tetracycline and off tetracycline reared flies ($p<0.005$); OX4483A and OX4483E crossed to OX4104F also produce significant results in the difference between the egg viability number between flies reared off and on tetracycline ($p<0.05$).

Twelve females of the double-heterozygotes from individual lines were crossed to six wild-type male flies to test if the observed sterility was male specific. The results confirmed that the OX4483 x OX4114 in medfly does not affect female fertility. The results are shown in Figure 3.26.

OX4483 x OX4104 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	380	351	92.3	0.5
	NT	413	399	82.2	
OX4483A x OX4104(m)	T	412	337	81.9	6.8×10^{-7}
	NT	496	238	49.1	
OX4483D x OX4104(m)	T	416	366	88.1	0.0009
	NT	406	250	61.3	
OX4483E x OX4104(m)	T	439	364	82.9	0.0007
	NT	420	243	59.9	
OX4483I x OX4104(m)	T	388	337	87.1	0.42
	NT	494	309	62.4	
OX4483Q x OX4104(m)	T	512	431	84.2	4.8×10^{-7}
	NT	413	183	43.7	
OX4483D x OX4104(f)	T	375	341	90.9	0.67
	NT	396	321	87.3	
OX4483E x OX4104(f)	T	399	350	87.6	0.88
	NT	390	337	86.5	
OX4483I x OX4104(f)	T	343	303	88.3	0.67
	NT	361	305	84.5	
OX4483Q x OX4104(f)	T	346	298	86.2	0.94
	NT	409	353	86.4	

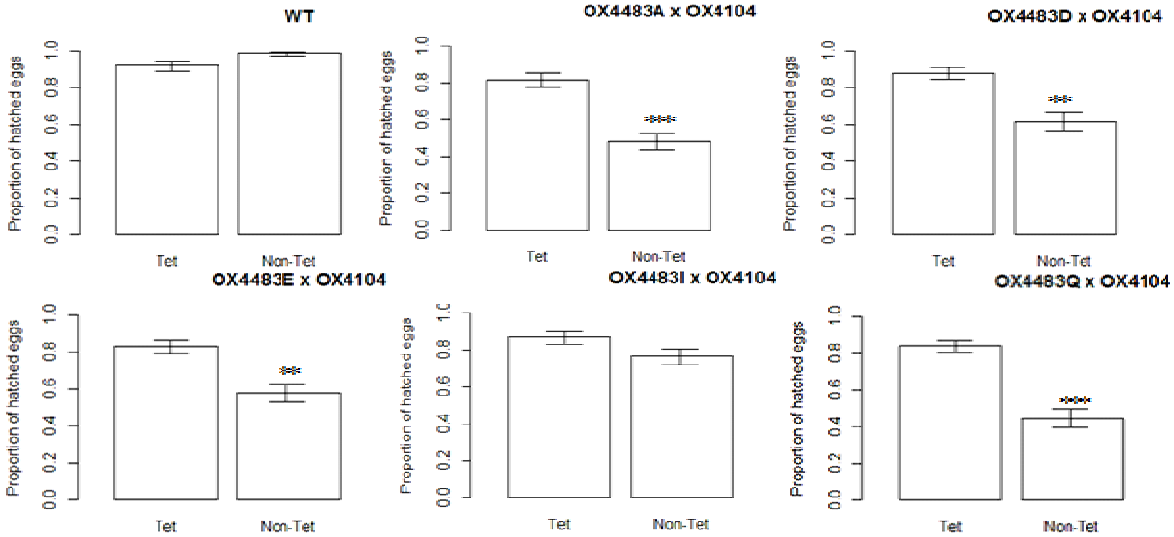


Figure 3. 26 The male and female fertility test for medfly lines carrying both *ß2-tubulins* driven *tTAV* strain (OX4483) and *tetO- ZnF* strain (OX4104F).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4483 (A, D, E, I and Q); OX4104F crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4483 (A, D, E, I and Q); OX4104F crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of OX4483 (A, D, E, I and Q); OX4104F crossed to wild-type females. In all cases of OX4483 (A, D, E, I and Q); OX4104F (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05) except for OX4483I x OX4104 (males) crossed to wild-type females. The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicates a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

The reduction in egg viability was also significant when OX4483E or OX4483Q was crossed to OX4763D4 ($p<0.05$), and highly significant for OX4483E or OX4483Q x OX4763D3, and OX4483A x OX4763D4 ($p<0.0005$) (Figure 3.27).

The double-heterozygotes females of above crosses were crossed to wild-type males to test if the observed sterility was male specific. As Figure 3.27 shows OX4483 x OX4763 in medfly does not generates male specific sterility, as results from female crosses showed significant reductions in the egg viability in the absence of tetracycline in the larval diet.

OX4483 x OX4763 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	385	359	93.2	0.05
	NT	392	344	87.7	
OX4483A x OX4763D3(m)	T	395	281	71.1	2.8×10^{-10}
	NT	354	99	27.9	
OX4483E x OX4763D3(m)	T	416	330	79.3	1.4×10^{-10}
	NT	411	229	55.7	
OX4483Q x OX4763D3(m)	T	447	357	79.8	6.6×10^{-10}
	NT	440	246	55.9	
OX4483A x OX4763D4(m)	T	463	396	85.5	6.3×10^{-10}
	NT	416	160	38.4	
OX4483E x OX4763D4(m)	T	420	255	60.7	0.0006
	NT	411	85	20.6	
OX4483Q x OX4763D4(m)	T	414	309	74.6	0.007
	NT	408	112	27.4	
OX4483E x OX4763D3(f)	T	463	348	75.1	0.040
	NT	475	291	61.2	
OX4483Q x OX4763D3(f)	T	463	384	82.9	0.050
	NT	475	291	61.2	
OX4483E x OX4763D4(f)	T	417	331	79.3	0.0004
	NT	413	222	53.7	
OX4483Q x OX4763D4(f)	T	447	357	79.8	0.0008
	NT	440	246	55.9	

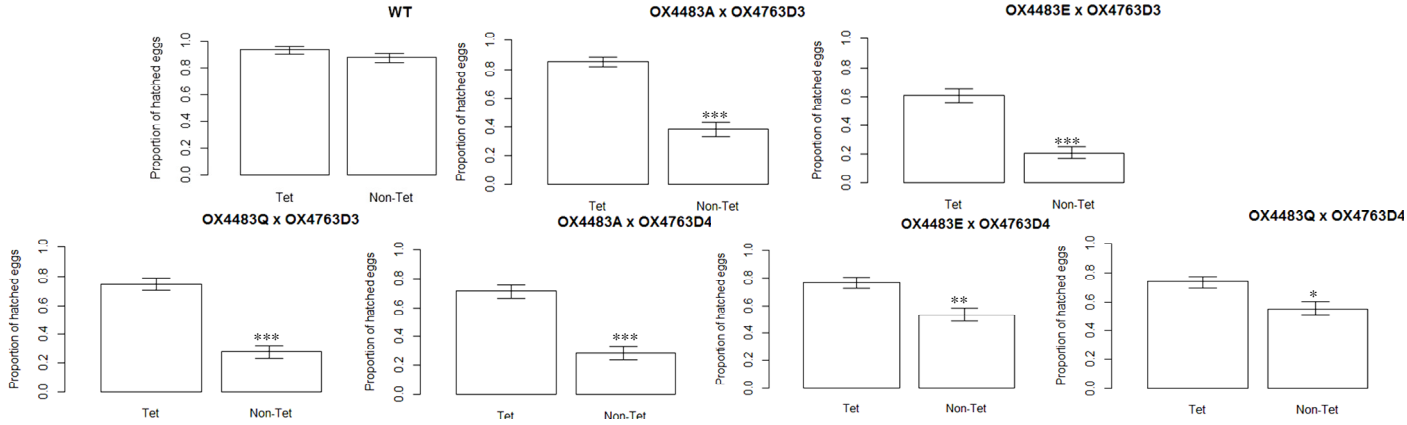


Figure 3. 27 The male and female fertility test for medfly lines carrying both *ß2-tubulin* driven *tTAV* strain (OX4483) and *tetO- EcoRI* strain (OX4763).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4483 (A, E and Q); OX4763 (D3 and D4) crossed to wild-type females are presented with an (m) in front of them while the female flies of OX4483 (A, E and Q); OX4763 (D3 and D4) crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of the OX4483 (A, E and Q); OX4763 (D3 and D4) crossed to wild-type females. In all cases of the OX4483 (A, E, and Q); OX4763 (D3 and D4) (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

Although significant reductions in male fertility were found for many of these *β2-tubulin - tTAV* x effector combinations, the most dramatic effect was OX4483Q x OX4112, which reduced male fertility to 17.3% of control levels. This falls short of our ideal goal of 100% sterility.

3.3.6. The combination of the *topi-tTAV* construct (OX4485) with effector strains (*tetO-I-ppoI* (OX4112I), *tetO-ZnF* (OX4104F) and *tetO-EcoRI* strain (OX4763)) has limited effects on male fertility

Six transgenic lines were obtained from injection of OX4485 into medfly eggs. All lines were crossed to flies of the opposite sex containing the effector constructs (*tetO-I-ppoI* (OX4112I), *tetO-ZnF* (OX4104F) and *tetO-EcoRI*). OX4485 has *topi* as the promoter that controls expression of *tTAV* while the effectors' expression is driven by *tetO*. The progeny of these crosses shouldn't be fertile in the absence of tetracycline in the larval diet if *topi* driven expression of effectors in the male germline causes cleavage of DNA and thus non-functional sperm.

Eight females from OX4485 were crossed to four OX4112I, OX4104F or OX4763D3 or D4 males. Male fertility was assessed in the absence and presence of tetracycline in the larval diet (100ng/µl) as the expression of the effector should be repressed by addition of tetracycline.

To check for possible detrimental effect on viability the number of transgenic and wild-type progeny of the OX4485 (Da, Db, E, Ja and Jb) flies crossed to the OX4112I, OX4104F or OX4763D3 or D4 flies were scored.

As shown in Figure 3.28 in all lines of OX4485 x OX4112, the presence of both transgenes in a single organism seems to be detrimental as the number of the individuals containing both constructs is lower in all crosses.

OX4485 x OX4112 crosses	No. of wild-type progeny (%)	No. of OX4112 progeny (%), *P* Value	No. of OX4485 progeny (%), *P* Value	No. of OX4485 x OX4112 progeny (%), *P* Value
OX4485Da x OX4112	115(30.9)	89(23.9), 0.34	92(24.7), 0.40	76 (20.4), 0.14
OX4485Db x OX4112	102(30.9)	76(23), 0.28	89(26.9), 0.60	14(19.1), 0.09
OX4485E x OX4112	65(35.9)	42(23.2), 0.09	51(28.1), 0.33	38(12.7), 0.0008
OX4485Ja x OX4112	63(34.8)	52(28.7), 0.44	46(25.4), 0.22	26(11), 0.0004
OX4485Jb x OX4112	146(32.1)	112(24.6), 0.32	105(23.1), 0.22	92(20.7), 0.10

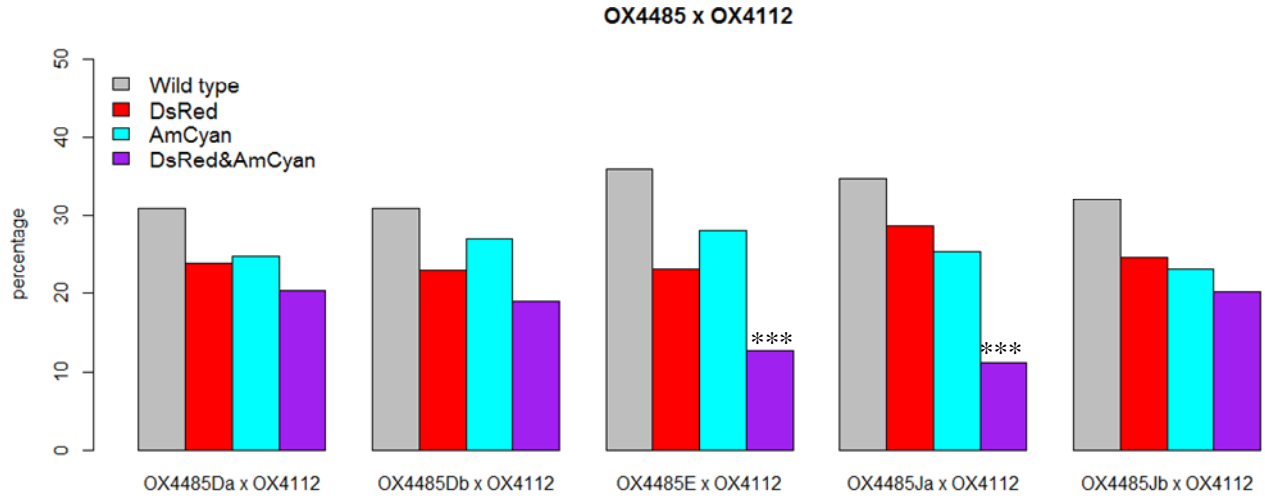


Figure 3. 28 The results from fluorescence screening of the progeny from crosses of the OX4485 strain (*topi*) with OX4112I strain (*IppoI*).

The table shows the actual number and percentage of wild-type and transgenic progeny of the OX4485 (*AmCyan*), OX4112I (*DsRed2*) and OX4485XOX4112I (both markers for double-heterozygotes) resulting from the crosses of OX4485XOX4112I (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4485 and OX4112I transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

No detrimental effects were detected and the observed segregation of different fluorescent phenotypes for OX4485 x OX4104F suggests a single insertion of each transgene, as shown in Figure 3.29.

OX4485 x OX4104 crosses	No. of wild-type progeny (%)	No. of OX4104 progeny (%), *P* Value	No. of OX4485 progeny (%), *P* Value	No. Of OX4485XOX4104 progeny (%), *P* Value
OX4485Da x OX4104	59(25.2)	55(23.5), 0.80	61(26.1), 0.90	59 (25.2), 0.66
OX4485Db x OX4104	63(24.1)	71(27.2), 0.78	68(26), 0.82	59(22.6), 0.85
OX4485E x OX4104	78(25.4)	74(24.1), 0.75	85(27.7), 0.49	69(22.5), 0.75
OX4485Ja x OX4104	73(29.5)	69(27.9), 0.87	56(22.6), 0.16	49(19.8), 0.78
OX4485Jb x OX4104	43(22.9)	52(27.8), 0.70	47(25.1), 0.86	45(24.1), 0.95

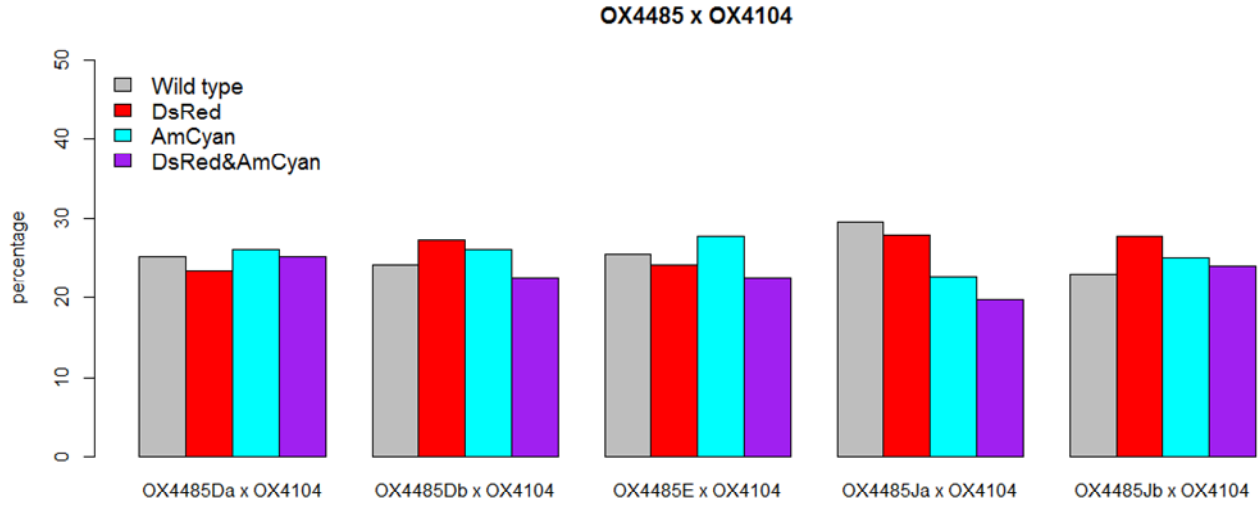


Figure 3. 29 The results from fluorescence screening of the progeny from crosses of the OX4485 strain (*topi*) with OX4104F strain (*ZnF*).

The table shows the actual number and percentage of wild-type and transgenic progeny of the OX4485 (*AmCyan*), OX4104F (*DsRed2*) and OX4485 x OX4104F (both markers for double-heterozygotes) resulting from the crosses of OX4485 x OX4104F (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4485 and OX4104F transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class.

Figure 3.30, shows the result of the OX4485 x OX4763 cross, and reveals that the presence of both transgenes in a single organism seems to be detrimental. As noted earlier, OX4763D4 is very dim, screening can therefore be difficult and human error could influence the results.

OX4485 x OX4763 crosses	No. of wild-type progeny (%)	No. of OX4763 progeny (%), *P* Value	No. of OX4485 progeny (%), *P* Value	No. of OX4485 x OX4763 progeny (%), *P* Value
OX4485Da x OX4763D3	64(28.7)	59(24.9), 0.77	71(22.9), 0.70	63(24.5), 0.95
OX4485E x OX4763D3	65(24.5)	69(25.4), 0.82	62(27.1), 0.86	59(23.1), 0.73
OX4485Ja x OX4763D3	55(28.2)	61(25.7), 0.70	48(28.5), 0.82	50(23.3), 0.63
OX4485Da x OX4763D4	72(33.8)	38(34.9), 0.81	68(18.4), 0.02	28(13.5), 0.002
OX4485E x OX4763D4	67(41.2)	35(16.5), 0.02	71(33.6), 0.81	38(18), 0.03
OX4485Ja x OX4763D4	70(32)	33(17.2), 0.008	68(35.6), 0.90	20(10.4), 0.0001

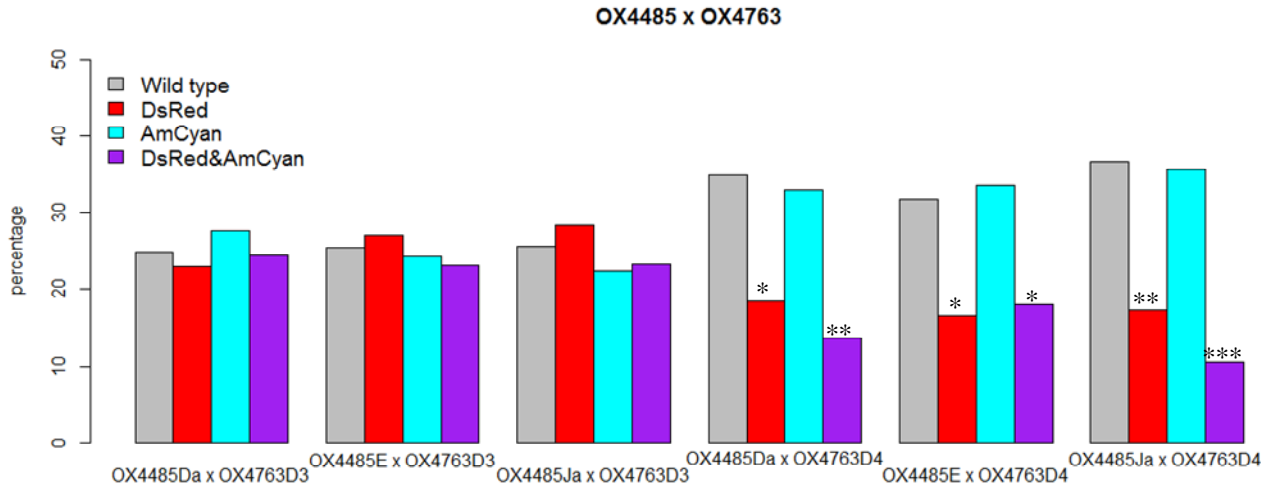


Figure 3. 30 The result from fluorescence screening of the progeny from crosses of the OX4485 strain (*topi*) with OX4763 strain (*EcoRI*).

The table shows the actual number and percentage of wild-type and transgenic progeny of the OX4485 (*AmCyan*), OX4763 (*DsRed2*) and OX4485 x OX4763 (both markers for double-heterozygotes) progeny resulting from the crosses of OX4485 x OX4763 (progeny were fed on tetracycline diet (100ng/ul) at larval stage). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1:1:1 ratio expected from the segregation of a single insertion of the OX4485 and OX4763 transgene. The bar chart illustrates percentage ratio of observed progeny. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion. The Y axis shows the percentage of pupae in each phenotypic class. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

The progeny from the OX4485 x OX4112I, OX4104F or OX4763D3 or D4 crosses were screened and pupae expressing both markers were collected (double-heterozygotes). To check for male fertility, 6 males from each line were crossed to 12 wild-type virgin females. Two of the OX4485 strain (Da and Ja) crossed to the OX4112I strain produce significant reduction in egg viability (p value is less than 0.05) but this at best 47.5%, still very far from 100% (Figure 3.31). The remaining crosses showed no significant reduction in egg viability; thus, most of the OX4485 strains crossed to the OX4112I were unable to produce significant results when reared off tetracycline. Females from individual lines were crossed to wild-type males to check if the sterility was male specific. Results from female crosses showed no significant reductions in egg viability in the absence of tetracycline. The double-heterozygotes of the OX4485 x OX4112I in medfly have no effect on female fertility. The results are shown in Figure 3.31.

OX4485 x OX4112 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	461	367	80.6	0.06
	NT	353	294	83.1	
OX4485Da x OX4112(m)	T	486	419	85.6	0.01
	NT	488	331	67.9	
OX4485Db x OX4112(m)	T	457	365	80.1	0.38
	NT	439	260	59.2	
OX4485E x OX4112(m)	T	490	316	64.1	0.05
	NT	486	256	52.5	
OX4485IJa x OX4112(m)	T	394	304	76.4	0.04
	NT	415	255	60.8	
OX4485Jb x OX4112(m)	T	450	332	73.4	0.05
	NT	416	226	54.8	
OX4485Da x OX4112(f)	T	364	283	77.8	0.53
	NT	341	247	72.4	
OX4485Db x OX4112(f)	T	388	294	75.8	0.48
	NT	355	296	83.3	
OX4485IJa x OX4112(f)	T	411	316	77.1	0.93
	NT	413	315	76.3	
OX4485Jb x OX4112(f)	T	367	271	73.8	0.95
	NT	413	307	74.6	

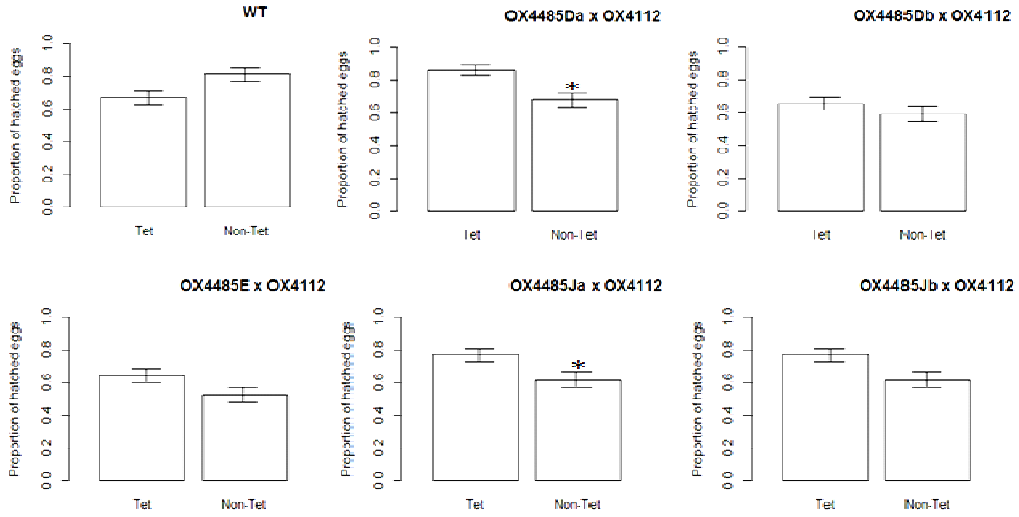


Figure 3. 31 The male and female fertility test for medfly lines carrying both *topi* driven *tTAV* strain (OX448) and *tetO- I-ppoI* strain (OX4112).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4485 (Da, Db, E, Ja and Jb); OX4112 crossed to wild-type females are presented with an (m) in front of them while the female flies of the OX4485 (Da, Db, E, Ja and Jb); OX4112 crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of the OX4485 (Da, Db, E, Ja and Jb); OX4112 crossed to wild-type females. In non of the cases of the OX4485 (Da, Db, E, Ja and Jb), OX4112 (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value>0.05), except for OX4485Da or Ja x OX4112 (males) crossed to wild-type females. They axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

Figure 3.32 presents the results from the male fertility test of OX4485 x OX4104F. The reduction in egg viability in most cases was not significantly different between the flies reared on tetracycline vs. non tetracycline, as the P values are greater than 0.05. The OX4485E and Ja lines are exceptions as they showed a significant reduction of $p<0.05$ in the egg viability. Again the reduction, while significant, is only at best 43.7%.

The female flies were also assed for fertility; twelve double-heterozygotes females were crossed to six wild-type males. The crosses showed no significant reduction in egg viability in all cases. This indicates that the transgene does not impair the female fertility. The results are shown in table 3.32.

OX4485 x OX4104 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	482	440	91.2	0.50
	NT	390	336	86.2	
OX4485Da x OX4104(m)	T	497	431	86.7	0.002
	NT	476	280	59.3	
OX4485Db x OX4104(m)	T	409	306	74.9	0.26
	NT	494	298	60.6	
OX4485E x OX4104(m)	T	363	281	77.3	0.23
	NT	500	303	60.6	
OX4485Ja x OX4104(m)	T	457	36	80.4	0.0004
	NT	449	249	55.5	
OX4485Jb x OX4104(m)	T	474	456	75.7	0.00002
	NT	455	205	45	
OX4485Da x OX4104(f)	T	384	318	83.2	0.49
	NT	453	402	88.7	
OX4485Db x OX4104(f)	T	389	335	86.6	0.99
	NT	379	326	86.3	
OX4485Ja x OX4104(f)	T	388	330	85.1	0.91
	NT	454	382	84.1	
OX4485Jb x OX4104(f)	T	394	350	89.1	0.72
	NT	391	335	85.8	

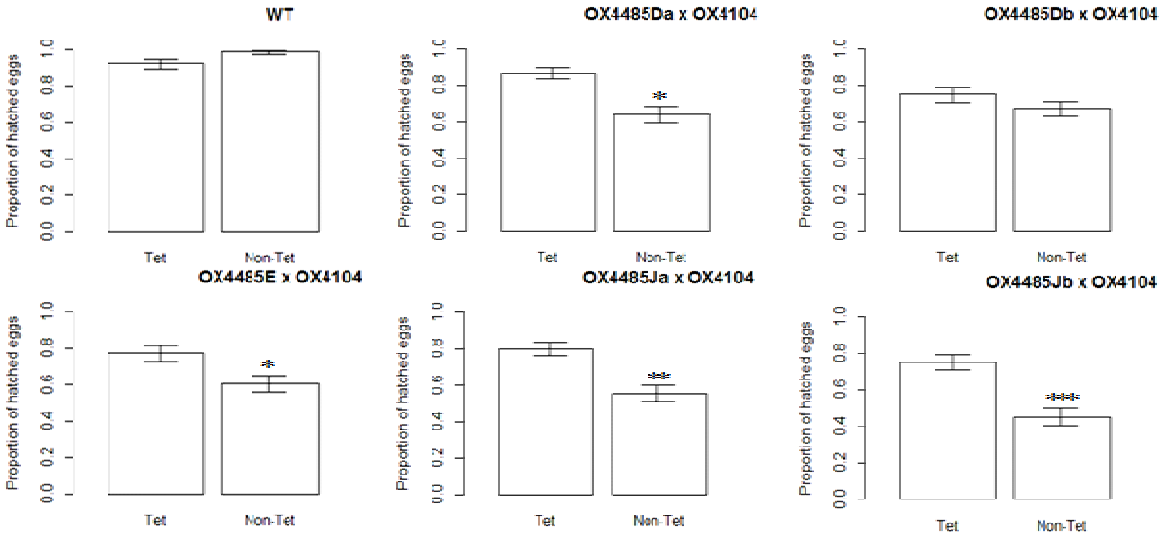


Figure 3. 32 The male and female fertility test for medfly lines carrying both *topi* driven *tTAV* strain (OX4485) and *tetO-ZnF* strain (OX4104F).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males and females of each cross to wild-type (WT) of the opposite sex. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The male flies of OX4485 (Da, Db, E, Ja and Jb); OX4104F crossed to wild-type females are presented with an (m) in front of them while the female flies of the OX4485 (Da, Db, E, Ja and Jb); OX4104F crossed to wild-type males are presented with an (f). The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of the OX4485 (Da, Db, E, Ja and Jb); OX4104F crossed to wild-type females. In all cases of the OX4485 (Da, Db, E, Ja and Jb), OX4104F (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05), except for OX4485Db x OX4104F (males) crossed to wild-type females. They axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

The reduction (45% at best) in egg viability for the males from the OX4485 x OX4763 crossed to wild-type females is significant when the males were reared off tetracycline compared to on tetracycline. All the P values are less than 0.05 except for the males of the OX4485E x OX4763D3 crossed to wild-type females ($p>0.05$). The results are shown in Figure 3.33.

The combination of the OX4485Da or Ja x OX4763D3 in medfly produce male specific sterility, as results from the female crosses do not give a significant reduction in egg viability in the absence of tetracycline in the larval diet. However, sterility does not appear to be male specific in the OX4485Da or Ja x OX4763D4 ($p<0.05$) (Figure 3.34).

OX4485 x OX4763 crosses		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	385	359	93.2	0.50
	NT	392	344	87.7	
OX4485Da x OX4763D3(m)	T	400	312	78	0.0002
	NT	410	212	51.7	
OX4485E x OX4763D3(m)	T	431	350	81.2	0.38
	NT	399	291	72.9	
OX4485Ja x OX4763D3(m)	T	309	255	82.5	$2.2\ 10^{-14}$
	NT	373	64	17.1	
OX4485Da x OX4763D4(m)	T	469	311	66.3	1.4×10^{-6}
	NT	410	136	33.1	
OX4485E x OX4763D4(m)	T	447	288	64.4	3.2×10^{-7}
	NT	462	146	31.6	
OX4485Ja x OX4763D4(m)	T	396	349	93.2	0.0001
	NT	374	202	87.7	

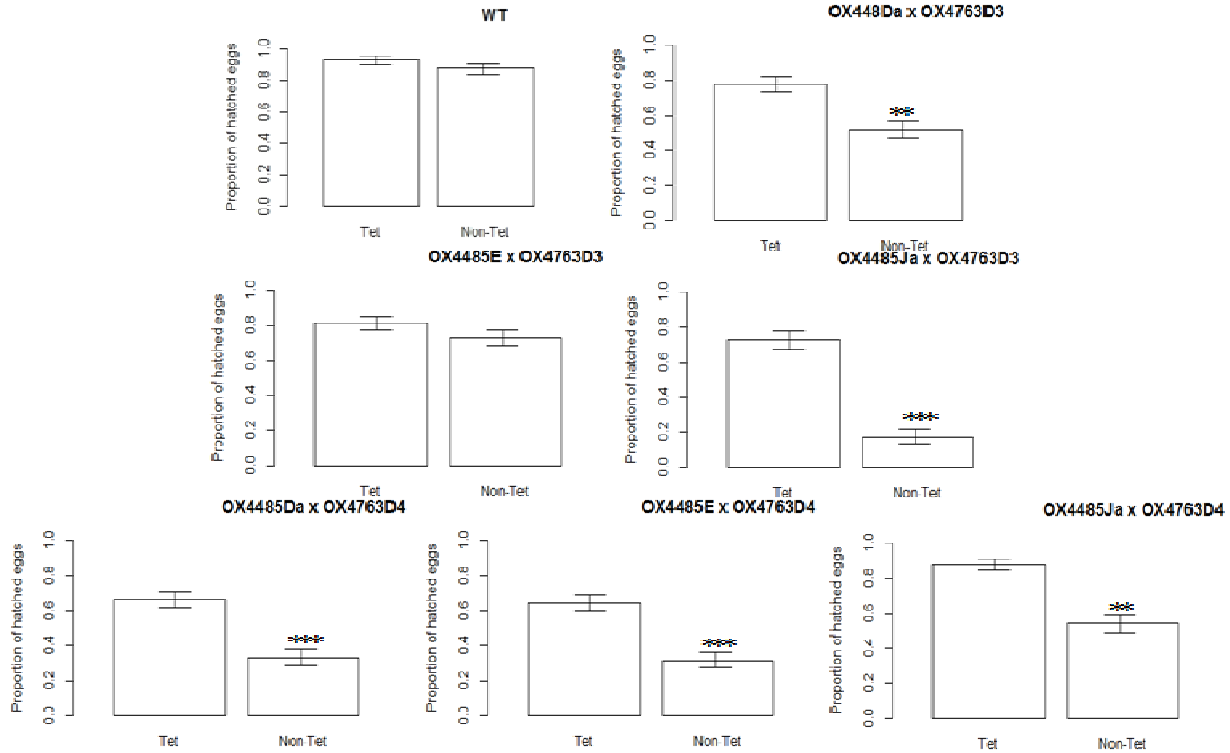


Figure 3. 33 The male fertility test for medfly lines carrying both *topi* driven *tTAV* strain (OX4485) and *tetO- EcoRI* strain (OX4763).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes females of each cross to wild-type (WT) females. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The *P* values from a Chi Squared test are also shown. The bar chart illustrates proportion of hatched eggs to non-hatched eggs in males of the OX4485 (Da, E and Ja); OX4763 (D3 and D4) crossed to wild-type females. In all cases of the OX4483 (Da, E and Ja); OX4763 (D3 and D4) (males) crossed to wild-type females; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

OX4485 x OX4763 crosses (Female)		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	385	359	93.25	0.50
	NT	392	344	87.76	
OX4485Da x OX4763D3	T	424	317	74.76	0.29
	NT	483	324	67.08	
OX4485Ja x OX4763D3	T	459	354	77.12	0.40
	NT	406	293	72.17	
OX4485Da x OX4763D4	T	420	325	77.38	0.0008
	NT	334	180	53.89	
OX4485Ja x OX4763D4	T	415	305	73.49	0.002
	NT	470	240	51.06	

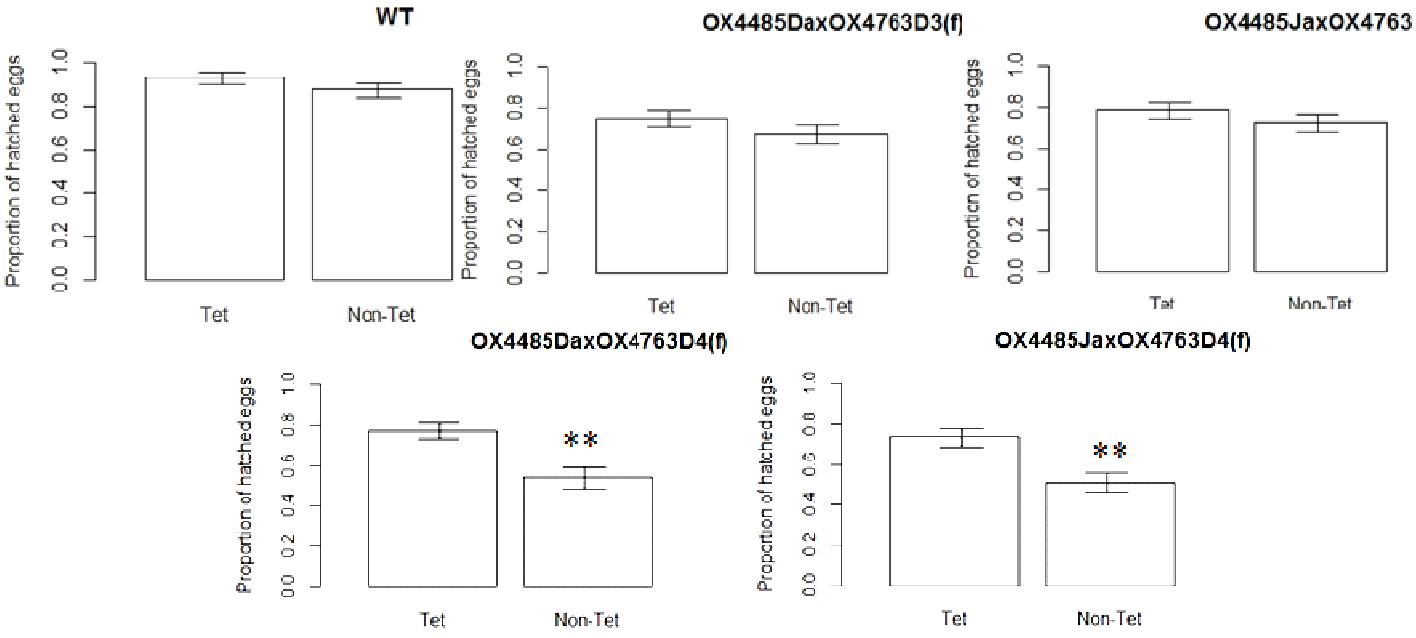


Figure 3. 34 The female fertility test for medfly lines carrying both *topi* driven *tTAV* strain (OX4485) and *tetO- EcoRI* strain (OX4763).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes females of each cross to wild-type (WT) males. The table presents the total number of eggs, The bar chart illustrates proportion of hatched eggs to non-hatched eggs in females of the OX4485 (Da and Ja); OX4763 (D3 and D4) crossed to wild-type males. In the OX4483 (Da and Ja); OX4763 D3 (females) crossed to wild-type males; the number of hatched eggs didn't differ significantly between Tet and Non-Tet (p-value>0.05). In the OX4483 (Da and Ja); OX4763 D4 (females) crossed to wild-type males; the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence intervals.

3.4. Conclusion and future work

Currently SIT is desirable method of pest control as it is species specific, environmentally friendly and cost effective. One important factor to consider is the level of sterility needed for an effective SIT programme. To decrease the number of the releases for population suppression, a high level of sterility has been proposed (99-100% sterility) (Klassen & Creech 1971). To achieve a high level of sterility, a high dose of radiation is needed, and this then imposes a fitness cost on irradiated males. The irradiated males have reduced mating competitiveness, reduced capability for successful sperm transfer and a shorter lifespan (Barry et al. 2003). All of the above issues cause a 4 to 10 fold decrease in the fitness of irradiated males compare to wild males (Alphey 2002). Therefore it is crucial to keep a balance between sterilising all released flies and reduce the fitness cost to a minimum (Bhattacharyya 2014).

Partially sterile insects have been considered for the SIT programmes, to reduce the fitness cost of radiation on irradiated flies. The radiation-induced partial sterility of Drosophila has been studied by Serebrovskii in 1940. A radiation-induced mutation, including dominant lethal which would be passed on to the offspring, was discovered. This paternal effect lethality mechanism is attractive for SIT, as it is an effective mechanism to achieve population suppression with a lower release ratio (Carpenter et al. 2005).

To enhance capitalise on the advantages of SIT while avoiding the adverse effects of radiation, a genetic based SIT has been proposed (Thomas et al. 2000). This chapter studied the development of a transgenic based paternal effect lethal strain of medfly. For purpose of this study, 100% sterility has been considered highly desirable, if not essential. This should achieve suppression in the targeted population with fewer releases, which will make the project cost efficient. Furthermore, releases of partially sterile males would mean more larvae

developing inside fruit, with consequent economical impacts. Finally, partial sterility could allow the transgene to enter the wild gene pool (although it would be highly selected against), and therefore it would be more difficult to achieve acceptance from the regulatory bodies and public for a partially sterile strain than for a strain with fully penetrant sterility.

The flies carrying the *topi* based (OX4391 and OX4391) or *β2-tubulin* based (OX4282) *tTAV* linked promoter constructs were crossed to flies of the opposite sex carrying various effector based insertions (*IppoI*, OX4112I; *ZnF*, OX4104F; *ProtamineFokI*, OX4458 and *EcoRI*, OX4763 where *tetO* controls expression of these effectors) according to the experimental design described at section 3.2. For the purposes of this report, transgenic strains or individuals from such strains, containing a specific effector or promoter insertion will be referred to by the name of the promoter or effector, respectively. As described above, I performed the tests for I-ppoI, ZnF and EcoRI, while the crosses of *ProtamineFokI* based insertion lines to lines carrying either *topi* or *β2-tubulin* based constructs were performed by Michal Bilski (PhD at Oxford University). I reference them in this report is purely for purposes of comparison to other crosses.

The results from the various promoter x effector crosses described in this chapter are summarised in Figures 3.35, 3.36, 3.37 and 3.38. The results demonstrate that, of all the combinations tested, *β2-tubulin* crossed to *ProtamineFokI* was able to provide the highest level of male-specific sterility.

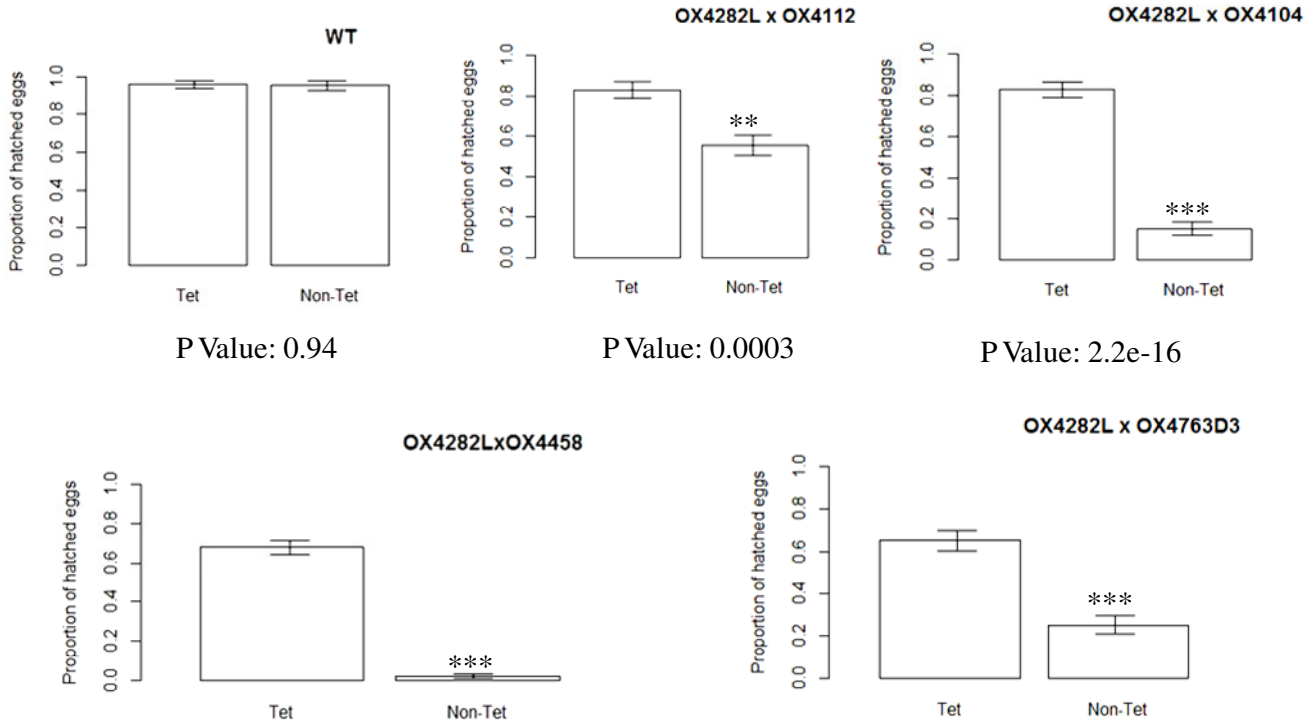


Figure 3. 35 The male fertility test for medfly lines carrying both a *β2-tubulin* based strain (OX4282) crossed to different effector based strains (OX4112I, OX4104F, OX4458 and OX4763).

The effect and repressibility of the transgene expression were tested by crossing the double-heterozygotes males wild-type (WT) of the opposite sex. The proportion of hatched eggs to non-hatched eggs in males of the OX4282L x (OX4112I, OX4104F, OX4458 and OX4763) crossed to wild-type females compared in presence and absence of tetracycline (100ng/ul) in the larvae diet. The *P* values from a Chi Squared test are also shown. In all cases the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisk on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence level. 2.2e-16=2.2 x 10^{-16}.

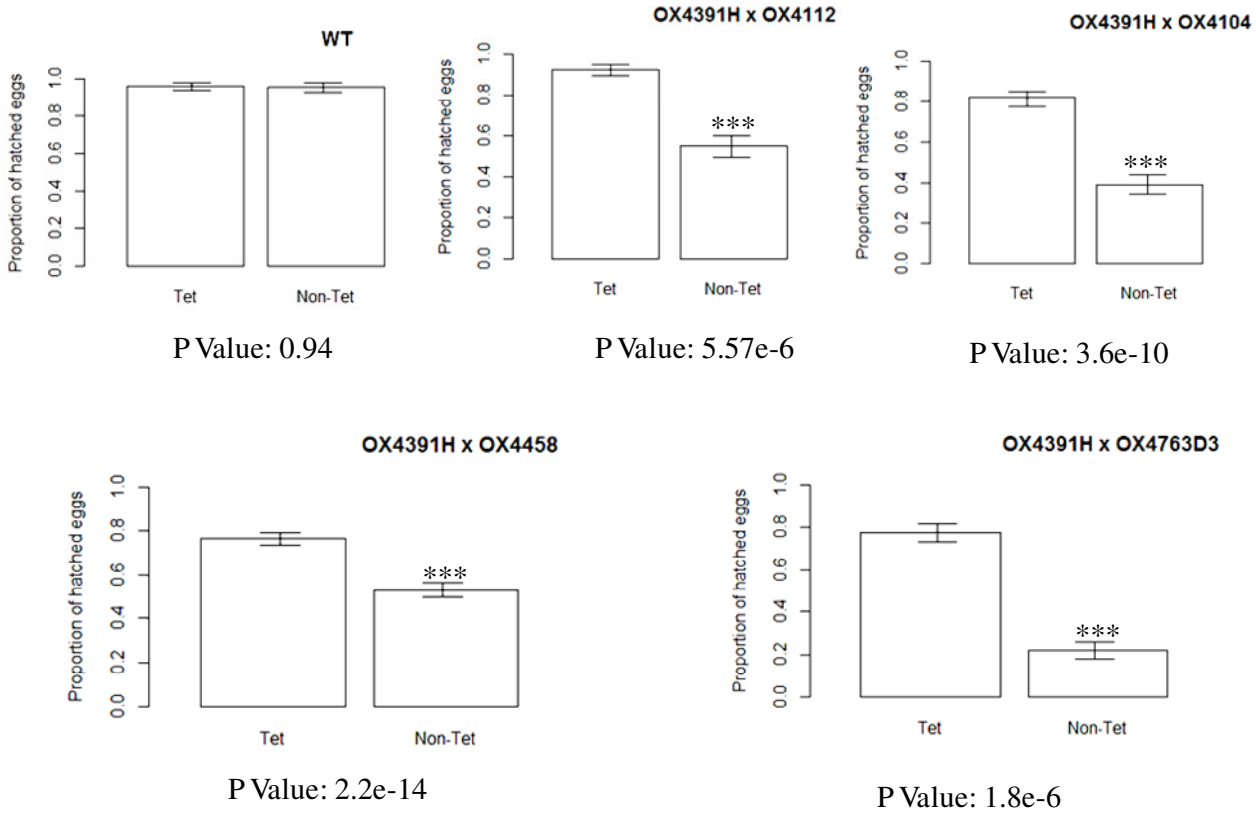


Figure 3. 36 The male fertility test for medfly lines carrying both a *topi* based strain (OX4391H) crossed to different effector based strains (OX4112I, OX4104F, OX4458 and OX4763).

The effect and repressibility of the transgene expression were tested by crossing double-heterozygotes males wild-type (WT) of the opposite sex. The proportion of hatched eggs to non-hatched eggs in males of the OX4391H x (OX4112I, OX4104F, OX4458 and OX4763) crossed to wild-type females compared in presence and absence of tetracycline (100ng/ul) in the larvae diet. The *P* values from a Chi Squared test are also shown. In all cases the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisk on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence level.

Most of the lines tested are heterozygous for the transgene insertion, whereas any product line using this technology will be homozygous for the insertion. In a product line both driver and effector will be placed onto a single construct, therefore homozygotes of this will have two driver elements and two responder elements. There is a strong chance that with two copies of the transgene, one might obtain much higher male sterility.

The fertility assay for the *topi* based strain (OX4485 strain) and *β-2tubulin* based strain (OX4483 strain) crossed with all effector lines (*Ippo1*; OXOX4112I, *ZnF*; OX4104F *ProtamineFokI*; OX4458 and *Eco*R*I*; OX4763 where *tetO* controls expression of these effectors) indicate that the males from those crosses were not completely sterile. One possibility is that neither of the promoters used drive this effector very strongly, thus the effector does not operate as well as expected. The other possibility is that the promoters are adequate, but the effectors are insufficient due to a low level of activity. These possibilities are not mutually exclusive. Despite the failure to achieve full sterility, the partial sterility achieved suggest that *β2-tubulin* is stronger than *topi*, and is a suitable promoter for further development for the purposes of developing a paternal effect lethal RIDL strain in medfly. The summary of results is shown in Figure 3.37 and 3.38.

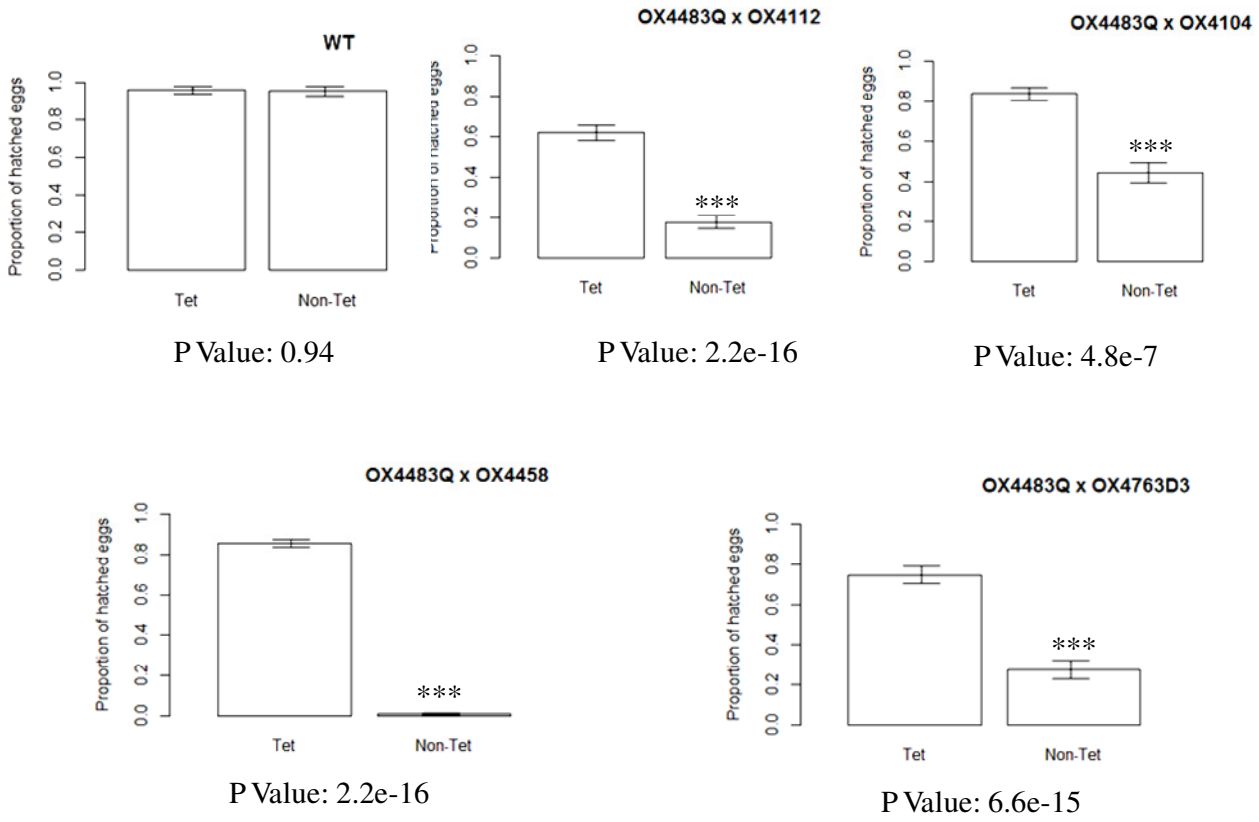


Figure 3. 37 The male fertility test for medfly lines carrying both a *β2-tubulin* based strain (OX4483Q) crossed to different effector based strain (OX4112I, OX4104F, OX4458 and OX4763).

The effect and repressibility of the transgene expression were tested by crossing double-heterozygotes males wild-type (WT) of the opposite sex. The proportion of hatched eggs to non-hatched eggs in males of the OX4483Q x (OX4112I, OX4104F, OX4458 and OX4763) crossed to wild-type females compared in presence and absence of tetracycline (100ng/ul) in larvae diet. The *P* values from a Chi Squared test are also shown. In all cases the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisk on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence level.

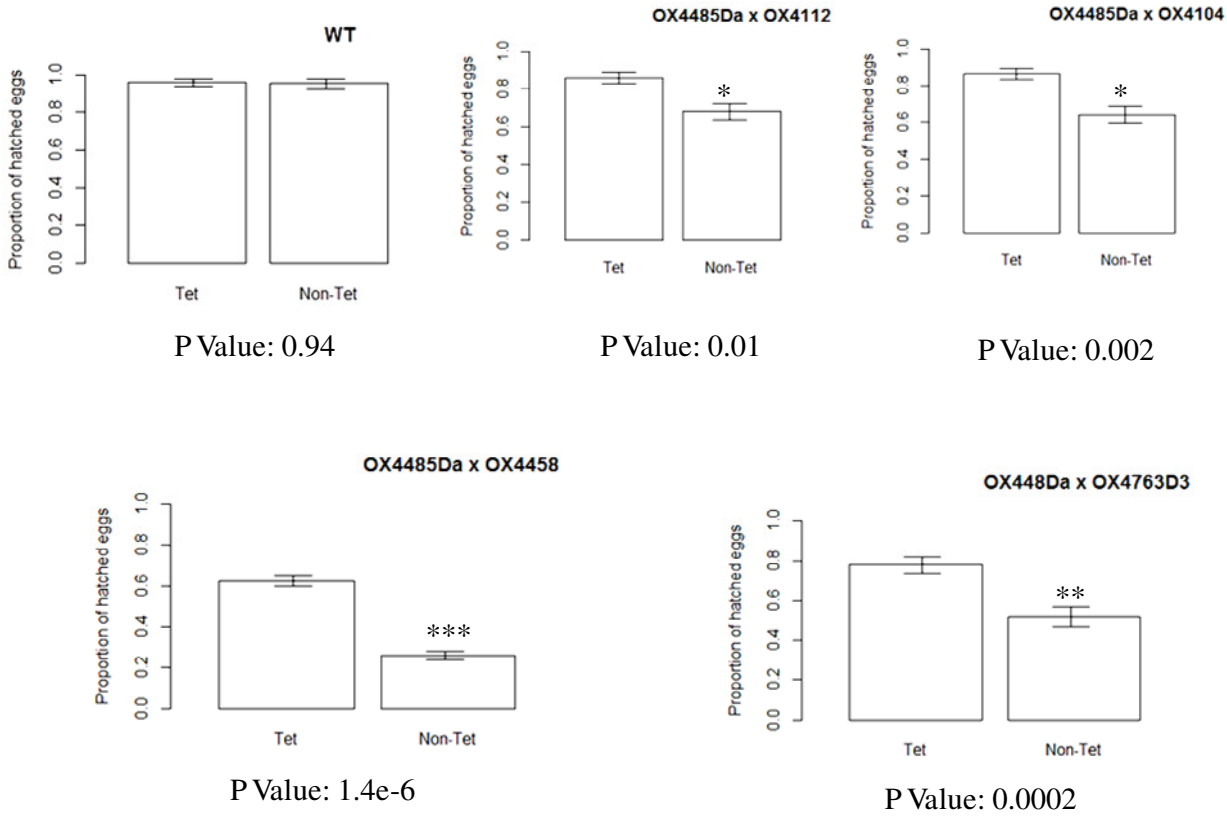


Figure 3. 38 The male fertility test for medfly lines carrying both a *topi* based strain (OX4485Da) crossed to different effector based strains (OX4112I, OX4104F, OX4458 and OX4763).

The effect and repressibility of the transgene expression were tested by crossing double-heterozygotes males wild-type (WT) of the opposite sex. The proportion of hatched eggs to non-hatched eggs in males of the OX4485Da x (OX4112I, OX4104F, OX4458 and OX4763) crossed to wild-type females compared in presence and absence of tetracycline (100ng/ul) in larvae diet. The *P* values from a Chi Squared test are also shown. In all cases the number of hatched eggs differed significantly between Tet and Non-Tet (p-value<0.05). The Y axis shows proportion of hatched eggs. The asterisk on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show confidence intervals which was set at 95% confidence level.

Previous results obtained from crossing OX4282 strains to the same effector strains resulted in greater male sterility, as measured by the number of viable eggs produced from those crosses. As mentioned before; *tetO* is a bidirectional promoter which might lead to a greater expression of the effector by creating a positive feedback loop whereby more tTAV is produced. The positive feedback loop was used in the designing of a female specific lethal strain where an excess level of tTAV causes death in females (Fu et al. 2007). The results from crosses of modified *topi* and *β2-tubulin* confirms this hypothesis. Comparing Figure 3.35 and 3.36 with Figure 3.37 and 3.38; sterility where the feedback loop is created is much greater; therefore, presence of the feedback loop may promote greater expression of the effector gene in the germline. The feedback loop may also cause non-germline specific defect such as malformations of the male genital tract, which may be the reason for the observed higher sterility in these lines.

The timing of promoter expression in relation to spermatogenesis is a very important factor in promoter selection for the development of a paternal effect lethal strain. The promoter should act relatively early (before cessation of transcription as cells enter the meiotic divisions), so that enough *tTAV* is produced in order to drive the expression of the *tetO*-effector. *topi* expresses early in spermatogenesis but its expression level is relatively low. On the other hand, *β2-tubulin* expression is abundant but is activated relatively late in primary spermatocytes. As said before, for the purpose of this work I have tried an altered form of *β2-tubulin* (by changing the 5'UTR to give earlier translation; 5'UTR from the Medfly *hsp83* gene to replace *β2-tubulin* 5'UTR) therefore the promoter that drives tTAV is strong while still translated early in primary spermatocytes. The *ProtamineFokI* construct consists of a single *FokI* cleavage domain where it is fused to *Protamine* from *D. melanogaster* under the transcriptional control of tetO. The Protamine component is predicted to locate the fusion

protein to the spermatid DNA, and thus generate non-specific cleavage of DNA by the *FokI* domain. The above results strongly suggest that an altered *β2-tubulin* promoter and 5'UTR linked to *tTAV* can successfully activate nuclease expression and cleave sperm DNA in a tetracycline controlled manner.

For purpose of continuing this project, *β2-tubulin* that drives tTAV x *ProtamineFokI* under the transcriptional control of tetO, was chosen. It generated the highest sterility of all the combinations tested (8% of controls).

Chapter 4-Development of Paternal Effect Lethality Strains in Medfly

4.1. Introduction

4.1.1. Requirements analysis for medfly paternal effect lethality strain

The ultimate aim of developing conditional sterility using RIDL technology is to produce paternal effect lethal strains of medfly, to use in the SIT programme. Radiating flies damages sperm DNA thus the flies produce sperm with dominant lethal alleles (i.e. they have a paternal effect lethal). Radiation damages somatic tissue as well as the germline, therefore the mating ability and longevity of irradiated flies is compromised. To eliminate the use of radiation, at Oxitec, biotechnology methods have been used to produce a paternal effect lethality strain.

This technology is based on the tet-off system which is a binary gene regulation system (based on the E. coli tetracycline-resistance system). In the absence of tetracycline in the larval diet, *tTAV* binds to *tetO*, which will then activate the transcription of the targeted gene in male germline. If the insect's diet (at larvae stage) contains tetracycline, *tTAV* will preferentially bind to it and thus it will suppress the activation of the targeted gene.

This system only targets specific tissues (in this case, male germline). The aim is to minimize detrimental effects on the other tissues. Ideally there won't be any fitness cost, therefore these flies will have better fitness and mating ability compare to irradiated flies.

In SIT, the quality and fitness of the paternal effect lethality strain can be determined by the relative mating competitiveness of that specific strain compared to the wild-type. Radiation, mass rearing and reduced genetic diversity of a fly strain have an impact on the fitness of a strain. It has been shown that the mating competitiveness of the irradiated medfly strain has been reduced approximately 4-10 fold (Whittier et al. 1994). Hence, it is of interest to

compare mating capability and competitiveness of a RIDL strain of medfly to wild-type medfly.

A further issue is re-mating of flies that have copulated with irradiated males where it has been shown that females mated irradiated flies are more likely to re-mate due to the low sperm production of irradiated flies (Taylor et al., 2001).

4.1.2. System components for medfly paternal effect lethality strain

In chapter 3, I investigated combinations of a germline promoter and a nuclease effectors to identify one that gave a high degree of male sterility. The results from chapter 3 suggest that an altered *β2-tubulin* promoter linked to *tTAV* can successfully activate nuclease expression via tetO. The best effector nuclease was a fusion of Protamine (from *Drosophila melanogaster*) to FokI (from *Flavobacterium Okeanokoites*). Together these constructs generate 1.8% male sterility, presumably by cleaving sperm DNA, in a tetracycline controlled manner.

In Oxitec's lead female specific lethal RIDL medfly strain, the effector is a positive feedback loop, where basal tTAV expression is amplified by tTAV binding to *tetO* and acting to increase *tTAV* expression (see section 1.9). The female specificity is engineered by sex specific splicing, and the entire system is repressed by tetracycline. The positive feedback loop caused an excess level of *tTAV* expression therefore resulted death in females. OX4353 have the tetO sequence on the promoter construct. This was originally included to drive expression of a fluorescent protein reporter, to monitor activity, however it is cloned head-to-head with the promoter-tTAV element, and thus could have a positive feedback effect. While the positive feedback may be useful in increasing tTAV expression, it may also cause spermatogenesis defects, or testis morphology defects. While this could explain the higher sterility in the lines with a positive feedback capacity, it is undesirable, as our aim is to produce a line that is sterile due to paternal effect lethality. I.e. the ideal line will produce sperm that will be transferred to the female on mating, but which will not be able to support embryonic development when it is used or fertilization.

The results from all crosses described in chapter 3 revealed that *β2-tubulin-tTAV* crossed to tetO-*ProtamineFokI* gave the highest male sterility; therefore, a construct containing both

promoter and effector in one plasmid was designed to check if they are able to perform well as a single transgene insertion.

4.2. Experimental design

Flies carrying the all-in-one paternal effect lethality construct (*β2-tubulin-tTAV-tetO-ProtamineFokI* as the main components of this design) were tested. The fertility and fitness of male and female flies carrying the paternal effect lethality construct were studied in the absence and presence of tetracycline in the larval diet (100ng/µl). To check if there is any expression in somatic tissue of male and female flies, RT-PCR and Q-RT-PCR was performed.

Males of the paternal effect lethality strain were crossed to females of an existing female specific lethal strain to evaluate the performance of flies when they are carrying both constructs in their genome. Male double-heterozygote transgenic flies were crossed to wild-type females. The male fertility was assessed in the absence and presence of tetracycline in the larval diet (100ng/µl) as the expression of the effector should be repressed by the addition of tetracycline in the larval diet.

A combined construct with both paternal effect lethality and female specific lethal modules was designed. The fertility and fitness of male and female flies carrying such a construct in their genome was studied in the absence and presence of tetracycline in the larval diet (100ng/µl).

To more stringently test the performance of the transgenic males, a mating competition experiment was designed. The same numbers of male flies from a targeted strain, a wild-type strain and female flies from wild-type strain were placed in an insect cage. Both types of male flies compete for mating and mating pairs were removed to identify which strain they belong to. For the re-mating experiment, females mated to one of the strains will have the option of mating to fresh males and the re-mating ratio will be observed.

4.3. Results and discussion

4.3.1. The OX4353 strain produces partial or full sterility in males in the absence of tetracycline in the larval diet

The paternal effect lethality construct; OX4353 (Figure 2.10) has *β2-tubulin* promoter which drives the expression of *tTAV* in the male germline and results in expression of *ProtamineFokI* as an effector molecule under the control of *tetO*. Flies carrying this construct are predicted be sterile when reared in the absence of tetracycline in their diet, due to DNA damage in the sperm nuclei.

The construct was made by molecular team at Oxitec. I injected it into pre-blastoderm medfly embryos and obtained five independent lines. To estimate any possible detrimental effects to the individuals carrying OX4353 construct in their genome, and to check the number of transgene insertions in each line of OX4353, males with OX4353 insertions were crossed to wild-type and ratio of progeny was observed. The numbers of wild-type and transgenic were scored. .All OX4353 lines had a pupation rate of about 70 to 85% (from eggs to pupae) and emergence (from pupae to adult 80-90%) when reared on tetracycline diet provided in their larval stage (similar to wild-type 80-100% (Duarte et al. 1993)). In the absence of tetracycline in the larval diet, pupation and emergence of OX4353F and B were similar that seen when they were reared on tetracycline-containing diet (Table 4.1). However, in the first experiment, only two females and two males were emerged from OX4353Awhile OX4353D yielded only three adult males and one adult female (the female died soon after emergence). For OX4353C no adults emerged when the diet did not contain tetracycline.

To confirm that the poor survival was not due to human error (poor rearing practice) and rather represented a detrimental effect of the transgene, the experiment was repeated. In this experiment, approximately 200 progeny embryos were collected in total (100 embryos in two

collections) for each individual line, therefore there were more survivors. When OX4353A line was reared in the absence of tetracycline, from approximately 200 embryos that were collected, the survival number was increased to 25 males and 22 females but they lacked general fitness compared to the same line when it was reared on the tetracycline-containing diet. For the OX4353C line, from approximately 200 embryos that were collected 21 males and 24 females survived, while 15 males and 13 females survived for the line OX4353D; however in both cases their general fitness was poor. This shows that the OX4353 transgene affects viability in medfly in the absence of tetracycline in the larval diet. The results are shown in Table 4.1.

Table 4. 1 The OX4353 strain has fitness cost on medfly.

The table shows the actual number of transgenic males and females when males of all individual OX4353 lines were crossed to wild-type females with the progeny collected in the absence and presence of tetracycline in the larval diet (100ng/ul). Approximately 200 embryos were collected in two batches for each individual line. The experiments are the comparison between survival rates of all OX4353 lines when reared in the absence and presence of tetracycline in the larval diet. T: presence of tetracycline in the larval diet. NT: absence of tetracycline in the larval diet.

Lines		Experiment 1		Experiment 2	
		Male	Female	Male	Female
Wild type		76	71	82	78
OX4353A	T	99	61	67	64
	NT	2	2	21	24
OX4353B	T	73	70	74	75
	NT	66	69	71	73
OX4353C	T	58	55	61	60
	NT	0	0	15	13
OX4353D	T	63	67	72	70
	NT	3	1	25	22
OX4353F	T	79	83	84	85
	NT	91	86	87	82

To determine the number of copies inserted in the genome of each lines, both wild-type and transgenic progeny were counted. The table 4.2 shows the number of transgenic versus wild-type progeny in males of all individual OX4353 lines when they were crossed to wild-type females. Around 200 progeny in pupal stage were counted for each line. The results show that four lines of flies carrying the OX4353 construct are only carrying a single transgene in their genome. On the other hand, line OX4353F has more than one insertion (p= 0.003).

Table 4. 2 The results from fluorescence screening of progeny of the OX4353 strain (*ß2-tubulin*- and *ProtamineFokI*).

The progeny of the OX4353 strain were screened for *AmCyan*. According to their history and inheritance pattern; all of them seem to have one independent autosomal insertion except for OX4353F line which seems to have two insertions.

OX4353 strains	No. of wild -type progeny (%)	No. of OX4353 progeny (%) , *P* Value
OX4353A	76(49.03)	63(51.87), 0.37
OX4353B	71(51.45)	67(48.55), 0.91
OX4353C	83(57.24)	62(42.76), 0.05
OX4353D	109(53.69)	95(46.31), 0.28
OX4353F	74(38.74)	117(61.26), 0.003

To evaluate any reduction in reproductive capacity of males carrying the OX4353 construct, the fertility assay (in the form of an egg hatching experiment) was carried out. The fertility assay was performed in duplicate to check reproducibility of data. Six males of each OX4353 strain were crossed to twelve wild-type females. Reduction of sperm function will result in fertilization of fewer eggs. Since the expression of *FokI* (the effector) should be repressed by the addition of tetracycline in the larval diet, I compared the male fertility in the absence and presence of dietary tetracycline (100ng/µl).

Wild-type females that mated with males of OX4353A (reared with tetracycline in their diet at larval stage), produced 58% viable embryos while wild-type females mated with OX4353A males (reared in absence of tetracycline in their larval stage) had no viable embryos. The embryo viability from wild-type females that were copulated with tetracycline-reared males of OX4353B was 82% while it was only 8% for wild-type females that were copulated with off-tetracycline reared OX4353B males. OX4353D on-tetracycline males crossed to wild-type females gave 50% embryonic viability, and this line gave 0% embryonic viability for wild-type females copulated with males reared off-tetracycline. 86% of the embryos from wild-type females that were copulated with males of on-tetracycline reared OX4353F were viable but this number significantly decreased to 12% when wild-type females were copulated with off-tetracycline reared OX4353F males (Fig. 4.1 & table 4.3). A Chi Squared test was performed and the P value is 2.2×10^{-16} for all lines.

Overall, male flies of OX4353 which were reared without having tetracycline in their diet at larval stage, showed significant reduction in fertility in compare to the males of the OX4353 strain that were reared with tetracycline in their diet at larval stage. With the presence of tetracycline in the larval diet of males, the proportion of viable embryos from wild-type

females copulated with OX4353 males in most lines is very similar to the wild-type control, although wild-type females mated with the OX4353A and OX4353D lines showed some reduction, meaning that these two lines have some sterility even with the presence of tetracycline in the diet at larval stage (Figure 4.1 and table 4.3). This shows tetracycline does not completely suppress the effect of OX4353.

Table 4. 3 The fertility test for male flies of the OX4353A, B, D & F (*ß2-tubulin* driven *tTAV* which controls *tetO-ProtamineFokI*) crossed to wild-type females in medfly.

The effect and repressibility of the transgene expression were tested by crossing OX4353 males of each line to wild-type (WT) females. The table presents the total number of eggs, eggs that hatched and percentage of eggs that hatched in three replicas (R1, R2, and R3). The proportion of eggs that hatched to eggs that failed to hatch in males of OX4353 crossed to wild-type females compared in the absence and presence of tetracycline in the larval diet (100ng/ul). The *P* values from a Chi Squared test are also shown.

Paternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	399	286	71.40	0.88
	NT	404	285	70.74	
OX4353A	T	333	187	56.04	2.2×10^{-16}
	NT	271	0	0	
OX4353B	T	409	347	85.58	2.2×10^{-16}
	NT	481	25	5.21	
OX4353D	T	347	194	56.03	2.2×10^{-16}
	NT	334	3	0.80	
OX4353F	T	176	154	87.48	2.2×10^{-16}
	NT	266	23	10.36	

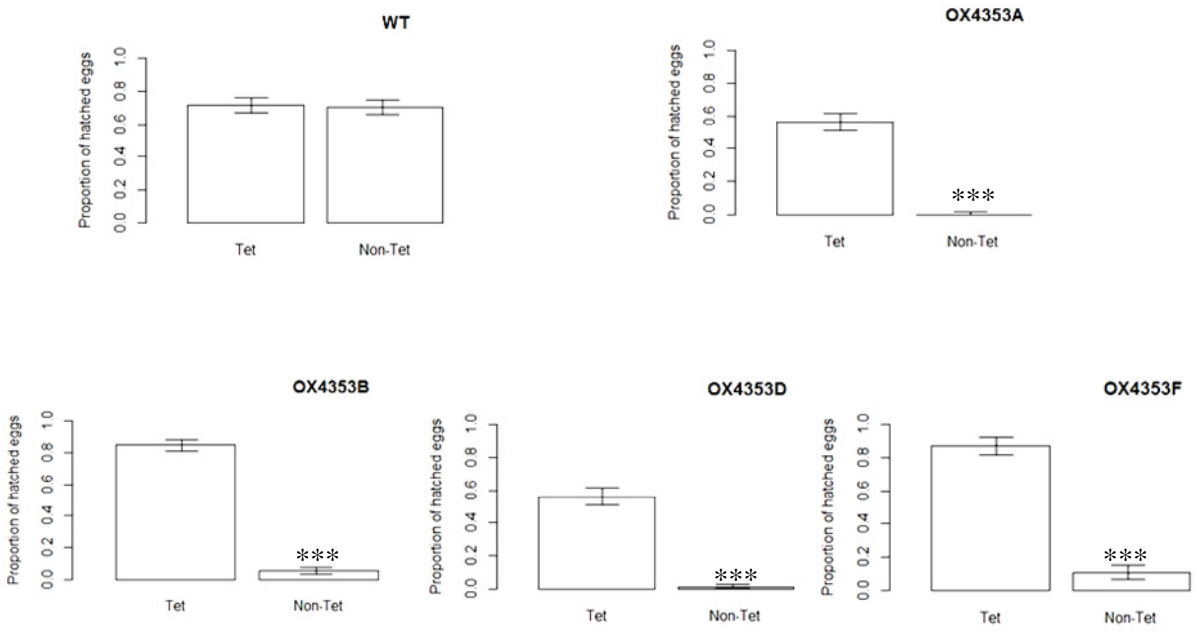


Figure 4. 1 The male fertility test of medfly lines carrying OX4353 (*β2-tubulin* driven *tTAV* which controls *tetO-ProtamineFokI*) crossed to wild-type females.

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in males of the OX4353 crossed to the wild-type females. A Chi Squared (X^2) test was done as statistical analysis; the p value for each set is a comparison of proportion of eggs that hatched to eggs that failed to hatch in males of the OX4353 crossed to wild-type females on and off tetracycline (at larval stage). The Y axis shows the proportion of eggs that hatched. The asterisks on bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show 95% confidence intervals.

To check for reproducibility of the data, the experiment was repeated. As it shows in Figure 4.2 and Table 4.4, in the second experiment, male flies of OX4353A, reared in the presence of tetracycline in their larval diet, and mated with wild-type females produced 70% embryo viability, while 0% of embryos were viable for wild-type females mated with males of OX4353A reared in the absence of tetracycline in their larval diet. Wild-type females crossed with male OX4353B flies reared in the presence of tetracycline in their larval diet had 86% viable embryos, but only 8.8% embryos were viable when wild-type female were mated with males from the same line reared in the absence of tetracycline in their larval diet. OX4353C males reared in the presence of tetracycline (in their larval diet) and crossed to wild-type females gave 58.6% viable eggs, while for those reared in the absence of tetracycline, only 1.9% of their eggs were viable. In the presence of tetracycline in their diet at larval stage, male flies of OX4353D crossed to wild-type females had 71% of viable eggs but only produced 2% viable eggs when wild-type females were crossed to males of the same strain reared in the absence of tetracycline. 83.5% of eggs were viable for male flies of the 4353F line reared in the presence of tetracycline (in their larval diet) while about 13% of their eggs were viable when reared in the absence of tetracycline. A Chi Squared test was performed and the P value of 2.2×10^{-16} was observed for all the lines of this strain.

Table 4. 4 The second fertility test for male flies of the OX4353A, B, C, D & F (*ß2-tubulin* driven *tTAV* which controls *tetO-ProtamineFokI*) crossed to wild-type females in medfly.

The effect and repressibility of the transgene expression were tested by crossing OX4353 males of each line to wild-type (WT) females. The table presents the total number of eggs, eggs that hatched and percentage of eggs that hatched in three replicas (R1, R2, and R3). The proportion of eggs that hatched to eggs that failed to hatch in males of OX4353 crossed to wild-type females compared in the absence and presence of tetracycline in larval diet (100ng/ul). The *P* values from a Chi Squared test are also shown.

Paternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	366	346	94.49	0.88
	NT	401	363	90.64	
OX4353A	T	336	247	73.33	2.2×10^{-16}
	NT	389	0	0	
OX4353B	T	405	344	84.90	2.2×10^{-16}
	NT	433	39	8.94	
OX4353C	T	320	186	58.36	2.2×10^{-16}
	NT	433	7	1.61	
OX4353D	T	409	293	71.49	2.2×10^{-16}
	NT	387	11	2.87	
OX4353F	T	476	394	87.68	2.2×10^{-16}
	NT	410	75	18.24	

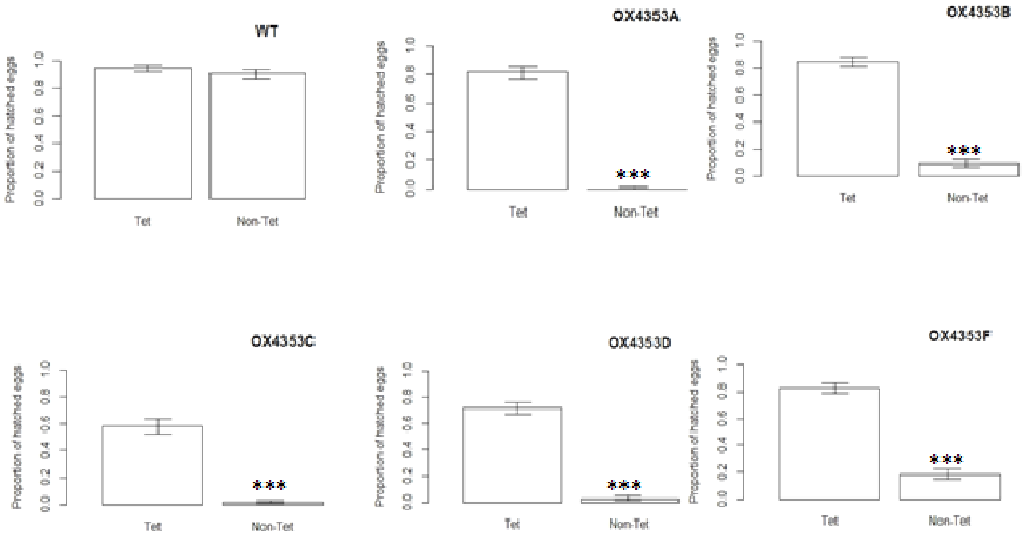


Figure 4. 2 The second male fertility test of medfly lines carrying OX4353 (*ß2-tubulin* driven *tTAV* which controls *tetO-ProtamineFokI*) crossed to wild-type females.

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in males of the OX4353 crossed to the wild-type females. A Chi Squared (X^2) test was done as statistical analysis; the p value for each set is a comparison of proportion of eggs that hatched to eggs that failed to hatch in males of the OX4353 crossed to wild-type females on and off tetracycline (at larval stage). The Y axis shows the proportion of eggs that hatched. The asterisks on bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001). The error bars show 95% confidence intervals.

Comparing the results of the two experiments reveals that the results are highly reproducible. The above data shows a significant reduction in proportion of viable embryos when males from all lines of this strain are reared in the absence of tetracycline in their larval diet. That means the males of the OX4353 strain are producing reduced male fertility in the absence of tetracycline in their diet at larval stage, however some transgenic lines are still sub-fertile even in the presence of tetracycline in the larval diet.

As mentioned above, the fertility test was performed for the females of flies carrying the OX4353 construct in their genome to check if the observed sterility was male specific. Twelve females from each line were crossed to six wild-type males.

Figure 4.3 and Table 4.5 shows the fertility test for females carrying the OX4353 construct in their genome, in the absence and presence of tetracycline in the larval diet. Some reduction in proportion of viable eggs can be seen when females of these lines were reared in the absence of tetracycline in their larval diet however this reduction was not significant. Looking at Figure 4.3 which compares the proportion of viable eggs in the absence and presence of tetracycline, the transgenic females of lines OX4353A and OX4353B are also seem to possess some sterility irrespective of the type of diet.

Table 4. 5 The fertility test for female flies of the OX4353 strain crossed to wild-type males in medfly.

The effect and repressibility of the transgene expression were tested by crossing OX4353 females of each line to wild-type (WT) males. The table presents the total number of eggs, eggs that hatched and percentage of eggs that hatched in three replicas (R1, R2, and R3). The proportion of eggs that hatched to eggs that failed to hatch in females of the OX4353 crossed to wild-type males compared in the absence and presence of tetracycline in the larval diet (100ng/ul). The *P* values from a Chi Squared test are also shown.

Maternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	366	346	94.49	0.88
	NT	401	363	90.64	
OX4353A	T	383	305	79.95	0.003
	NT	330	185	54.99	
OX4353B	T	380	361	95.02	0.06
	NT	362	281	77.73	
OX4353C	T	382	306	81.25	0.41
	NT	354	310	87.61	
OX4353D	T	379	309	81.70	0.70
	NT	448	351	78.89	
OX4353F	T	327	285	87.19	0.23
	NT	434	332	76.85	

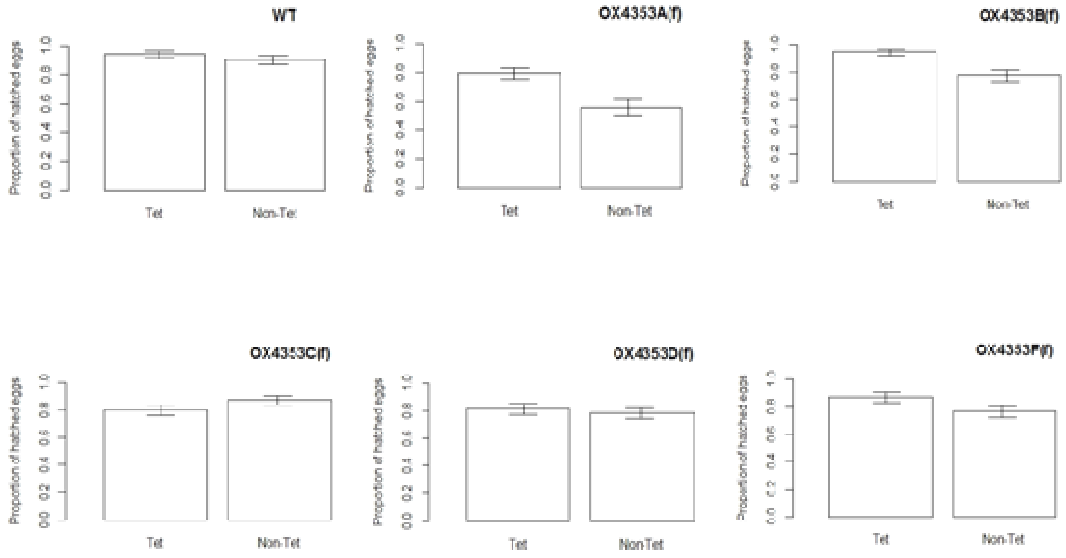


Figure 4. 3 The female fertility test of medfly lines carrying OX4353 (*ß2-tubulin* driven *tTAV* which controls *tetO-ProtamineFokI*) crossed to wild-type males.

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in females of the OX4353 crossed to wild-type males. A Chi Squared (X^2) test was done as statistical analysis; the p value for each set is a comparison of proportion of eggs that hatched to eggs that failed to hatch in females of the OX4353 crossed to wild-type males on and off tetracycline (at larval stage). The number of hatched eggs didn't differ significantly between Tet and Non-Tet (p- >0.05).The Y axis shows the proportion of eggs that hatched. The error bars show 95% confidence intervals.

4.3.2. Crossing male flies of OX4353 strain to female flies of OX4353 strain generates similar results to heterozygous males of OX4353

The male flies of the OX4353B and F were crossed to the female flies from the same line in an attempt to produce homozygous lines. This was done to investigate the fitness and survivability of flies when homozygous, and also to check if repressibility changes. Fertility was assessed in the absence and presence of tetracycline in the larval diet (100ng/µl) as before. Obtaining homozygous strains by simply using fluorescence intensity to sort homozygotes from heterozygotes is not reliable, as the intensity does not increase in homozygotes. The fluorescent progeny of OX4353 males crossed to OX4353 females could have been homozygous or heterozygous and the test populations were most likely a mixture of both. The fertility and viability of these test groups were similar to those seen for known heterozygotes (Figure 4.4 and Table 4.6).

Table 4. 6 The male fertility test for male flies of (OX4353♂ x OX4353♀) crossed to wild-type females in medfly.

The effect and repressibility of the transgene expression were tested by crossing the OX4353; OX4353 males to wild-type females. The table presents the total number of eggs, eggs that hatched and percentage of eggs that hatched in three replicas (R1, R2, and R3). The proportion of eggs that hatched to eggs that failed to hatch in males of OX4353; OX4353 crossed to wild-type females compared in presence and absence of tetracycline in the larval diet (100ng/ul). OX4353B x OX4353B: male flies of (OX4353B♂ x OX4353B♀) crossed to wild-type females. OX4353F x OX4353F: male flies of (OX4353F♂ x OX4353F♀) crossed to wild-type females. The *P* values from a Chi Squared test are also shown.

Paternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	357	330	92.43	0.87
	NT	335	274	94.02	
OX4353B x OX4353B	T	320	274	85.62	2.2×10^{-16}
	NT	221	12	5.42	
OX4353F x OX4353F	T	312	257	82.37	2.2×10^{-16}
	NT	330	30	10	

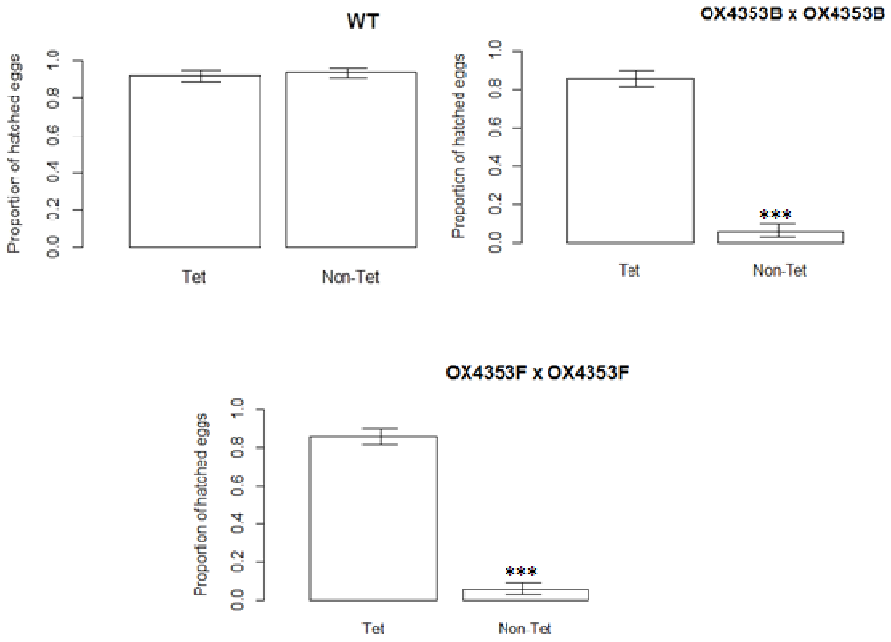


Figure 4. 4 The male fertility test of medfly lines carrying (OX4353♂ x OX4353♀) crossed to wild-type females (*β2-tubuli*n driven *tTAV* which controls *tetO-ProtamineFokI*).

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in males of the OX4353 crossed to the wild-type females. A Chi Squared (X^2) test was done as statistical analysis; the p value for each set is a comparison of eggs that hatched to eggs that failed to hatch in males of OX4353 crossed to females of the same line on and off tetracycline. The number of hatched eggs differed significantly between Tet and Non-Tet as p-value<0.05.The Y axis shows the proportion of eggs that hatch. The asterisks on bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).The error bars show confidence intervals which was set at 95% confidence interval.OX4353B x OX4353B: male flies of (OX4353B♂ x OX4353B♀) crossed to wild-type females. OX4353F x OX4353F: male flies of (OX4353F♂ x OX4353F♀) crossed to wild-type females.

4.3.3 Male flies of OX4353 crossed to females flies of OX3467/ OX3864, generate strains that are female specific lethal and male sterile in the absence of tetracycline

To assess interaction of the paternal effect lethality strain with the female specific lethal strain, and the possible effects on the performance of a final strain that contains both transgenes, an experiment was set up where OX4353 males (two lines were selected, B and F) were crossed to females of a female specific lethal strains (Figure 4.5 and Table 4.7). OX3864 and OX3467 are both female specific lethal strains of medfly, having DsRed2 as their fluorescence marker. Fu et al, 2007 studies such a strain in *medfly*. The same construct was used to develop the OX3864 and OX3467 strains. Although there are five lines of OX4353 available only two lines (OX435B and OX4353F) were selected as they have higher surviving numbers F1 progeny (in the absence of tetracycline in the larval diet) in comparison to the others. Individuals containing both insertions were selected according to their fluorescent phenotypes. These were tested for male fertility and female lethality assays. All the line used express the fluorescence marker strongly, so it is easy to pick the progeny carrying both markers. In the absence of tetracycline in the larval diet, the progeny of this cross should be exclusively male, and these males should be sterile. Both these phenotypes should be tetracycline repressible. The male fertility and female lethality was assessed in the absence and presence of tetracycline in the larval diet (100ng/µl). The result confirmed that no female progeny were produced in the absence of tetracycline in the larval diet where as a normal 50:50 male to female ratio was obtained when larvae were grown on a diet containing tetracycline at larval stage. Male flies containing both insertions were crossed to wild-type and male fertility was assessed as in described in the previous sections. The results are presented in Figure 4.5 and Table 4.7.

Table 4. 7 The male fertility test for males flies of (OX4353♂ x OX3467or OX3863♀) crossed to wild-type females in the males carrying paternal effect lethality strain combined with female specific lethal strain.

The effect and repressibility of the transgene expression were tested by crossing the OX4353; OX3467or OX3863 males to wild-type females. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The proportion of eggs that hatched to eggs that failed to hatch in males of the OX4353; OX3467or OX3863 crossed to wild-type females compared in presence and absence of tetracycline in the larval diet (100ng/ul). The OX4353B x OX3467: male flies of (OX4353B♂ x OX3467♀) crossed to wild-type female. OX4353B x OX3864: male flies of (OX4353B♂ x OX3864♀) crossed to wild-type female. OX4353F x OX3864: male flies of (OX4353F♂ x OX864♀) crossed to wild-type female. OX4353F x OX3467: male flies of (OX4353F♂ x OX3467♀) crossed to wild-type female. The *P* values from a Chi Squared test are also shown.

Paternal genotype		**Total eggs** (R1+R2+R3)	**Hatched eggs** (R1+R2+R3)	**% hatched eggs** (R1+R2+R3)	***P* Value**
Wild type	T	457	330	92.43	0.87
	NT	335	315	94.02	
OX4353B x OX3467	T	341	285	84.90	2.2×10^{-16}
	NT	206	6	2.91	
OX4353F x OX3467	T	226	201	88.93	2.2×10^{-16}
	NT	345	47	13.62	
OX4353B x OX3864	T	335	285	85.07	2.2×10^{-16}
	NT	395	16	4.05	
OX4353F x OX3864	T	313	253	80.83	2.2×10^{-16}
	NT	345	47	13.66	

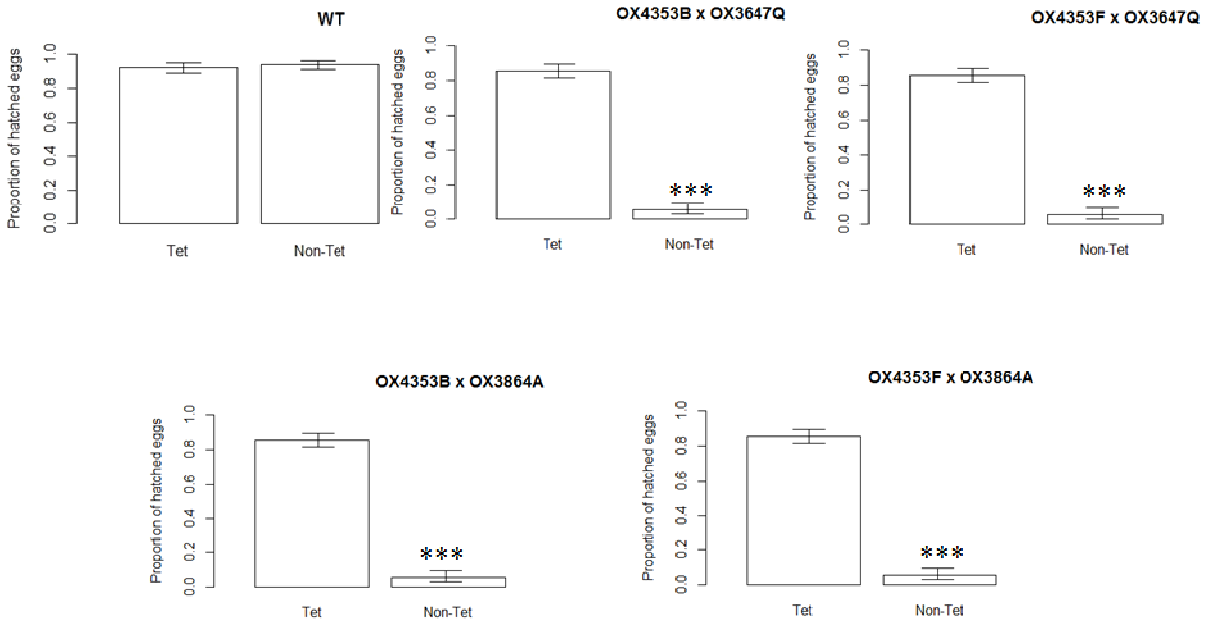


Figure 4. 5 The male fertility test for the male medflies carrying (OX4353♂ x OX3467♀ or OX3864♀) crossed to wild-type females.

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in males of the (OX4353♂ x OX3467♀ or OX3864♀) crossed to the wild-type females. A Chi Squared (X^2) test was done as statistical analysis; the p value for each set is a comparison of eggs that hatched to eggs that failed to hatch in males of (OX4353♂ x OX3467♀ or OX3864♀) crossed to wild-type females on and off tetracycline (at larval stage). The number of hatched eggs differed significantly between Tet and Non-Tet as p-value<0.05.The Y axis shows the proportion of eggs that hatched. The asterisks on bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).The error bars show confidence intervals which was set at 95% confidence interval. OX4353B x OX3467: male flies of (OX4353B♂ x OX3467♀) crossed to wild-type female.OX4353B x OX3864: male flies of (OX4353B♂ x OX3864♀) crossed to wild-type female. OX4353F x OX3864: male flies of (OX4353F♂ x OX864♀) crossed to wild-type female. OX4353F x OX3467: male flies of (OX4353F♂ x OX3467♀) crossed to wild-type female.

Comparing the data from Figure 4.5 to data from section 4.3 shows that paternal effect lethality of flies carrying both construct in their genome remains unaffected by the presence of a female specific lethal strain's positive feedback mechanism; indeed it has been increased slightly further although this can be attributed to stochastic variation.

4.3.4. RT-PCR on the male germline and somatic tissue of the OX4353 reveals that *tTAV* and *FokI* are expressed in somatic tissue as well as the male germline.

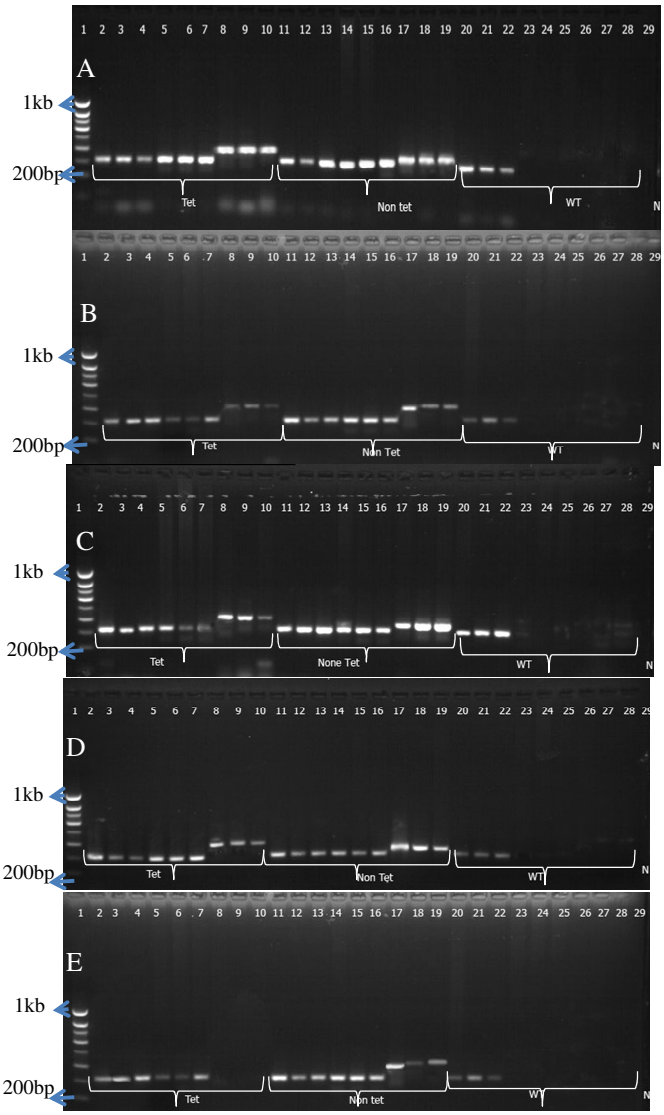
Using radiation to sterilise flies damages their somatic tissue as well as their germline therefore fitness of sterile flies is affected (Thomas et al. 2000). On the other hand RIDL technology is designed to eliminate such an effect as this technology allows for tissue-specific targeting. Although OX4353 is designed to be male germline-specific some of the OX4353 lines have impaired viability, and longevity. I hypothesised that the *ProtamineFokI* effector has leaky expression in soma, resulting in impaired fitness in those flies. To address this; an rt-PCR was performed on testis, male carcasses and females (whole body) of adult flies that were reared without tetracycline in their diet. As a control, testis, male carcasses and females (whole body) of flies that were reared on tetracycline at larval stage, was used. As a negative control, testis, male carcasses and females (whole body) of wild-type flies were used. Primers specific for *tTAV* and *FokI* were used to reveal expression of these components of the construct, while *tra*2 was used as a positive control (*tra*2 expresses in all tissues of medfly, both male and female). The over expression of *tTAV* is lethal to flies, additionally expression of *FokI* in somatic tissue can be lethal. Therefore, somatic expression of either of these sequences (or both of them) could underlie the reduced viability phenotype. RNA was extracted from testis, male carcass and female flies of all individual lines reared off and on-tetracycline at larval stage, and also wild-type flies followed by cDNA synthesis (see section 2.6).

tTAV expression controls expression of *FokI* in the germline tissue of the OX4353 strain therefore in the absence of tetracycline it produces infertile sperm in flies. The OX4353 construct was designed to express *tTAV* and *FokI* just in germline tissue where, in the absence of tetracycline, infertile sperm is produced. I confirmed that both *tTAV* and *FokI* are

expressed in the germline, but found that they both also express in the somatic tissue in all lines examined. Expression was seen when they were reared both on- and off-tetracycline.

Figure 4.6 shows rt-PCR results for medfly strains OX4353A, B, C, D and F. The expression of both *tTAV* and *FokI* was detected in somatic tissue as well as in the germline in all OX4353 lines when tetracycline was not present and also in presence of tetracycline in the larval diet.

The results from rt-PCR confirm the leakiness of the transgene into the flies' somatic tissue. This experiment used 35 cycles of PCR, and therefore is not quantitative. However, if there are dramatic differences between expression in the samples, some differences in the total amount of PCR product can be detected. In this experiment I indeed found that the *tTAV* and *FokI* rt-PCR product levels varied, and that the variation in observed expression level of *tTAV* and *FokI* correlated with the sterility that was observed earlier in the different lines analysed.



1-Ladder
2-T-Testis-*tra2*
3-T-Male carcass-*tra2*
4-T-Female carcass-*tra2*
5-T-Testis-*tTA*
6-T-Male carcass-*tTA*
7-T-Female carcass-*tTA*
8-T-Testis-*Fok1*
9-T-Male carcass-*Fok1*
10-T-Female carcass-*Fok1*
11-NT-Testis-*tra2*
12-NT-Male carcass-*tra2*
13-NT-Female carcass-*tra2*
14-NT-Testis-*tTA*
15-NT-Male carcass-*tTA*
16-NT-Female carcass-*tTA*
17-NT-Testis-*Fok1*
18-NT-Male carcass-*Fok1*
19-NT-Female carcass-*Fok1*
20-WT-Testis-*tra2*
21-WT-Male carcass-*tra2*
22-WT-Female carcass-*tra2*
23-WT-Testis-*tTA*
24-WT-Male carcass-*tTA*
25-WT-Female carcass-*tTA*
26-WT-Testis-*Fok1*
27-WT-Male carcass-*Fok1*
28-WT-Female carcass-*Fok1*

Figure 4. 6 The **reverse transcriptase PCR on the flies of OX4353 strain (reared on and off tetracycline) to evaluate expression level of *tTAV* and *FokI* in the germline and somatic tissue of these flies.**

A: OX4353A, B: OX4353B, C: OX4353C, D: OX4353D and E: OX4353F. The gel photo showing rt-PCR of *tTAV*, *FokI* and *tra2* specific primer sets. For each sample a PCR has been carried out with cDNA of male (minus testes), female (whole body) and testis that were reared off and on tetracycline. All samples (off-tetracycline) gave products of expected sizes with the *tTAV* and *FokI* primers. All samples on tetracycline samples those from OX4353F gave products for *tTAV* and *FokI*. No-RT negative controls were run for each sample and these produced no product, confirming no genomic DNA contamination. Left and right lanes show DNA size standards. Sizes are as follows: from 200bp to 1000bp (Eurogentec Smart ladder). T: on-tetracycline, NT: off-tetracycline. Expected product size for *tra2* is 420bp, *tTAV* is 425bp and 615bp for *FokI*.

The results from rt-PCR confirmed that *tTAV* and *FokI* have leaky expression into the somatic tissue in males and females but it did not quantify the exact level of fold expression in each individual samples. To quantify the expression levels of *tTA* and *FokI* in the different lines of the OX4353, Q-RT-PCR was performed on samples of cDNA of testis, male carcasses and females on and off tetracycline. Wild-type cDNA samples served as a negative control.

4.3.5. The real-time PCR reveals that high expression of *tTAV* and *FokI* in somatic tissue correlates with reduced fitness of OX4353A, C and D

Section 4.3.1 studies the survival rate of different lines of the OX4353 strain. The OX4353A, C and D have a low pupal and adult survival rate when reared off-tetracycline compare to OX4353B and F. Real-time PCR was used here to evaluate the expression level of *FokI* in germline and somatic tissue of the OX4353 lines. It is of an interest to know the level of expression of these genes as different amount might have different effect on the fertility and fitness of the flies from different lines. Real-time PCR confirms a high expression level of *FokI* in somatic tissue as well as male germline. The *tTAV* expression was reduced in somatic tissue in comparison to the germline. As *ProtamineFokI* is driven by a minimal promoter (*hsp70*), its expression could be affected by insertion-site specific effects, for example enhancers that could act through this promoter independent of *tTAV*. The results are shown in Figure 4.7.

Flies from the OX4353B line have a high survival rate when reared off tetracycline; the expression level of *FokI* increased in the germline off tetracycline but the expression of *FokI* in somatic tissue was relatively low. The expression of *tTAV* was lower in the somatic tissue in comparison to the male germline. The survival rate of flies from the OX4353C line was low when reared off tetracycline; high expression of *FokI* in somatic tissue was observed in addition to its expression in the male germline. *tTAV* expression increased in somatic tissue as well as in the male germline. The OX4353D flies have low survival rate when reared off tetracycline; high expression of *FokI* in somatic tissue was observed in addition to its expression in the male germline. *tTAV* expression was also robustly detected in somatic tissue as well as male germline.

The survival rate of the OX4353F flies is high when reared off tetracycline diet; the expression level of *FokI* was low in somatic tissue off tetracycline food in comparison to the germline. The expression of *tTAV* was also lower in somatic tissue in comparison to the germline. In lines where the expression of *tTAV,* or *FokI,* or both is high, the survival rate is compromised as observed in flies from lines OX4353A, C and D. Although some expression of *tTAV* and *FokI* is observed in flies of lines OX4353B and F, the level of expression is low enough to allow for normal development in those flies when reared off-tetracycline. All the lines, in which the expression of *tTAV* and *FokI* was high in male germline tissue, also had high expression in somatic tissue explaining the correlation between compromised fitness and higher sterility male sterility.

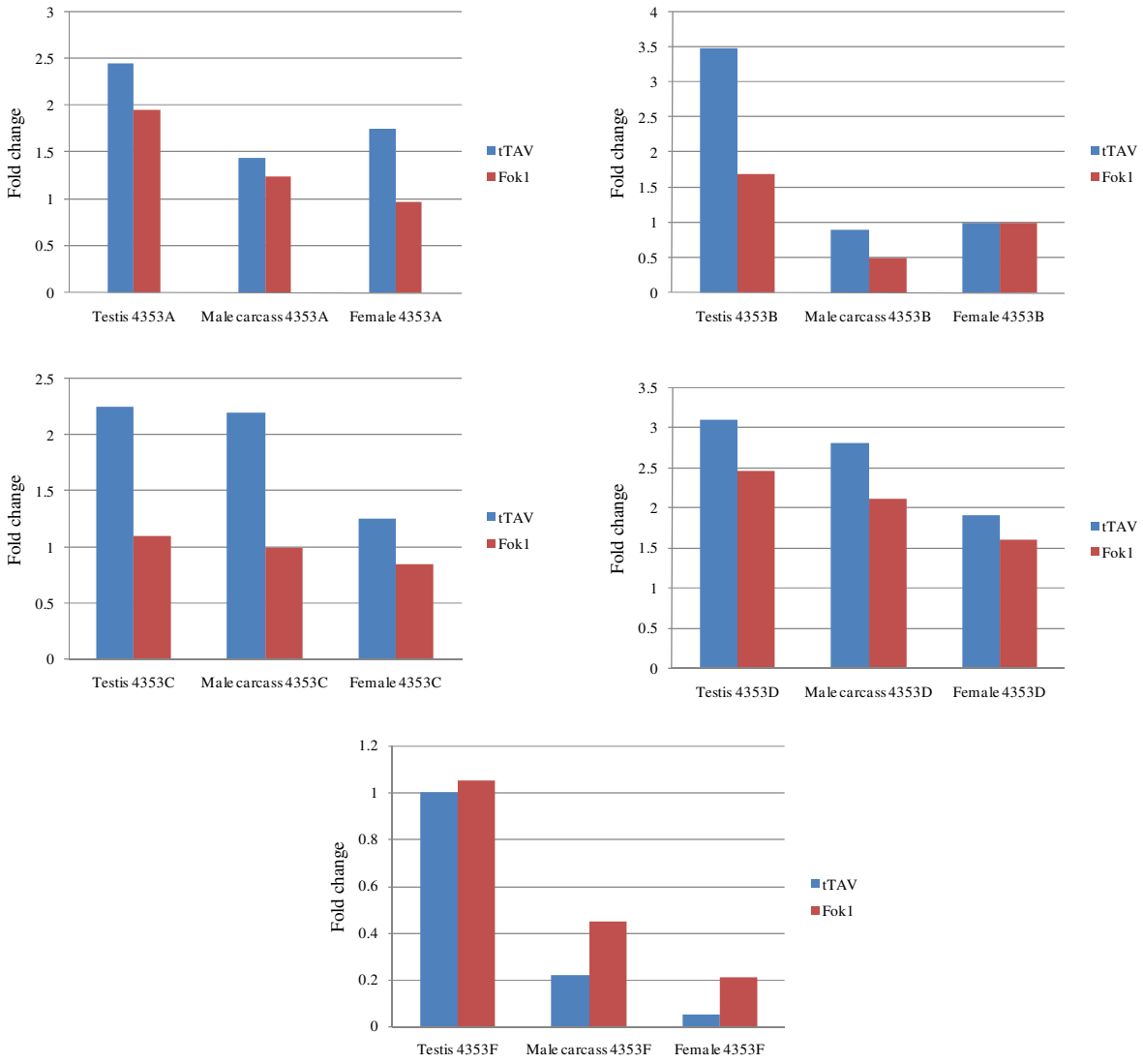


Figure 4. 7 The Quantitative reverse transcriptase PCR on flies of the OX4353 strain when reared on and off tetracycline.

The graphs show Expression Ratio=2-$^{\Delta\Delta CT}$, of *tTAV* and *FokI* normalised using *tra2* where ΔΔCT= (CT (*tTAV* or *FokI*, reared off-tetracycline) – CT (*tTAV* or *FokI*, reared on-tetracycline)) – (CT (*tra2*, reared off-tetracycline) – CT (*tra2*, reared on-tetracycline). For each sample a Q-PCR has been carried out with cDNA of male carcass, female and male testis when reared off and on tetracycline. In all the lines *tTAV* and *FokI* were expressed in the male germline as well as somatic tissue. The expression ratio of *tTAV* and *FokI* are higher in the OX4353A, C and D compare to the OX4353B and F.

4.3.6. Sperm morphology is unaffected by presence of the OX4353 transgene

Sperm produced by irradiated males on average have shorter heads than those produced by wild-type flies (Barry et al. 2003). In theory, the RIDL technique used for generating paternal effect lethality strains should not negatively impact sperm morphology.

To test whether OX4353 generates any morphologically normal sperm, twenty males were dissected from each line of the OX4353 (reared off-tetracycline). Testis, spermatid and sperm morphology was assessed by phase contrast microscopy, and nuclear morphology was additionally assessed by labelling of DNA using the vital dye Hoechst 33342. No morphological differences were detected between transgenic and wild-type sperm (Figure 4.8).

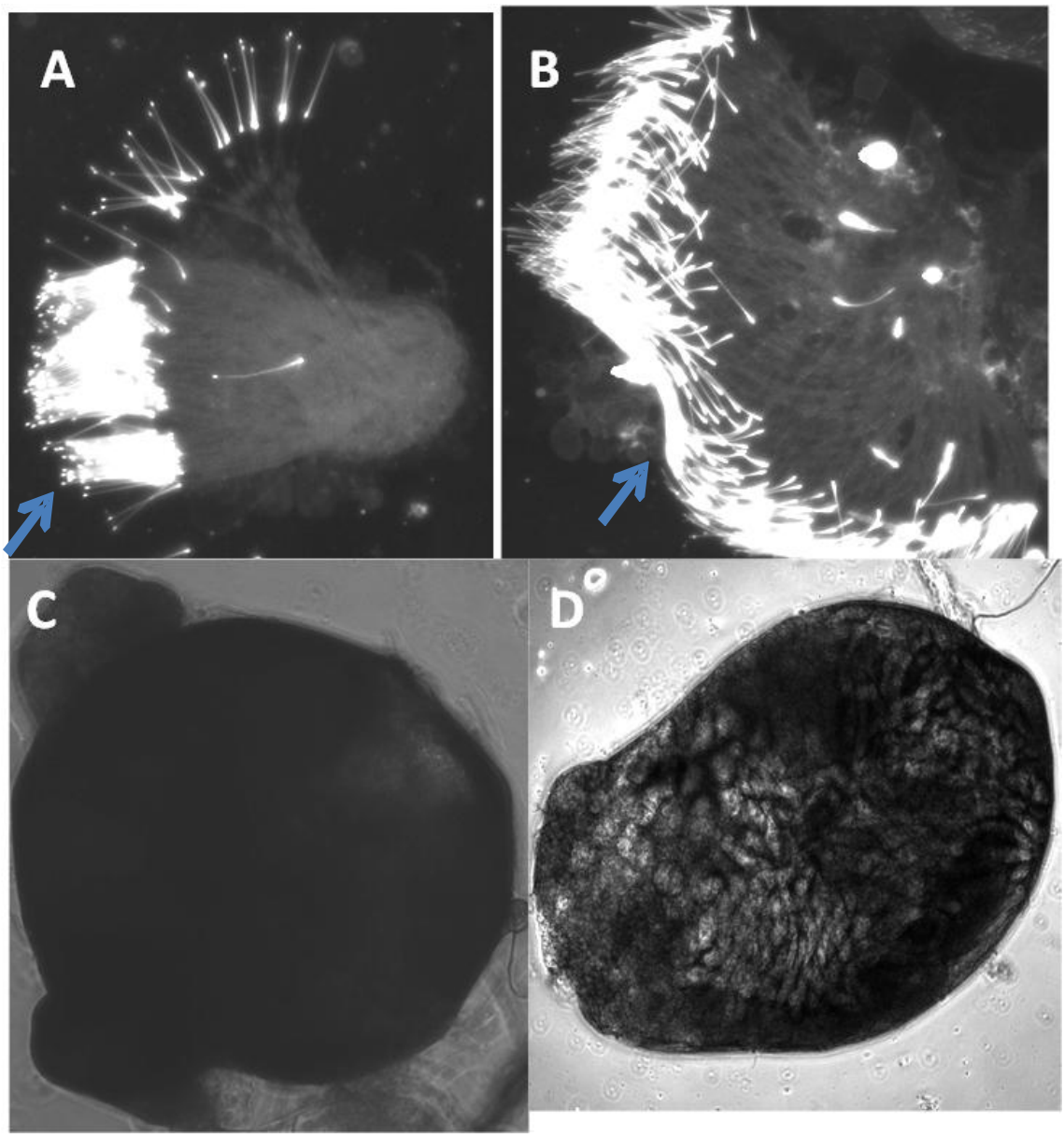


Figure 4. 8 The microscopic photograph of spematid and testis.

A comparison between spermatid and testis of flies from the OX4353B strain (reared off-tetracycline) and wild-type. There is no morphological difference between spermatid nuclei from the OX4353 strain (reared off-tetracycline) of medfly and wild-type (A and B). Blue arrows indicate spermatid nuclei. Testes of the OX4353 strain showed developmental defects; testes from wild-type male had an ovoid shape while testes from transgenic male were more rounded (D and C). A: Wild-type spermatid stained with Hoechst 33342; B: Transgenic spermatid stained with Hoechst 33342); C: Transgenic testis viewed by phase contrast; D: Wild-type testis viewed by phase contrast.

However, during dissections it was apparent that some of the testis of flies of OX4353 strain had developmental defects (Figure 4.8). The genital system in male medfly consists of a pair of testes, two vas deferens, an ejaculatory duct, vesicles, several pairs of accessory glands, a sperm pump (ejaculatory apodeme) and an aedeagus. Testes have an ovoid shape at full sexual maturity (Aguilar et al. 2003). Although spermatogenesis had proceeded, in some males the testes had not associated with the rest of the male genital tract during metamorphosis. This would result in sperm being trapped in the testis and not able to be transferred from the male to the female during mating. Thus the high level of male sterility that was achieved with this strain might result from gross developmental testis defects of rather than impaired sperm function and paternal effect lethality.

50 flies were dissected from all lines of the OX4353 strains (both when reared on and off tetracycline) to quantify how the frequency with which the testes fail to connect to the rest of the genital tract in this strain compared to wild-type. In some flies only one of the testes is unconnected while in others both testes are unconnected. Comparing to wild-type reared off-tet, the number of unconnected testes in OX4353C significantly more (P value= 0.01) and this number for OX4353D is highly significant (p value=0.001). The results are shown in Figure 4.9 and table 4.11.

Table 4. 8 The effect of transgene on the testis development of flies of the OX4353 strain.

The table presents the number of fully developed testis from 50 flies that were dissected from each category in the absence and presence of tetracycline in the larval diet (100ng/ul).The numbers are representing individual testis. The *P* values from a Chi Squared test are also shown.

Strains	No. of "connected" testis on-tetracycline	No. of "connected" testis off -tetracycline	*P* Value
WT	99	97	0.88
OX4353A	88	76	0.11
OX4353B	98	86	0.37
OX4353C	89	67	0.01
OX4353D	79	58	0.001
OX4353F	99	92	0.71

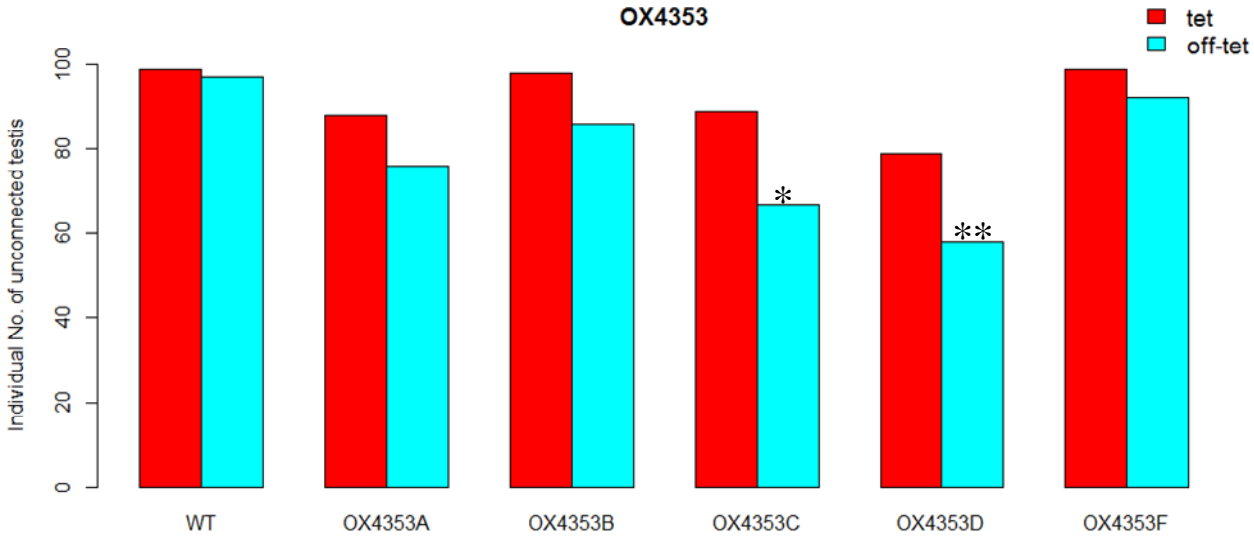


Figure 4. 9 The effect of the transgene on the testis development of flies of the OX4353 strain.

100 flies were dissected to compare the testis development in flies carrying the OX4353 construct in their genome when reared in the absence and presence of tetracycline. The number of unconnected testes in OX4353C significantly more (P value= 0.01) and this number for OX4353D is highly significant (p value=0.001) comparing to wild-type. The Y axis shows the actual number of developed testis. The asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

4.3.7. Generation of a potential paternal effect lethality, female specific lethal and sperm marking all-in-one construct strain of medfly (OX4751)

The OX4751 construct (Figure 2.20) is a modified version of the OX4353 construct. OX4751 contains a dominant selectable transformation marker (DsRed, a fluorescent protein, driven by the muscle actin promoter (mActin from the Mexican fruit fly)) whereas AmCyan in the OX4353 construct is driven by Hr5-IE1. As studied before, the muscle actin promoter (from the Mexican fruit fly) gives a stronger expression of the fluorescent protein which is helpful for detection purposes. Expression of *Protamine-mCherry-nuclease* is under the control of *tetO* (and thus tTAV). The effector protein in the previous design was Protamine-nuclease. Inclusion of the fluorescent protein, mCherry, should allow the sperm nuclei to be directly visualised. It also has *tra2* for "female-specific" splicing and *tTAV* positive feedback and this combination produces female specific lethal. The rest of the design is very similar to the OX4353 strain, specifically both strains have (*β2-tubulin* promoter which drives the expression of *tTAV* in the male germline and results in expression of *ProtamineFokI* as an effector molecule under the control of *tetO).* The fluorescence difference between the current RIDL female specific lethal strain (OX3864A) and the OX4751 strain of medfly is shown in Figure 4.10.

Freshly collected medfly embryos were injected with OX4751 plasmid and *piggyBac* mRNA and the survival rate from embryo to adult was 11%. Around 32% of these pupae displayed the *DsRed2* transient expression that confirmed the presence of *piggyBac* transposition in those individual injections. The results are shown in Table 4.12.

The surviving adults from this injection were crossed to wild-type flies (ten G0 male survivors crossed to 30 wild-type females and 20 G0 female survivors crossed to ten wild-type males). The G1 progeny from individual crosses were collected and screened for

expression of the transformation marker (*DsRed* fluorescence). Three independent lines were obtained from injections with OX4751. Lines D and D2 were generated from the same cage but since they had different expression levels and patterns, and also different characteristics, they have been identified as two different insertions.

Table 4. 9 Generation of the OX4751 transgenic strain in medfly.

The OX4751 construct was injected into freshly collected medfly eggs. Three transgenic lines were obtained.

Construct No.	No. of eggs injected	G0 larvae	G0 adults	No. Of transgenic lines obtained
OX4751	500	197	53	3 lines (D, D2 and E)

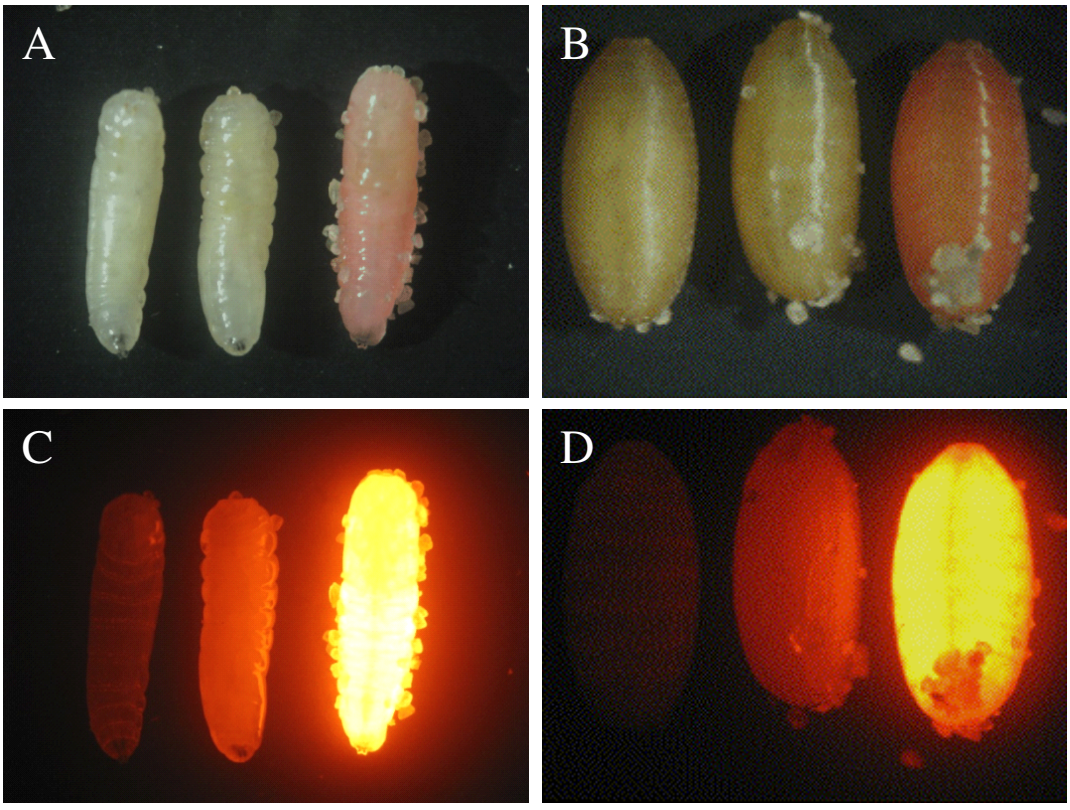


Figure 4. 10 Comparison of fluorescence microscopy of transformation markers in OX3864A, OX4751D at larval and pupal stages.

The photographs are under (upper panels) bright-field illumination and (lower panels) red fluorescence. Each panel shows the wild-type to the left, OX3864A to the middle and the OX4751D to the right: The OX3864A, OX4751D and wild-type (A, C) larva, (B, D) pupa.

4.3.8. OX4751 strains are male sterile and female specific lethal.

Flies carrying the OX4751 construct should produce male sterility (ideally via paternal effect lethality) and female specific lethality in the absence of tetracycline in larval diet. The promoter (*β2-tubulin*) drives the expression of tTAV and thus, the effector (Protamine-mCherry-*FokI*) in the testes, which should cleave sperm DNA. Separately, a basal level of tTAV is expressed from tetO and a minimal promoter. This transcription unit contains the sex-specifically spliced intron from the *tra* gene. In females the intron will be spliced out, permitting full length tTAV expression, which then feeds back to tetO to increase tTAV expression. In males, failure to remove the intron generates an mRNA which does not encode functional tTAV. This means that male survival is independent of tet, while female survival depends on the presence of tet to block the positive feedback loop.

Six males from each lines of the OX4751 strain were crossed to 12 wild-type females. Approximately 200 progeny pupae were counted for each line to determine whether the lines have a single insertion or multiple insertions. Table 4.13 shows the number of transgenic versus wild-type progeny in all OX4751 lines when they were crossed to wild-type. All OX4751 lines had a single transgene. Males of OX4751E were completely sterile and the line was not maintained.

Table 4. 10 The results from fluorescence screening of progeny from the OX4751.

The table shows the actual number and percentage of wild-type and transgenic progeny resulting from the OX4751 strain crossed to wild-type, with the progeny collected in the presence of tetracycline in the larval diet (100ng/ul). A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1 ratio expected from the segregation of a single insertion of the OX4751 transgene.

OX4751 strains	**Number of wild-type progeny (%)**	**Number of OX4751 progeny (%) , *P* Value**
OX4751D	96(48)	104(52), 0.68
OX4751D2	102(51)	98(49), 0.84

In the presence of tetracycline in the larval diet of the OX4751 lines, the proportion of viable embryos from wild-type females mated with males of this strain was the same as that of the wild-type female mated wild-type males. The proportion of viable embryos was significantly reduced when wild-type females were mated with OX4751 males reared in the absence of tetracycline. 85% of embryos of wild-type female mated with males of OX4751D were viable (when OX4751D was reared on tetracycline) but no viable embryos were produced from wild-type female mated with males of OX4751D (when OX4751D was reared off-tetracycline). Wild-type females mated with males of OX4353D2 showed 82% embryo viability in the presence of tetracycline in the larval diet of OX4751D2 while about 23% of embryos were viable in the absence of tetracycline in the larval diet of OX4751D2. The results are shown in Figure 4.11 and Table 4.11.

Table 4. 11 The fertility test for the OX4751 males crossed to wild-type females in medfly.

The effect and repressibility of the transgene were tested by crossing OX4751 males of each line to virgin wild-type (WT) females. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The proportions of eggs that hatched to eggs that didn't hatch were compared in the absence and presence of tetracycline in the larval diet (100ng/ul). The *P* values from a Chi Squared test are also shown.

Paternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	358	318	88.82	0.88
	NT	365	334	91.50	
OX4751D	T	354	309	87.28	2.2×10^{-16}
	NT	326	0	0	
OX4751D1	T	326	296	90.79	2.2×10^{-12}
	NT	337	89	26.40	

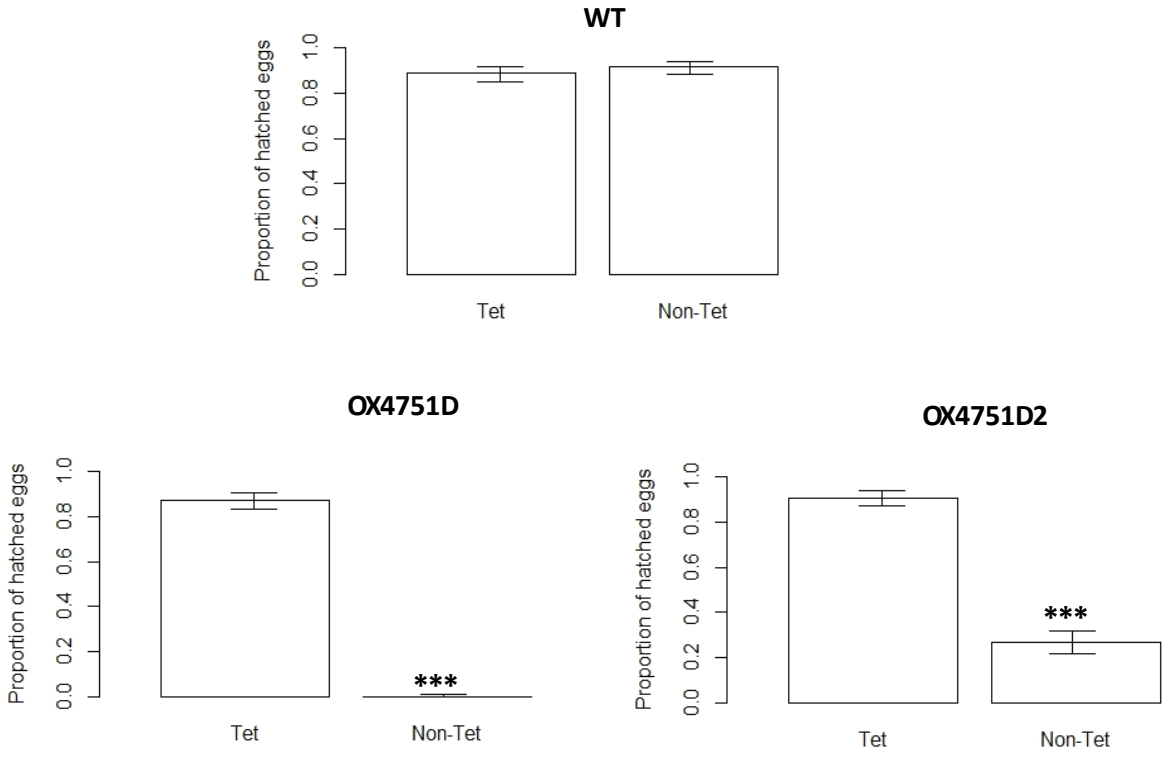


Figure 4. 11 The male fertility test for males of medfly lines carrying the OX4751 construct in their genome.

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs after OX4751 males were crossed to wild-type females. A Chi Square (X^2) test was done as statistical analysis; the p value for each set is comparisons of proportion of eggs that hatch to eggs don't hatch when OX4751 was reared on and off tetracycline. The number of hatched eggs differed significantly between Tet and Non-Tet (p-value <0.05). The Y axis shows the proportion of hatched eggs. Asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

Both lines were evaluated for female specific lethality penetrance in the absence of tetracycline in the larval diet and also for the repressibility of phenotype in the presence of tetracycline in the larval diet. The transgenic adults were sexually identified post-emergence, and the numbers of males and females were scored. The results are shown in Table 4.12 and Figure 4.12.

Table 4. 12 The test for the female specific lethal penetrance for the OX4751 strain.

The transgene penetrance and repressibility were evaluated in the absence and presence of tetracycline in the larval diet. The adult progeny's sex ratio is presented for both lines. A Chi Squared test was performed; the sex ratio of both lines was compared to the sex ratio of wild-type flies when reared on or off-tetracycline.

Strains		Male	Female	P value
Wild-type		174	178	-
OX4751D	T	161	163	0.946
	NT	159	0	2.2×10^{-16}
OX4751D2	T	169	174	0.966
	NT	183	168	0.885

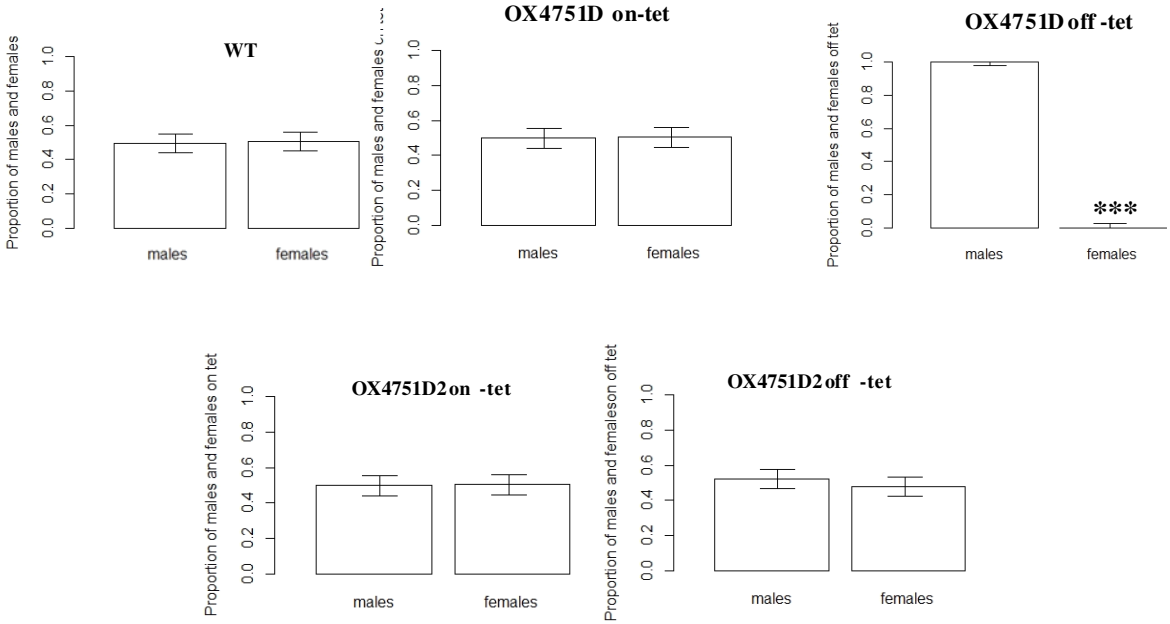


Figure 4. 12 The female specific lethal penetrance test for the OX4751 strain.

The bar chart illustrates the proportion of male and female transgenic progeny of OX4751 males crossed to wild-type females. A Chi Squared (X^2) test was done as statistical analysis; the p value for each set is a comparison of proportion of female and male when OX4751 was off and on tetracycline. The Y axis shows the proportion of male and female when the strain was reared off and on tetracycline. Asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

4.3.9. Sperm morphology was affected by the presence of transgene in OX4751D but not OX4751D2

In addition to generating male sterility and female lethality, this strain was designed to incorporate a fluorescent marker into the sperm heads. To check if the sperm head of the OX4751 strain were marked, the male flies (grown in the presence or absence of tetracycline) were selected. To check if sperm head was fluorescently marked, testes and genital tract were dissected and examined by microscopy for the presence of fluorescence protein in sperm head. OX4751D showed fluorescence in spermatocytes. No differentiation was observed beyond this stage in this line, i.e. it showed meiotic arrest. No fluorescence was observed for OX4751D2 at any stage. The results are shown in Figure 4.13.

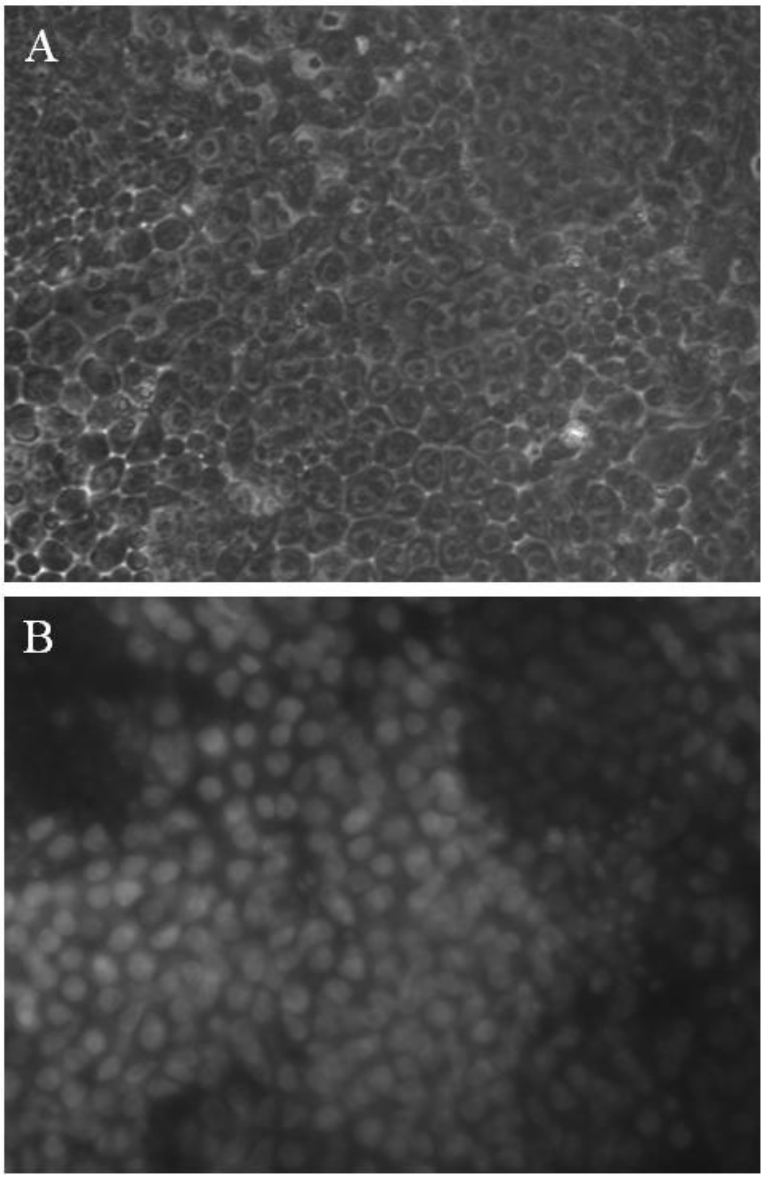


Figure 4. 13 OX4751D showed fluorescent spermatocytes, and a block in differentiation in the absence of tetracycline.

A: phase contrast; B: red fluorescence. Spermatocytes of OX4751D males have red fluorescent nuclei, but no spermatids are present.

4.3.10. The tetracycline concentration assay for flies the OX4751D line.

Given that the OX4751D line is viable and fertile in the presence of 100ng/µl tetracycline in the larval diet, while being fully penetrant female lethal and male sterile in the absence of tetracycline, I hypothesized there is a minimum tetracycline concentration which would suppress the male sterility and sperm fluorescence, and the female specific lethality. An experiment was designed to evaluate the effect of tetracycline concentration on the penetrance of the transgene-induced phenotypes in the OX4751D strain. In this experiment, males of OX4751D were reared on tetracycline and crossed to wild-type females and progeny were collected on concentrations of 0, 0.1, 0.3, 0.5, 1, 3, 5, 10, 20, 30, 50 and 100ng/µl of tetracycline in the larval diet. The results show that repressibility of transgene is initially detected at 20ng/µl of tetracycline and full repressibility of the transgene requires 100ng/µl Figure 4.14.

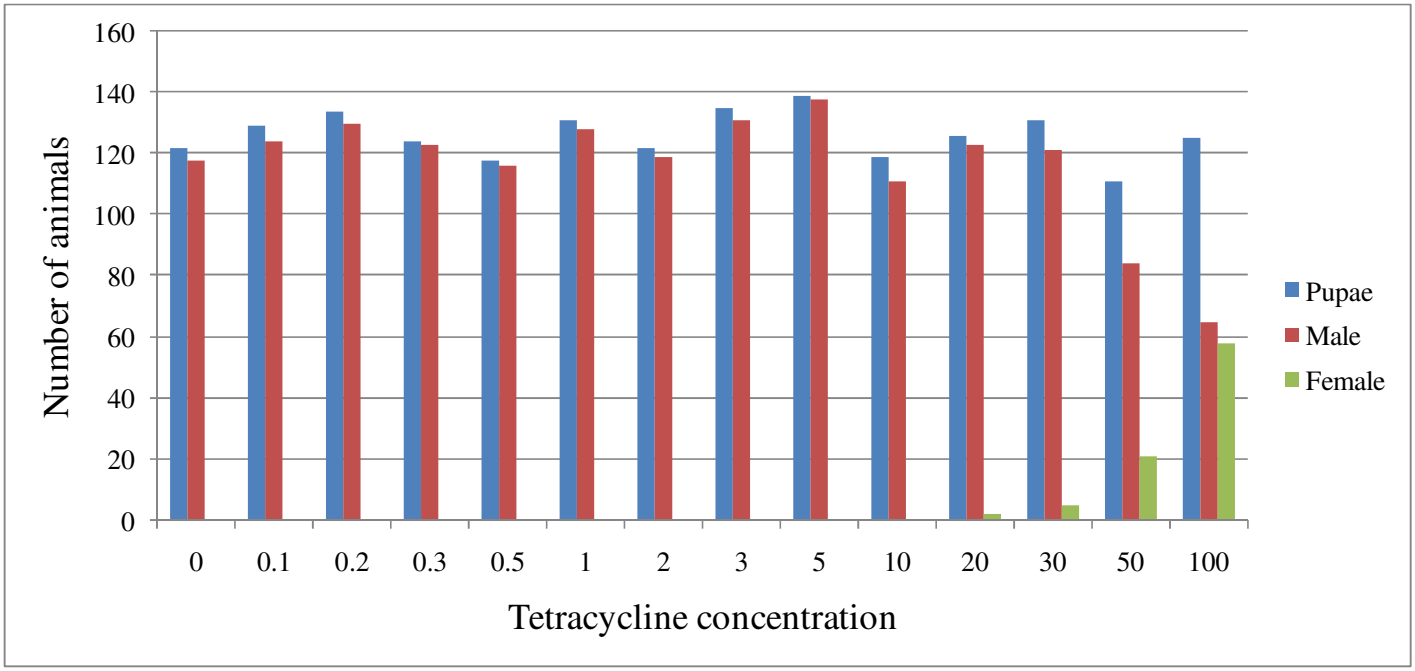


Figure 4. 14 The effect of tetracycline concentration on penetrance and repressibility of OX4751D-induced phenotypes.

The penetrance and repressibility of the phenotypes were tested by crossing OX4751D males of each strain to virgin wild-type (WT) females and culturing their progeny in the presence of 0, 0.1, 0.3, 0.5, 1, 3, 5, 10, 20, 30, 50 and 100ng/µl of tetracycline in the larval diet. The female specific lethality occurs before the 3rd instar larval stage, therefore all the pupae progeny emerge as males unless this lethality is suppressed. The Y axis is actual number of animals.

4.3. 11. Mating competition and re-mating assay

OX4751D males were tested to evaluate their ability to compete with wild-type males for mating to wild-type females. Additionally, the re-mating propensity of wild-type female mated with OX4751D males was compared to that of wild-type females mated with wild-type males.

Both experiments were designed according to guidelines for mating competition tests and re-mating tests (IAEA/USDA/FAO 2003). These tests were designed to be carried out in medium cages (30x30x30cm, because of the numbers of the flies that have been used here, the medium size cages were used). For the mating competition test, the cage contained 200 males of OX4751, 200 wild-type males and 200 wild-type females for duration of 5 hours. Mating pairs were removed and were screened using a fluorescence microscope to genotype the mating males (see section 2.7 for more details).

The relative sterility index (RSI) is a parameter that has been used to evaluate the mating capability of a fly strain. The RSI is the proportion of the total mating that the targeted strain achieved in the mating test. In this experiment, the OX4751D males, reared in the absence of tetracycline, successfully achieved 47% (RSI: 0.47) of the mating compared to wild-type males who achieved 53% (RSI: 0.53). There is no statistical difference between the RSI values for the OX4751D and wild-type males. The results suggest that OX4751D has no reduction in mating competitiveness relative to wild-type males.

OX4751D males reared in the absence of tetracycline do not produce any sperm. Female medfly that mated with a sperm-less male medfly is more likely to re-mate (Mossinson & Yuval 2003). Therefore, in the second part of this experiment; the effect of the genotype of the first male on wild type female re-mating was tested.

Two separate cages were set up: the first cage contained 100 mature wild-type females (four days old) along with 100 mature wild-type males (four days old). The second cage contained 100 mature wild-type females (four days old) along with 100 mature OX4751D males (four days old, reared without tetracycline).

Ninety eight mating pairs were collected from the first cage (WT females + WT males). Males were discarded, and after three days fresh wild-type males and OX4751D males were introduced to mated-females, ratio 1:1:1 for fifteen days. 51 of the females re-mated; 27 of them re-mated to wild-type and 24 of them re-mated to OX4751D. From the second cage (WT female + OX4751D males), 97 mating pairs were collected. Males were discarded and fresh wild-type males and OX4751D males were introduced to mated-females (ratio 1:1:1) after three days, for fifteen days. 55 of the females re-mated; 31 of them re-mated to wild-type and 24 of them re-mated to OX4751D. In both cages the number of re-mating was relatively high, most likely this is because they were kept in a small size cage for a long time period.

4.4. Conclusion

The results from all crosses described in chapter 3 revealed that *β2-tubulin-tTAV* crossed to tetO-*ProtamineFokI* gave the highest male sterility. The purpose of this chapter was to test a construct containing both promoter and effector, to determine if they are able to perform well as a single transgene insertion.

The simplest version, OX4353 contains the *β2-tubulin-tTAV* and tetO-*ProtamineFokI* elements from OX4282 and OX4458 respectively. The head-to-head orientation of these elements is such that tTAV expression can enhance expression not only of the *ProtamineFokI* effector, but also of itself, via a positive feedback loop. Reassuringly, and as predicted, all the OX4353 lines gave a male sterile phenotype in the absence of tetracycline. Although males of OX4353 are sterile in the absence of tetracycline, the transgenic males also showed some sterility even in the presence of tetracycline.

The positive feedback loop generated between the *tTAV* and the *tetO* in this OX4353 construct, might lead to adverse effects on the fitness of the insects if it is expressed outside the germline, since a similar loop is used for female specific lethal in several fruit fly lines (Fu et al. 2007). Real time PCR was performed on the testis and carcasses of flies on and off tetracycline to quantitate the level of *tTAV* and *FokI* expression in testis and carcasses which supported this hypothesis (Sections 4.8 and 4.9). There was no significant reduction in the fertility of OX4353 females in the absence of tetracycline, indicating that the construct is either not expressed (even at a basal level) in the female germline or genital tract, or that the minimal expression in these tissues is not detrimental to female fertility.

The OX4353 strain which contains both *β2-tubulin* and *ProtamineFokI* in one construct demonstrated the compatibility of promoter and effector in one construct. In some lines of OX4353 there are defects of testis morphology meaning that the sterility was not achieved in all lines via the desired mechanism. Our aim was to generate a strain with excellent sterility, achieved via a paternal effect lethality mechanism, and with no somatic defects.

In light of the promising results from OX4353, a further set of lines, containing a new construct OX4751 was developed. One of the aims to produce this strain was to check the adverse effect of a transgene which produces both targeted phenotypes in one construct (paternal effect lethality and female specific lethal). An additional aim in this experiment was to incorporate a marker on the effector, to label sperm from transgenic males. Three lines were generated, one could not be cultured because of dominant, non-repressible sterility. One gave some male sterility but no female specific lethality, and finally one line of this strain showed both male sterility and female specific lethality (OX4751D). OX4751D flies have no sperm when grown in the absence of tetracycline, but spermatocytes nuclei are marked. This suggests that the effector is being translated early in spermatogenesis (before meiosis), and early expression of this nuclease is likely to negatively impact on normal sperm production.

To achieve the ultimate purpose which is paternal effect lethality strain in combination with "Female-specific lethality" strain, another approach can be taken. Instead of having both in one construct, two separate insertions can be characterised and later on crossed together. This was described in section 4.3.2 and it demonstrated that two separate insertions work well.

Chapter 5-Development of Paternal Effect Lethality Strains in Olive Fly (*Bactrocera oleae*)

5.1 Introduction

5.1.1 Requirements analysis for generation of an olive fly paternal effect lethality strain

Bactrocera oleae; olive fly is the single most important pest species for olive plantations worldwide. Currently, control of the population of the olive fly relies on the heavy use of chemical pesticides (Başkurt et al. 2011). Radiation-based SIT was not successful since radiation adversely affects the fitness of the released insects and their ability to mate successfully with the wild population (Economopoulos 2002). Oxitec has developed a genetic variant of SIT which could potentially overcome the negative aspects of using the radiation-based SIT. This new technology provides a sexing mechanism which previously a study that was undertaken in south-western Guatemala, was shown to increase the efficacy of control programmes three to five fold in medfly (Rendón et al. 2004). This is particularly important for the olive fly SIT where a male-only release strategy is now considered essential. This is because of the olive fly photo-periodic mating behaviour (the laboratory reared olive fly mate at a different time of the day than the wild olive fly), which is hypothesised, to be the result of an involuntary artificially selected change away from the natural mating (Zervas & Economopoulos 1982). This behaviour wasn't observed when females were removed from the experimental cages (Estes et al. 2012). A sex-specifically spliced intron from the medfly (*cctra*) *transformer* gene has been used to engineer female-only protein expression (Keyes et al. 1992; Fu et al. 2007).

Oxitec have developed a set of conditional female specific lethal strains of olive fly (Ant et al. 2012). These strains could be improved by rendering the released males sterile. Released

males of the female specific lethal strain mating with wild type females would produce fully viable, fertile, male progeny while female progeny would die at a larval stage. The regulatory licensing and public acceptance is likely to be considerably eased by providing a genetic sterilisation trait to males in addition to the female specific lethality.

The olive fly larvae cause extensive damage to the olive fruit, hence there is a need to develop a dominant lethal system that induces lethality as early as possible. Male sterility, via engineered paternal effect lethality acts early - eggs are fertilised but no viable zygote are produced. Most normal sperm functions such as motility are retained, to allow sperm to reach the female's spermatheca. The re-mating desire of females with either no sperm, or a small number of sperm in their spermathecae is greater than those with spermathecae full of sperm (Taylor et al. 2001). Although sperm of a paternal effect lethal fly should be capable of delivering its genetic material to the eggs, it will have damaged DNA which will not be capable of fertilising eggs or alternatively it will cause lethality in the earliest embryonic stage. This system should not have any impact on the male's mating ability. If the sterile males are not able to mate satisfactorily with wild females, the wild females will look for another mate as females are capable of mating more than once (Lux et al. 2002). Therefore, the mating competitiveness and sperm competition should both be considered when developing paternal effect lethal strains.

5.1.2. System components for engineering paternal effect lethality in olive fly

It has been shown that the female specific lethal construct based on the medfly *cctra* intron produces female specific lethality in medfly (Fu et al. 2007), mexfly (Stainton et al. unpublished data) and the Caribbean fruit fly, *Anastrepha suspensa* (Schetelig & Handler 2012). In light of this conservation, we predicted that the paternal effect lethal system, that was developed based on the medfly male germline promoters (Chapters 3 and 4) should work in olive fly.

5.2. Experimental design and constructs

Strains of olive fly carrying a potential paternal effect lethal construct based on *C. capitata β2-tubulin-tTAV-tetO- Protamine-mCherry-FokI* were generated and tested. Sterility and fitness tests on male and female flies in the absence and presence of tetracycline in the larval diet (100ng/µl) were carried out. RT-PCR was performed to check if there is any leakiness of the expression in somatic tissue and females, and also to test whether the fusion mRNA is correctly transcribed.

5.3. Results and discussion

5.3.1. The generation of OX4705 olive fly strain

OX4705 is a modified version of OX4353 (Figures 2.10 and 2.19). The OX4705 construct contains a dominant selectable transformation marker (*DsRed*, a fluorescent protein, driven by the muscle *actin* promoter (*mActin* from Mexican fruit fly)) whereas AmCyan in the OX4353 construct is driven by Hr5-IE1. The muscle *actin* promoter (from the Mexican fruit fly) gives a stronger fluorescent protein expression which is helpful for detection purposes. *tetO* drives the expression of *Protamine* (dProtB chain from *D. Melanogaster*) fused to *mCherry* and *FokI*. This design was intended to generate a fusion protein which is both a nuclease, and fluorescent, that localises on spermatid and sperm DNA.

Olive fly embryos were injected with the OX4705 plasmid and a *piggyBac* transposase mRNA; two different sets of injections were done. The germline transformation rate in olive fly is low, to increase the chance of transformation *piggyBac* transposase mRNA was used. From the first set of injections, the egg to adult survival rate was 0.7%. In the second set of injections the egg to adult survival rate was 2%. In both sets of injections, an average of 27% of these pupae displayed the *DsRed* transient expression that confirmed the presence of plasmid in those individual injections. The results are shown in Table 5.1. To repress the effect of the transgene all lines were reared on tetracycline at all times. The olive fly lab strain (at Oxitec) has very low egg hatching rate, furthermore olive fly's egg has low tolerance toward injection, therefore after injection a high level of death occurs among injected eggs.

The surviving adults from both injections were back crossed to wild-type flies (10 males of G0 survivors back crossed to 30 wild-type females and 20 females of G0 survivors back

crossed to 10 wild-type males). The cages were labelled alphabetically (A, B, C…). G1 progeny were collected and screened for expression of the transformation marker (*DsRed*). Ten independent lines were obtained from the injections with the OX4705 construct, all of them were generated from the second round of the micro-injection. The obtained lines were OX4705A, A1, A2, A3, B, B1, F, F1, K, and P. The OX4705A, A1, A2 and A3 strains were produced from the same cage. They all showed different fluorescence pattern so they were separated as four different strains. All strains were observed for few generations, they continuously had distinct fluorescence pattern from each other but there was very little within strain variation in fluorescence pattern. The same was detected in the OX4705B and B1 and OX4705F and F1 strains.

Table 5. 1 Generation of the OX4705 transgenic strains.

OX4705 (*β2-tubulin-* and *ProtamineFokI*-based construct) plasmid DNA was injected into freshly collected olive fly eggs, along with transposase mRNA. Ten transgenic lines were obtained from the micro-injection of the OX4705 construct into olive fly.

Injections	No. of eggs injected	G0 larvae	G0 adults	No. Of transgenic lines obtained
1st	5000	150	35	0
2nd	7000	710	195	10

mActin is expressed in every muscle in the fly body; in this construct it drives the expression of *DsRed*. Depending on the position of the transgene inserted in the fly chromosome, the expression level of *DsRed* will vary. In three of the lines obtained (OX4705B, F and P), the *mActin-DsRed* expressed so strongly that the pupae from those lines were bright pink, even in normal light. Two of the lines produced pale pink pupae (OX4705A and F1). The other five lines were not as strong (OX4705A1, A2, A3, B1 and K); the pupae were normal colour but their red fluorescence was very strong under the fluorescence microscope.

5.3.2. In the absence of tetracycline in the larval diet of the OX4705 strain, the male flies of this strain are sterile

If the OX4705 construct works as expected, males will be sterile, and have red fluorescent sperm nuclei when raised in the absence of tetracycline in their larval diet. To estimate the number of copies inserted in the genome of all ten lines and also to assess any detrimental effect of the transgene on flies, wild-type and transgenic progeny were counted at pupal stage. Figure 5.3 shows the number of transgenic versus wild-type pupae in all the OX4705 lines when they were crossed to wild type. Around 200 progeny were counted for each line. The results indicate that all lines of flies carrying the OX4705 construct apparently have a single transgene in their genome. This test also revealed whether the insertion was autosomal or X or Y-chromosome linked. Nine of the lines had an autosomal transgene insertion, while the transgene in line OX4705K was X-linked. Transgenic males from OX4705K crossed to wild-type females only produced female transgenics.

From some of the G0 cages, different individuals showed different fluorescence phenotypes (the expression level of *DsRed* was varied), possibly indicating independent insertions. The numbers next to alphabet associate with these different phenotypes (Table 5.2).

Table 5. 2 Fluorescence screening of the progeny from of OX4705 males (*β2-tubulin*- and *ProtamineFokI*-based construct) crossed to wild type females.

The table shows the actual number and percentage of wild-type and transgenic progeny resulting from males of the OX4705 crossed to wild type females, with the progeny collected in the presence of tetracycline in larval diet (100ng/ul). The progeny of the OX4705 strain were screened for *DsRed*. According to their history and inheritance pattern; all of them seem to have one autosomal insertion except the OX4705K which has one X-chromosome insertion. A Chi Squared test was performed to obtain the *P* value (next to the percentage) which is the comparison of the observed ratio with a theoretical 1:1 ratio expected from the segregation of a single insertion of the OX4705 transgene.

OX4705 strains	Number of wild-type progeny (%)	Number of OX4705 progeny (%), *P* Value
OX4705A	80(47.9)	87(52.1), 0.67
OX4705A1	83(47.7)	91(52.3), 0.64
OX4705A2	78(48.1)	84(51.8), 0.71
OX4705A3	86(48)	93(51.9), 0.69
OX4705B	93(47.9)	101(52.1), 0.67
OX4705B1	87(49.4)	89(50.5), 0.90
OX4705F	90(48.6)	95(51.3), 0.71
OX4705F1	88(48.3)	94(51.6), 0.69
OX4705K	79(47.5)	87(52.4), 0.62
OX4705P	93(51.9)	86(48), 0.69

To test the male fertility, OX4705 males were crossed to wild-type females. Since the expression of *FokI* (the effector) in males should be repressed by the addition of tetracycline, male fertility was evaluated in the absence and presence of dietary tetracycline in the larval diet (100ng/µl). Six males reared off and six males reared on-tetracycline from all lines and were crossed with twelve wild-type females. The proportion of eggs viability was monitored in all the crosses (Table 5.3 and Figure 5.1). In the absence of tetracycline in the larval diet of the OX4705 strain, the male fertility was significantly reduced in comparison to the controls (presence of tetracycline in the larval diet). The reduction indicates that the male flies carrying the OX4705 construct are fully, or almost, sterile in the absence of tetracycline.

The male fertility assessment of the OX4705 strain when reared off-tetracycline indicates a high effect of the transgene in all lines; 0-2.4% viable progeny, while the male fertility remained high when reared on tetracycline. As shown in Figure 5.1, almost 85% of eggs were viable in all OX4705 lines when tetracycline was provided to the father's diet at the larval stage.

Table 5. 3 Fertility of OX4705 males crossed to wild-type females.

The effect and repressibility of the transgene were tested by crossing males of the OX4705 strain to virgin wild-type (WT) females. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The proportion of eggs that hatched to eggs didn't hatch was compared in the absence and presence of tetracycline (in the larval diet) (100ng/ul). The *P* values from a Chi Squared test are also shown.

Paternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	481	454	94.46	0.96
	NT	463	435	9386	
OX4705A	T	394	366	93.05	2.2×10^{-16}
	NT	299	0	0	
OX4705A1	T	288	268	84.90	2.2×10^{-16}
	NT	321	2	0.54	
OX4705A2	T	407	377	92.61	2.2×10^{-16}
	NT	379	5	1.23	
OX4705A3	T	254	237	93.53	2.2×10^{-16}
	NT	491	6	1.15	
OX4705B	T	348	323	92.81	2.2×10^{-16}
	NT	345	6	1.73	
OX4705B1	T	309	286	92.50	2.2×10^{-16}
	NT	343	2	0.49	
OX4705F	T	329	307	93.00	2.2×10^{-16}
	NT	281	5	1.76	
OX4705F1	T	408	387	94.95	2.2×10^{-16}
	NT	263	1	0.25	
OX4705K	T	388	358	92.50	2.2×10^{-16}
	NT	377	11	2.91	
OX4705P	T	333	315	94.45	2.2×10^{-16}
	NT	336	8	2.47	

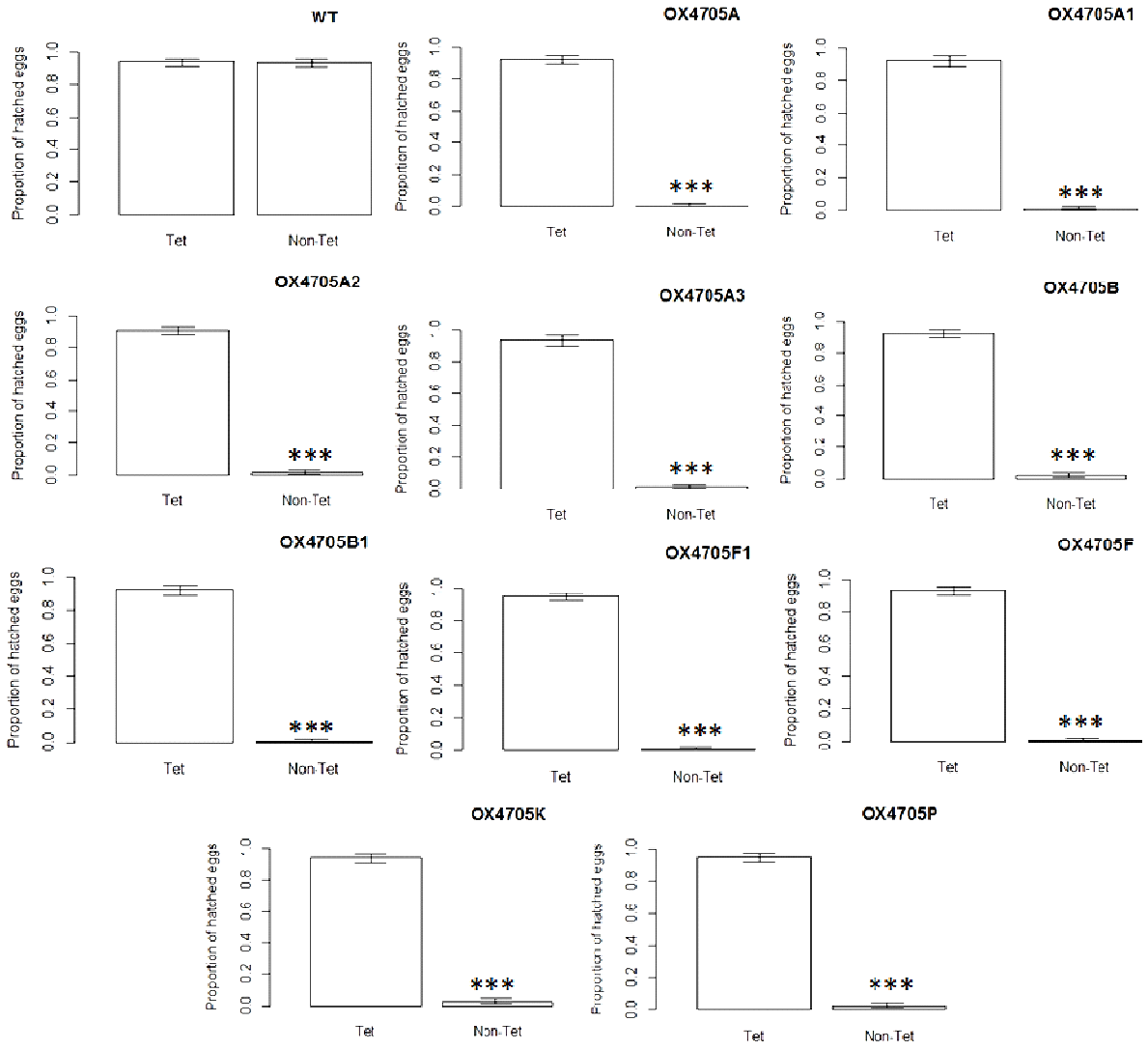


Figure 5. 1 The male fertility test of the OX4705 strain in olive fly.

The bar charts illustrate the proportion of hatched eggs to non-hatched eggs in progeny of OX4705 males crossed to the wild-type females. Chi Squared (X^2) tests were performed; the p-value for each set is a comparison of eggs viability on and off tetracycline in the larval diet of the OX4705 males mated with wild-type (WT) females. In all lines the number of hatched eggs differed significantly between Tet and Non-Tet. The Y axis shows the proportion of hatched eggs. Asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

The female fertility for all OX4705 lines was assessed to test whether the effect of the transgene was male-specific. The female fertility was not affected in any of the transgenic strains tested (Figure 5.2 and Table 5.4).

Table 5. 4 OX4705 females have equal fertility in the presence or absence of tet.

The effect and repressibility of the transgene were tested by crossing of the OX4705 females to wild-type (WT) males. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The proportion of eggs hatched compared in the absence and presence of tetracycline (in the larval diet of patents) (100ng/ul). The *P* values from a Chi Squared test are also shown.

Paternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	481	454	94.4	0.96
	NT	463	435	93.8	
OX4705A	T	394	366	93	2.2×10^{-16}
	NT	299	0	0	
OX4705A1	T	288	268	84.9	2.2×10^{-16}
	NT	321	2	0.5	
OX4705A2	T	407	377	92.6	2.2×10^{-16}
	NT	379	5	1.2	
OX4705A3	T	254	237	93.5	2.2×10^{-16}
	NT	491	6	1.1	
OX4705B	T	348	323	92.8	2.2×10^{-16}
	NT	345	6	1.7	
OX4705B1	T	309	286	92.5	2.2×10^{-16}
	NT	343	2	0.4	
OX4705F	T	329	307	93	2.2×10^{-16}
	NT	281	5	1.7	
OX4705F1	T	408	387	94.9	2.2×10^{-16}
	NT	263	1	0.2	
OX4705K	T	388	358	92.5	2.2×10^{-16}
	NT	377	11	2.9	
OX4705P	T	333	315	94.4	2.2×10^{-16}
	NT	336	8	2.4	

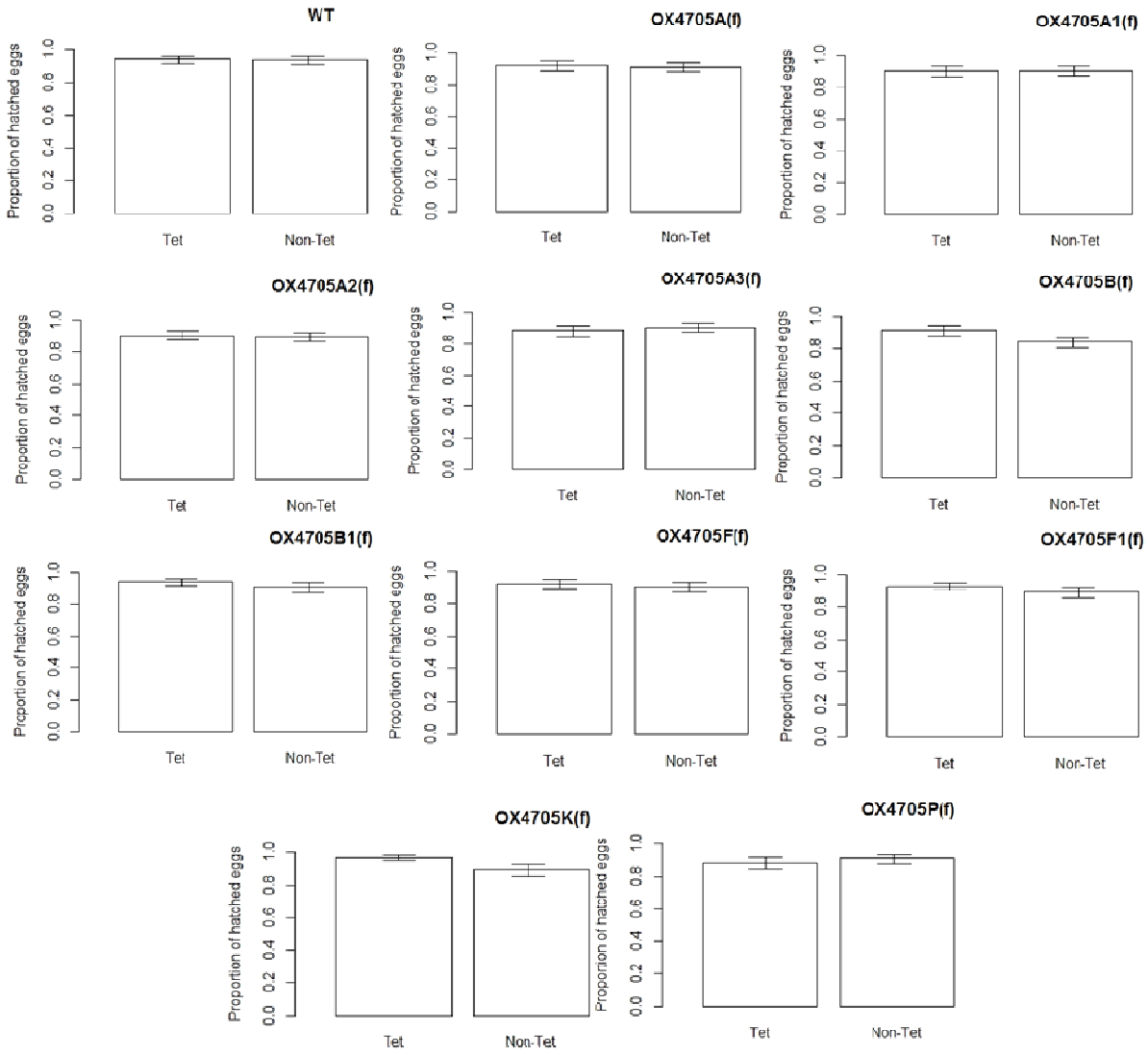


Figure 5. 2 The fertility test for OX4705 females.

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in laid by OX4705 females crossed to wild-type males. A Chi Squared (X^2) test was performed; the p-value for each set is a comparison of eggs viability on and off tetracycline in the larval diet of the OX4705 females mated with wild-type (WT) males. In all lines the number of hatched eggs didn't differ significantly between Tet and Non-Tet ($p > 0.05$). The Y axis shows the proportion of hatched eggs.

5.3.3. OX4705-bearing male do not produce sperm with fluorescent nuclei in the absence of tetracycline

One aim in designing of this construct, besides generating male sterility (paternal effect lethality), was to fluorescently mark sperm nuclei. Eggs were collected and reared in the absence and presence of tetracycline, to check if nuclei of sperm produced by OX4705 males were fluorescent. The testes and genital tract from the males were dissected and examined for the presence of DsRed fluorescent protein in spermatid or sperm nuclei. No fluorescence was observed in any of the lines, meaning that their sperm nuclei were not successfully marked (Figure 5.3).

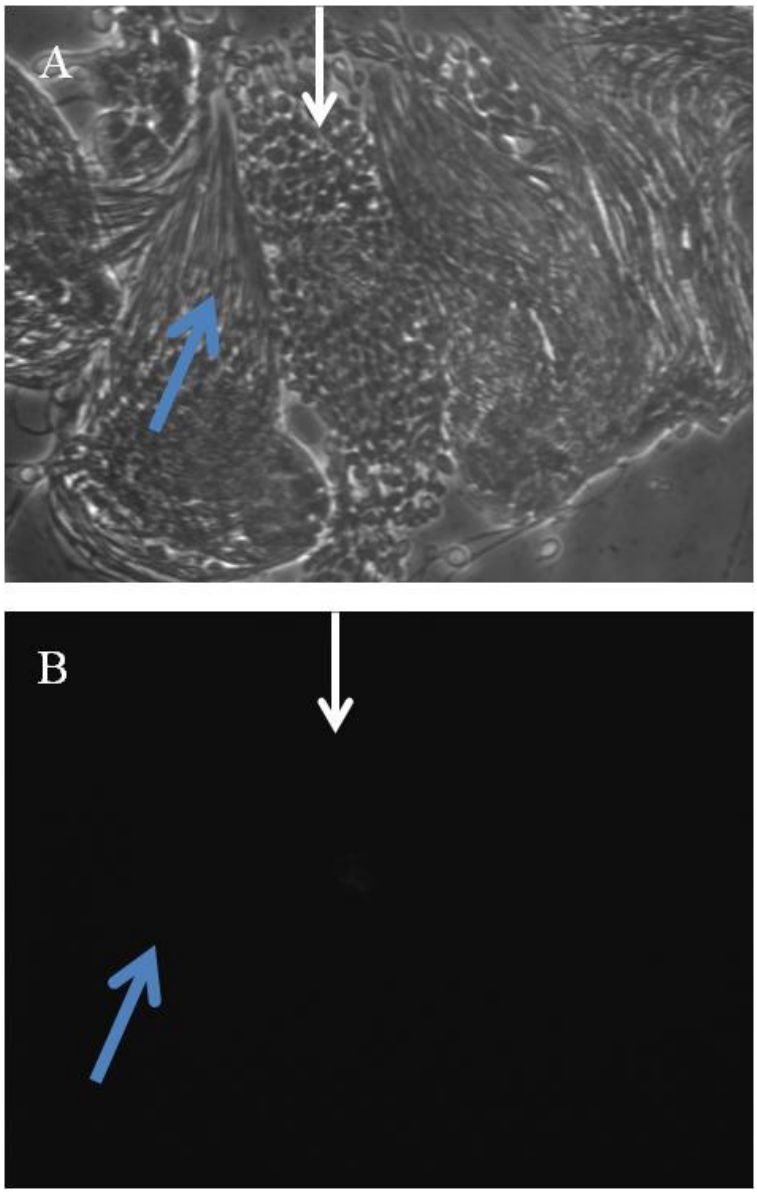


Figure 5. 3 Microscopy of cells from the OX4705F testes.

A: spermatocytes, spermatid bundles viewed by phase contrast B: spermatocytes, spermatid bundles RFP fluorescence. The white arrows show spermatocytes, blue arrows show spermatid bundles, no fluorescence was detected at either stage.

5.3.4. RT-PCR reveals that mCherry is expressed in OX4705 testes

Radiating flies damages somatic tissue as well as their germline so the fitness of sterile flies is compromised. RIDL flies are assumed to eliminate such an effect as this technology allows for tissue-specific targeting in species. To determine if the effector had leaky expression in somatic tissue, RT-PCR was performed using RNA extracted from testes, male carcasses and whole body of females of olive fly of three of the OX4705 strains that were reared on and off-tetracycline. cDNA synthesis was using oligo dT primers. Negative control samples omitted RNA polymerase from the cDNA synthesis reaction. For the test RT-PCRs, *tTAV* and *mCherry-FokI* primers were used, while the *ADH* primers were used as a control (the *ADH* gene expresses in all tissues of the olive fly, both in the males and females (Brogna et al. 2001)).

Three OX4705 lines were chosen for RT-PCR analysis; OX4705A (weak expression of the transformation marker), OX4705B (relatively strong expression of the transformation marker) and OX4705F (extremely strong expression of the transformation marker). All samples gave bands of the expected size with the *ADH* primers. RT-PCR on RNA of the OX4705A and OX4705B showed that both testes and male carcasses (off and on tetracycline) samples gave bands of the expected sizes with the *tTAV* primers. For OX4705F, only in testes (off and on tetracycline) samples gave the expected product using *tTAV* primers. Only testis (off and on tetracycline) and male carcass (only off tetracycline) from OX4705A gave the band of right size for *mCherry-FokI*. Clearly for this line at least, mRNA of the mCherry is present in testes despite our inability to detect any fluorescence. The RNA could be degraded before translation, could remain stable but untranslated, or could be translated but the fusion protein doesn't fold correctly or the protein gets degraded soon after translation. The results are shown in Figure 5.4.

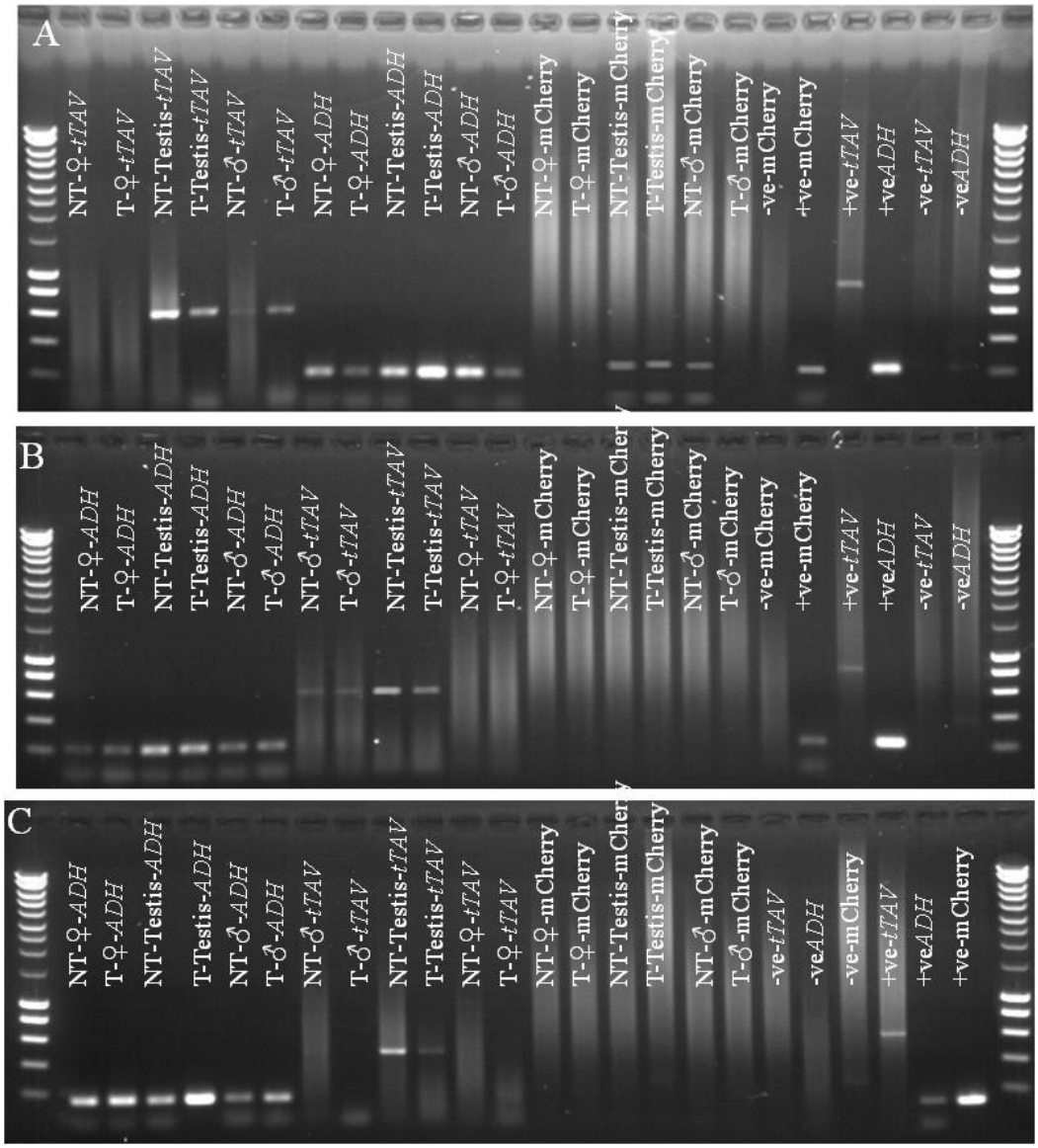


Figure 5. 4 The reverse transcriptase PCR on OX4705 lines reared on and off tetracycline.

A: OX4705A, B: OX4705B, C: OX4705F. RT-PCR using *tTAV*, *mCherry-FokI* and *ADH* specific primer set of cDNA generated from male carcass, female and testes when reared off and on tetracycline, as labelled. Both testes and male (off and on tetracycline) samples gave products of the expected sizes with the *tTAV* primers (except in C; No product for male samples). None of the samples except testis (off and on tetracycline) and male (only off tetracycline) from OX4705A gave the expected product for *mCherry-FokI*. No-RT controls were run for each sample but produced no product confirming no genomic DNA contamination. The left and right lanes show the DNA size standards from 200bp to 10000bp (Eurogentec Smart ladder). T: reared on-tetracycline, NT: reared off-tetracycline. The expected product size for *ADH* is 190bp, *tTAV* is 490bp and 195bp for *FokI*.

5.3.5. Addition of short peptide linkers within the Protamine-mCherry-Fok1 fusion protein – generation and testing of OX4801

In the second part of this chapter, peptide linkers were used, this was part of an attempt to fuse the nuclease to a fluorescent protein. The peptide linkers are small peptide chains that naturally are located between protein domains. Fusion of protein domains without the use of a peptide linker may have some adverse outcomes in the forming of the final product, including mis-folding of both domains of the fusion protein, lowering the production of protein and also damaging the bioactivity of the protein (Chen et al. 2013).

The peptide linkers are not inherently flexible, but they are often found in flexible, inherently unstructured, protein regions. Short linkers rich in glycine and serine (5 amino acid) assist the nearby protein domains move more freely comparatively to one another. Longer linkers (35-40 amino acid) can be used when it is essential to make sure that two flanking domains do not sterically interfere with each other (Minczuk et al. 2008).

After failing to detect sperm marking in OX4705 flies, a modified version of the OX4705 construct was designed (Figure 2.22). One of the possible explanations for the lack of sperm nuclear fluorescence, despite the presence of the transcript, and male sterility, is that of the Protamine-mCherry-Fok1 fusion protein did not fold correctly. To improve protein folding, G4S linkers (four residues of glycine and one serine) were added N-terminal and C-terminal of the mCherry sequence (Chen et al. 2013). The presence of linkers should help all three protein domains of the fusion protein to fold properly, and have normal function.

Olive fly embryos were injected with the OX4801 plasmid and *piggyBac* mRNA in two rounds. From the first set of injections, embryo to adult survival rate was 5%. In the second set of injections embryo to adult survival rate was 6%. In the both sets of injections, an

average of 28% of injected embryos displayed the *DsRed* transient expression at pupal stage. Six independent lines (OX4801A, C, D, F, I, G) were obtained from injections with the OX4801 construct (Table 5.5). They all had distinct fluorescence patterns, as well as coming from different pools of G0 flies.

Table 5. 5 The generation of the OX4801 strain in the olive fly.

The OX4801 construct (*β2-tubulin-* and *ProtamineFokI*-based construct) was injected into freshly collected olive fly embryos. Six transgenic lines were obtained.

Injections	No. of eggs injected	G0 larvae	G0 adults	No. Of transgenic lines obtained
1st	2500	450	125	3
2nd	3000	560	201	3

5.3.6. OX4801 male have reduced fertility in the absence of tetracycline in the larval diet.

Olive flies carrying the OX4801 construct should have a tetracycline-repressible system conferring paternal effect lethality. This is achieved by the expression of a *Protamine-mCherry-FokI* fusion protein, with short linkers between the protein domains, in the male germline. If the construct works as expected, males will be sterile, and have red fluorescent sperm nuclei when raised in the absence of tetracycline in their larval diet.

To determine the number of the transgene copies inserted in the genome of each individual lines and also to check for any adverse effect of the transgene on viability of flies in the presence of tetracycline, wild-type and transgenic progeny from OX4801 males crossed to wild-type females were scored. Table 5.6 shows the number of the wild-type versus transgenic progeny at the pupal stage in all OX4801 lines. Approximately 200 pupae progeny were counted for each line. The numbers of wild-type and transgenics were similar in all cases ($p>0.05$), therefore the results indicate that all the OX4801 lines had a single autosomal transgene insertion.

Table 5. 6 The result from fluorescence screening of the progeny from the OX4801 strain (*β2-tubulin*- and *ProtamineFokI*-based construct).

The table shows the actual number and percentage of wild-type and transgenic progeny resulting from OX4801 (males) x WT (females) crosses with the progeny collected in the presence of tetracycline (100ng/ul). The progeny of the OX4801 strain were screened for *DsRed*. All of them seem to have one autosomal insertion. A Chi Squared test was performed to obtain the *P* value (next to percentage) which is the comparison of the observed ratio with a theoretical 1:1 ratio expected from the segregation of a single insertion of the OX4801 transgene.

OX4801 strains	Number of wild-type progeny (%)	Number of OX4801 progeny (%) , *P* Value
OX4801A	91(51.4)	86(48.5), 0.77
OX4801C	93(49.4)	95(50.5), 0.91
OX4801D	89(48.9)	93(51.1), 0.82
OX4801F	99(51)	95(48.9), 0.83
OX4801I	78(51.6)	73(48.3), 0.73
OX4801G	81(47.3)	90(52.6), 0.59

The male and female fertility of OX4801 transgenic flies was assessed by crossing them to wild-type flies of the opposite sex. The male and female fertility was evaluated in the absence and presence of the dietary tetracycline in their larval diet since the expression of *FokI* (the effector) in males should be repressed by the addition of tetracycline (100ng/µl). Female fertility should be unaffected in both cases. The proportion of viable progeny (eggs) was monitored in all crosses (Figure 5.5 and Table 5.7).

In the absence of tetracycline, the males from the OX4801 strain showed a moderately strong reduction in male fertility, giving 10-24% viable progeny. The male fertility remained high in presence of tetracycline in the larval diet. As shown in Figure 5.5, nearly 85% progeny were viable for all OX4801 lines when they were reared with tetracycline in their diet.

Table 5. 7 The males of the OX4801 strain crossed to wild-type females in olive fly.

The effect and repressibility of the transgene was tested by crossing the males of each line to virgin wild-type (WT) females. The table presents the total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The proportion of eggs that hatched to eggs that failed to hatch were compared in the absence and presence of tetracycline (100ng/ul). The *P* values from a Chi Squared test are also shown.

Paternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	323	285	88.3	0.66
	NT	361	317	87.6	
OX4801A	T	309	278	89.8	2.2×10^{-16}
	NT	337	40	11.8	
OX4801C	T	334	288	86.2	2.2×10^{-16}
	NT	326	77	23.7	
OX4801D	T	333	297	89	2.2×10^{-16}
	NT	337	36	10.6	
OX4801F	T	312	269	86.3	2.2×10^{-16}
	NT	324	66	19.7	
OX4801G	T	345	302	87.1	2.2×10^{-16}
	NT	320	60	19.7	
OX4801I	T	302	269	88.9	2.2×10^{-16}
	NT	327	65	19.6	

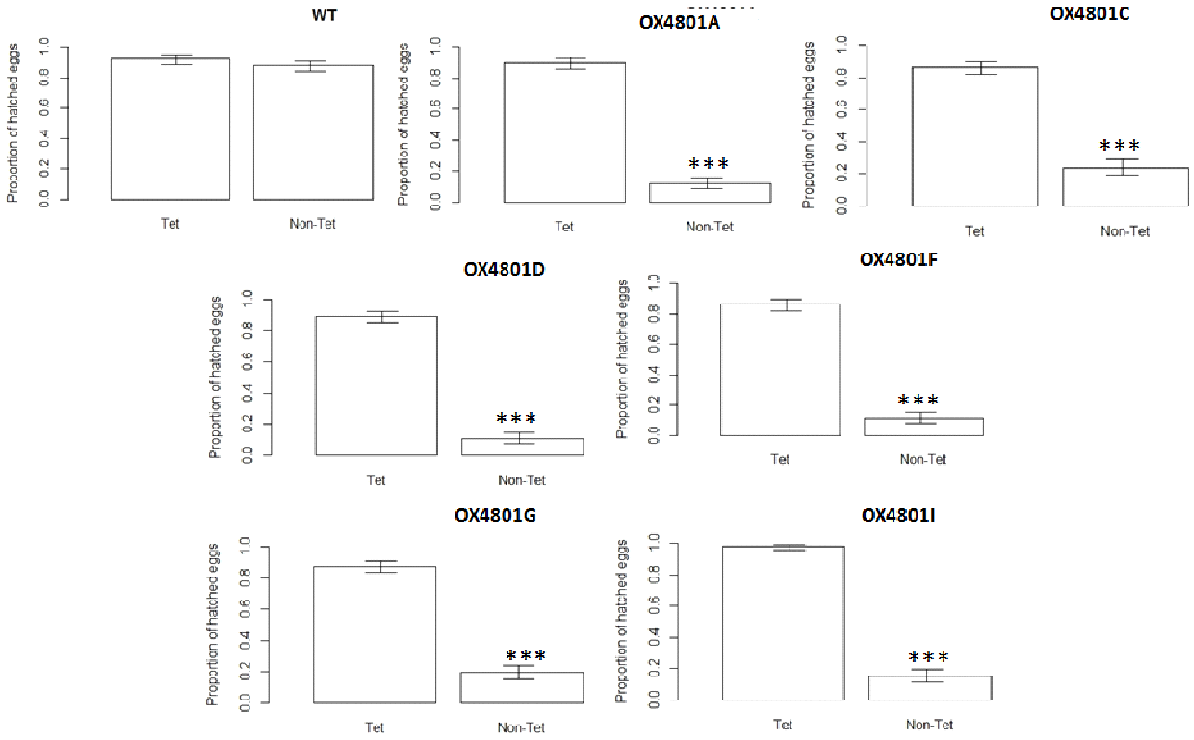


Figure 5. 5 The male fertility test for the OX4801 strain in olive fly.

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs after OX4705 males were crossed to wild-type females. The p-value for each set is a comparison of eggs viability on and off tetracycline in the larval diet of the OX4801 males mated with wild-type (WT) females. The Y axis shows the proportion of hatched eggs. Asterisks on the bar chart indicate a statistically significant difference, revealed by the Chi Squared (X^2) test (*: P<0.05, **: P<0.001, ***: P<0.0001).

Female fertility for all OX4801 lines was assessed to test if the effect of the transgene is male-specific. Female fertility was not affected in any of the transgenic strains tested (Figure 5.6 and Table 5.8).

Table 5. 8 The fertility test for females from the OX4801 strain crossed to wild-type males in olive fly.

The effect and repressibility of the transgene were tested by crossing males of each line to virgin wild-type (WT) females. The table presents total number of eggs, hatched eggs and percentage of hatched eggs in three replicas (R1, R2, and R3). The proportion of egg hatched to eggs that failed to hatch was compared in the absence and presence of tetracycline (100ng/ul). The *P* values from a Chi Squared test are also shown.

Maternal genotype		Total eggs (R1+R2+R3)	Hatched eggs (R1+R2+R3)	% hatched eggs (R1+R2+R3)	*P* Value
Wild type	T	323	285	88.34	0.66
	NT	320	277	86.41	
OX4801A	T	329	292	88.68	0.44
	NT	320	277	86.41	
OX4801C	T	358	330	92.19	0.53
	NT	319	271	84.92	
OX4801D	T	357	309	86.64	0.72
	NT	328	284	86.53	
OX4801F	T	350	307	87.72	0.88
	NT	321	280	87.21	
OX4801G	T	337	301	89.35	0.80
	NT	306	269	87.76	
OX4801I	T	314	265	84.20	0.74
	NT	307	272	88.63	

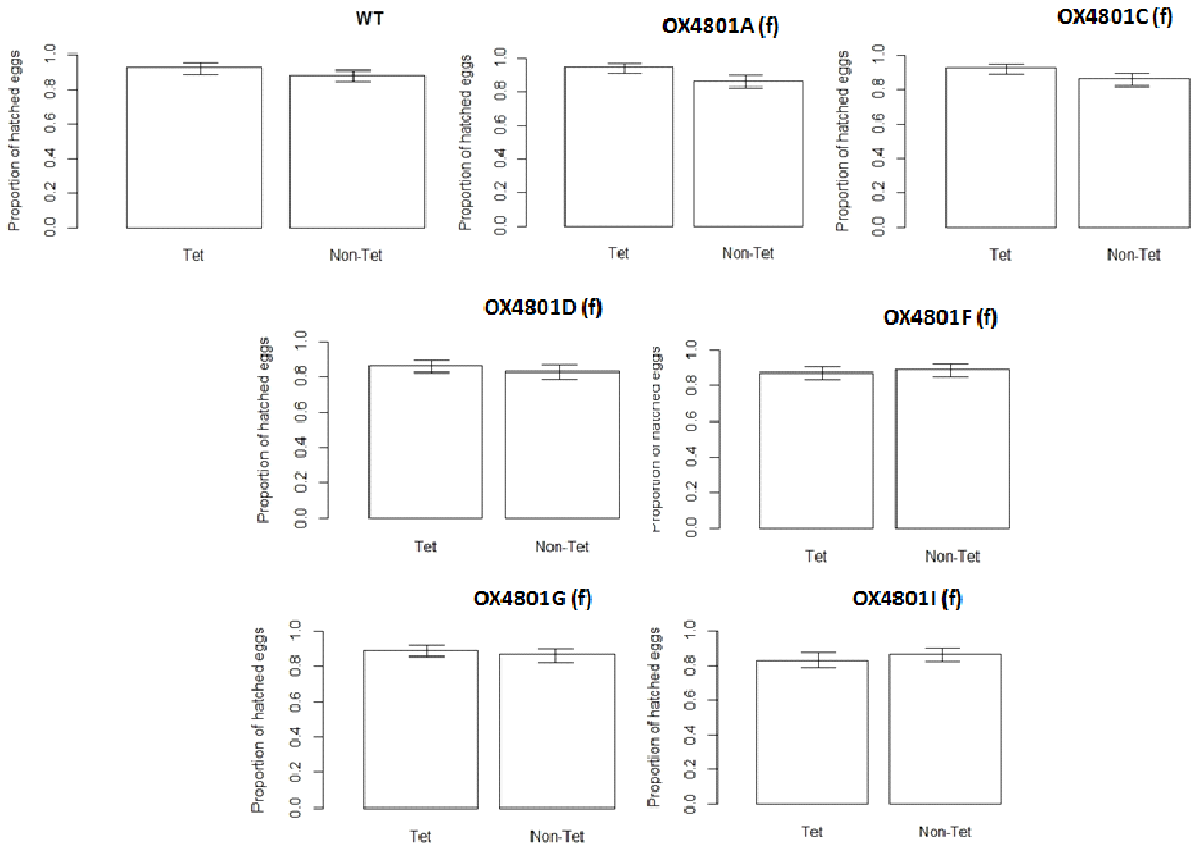


Figure 5. 6 The female fertility test for females of the OX4801 strain in olive fly.

The bar chart illustrates the proportion of hatched eggs to non-hatched eggs in OX4705 females crossed to wild-type males. A Chi Squared (X^2) test was done as statistical analysis; the p-value for each set is a comparison of eggs viability on and off tetracycline in the larval diet of the OX4801 females mated with wild-type (WT) males. In all lines the number of hatched eggs didn't differ significantly between Tet and Non-Tet as the p-value>0.05. The Y axis shows the proportion of hatched eggs.

5.3.7. The males of the OX4801 strain in olive fly produce a red fluorescence sperm

One of the aims in designing this construct was to modify the OX4705 construct so that sperm nuclei would be fluorescently marked. To check if the sperm heads of the OX4801 were marked in the absence of tetracycline in the larval diet, embryos were collected and reared in the absence and presence of tetracycline. Testes and genital tracts from the males were dissected and examined for the presence of fluorescence protein in spermatid nuclei and the heads of mature sperm. Fluorescence was observed in spermatid and sperm heads generated by all OX4801 lines (Figure 5.7). The fluorescent protein that was detected in all lines of this strain was faint.

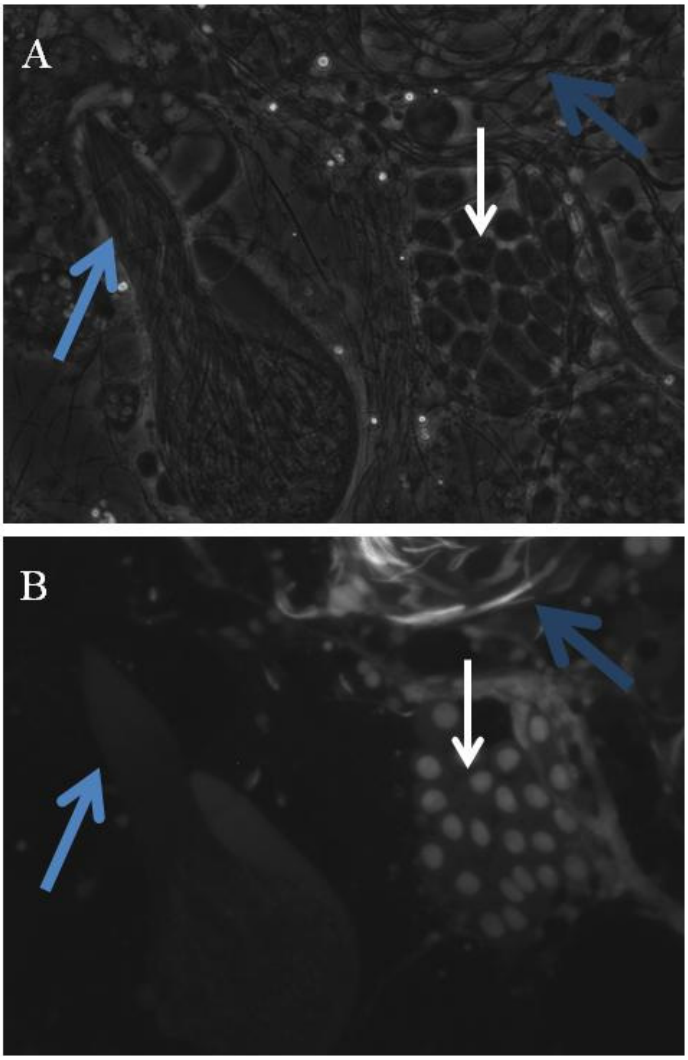


Figure 5. 7 Fluorescence and phase contrast microscopy of spermatocytes, spermatid bundles and individual sperm of the OX4801F line (in the absence of tetracycline in larval diet).

A: Phase contrast image of spermatocytes, spermatid bundles and individual sperm of OX4801F. B: RFP fluorescence of the same field of view showing fluorescence in the nuclei of spermatocytes, spermatids and individual sperm of OX4801F. The white arrows show spermatocytes, blue arrows show spermatid bundles and dark blue arrows show individual sperm.

5.4. Conclusion and future work

This chapter describes progress towards the generation of a paternal effect lethal strain of olive fly. Such a strain is designed to be sterile in the absence of tetracycline in larval diet. Additionally it is designed to fluorescently mark the sperm head in the absence of tetracycline in the larval diet.

The design of the OX4705 construct was based on the structure of OX4353 except that the transformation marker in OX4705 construct, is DsRed2 driven by the mexfly muscle actin promoter. In OX4705, also *mCherry* was added, between *Protamine* and *FokI* in the effector fusion protein in an attempt to mark sperm heads (see Figure 2.19 and 2.22). The lines that were generated using OX4705 produced highly penetrant, repressible male sterility, however their sperm wasn't fluorescent.

As this strain didn't successfully produce fluorescent sperm heads, the design of the construct was modified. It was assumed that there is a problem in folding of the fusion protein. To separate functional domains of the fusion protein, a SG4 linker was used. The new construct (OX4801) plasmid, is essentially the same as OX4705 except for having linkers at the either side of the *mCherry* sequence which is located in between *Protamine* and *FokI* (see Figure 2.22). With these SG4 linkers the sperm head was successfully marked with mCherry protein, suggesting that the linkers indeed improved the folding of the fusion protein.

The OX4801 strains generate male sterility, although with a lower penetrance than seen with the OX4705 strains. Although the linkers improved the function of mCherry protein but they could also affect the function, expression or stability of *ProtamineFokI*.

To improve the function of all proteins in the fusion protein, the design of the construct can be rearranged. To do that I propose to move the *mCherry* sequence to the N-terminus of the

fusion protein, upstream of the *ProtamineFokI* sequence. This could improve performance of the whole fusion protein. Ideally this should be tested with and without the linkers between the mCherry and ProtamineFok1 modules.

Chapter 6- Testing an alternative testis specific promoter in medfly to generate a paternal effect lethal strain

6.1. Introduction

To develop a paternal effect lethal strain, a male germline specific promoter is needed to drive the expression of an effector in testis. In previous chapters, *topi* and *β2-tubulin* were used to generate putative paternal effect lethal strains, and the performance of such strains was widely studied (See chapter 3). Jin 2011 showed that expression of *topi* in medfly testis is not very strong and *β2-tubulin* expression is late in spermatogenesis (see section 1.6 for more information). Therefore it would be ideal to find another gene that has high expression in testis and expresses early in spermatogenesis. Furthermore, a broad collection of testes-specific promoters provides us with a chance of producing a wide range of paternal effect lethal strains, and would advance the chance of generating a strain with desirable characteristics (e.g. fitness, mating competitiveness).

The level of transcription increases during the primary spermatocyte stage (Reinke et al., 2000). Some of these genes are germline specific and some have a germline specific transcription start site (White-Cooper 2010)(see section 1.6 for more information). There are few genes from *D. melanogaster,* such as *hsp70* and *topi*, that were previously used to guide design of constructs in medfly (Jin 2011). The *cchsp83* is a medfly gene that encodes a heat shock protein 83 in medfly which expresses in all stages of the medfly development. Depending on a temperature and developmental stage of medfly, the *cchsp83* gene is induced from a low to high level in cells of medfly (Theodoraki & Mintzas 2006). The hsp83 protein in *D. melanogaster* and medfly is heat shock induced in somatic tissue and it is constitutive in the germline (Theodoraki & Mintzas 2006). In the male germline of *D. melanogaster*, the

hsp83 gene is transcribed in early primary spermatocytes, and the transcript persists to early elongation (http://www.fly-ted.org/311/). The transcription start site of *hsp83* in the male and female germline is different from that in the somatic tissue (Jin 2011).

6.2. Experimental design

Three different constructs using the *cchsp83* based promoters were designed and injected into medfly eggs (See material and methods). The *cchsp83* gene in medfly has two overlapping promoter sequences with two overlapping 5'UTR sequences, one for germline (longer as it extends upwards in the promoter) and one for somatic (short, which is principally the 3' end of the germline 5'UTR). Both transcripts share the same ATG-start (personal communication with Dr. Tarig Dafa'alla). This makes it difficult when defining the promoter sequence and the 5'UTR sequence in the designing of a construct therefore we designed three different versions.

The OX4324, OX4642 and OX4656 constructs have AmCyan protein as the transformation marker and DsRed2 protein as the marker of cchsp83 promoter activity. In OX4324 (Figure 2.9) the *cchsp83* promoter (1584 bp) containing the somatic 5'UTR of the *cchsp83* gene (the somatic 5'UTR is 122 bp and the intron in the 5'UTR which is just before the start-codon is not included) which drives expression of DsRed2. The aim of designing this construct was to use the *hsp83* promoter to drive the expression of DsRed2 in somatic tissue as well as the germline in medfly. In OX4642 (Figure 2.16) the *cchsp83* promoter and the germline specific 5'UTR of the *cchsp83* gene was used. The 5'UTR was mutated to delete the somatic TATA-box (promoter = 2599 bp, germline 5'UTR 554 + intron = 281 bp), therefore such a construct should drive the expression of *DsRed2* exclusively in the male germline. In OX4656 (Figure 2.17), the *cchsp83* promoter containing the germline specific 5'UTR, here in order to delete the somatic transcript without deleting its 5'UTR (which is part of the germline 5'UTR), a couple of point mutations to open the reading frame even before the transcription start for the somatic transcript was made and a KOZAK start was inserted to enable the translation from upstream to the somatic 5'UTR (promoter = 1153 bp, 5'UTR = 552 bp, intron = 281 bp and

part of exon 2 =134 bp). The new ORF is different from the original ORF for the endogenous Cchsp83-protein. The start codon for the endogenous Cchps83 is immediately after the intron in the 5'UTR, deleting or mutating it might affect the splicing of that intron. ATG-start untouched but is out of frame the new ORF i.e. is not going to translate DsRed. The new ORF is continuous with Ubiquitin and DsRed, and therefore supposed to result in the translation of the red fluorescence and this should happen only in germline cells.

Transgenic lines from each injection were obtained and stable lines were generated. To determine whether the constructs were working as expected ten flies from each transgenic line were dissected and their testis were studied under microscopically. Testis of wild-type medfly was used as a control. Testes were examined using an upright compound fluorescence microscope and pupae with a stereo fluorescence microscope.

6.3. Result and discussion

6.3.1. The generation of the OX4324, OX4642 and OX4656 strain in medfly

Medfly embryos were injected with the OX4324 (Figure 2.18), OX4642 (Figure 2.19) and OX4656 (Figure 2.20) plasmids along with a plasmid helper (OX3022). Embryo to adult survivor rate for the OX4324 and OX4642 injections was 16% and it was 27% for the OX4656 injection. The results of injections are shown in table 6.1.

Three lines OX4324 lines were generated: OX4324I, OX4324L and OX4324N. Only one transformant line was generated for OX4642 (OX4642I). All lines for these two strains have single autosomal insertions as they produce equal number of male and female progeny after crossing transgenic males to wild type females. Four lines, OX4656B, OX4656C, OX4656I and OX4656N were generated for OX4656. OX4656B, OX4656C and OX4656I all have Y-chromosome insertions, as all the progeny from a cross of transgenic males to wild type females were male. OX4656N produced equal numbers of male and female progeny in this test, therefore it has an autosomal insertion.

Table 6. 1 The generation of the OX4324, OX4642 and OX4656 transgenic strains in medfly.

All three constructs were injected into freshly collected medfly embryos and transgenic lines were obtained.

Construct No.	No. of eggs injected	G0 larvae	G0 Pupae	G0 adults	No. Of transgenic lines obtained
OX4324	600	165	109	98	3 lines
OX4642	1100	263	191	176	1 line
OX4656	2000	810	651	547	4 lines

6.3.3. The OX4324, OX4642 and OX4656 strains lacked fluorescence in testes

To check fluorescent protein expression in the somatic tissue and germline of flies of all these three strains, testes and genital tracts from the males were dissected from individual lines. No fluorescence was observed in the testes of any lines of these three strains. The *hsp83* promoter fragment used fails to drive the expression of DsRed2 in the male germline of any lines from these strains (Figure 6.1). The DsRed2 fluorescent protein was observed in the somatic tissue in all OX4324 lines but not in the lines for the other two constructs (Figure 6.2).

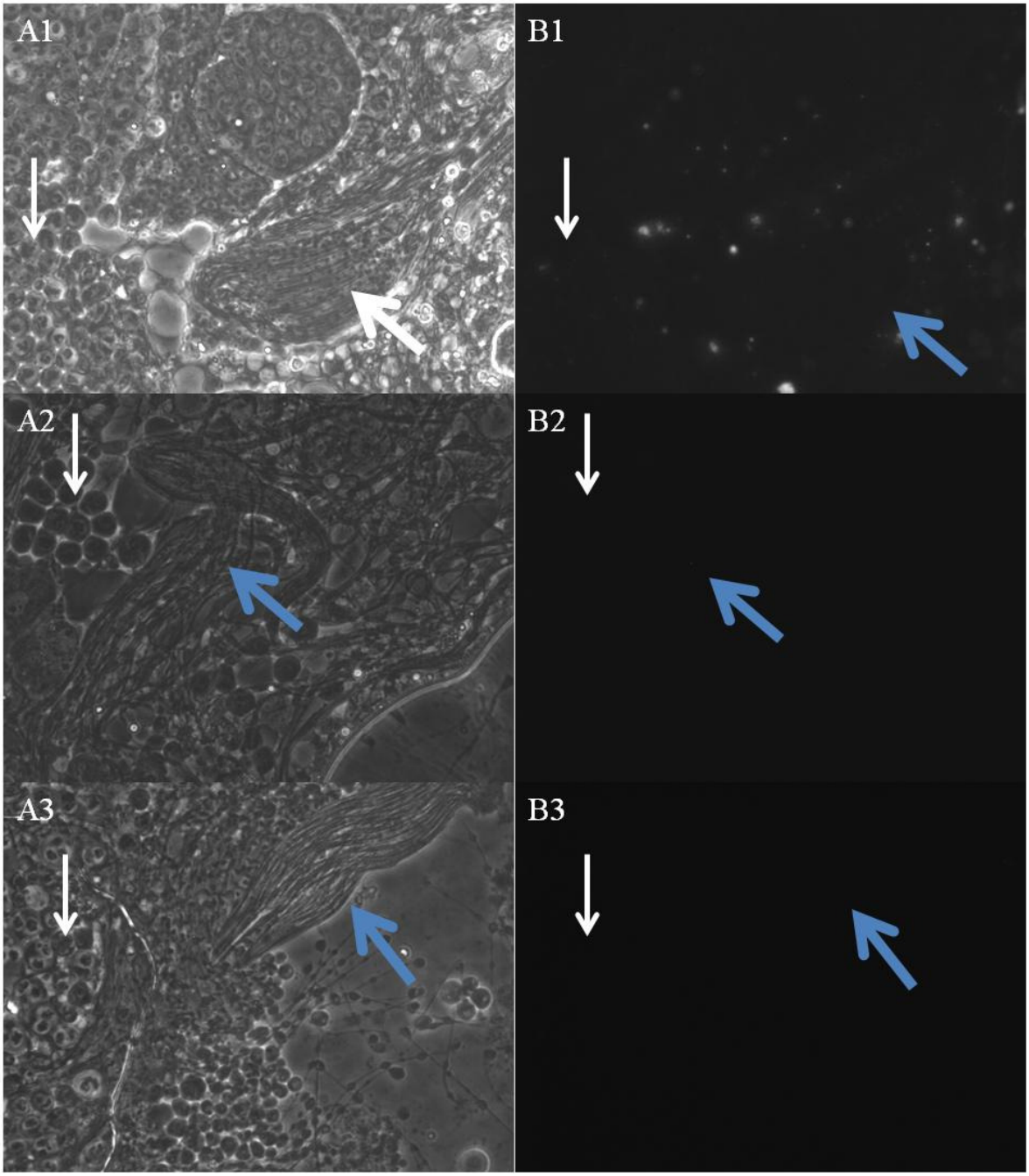


Figure 6. 1 Fluorescence microscopy of spermatid bundles and spermatocytes under phase contrast and RFP filter.

Panels A are under phase contrast and panels B are fluorescence of RFP (red fluorescent protein). A1 & B1: spermatid bundles and spermatocytes of the OX4324L strain; A2 & B2: spermatid bundles and spermatocytes of the OX4642I strain; A3 & B3: spermatid bundles and spermatocytes of the OX4656N strain. The white arrows show spermatocytes and blue arrows show spermatid bundles. In all strains, only a background level of fluorescence was observed.

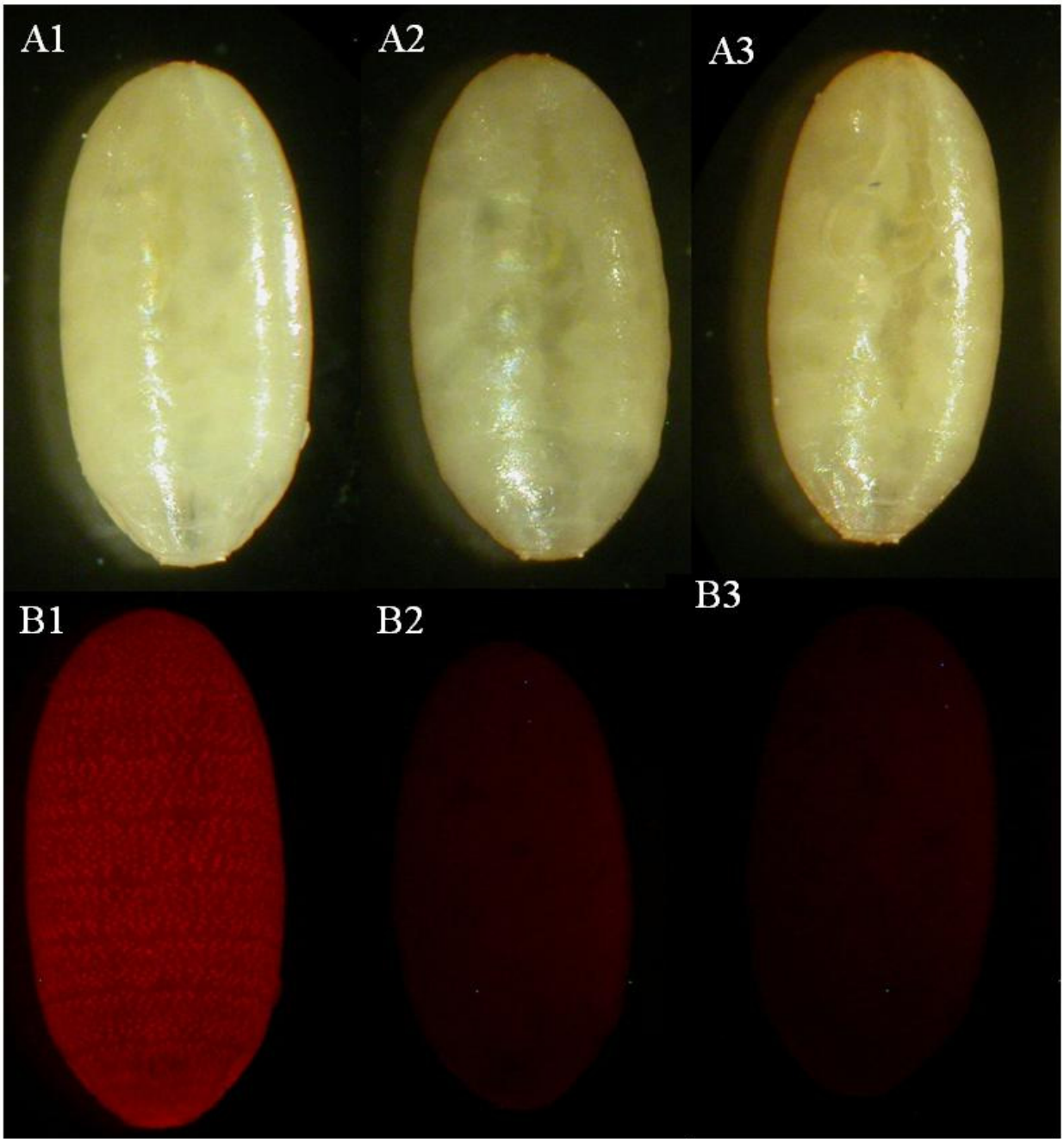


Figure 6. 2 Fluorescence microscopy of pupae from OX4324L, OX4642I and OX4656N strains under bright field and RFP filter.

Panel A are bright field and panel B are under RFP (red fluorescent protein) light. A1 & B1: The pupa of the OX4324L strain; A2 & B2: The pupa of the OX4642I strain; A3 & B3: The pupa of the OX4656N strain.

6.3.2. RT-PCR reveals the *hsp83* is expressing in the germline and somatic tissue of the OX4324, OX4642 and OX4656 strains

To check if the *hsp83*-consturct is expressed in somatic tissue and germline of all these three lines, RT-PCR was performed on cDNA samples from male, female, testis and ovary of all the lines of these three strains. Primers for the *hsp83* 5'UTR-DsRed2 sequence of different strains were chosen. Genomic DNA from a single medfly from each strain was used as positive control and No-RT was used as negative control. The *tra2* gene primers were used as a control to normalize the RT-PCR. For the OX4324 strain, cDNA from all samples generated a product of the expected size with the primer pairs for both *hsp83* and *tra2*. This shows the transcription of *hsp83* in all tissues (somatic tissue and gonad) of the male and female flies of this strain. The results are shown in Figure 6.3.

The product from the cDNA samples from testis and ovary of the OX4642 with the primer set for the *hsp83* is also the expected size. This indicates that the *hsp83*-driven reporter is transcribed in the germline of male and female flies of this strain. It also shows that the expression *hsp83* in somatic tissue of flies from this strain is successfully turned off (Figure 6.3).

The transgene insertions in three lines of OX4656 are on the Y-chromosome (OX4656B, C, I) so from those lines cDNA samples are only from a male carcass and testes. The remaining line, OX4656N has an autosomal insertion so both males and female soma and germline could be tested. The cDNA from all samples produced the predicted size product using the primer set for *hsp83*. This shows the *hsp83* is expressing in somatic tissue and germline of male but not female flies of this strain (Figure 6.3)

This shows that, despite the lack of expression of the reporter protein in the male germline, the *hsp83* promoter fragments do drive transcription in testes of all strains.

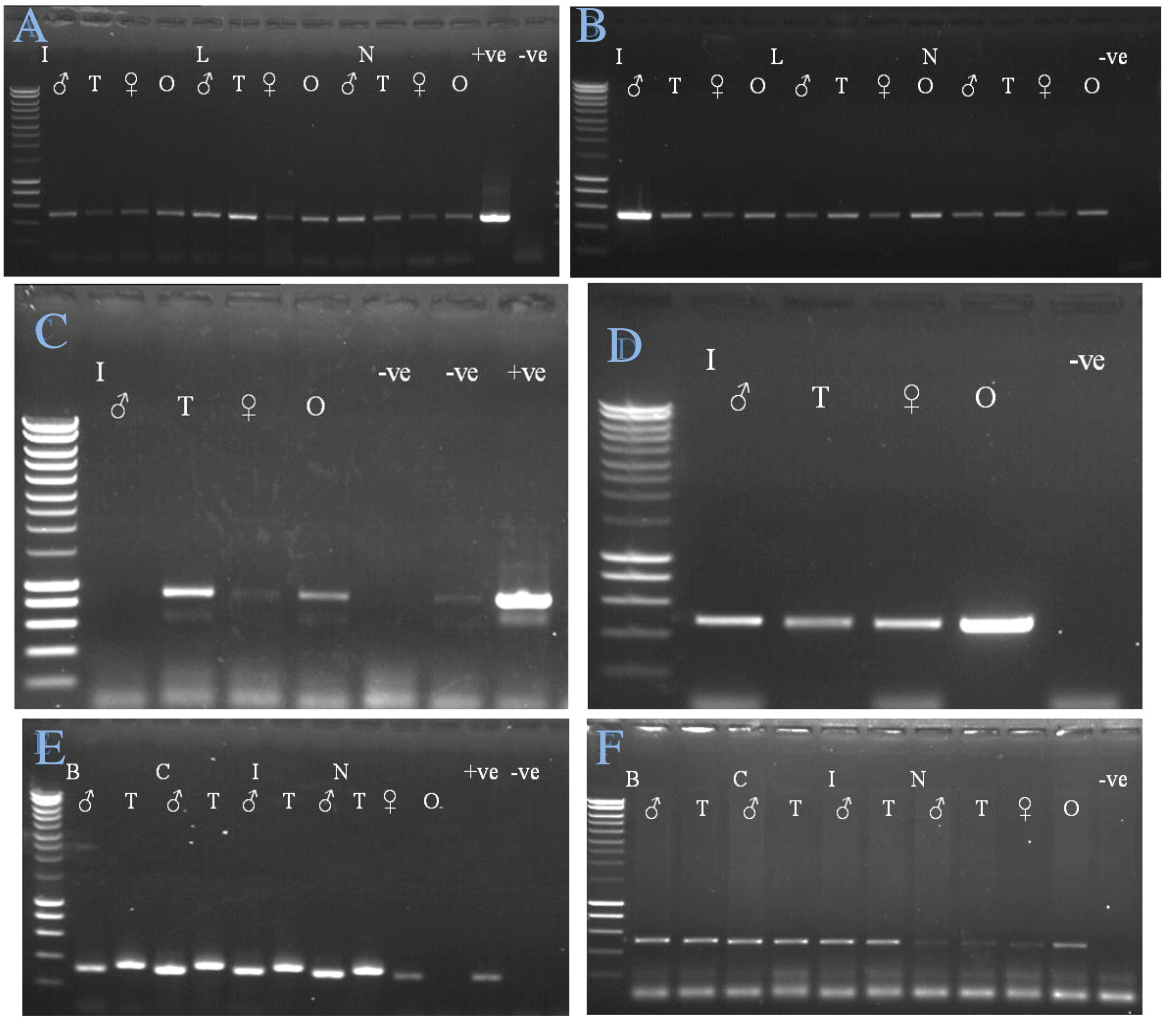


Figure 6. 3 RT-PCR on OX4324, OX4642, and OX4656 strains using the *hsp83* and *tra* primers.

For each sample a RT-PCR has been carried out with cDNA of male carcass, female carcass and testes and ovary. No-RT controls were run as negative control which gives no product, consistent with no genomic DNA contamination. Left lanes show DNA size standards, from 200bp to 10000bp (Eurogentec Smart ladder). A: The product from the *hsp83* primers on the OX4324 strains; B: The product from the *tra*2 primers on the OX4324 strains (I: OX4324I, L: OX4324L, N: OX4324N); C: The product from the *hsp83* primers on the OX4642I line; D: The product from the *tra*2 primers on the OX4642I line. E: The product from the *hsp83* primers on the OX4656 strains; F: The product from the *tra*2 primers on the OX4656 strain (B: OX4656B, C: OX4656C, I: OX4656I, N: OX4656N). ♂: male carcass, ♀: female carcass, T: testis, O: ovary, -ve: the negative control and +ve: the positive control. The expected product size for *tra2* is 450 bp, The expected product size for *hsp83* in OX4324 is 420bp, it is 815bp in OX4642 and 350bp for the OX4656 strain.

6.4. Conclusion

The transcription and expression of DsRed driven by the *cchsp83* promoter was studied in medfly to find an alternative male germline promoter to *topi* and *β2-tubulin* to be used in the paternal effect lethal system development. The *cchsp83* gene is expressed at all stages of medfly development although it is variable depending on the developmental stage and the temperature (heat shock status). The transcription start site of the *hsp83* promoter in the germline of male and female is different from that in the somatic tissue (Jin 2011). To check the utility of this in generating testis expression, three different constructs were designed.

The lines which were generated using these three constructs didn't successfully express fluorescent protein in the germline. The *hsp83* expression in somatic tissue of the OX4642 strain and the OX4656 strain was successfully turned off, while somatic expression was detected as expected in OX4324.

RT-PCR was performed to check the expression level of the *hsp83* reporter in the germline and somatic tissue. RT-PCR showed that in all lines of these three strains, the reporter is transcribed in the male gonad. It also transcribed in the somatic tissue of some of the strains (OX4324 and OX4656).

RT-PCR revealed transcription of the *hsp83* in the male gonad of these strains but the fluorescent protein doesn't expresses in the male germline. The fluorescent protein is not present in the spermatogenesis cells or spermatids. The *hsp83* signal detected in RT-PCR might just be from somatic tissue of the gonad. In the OX4642 strain there is no *hsp83* signal from the male and female carcasses, therefore the *hsp83* signal that detected in the gonad might actually be from germline in both sexes. Even if transcription is present in these

germline cells, I found no evidence for the translation of the protein in any stages of the male germline.

Chapter 7: Development of an olive fly sex specific marker

7.1. Introduction

In SIT applications, lab (or factory) reared insects are used. Lab-reared flies experience new environmental conditions, artificial diet, and artificial photoperiodic cycles compared to the same species living in the wild. The adaptation to lab conditions is a result of selection on behavioral traits, influencing the sexual behavior and mating competitiveness of the artificially reared flies. For example, mating competitiveness tests of olive fly in cage experiments showed that the wild olive flies mate primarily in the 2 hours before the onset of darkness, but the mating window for lab reared olive flies is longer; and they mate in the 4 hours before the onset of darkness (Economopoulos 2002). This difference in the mating behavior of lab-reared olive fly compared to wild flies caused problems in previous SIT attempts (Economopoulos 2002). In these trials individuals of both sexes were released, and the lab reared male olive flies primarily mated with the (co-released) lab reared female olive flies. One solution to this problem of olive fly SIT could be male only release of the irradiated olive flies.

Implementation of a male only release strategy in medfly SIT, showed improvement in the success of trials and gave a 3-7-fold performance improvement of the irradiated medflies (Rendón et al. 2004). In the medfly SIT, the sex separation was achieved by using a genetic sexing strain (TSL: Ttemperature-sensitive lethal mutation strain of medfly) (Willhoeft & Franz 1996; Morrison et al. 2010)

In this strain at higher temperatures (34°C) applied during the egg stage; the females die because they are homozygous for a recessive temperature-sensitive lethal mutation, while males survive because they have a wild type copy attached to the Y-chromosome by a (Y:5)

translocation. Temperature sensitive lethal gene (tsl) is located on chromosome 5 (Kerremans & Franz 1995). For more details see section 1.4.6.

Fu et al. 2007 generated a genetic sexing strain of medfly, in which sex specific alternative splicing was used to generate female only gene expression. The sex specifically spliced *tra* intron was used in the contest of the tetracycline repressible system, to result in expression of the effector only in females (see section 1.9 for more details). Ant et al. 2012 described a tetracycline repressible sexing strain of olive fly, but there is no RIDL independent genetic sexing strain available for olive fly. Thus male only release is not possible in the SIT programme without using the RIDL strain. In this study, the *tra* (*transformer*) gene was used to design a construct to facilitate sex separation in olive fly, to generate a RIDL-independent sexing strain. The *tra* gene is a sex determination gene in *D. melanogaster*, medfly and olive fly. *tra* is transcribed in both sexes, but its mRNA undergoes sex-specific splicing and encodes a functional tra protein only in females (Figure 5.1). The sex-specific splicing is controlled by regulatory elements found within the alternatively-spliced first intron.

Here we used the first intron of the *tra* gene of olive fly to design a construct which should differentially label males and females in olive fly (Figure 2.21). In such a strain, males and females express different fluorescent markers which allow the sex separation in the pupal stage.

7.2 Results and discussion - The sex specific marker OX4676

In OX4676 (PB-mActin-DsRed2-tra intron-ZsGreen-mActin3'UTR-PB) (Figure 2.18), the muscle-specific *Actin* promoter from Mexican fruit fly (*Anastrepha ludens* (Loew)) drives the expression of a transcript including coding regions for DsRed2 and ZsGreen. The olive fly *tra* first intron is included in this construct between the two fluorescent protein coding regions. In the females the first intron should be spliced out, producing an mRNA that encodes a fusion protein of DsRed2 and ZsGreen. The muscles of females should have red and green fluorescence. In the males the intron should not be spliced out, therefore males should only produce DsRed protein; thus their muscles will only have red fluorescence.

Olive fly embryos were injected with the OX4676 plasmid and helper plasmid (See section 2.2.1 for material and methods) from which 980 of them survived to first instar larva and seven different lines were generated (Table 7.1).

Table 7. 1 The generation of OX4676 transgenic strains.

Construct No.	No. of eggs injected	G0 larvae	G0 adults	No. Of transgenic lines obtained
OX4676	2700	980	178	7 lines

In the first few generations, all the transgenic flies that observed were females when the females from the OX4676 strains were crossed to wild-type males. Later, it was discovered that the male transgenic flies expressed the fluorescent protein but very weakly, so that they were phenotypically difficult to distinguish from non-transgenic siblings. Table 7.2 shows the number of transgenic males and females that were displaying DsRed2 fluorescence. The

number of transgenic males and females for all OX4676 lines were scored and the ratio of the males to females was calculated. In some lines of this strain, the ratio of males to females is not significantly different from the expected value of 1:1. However, in the OX4676Aa line, there were significantly fewer males scored as transgenic, and in two lines (OX4676C and Q) no fluorescent transgenic males were observed at all. The results are shown in Table 7.1 and Figure 7.2. OX4676C and Q lines could either have insertions that fail to express the marker in males, or they could have insertions that were male lethal. To test this, 20 random non-fluorescent males from both lines were crossed to wild-type females. The progeny of those crosses included fluorescent female transgenics. The results suggest that the OX4676C and Q lines have transgenic males even if the fluorescent marker expression is not sufficient to allow them to be identified. The OX4676L and P display the strongest fluorescence so correspondingly have the highest number of males with visible marker.

Table 7. 2 The number of DsRed fluorescent males and females in the OX4676 strains.

The number of transgenic progeny of a cross of transgenic females to wild type males (males and females) that were red fluorescent was counted, and the ratio of the males to females was calculated. A Chi Squared test was performed to obtain the *P* value which is the comparison of the observed ratio with a theoretical 1:1 ratio expected of male to female.

Line	Fluorescence Males (%)	Fluorescence Females (%)	*P* Value
OX4676A	17(41.46)	24(58.53)	0.08
OX4676Aa	17(39.53)	26(60.46)	0.03
OX4676C	0(0)	37(100)	2.2×10^{-16}
OX4676J	16(41.02)	23(58.97)	0.07
OX4676L	22(43.13)	29(56.86)	0.16
OX4676P	19(42.22)	26(57.77)	011
OX4676Q	0(0)	27(100)	2.2×10^{-16}

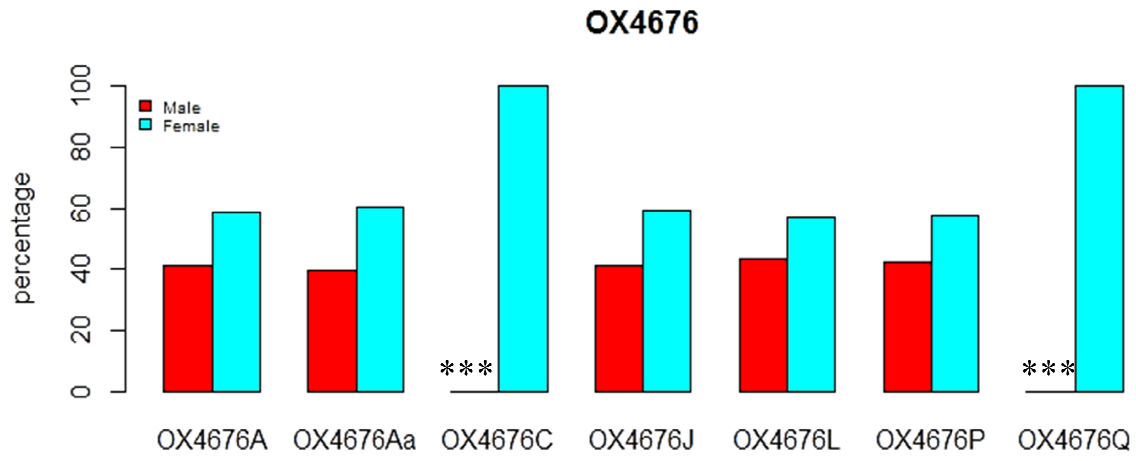


Figure 7. 1 The result from fluorescence screening of the progeny from the OX4676 strain.

The progeny of the OX4676 strain crossed to wild type were screened for the expression of the *DsRed2* for males and females and *ZsGreen* for females. The Y axis shows the percentage. An asterisk on the bar chart indicates a statistically significant difference. Asterisks on the bar chart indicate a statistically significant difference (*: P<0.05, **: P<0.001, ***: P<0.0001).

Figure 7.2 shows larvae and pupae from the OX4676L (female and male) compared to wild-type. The expression of DsRed2 in both genders, and ZsGreen in just females suggests that the construct works as designed and the *tra* intron splices out in the females and therefore allows the expression of the *DsRed2* and *ZsGreen*; failure of the intron to splice out in males results in expression of *DsRed2* alone.

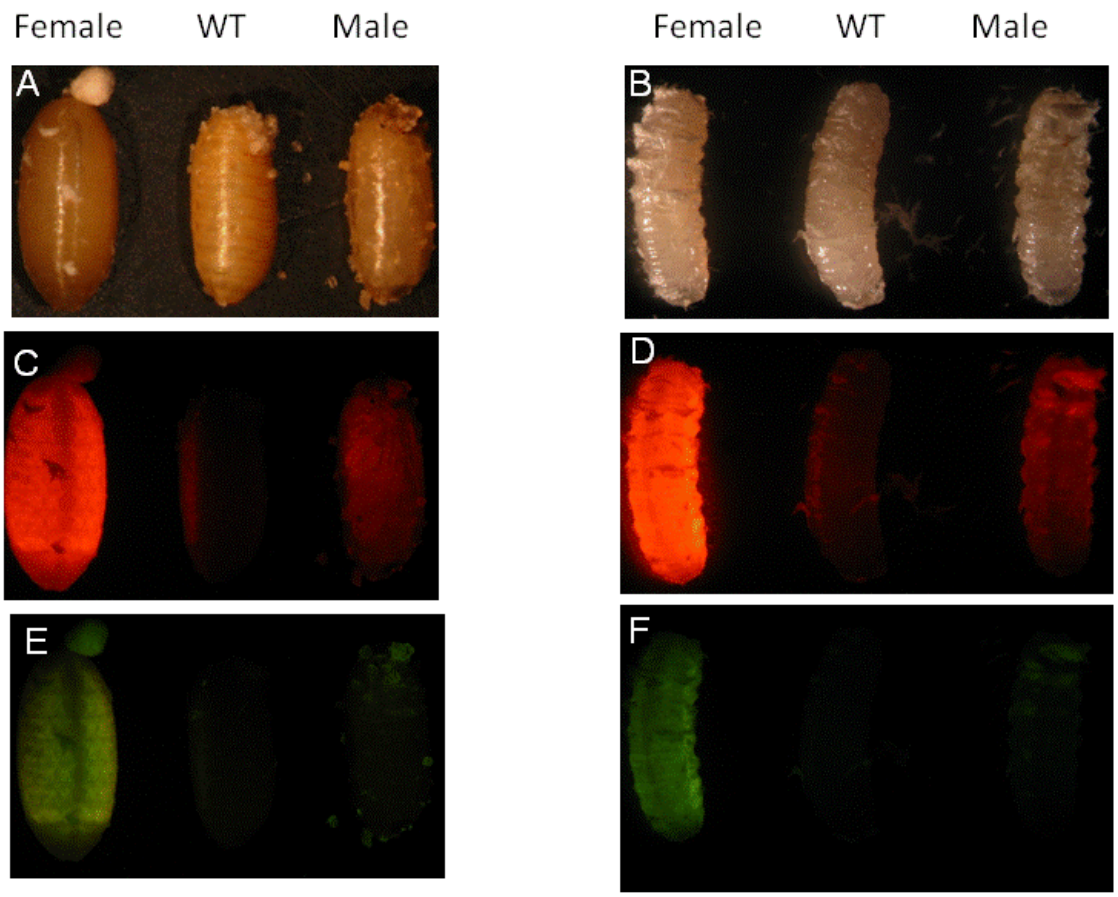


Figure 7. 2 Fluorescence microscopy shows the difference between fluorescence in the OX4676L from wild-type at larval (B, D and F) and pupal (A, C and E) stages.

Each panel shows a OX4676L female to the left, wild-type to the center, and a OX4676L male to the right. Expression of the *DsRed2* is clearly visible in the OX4676L female muscle and visible but weak in the male's muscle in larvae and pupae. The expression of the *ZsGreen* is clearly visible in the muscle of the OX4676L female (larvae and pupae) but is not detected in the males. Wild type animals have no only background levels of fluorescence.

The crosses described above are consistent with the hypothesis that males from the lines which only gave marked females were transgenic despite our inability to detect them by the fluorescence phenotype. To further test this hypothesis, PCR was performed to identify transgenic males in those lines that did not produce males that were phenotypically marked.

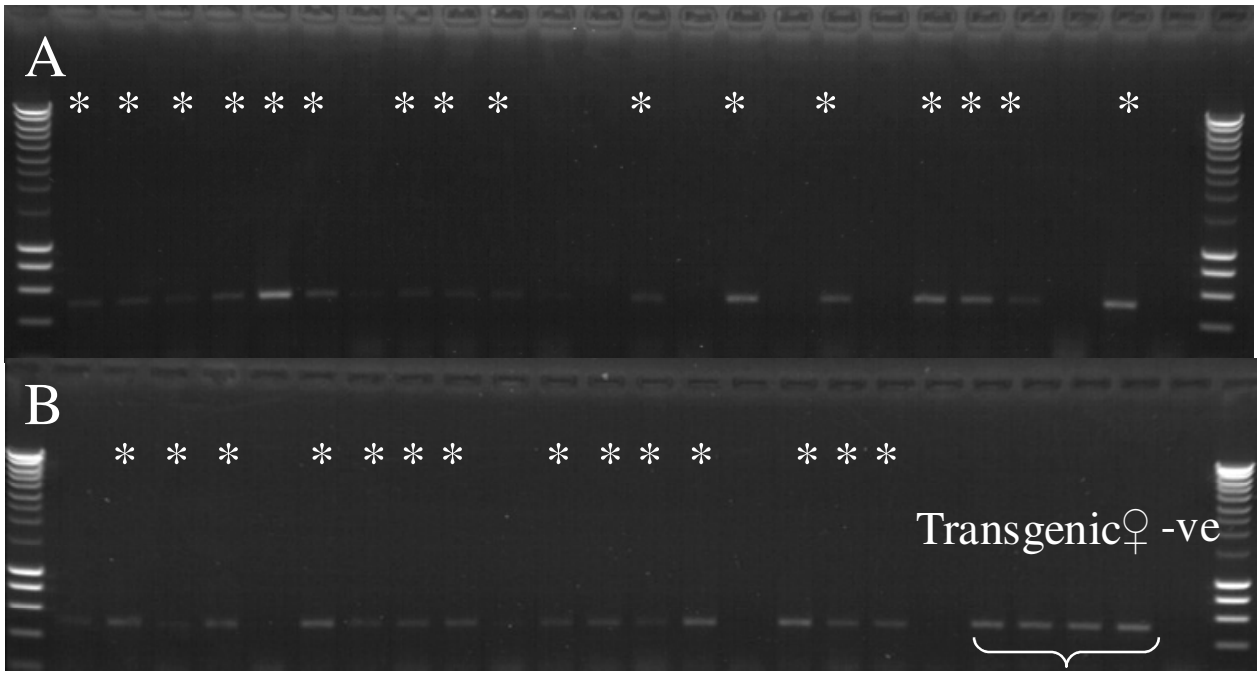


Figure 7. 3 PCR on the males of OX4676C and Q lines using DsRed2-mActin primers.

A: PCR on individual males derived from a cross of OX4676C females and wild-type males. This is expected to give a 1:1 mix of transgenic and non-transgenic male progeny. The positive bands represent the transgenic males. The transgenic males presented by an asterisk above them. B: PCR on mixture of transgenic and non-transgenic males derived from a cross of OX4676Q females and wild-type males. The transgenic males presented by an asterisk above them. The positive bands represent the transgenic males. The PCR products match the expected band size for DsRed2-mActin primers of 489bp.

To check the expression level of the marker gene in different lines of the OX4676 strain, an RT-PCR was performed. cDNA samples were made from transgenic females with strong fluorescence, males that express the fluorescence marker strongly and males that express the fluorescence marker weakly from all lines. RNA was extracted from the individuals followed by cDNA synthesis and PCR (35 cycles) (see chapter 2 for methods). Figure 7.4 shows RT-PCR performed on the OX4676 strain using primers to amplify the *DsRed* component of the transcript.

RT-PCR showed that samples from females of all lines generate a high level of product, consistent with their bright fluorescence. The males that had been selected because of the presence of the strong fluorescent marker also produced a strong band on the gel. The males that express the fluorescent marker weakly either produced a weak band or no band detectable on the gel.

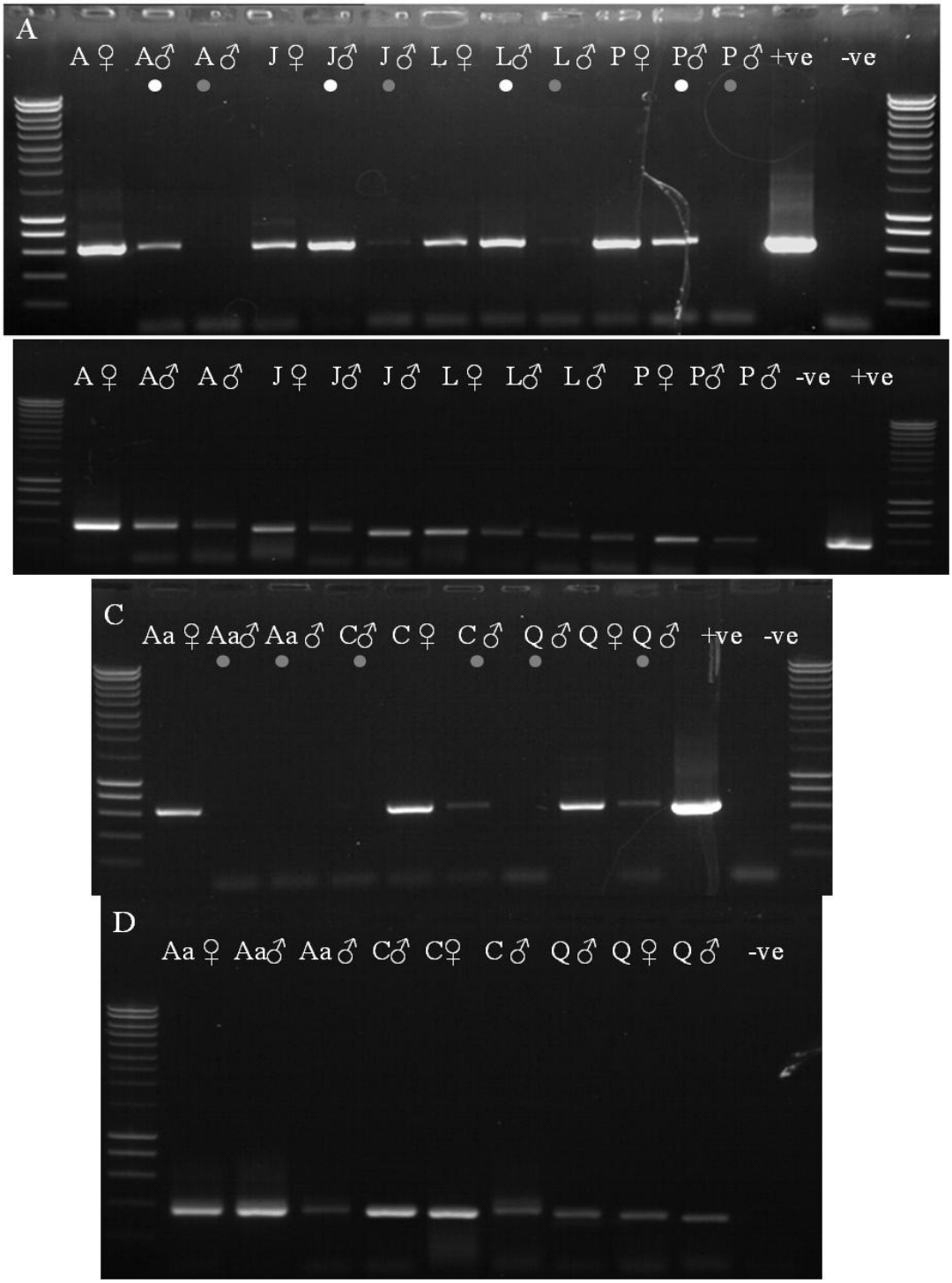


Figure 7. 4 RT-PCR on the OX4676J, L, P and Q using DsRed2 primers (males and females).

Female samples from all the lines produced strong bands on the RT-PCR gel, as did males with strong fluorescence. Samples from males with weak fluorescence either produced a weak band or no band on the RT-PCR gel. No-RT controls were used as negative control (-ve) which produce no product suggesting no genomic DNA contamination. Genomic DNA from the OX4676L was used as a positive control (+ve). The white dots above the lanes present the flies with a strong fluorescence and the grey dots above the lanes present the flies with a weak fluorescent. A & C: RT-PCR using DsRed primers; B & D: RT-PCR using ADH primers. The expected band size for the DsRed is 489bp. The expected product size for the ADH is 390bp.

7.3. Conclusions and future work

The *tra* gene from the olive fly was used to design an olive fly sexing strain. Seven lines were generated, from which robust sexing and marking of all transgenics was achieved in two of them. A homozygous line of either of these would generate males that are robustly red fluorescent, and females that are robustly both red and green fluorescent. In the other five strains, while the females were clearly marked the males did not show any transgenic phenotype. The lack of the marked males in the other lines suggests that the males were not expressing enough *DsRed2*, and therefore they appeared fainter. All lines of this strain differentially label the sexes, however, for the lines where the males are too faint only PCR is available to distinguish transgenic males from non transgenic males. If one of these weak or non-visible lines is made homozygous, simple sorting of fluorescent (female) vs non fluorescent (male) individuals would allow sorting of the two sexes.

One hypothesis for the failure of in some of the lines is that the transcript produced in males is rendered unstable because of inefficiencies in translation. To test this hypothesis RNA from individual males and females were extracted and rt-PCR was performed. rt-PCR showed that the low level of protein was due to a low level of transcript in these males. It remains to be determined whether this reduction in the transcript level is due to the reduction of the transcript stability in males, after the failure to splice out the long intron. An alternative hypothesis is that the failure in splicing feeds back to regulate the level of transcription of this transgene in the males.

The labeling of sexes using fluorescence protein successfully achieved in two lines therefore this construct was successful, but the variable penetrance, presumably caused by position effects, in males could be a problem. To achieve the aim of sex-marking of the olive fly with more consistent penetrance this construct needs to be redesigned in aim of increasing the level of the DsRed transcript in males. In the current design the first intron of the *tra* is placed between fusion protein of the "DsRed2 and ZsGreen". Another way of designing this construct is to have the male specific protein and the female specific protein as two independent cassettes. Therefore two fluorescent proteins could be expressed separately. The *tra* gene can be placed in a way that disrupts one of the proteins by a female-specific splicing and as a result it expresses only in females.

Chapter 8: The use of PCR technology to develop a monitoring method to evaluate the mating success of the transgenic flies in the field

8.1. Introduction

In SIT programmes, monitoring and tracking of the released insects in the field is very important. The purposes of monitoring include calculation of the ratio of the lab-reared released insects relative to the number of wild individuals in a given area, analyzing longevity and fitness of the released insects, and the determination of the mating success of the released insects.

Currently in medfly SIT programmes, the irradiated medfly are dusted with fluorescent dyes for field monitoring. As a result the irradiated medfly can be distinguished from the wild medfly when recaptured in traps within the release area. The use of fluorescent dyes has several disadvantages; they are expensive, they affect the quality of the irradiated insects, they are dangerous to human health in the mass rearing facilities, they are error prone and they cannot mark the mating partners of the released irradiated flies.

In this study we are interested in quantifying the mating success of the Oxitec's RIDL strains in the field, so that we can assess the efficacy of a RIDL SIT programme. We want to be able to determine whether a wild female has mated to a wild male, a released RIDL male or perhaps to both, by analyzing the trapped females. In the conventional SIT programmes, detection of the irradiated male mating success relies on microscopic dissection of the females to check for the absence or presence of sperm in their spermatheca. This can be achieved by comparing the head lengths of sperm in the spermathecae of mated females. Males sterilized with radiation produce sperm with shorter heads than wild males (Barry et al. 2003). This method is reliable and robust; however it is time consuming, labor intensive and

requires fresh specimens. This method is also not suitable for assessing the mating success of the RIDL males as the sperm of the RIDL medfly lead strain is not morphologically distinguishable from that of the wild types.

PCR was used to directly quantify mixed sperm stores in multiple mating females of the black field cricket, *Teleogryllus commodus* (Hall et al. 2010). In this project, we tested the hypothesis that Y-chromosome and RIDL-specific PCR assays can be used to determine mating and to differentiate the type of sperm DNA present in recently inseminated medfly and olive fly females. If the PCR method works, it can be applied to genetically modified insects that contain a heritable marker gene.

8.2. Experimental design

Males from the female-specific lethal strain, OX3864A (Figure 2.5), were crossed to wild-type females. Five cages were set up with 8 wild-type females and 3 transgenic males in each. To detect successful mating, females were dyed (using water based paint and based on IAEA instructions) with different colours. Each mating pair was removed from the cage immediately and the female was kept alive in a cage without an available oviposition surface. These females were sacrificed by freezing after 1, 3 or 5 days post-mating. A few females were left in the cage to die and samples were collected soon after they died. DNA (GeneJET Genomic *DNA* Purification *Kit* from Thermo Scientific) was extracted from the abdomen of females and PCR performed on the DNA using primers specific for the Y-chromosome and the transgene insertion present in the female specific lethal strain. The OX3864A primer pair targets the genomic insertion site in this strain – one primer site is in the transgene while the other is in the flanking sequence. DNA extracted from the OX3864A males were used as a positive control and DNA extracted from virgin wild-type females as negative control.

A similar experimental design was used for the olive fly female-specific lethal strain; OX3097D (Figure 2.5) males were crossed to wild-type females. Four different Y-chromosome primer pairs were designed according to (Gabrieli et al. 2011). All four were used on wild-type olive fly male and the best pair of primers was selected.

8.3. Result and discussion

8.3.1. Detection of the genotype of the mate of the wild-type medfly female using PCR

A preliminary experiment was performed to check the sensitivity of PCR using the Y-chromosome and the OX3864A-specific primers. Genomic was extracted from a single male's abdomen in 100µl of extraction buffer, and was diluted 1/2, 1/5, 1/10, 1/20, 1/50, 1/100, 1/150, 1/200, 1/250 (Figure 8.1) and PCR was carried out using the two different primer sets in two separate reactions. The expected size of PCR product using the Y-chromosome primers is 390 bp, whereas for the OX3864A primers it is 523 bp. Most diluted samples were successfully amplified with both primer sets, except for the 1:200 and 1:250 dilutions. This shows that standard PCR with 35 cycles of amplification (see section 2.5) is very sensitive and can readily detect up to 1/150 dilution of the male genomic DNA. In females after mating, sperm is present in spermathecae (receptacles and duct), the fertilisation chamber and the ovarian duct. Three hours post mating, a large number of sperm will be present in the female sperm storage apparatus (Marchini et al. 2001).

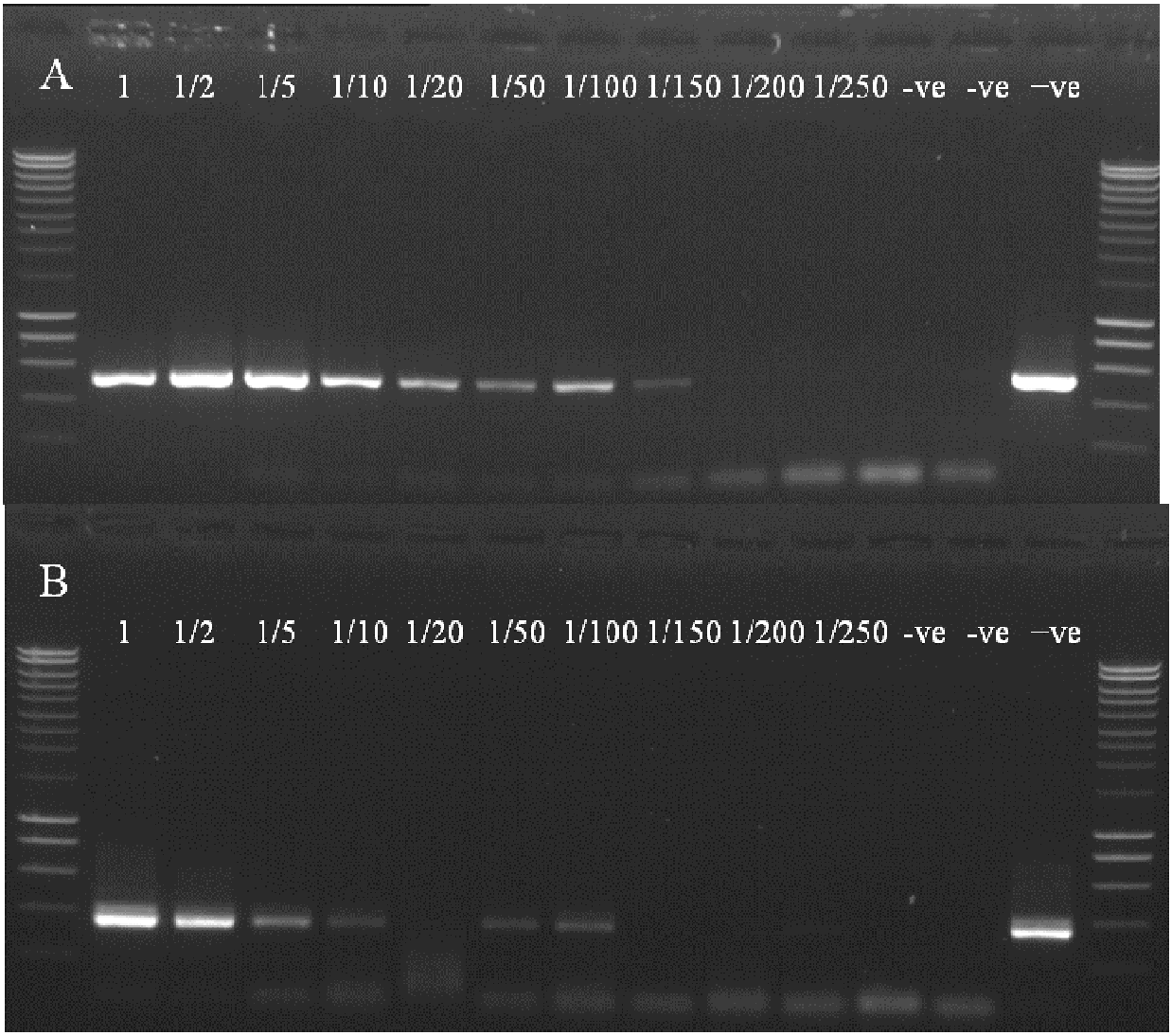


Figure 8. 1 PCR of diluted genomic DNA of the male of the OX3864A strain.

A. amplification using OX3864A primers on diluted gDNA (undiluted samples are one abdomen in 100µl); B. amplification using the Y-chromosome primers on diluted gDNA. Both primers were able to amplify samples as dilute as 1:150 dilution, but can't amplify more diluted than that (1 in 200). The DNA extracted from OX3864A males were used as a positive control and the DNA extracted from virgin females used as negative control.

Figure 8.2 shows PCR results from wild-type female mated to OX3864A males, where females were collected after 1, 3, 5 days post mating and also a dead female post mating. DNA was extracted from the abdomen of those females (20µl of extraction buffer was used to collect final product of DNA) followed by PCR. PCR optimisation included replicates with different quantities of the template and numbers of PCR cycles. Figure 8.2 shows the results of raising the PCR cycle number to 40, and using 2µl (~$1/10^{th}$ of an abdomen) of the template. All samples (females were collected after 1, 3, 5 days post mating and also a dead female post mating) were successfully amplified with both primer pairs. The product from the dead fly, while present, was very weak.

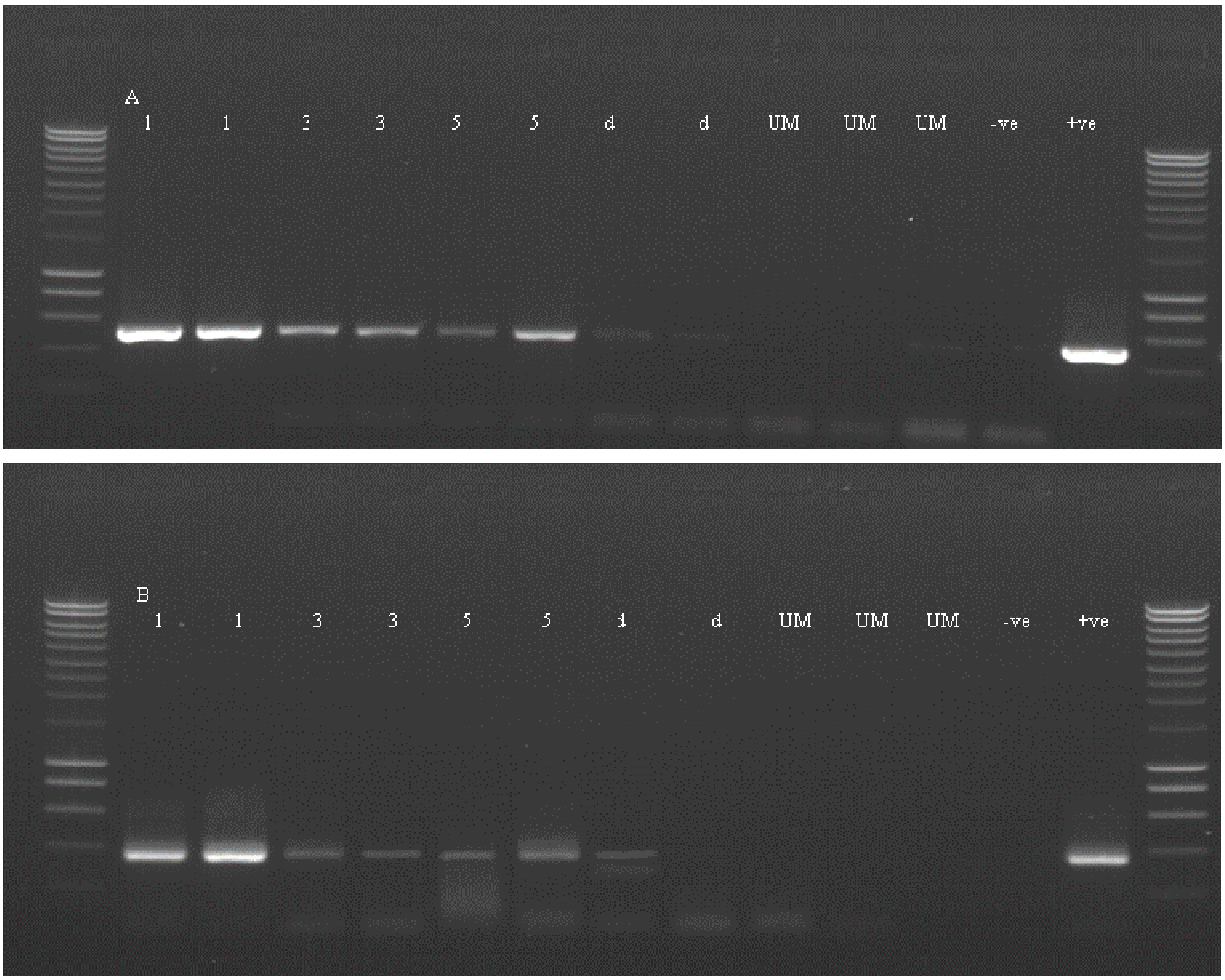


Figure 8. 2 Detection of sperm using PCR of wild-type females mated to OX3864A compared to unmated females.

A: PCR on all samples using the OX3864A primers. B: PCR on all samples using the Y-chromosome primers. 1, 1 day post mating, 3, 3 days post mating, 5, 5 days post mating, 8, 8 days post mating, d, a dead female post mating, um, unmated female. There is a faint band in one of the unmated females (A), which suggests contamination in that sample. DNA extracted from an OX3864A male was used as a positive control and DNA extracted from virgin wild-type females as negative control.

To test the system under more challenging conditions, mated females were left in the cage post mating to have an opportunity to oviposit and then they were collected after being dead for 7 days. As stated in section 8.2 DNA was extracted (into 20µl of extraction buffer) and PCR was performed on all samples. Initial PCR was performed using the best conditions determined above but there was no detectable product.

PCR was further optimized for as the dead sample previously produced only a very weak product. When the PCR cycle number was raised to 45 cycles and 4µl of DNA from each sample was used DNA samples from dead mated females gave successful amplification.

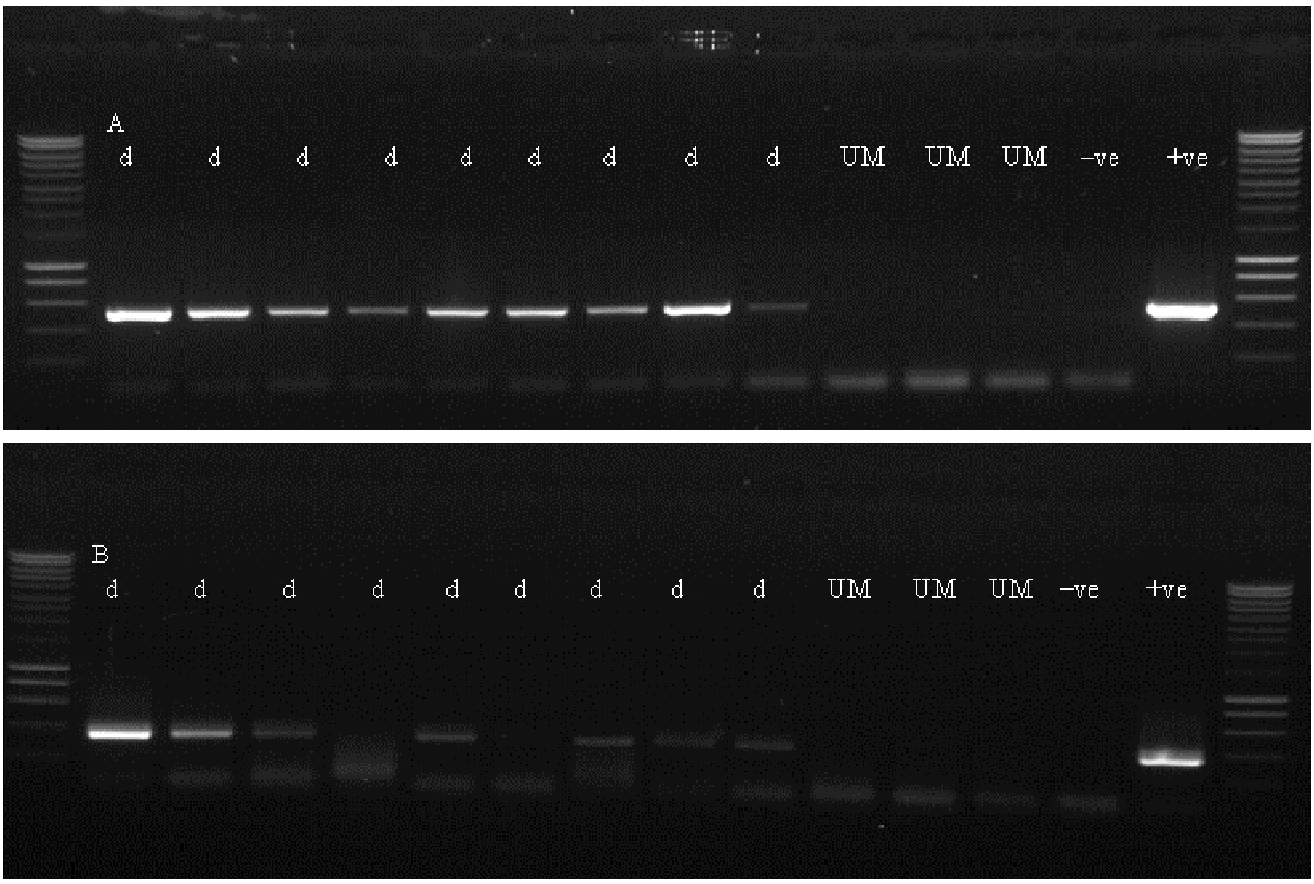


Figure 8. 3 Detection of sperm using PCR of mated wild-type females to the OX3864A males and unmated female after laying eggs.

A: PCR on all samples using the OX3864A primers. B: PCR on all samples using the Y-chromosome primers. All females were left on the sticky trap for a week post mating. d, dead female post mating, um, unmated female. DNA extracted from the OX3864A males were used as a positive control and DNA extracted from wild-type virgin females as negative control.

Some of DNA samples didn't produce sufficient product for detection (Figure 8.2 and 8.3), although most of the samples were successfully amplified from females mated to the OX3864A with both the OX3864A and Y-chromosome specific primers. Both experimental runs (Figure 8.2 and 8.3) demonstrated that it is not that easy to use this method on mated females as too many repeats were required.

8.3.2. Detection of the genotype of the mate of the wild-type olive fly female using PCR technology

To determine whether this PCR method also worked for olive fly a similar experiment was performed using olive fly females mated with OX3097D males and primers specific for the transposon sequence inserted in the OX3097D and the Y-chromosome specific primers. Four different pairs of the Y-chromosome primers were designed based on the Y-chromosome sequences that suggested on the Gabrieli et al. 2011. All of the primers were amplified using the genomic DNA from the olive fly male.

The Y-chromosome and the OX3097D primers were used to check how diluted DNA from the abdomen of an olive fly male (extracted in 100µl of extraction buffer) can be to be amplified. OX3097D genomic DNA was diluted 1/2, 1/5, 1/10, 1/20, 1/30, 1/40, 1/50 and 1/100 and 1/150 and used as a template in standard PCR (see section 2.5) with 35 PCR cycle. The results are shown in Figure 8.4. The expected DNA product for the Y-chromosome primers is 390 bp, whereas for the OX3097 primers it is 453 bp. Olive fly females post copulation, like medfly female, have sperm present in spermathecae (receptacles and duct), the fertilisation chamber and ovarian duct (Marchini et al. 2001).

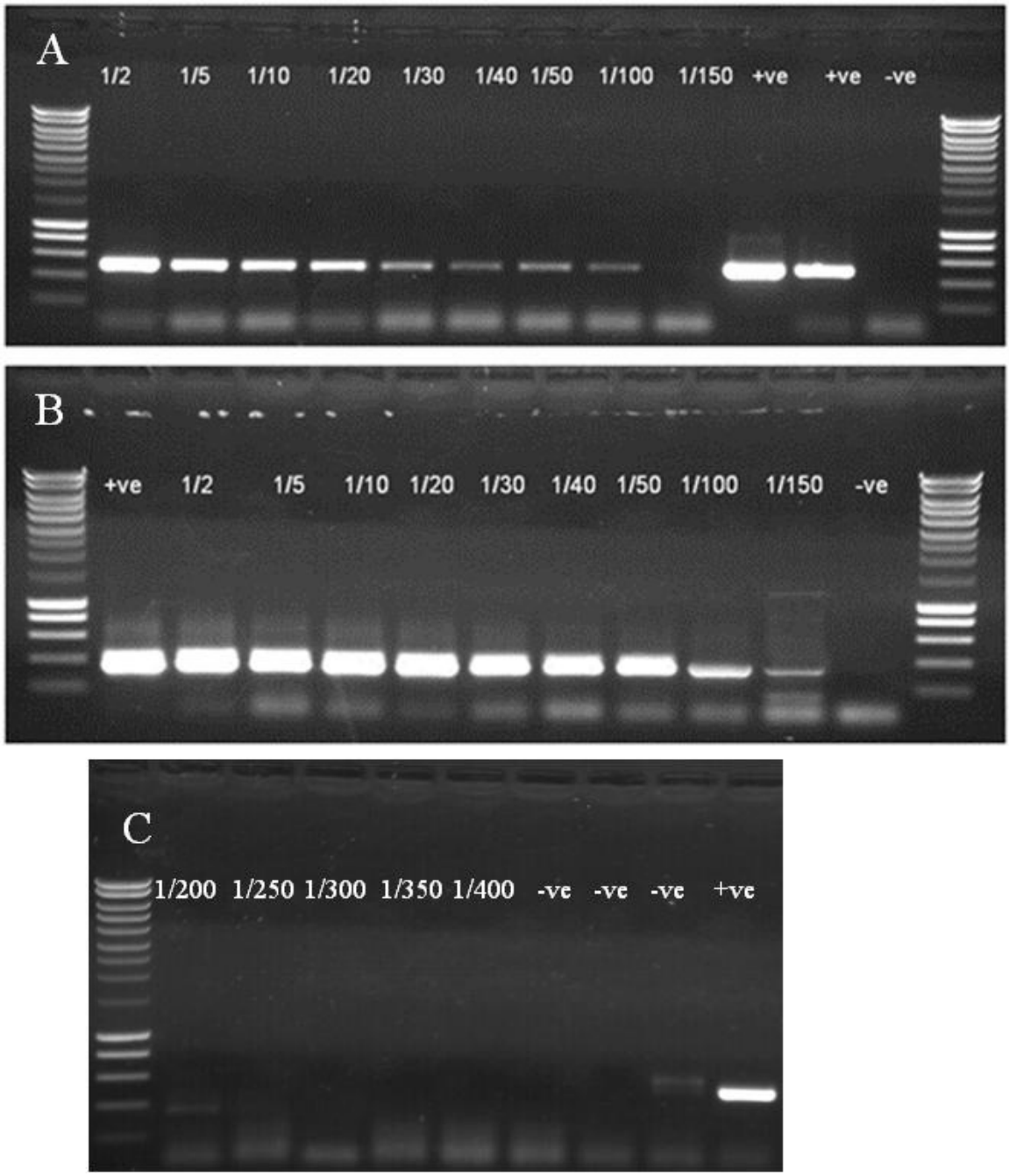


Figure 8. 4 Detection of sperm using PCR on the diluted genomic DNA of the OX3097D male.

A: The amplification of the OX3097D primers using the diluted samples (1 in 150). B and C: The amplification of the Y-chromosome primers using the diluted samples. Both primers are able to amplify samples as small as 1 in 150ul, but can't amplify more diluted than that (1 in 400). Higher dilutions were used for Y chromosome primers but not for OX3097D primers as OX3097D primers didn't produce any products at 1 in 150. The DNA extracted from OX3097D males were used as a positive control and the DNA extracted from virgin females used as negative control.

Figure 8.5 shows the PCR from wild-type olive fly females crossed to OX3097D males. The females were collected after storage with no oviposition surface for 0, 3, 6 days. Some females were also collected one week after death (see section 8.2 for experimental design). The DNA sample from 0, 3, 6 day post-mating females successfully produced products with both primers. The optimal PCR cycle was 38 cycles with 2µl of the template. The DNA sample from the dead female produced no product with OX3097D primers and both the expected product and a non-specific product, with the Y-chromosome primers.

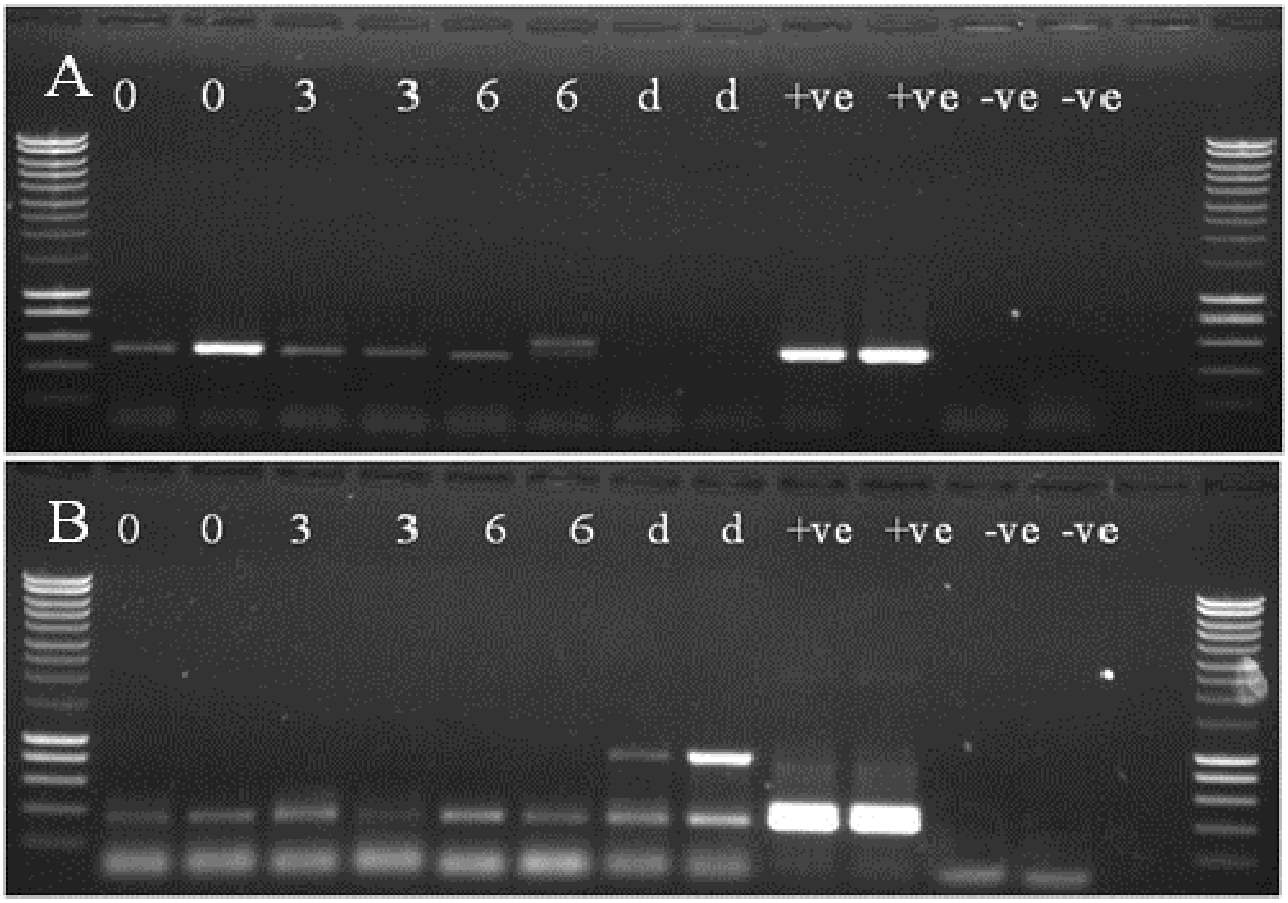


Figure 8. 5 Detection of sperm using PCR in mated female to OX3097A males before laying eggs and unmated females.

A: PCR on all samples using the OX3097D primers B: PCR on all samples using the Y-chromosome primers. 0, 0 day post mating, 3, 3 days post mating, 6, 6 days post mating, d, a dead female post mating, -ve is negative control and +ve is positive control. DNA extracted from the OX3097D male was used as a positive control and the DNA extracted from virgin wild-type females as negative control.

To test this technique under more challenging conditions, females were allowed to mate and left in the cage to oviposit their eggs for 3 days and then placed on a sticky trap. They were processed for DNA extraction after being dead for 7 days. DNA was extracted from all samples into 20µl of extraction buffer (see section 8.2) followed by PCR on all samples. In the previous section the dead samples gave no product or two different products therefore PCR was optimized further for this section. The PCR cycle was raised to 45 cycles and 3µl of DNA from each sample was used. In PCR for the presence of OX3097D; the number of PCR cycle was increased to 48 and amount of the template was increased from 3µl to 4µl. This resulted in having PCR product from dead samples. In the Y-chromosome PCR; the extension temperature was lowered in an attempt to reduce production of non specific products. Extension temperature was changed from 72°C to 68°C (see section 2.5 for PCR conditions). The DNA samples from all the dead samples of mated females produced products of the expected sizes in the PCR reactions, while unmated females gave no product, as expected. The results are shown in Figure 8.6.

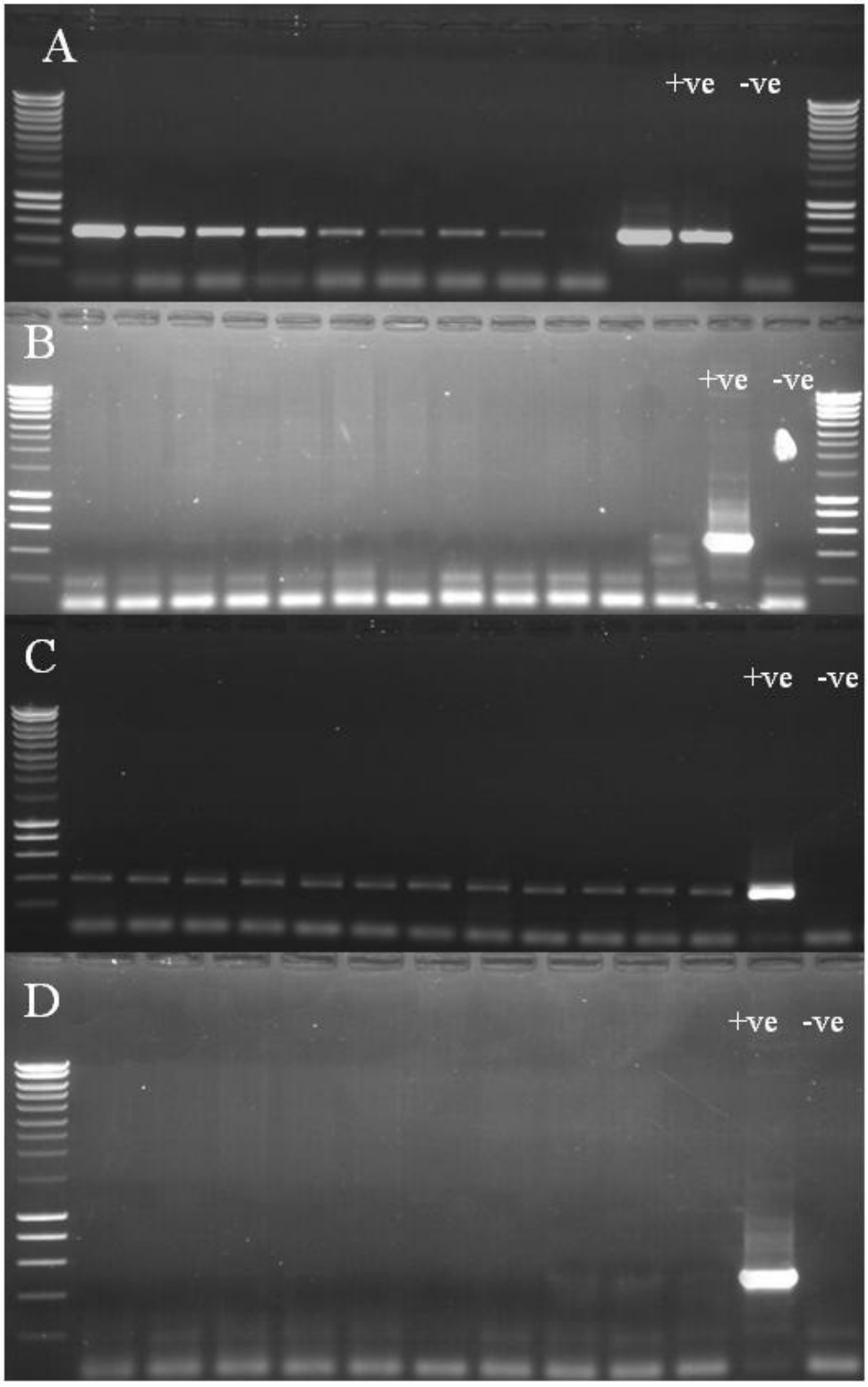


Figure 8. 6 Detection of sperm using PCR of the mated female to OX3097D males and unmated female.

A: PCR of post mated females using the OX3097 primers. B: PCR of unmated females using the OX3097D primers. C: PCR of post mated females using the Y-chromosome primers. D: PCR of unmated females using the Y-chromosome primers. All the females were left on a sticky trap for a week. +ve: positive control, -ve: negative control. DNA extracted from the OX3097D male was used as a positive control and the DNA extracted from virgin wild-type females as negative control.

8.4. Conclusion and future work

The detection of mating success after mass release of RIDL flies is very important to measure the success rate of the released males. In conventional SIT programmes, after releasing irradiated flies, dissection of females and microscopic analysis is being used to determine the absence or presence of mating. Examination of sperm in the spermatheca is informative, as the sperm heads are shorter in irradiated flies. Sperm of RIDL fly strains have no morphological difference from that of wild type sperm and therefore comparing sperm head size cannot be used. If the RIDL strain has a fluorescent sperm marker incorporated this can be used in microscopic analysis, however not all strains will have this, and a PCR based method would require less highly skilled analysis. In this project, we tested the hypothesis that the Y-chromosome and RIDL specific PCR assays can be successfully used to determine mating and to differentiate the type of sperm DNA present in recently inseminated female flies.

PCR was performed on the DNA sample of abdomen of females that mated with RIDL males. The results showed that the technique is possible, but there was some inconsistency. Most, but not all, replicate experiments with the Y-chromosome and RIDL primers on the DNA samples were successful. This preliminary data proved that it is possible to distinguish mated female from unmated female using PCR. After my initial work, Sarah Scaife from molecular team at Oxitec took over to do more investigation and development of this method.

She found out mated medfly females can be identified with two different Y chromosome primer sets. This means that Y-chromosome sequences in medfly has polymorphism. She also used different primer set for OX3864 PCR which gave better results, i.e. a more robust and reliable amplification. Additionally samples from the sticky trap were washed with acetone before DNA extraction. Component of the sticky trap were postulated to be inhibitors

of PCR. Indeed, PCR on samples washed with acetone produced more robust and more consistent results.

In this experiment we showed that sperm DNA from samples of abdomens of female can be amplified using the Y-chromosome primers and RIDL primers up to 7 days after mating and egg laying in both olive fly and medfly mated to RIDL males. While further development is clearly needed this is promising result, and suggests that molecular methods could be applied to analysis of the mating success of RIDL males in an SIT setting.

Chapter 9 -Discussion

This study describes the development of the parental effect lethality (pel) strains in medfly and olive fly using transgenic technology, with the aim of avoiding the need for the irradiation of the targeted insect in the SIT programmes. SIT technology is environmentally friendly and species specific therefore it has been considered a suitable replacement for chemical insecticide control of medfly and olive fly populations. This technology has been applied to medfly in different countries and the results confirm reduction in medfly populations (Vargas et al. 1995; Arita & Kaneshiro 1989). However, irradiation reduces the fitness of released males; mainly longevity and mating competitiveness, so that higher numbers are required for adequate population suppression. Irradiation induces double strand breaks in the DNA of germline cells of the targeted species, but also damages somatic tissue. The irradiation process also affects gut microbiota (Ben-Ami et al. 2010). These bacteria are important in fly's lifecycle therefore the elimination of them has an effect on survival and performance of the flies in the field (Ben-Yosef et al. 2008) such as their mating competitiveness (Hamden et al. 2013). Moreover, numerous studies have demonstrated the reduced ability of irradiated flies to compete with their wild counterparts for successful mating with the wild females (Chapman 2009; Lance et al. 2000; Lux et al. 2002; McInnis et al. 2002; Barry et al. 2003). The aim in the development of the pel strains was to limit this damage to the DNA of male germline to improve the performance of these flies in the field.

One of the issues that caused failure in olive fly SIT besides fitness costs due to irradiation is the problem of separating sexes for this insect. This is beneficial for a male-only release of sterile flies. In medfly SIT, a male-only release showed a 3-7-fold performance improvement of the irradiated release medfly cohort in comparison to an irradiated mixed-sex release. In medfly SIT, male only release was achieved by using a genetic sexing strain (TSL: Temperature-

sensitive lethal mutation strain of medfly) (Robinson & Van Heemert 1982; Franz 2002; Morrison et al. 2010). Although SIT has been successful against other tephritid pests, olive fly SIT trials have been historically unsuccessful. Contributing detrimental factors include altered diurnal mating-rhythms of the lab-reared insects resulting in assortative mating, high cost rearing and a low quality of radiation-sterilised mass-reared flies (Tsitsipis 1977; Estes et al. 2012). Male-only release is now considered essential for olive fly SIT success.

Fu et al. 2007 described the development of an fsl technology in medfly. This technology is an enhancement to radiation based SIT and also provides sexing mechanism for SIT. Ant et al. 2011 described the application of this technology in olive fly and Leftwich et al. 2014 described a similar application in the Mediterranean fruit fly. Further analysis on both stains (Ant et al. 2012; Leftwich et al. 2014) demonstrated their mating compatibility and competitiveness with their wild counterparts and also showed that sustained releases of such strains are capable of suppressing caged wild type populations of the respective insect pests. Furthermore, molecular analysis of these strains indicated that the damage is specific to the female tissue. The fsl strains can eliminate the need for radiation but the males of these strains are not sterile therefore not all the progeny of the wild female mated to such male, will die (only half of the progeny will die as the male progeny will survive). The surviving progeny will still damage fruits and cause some economic damage. Since the number of progeny originating from a wild female mated with an fsl male would be less than that of a wild female mated with a wild male, and also as an operational programme gradually reduces the number of wild females available for oviposition, the damage to fruits won't be extensive and most likely will be limited to the early phase of a control programme. However, the survival of male progeny in the fsl strains raises concerns regarding the regulatory and public acceptance. This is of especial concern as there could be genetically modified larvae in the fruit sold to customers.

Development of a strain that induces lethality at an earlier stage (embryos or early larvae) is considered beneficial in order to minimize crop damage. Schetelig et al. 2009 developed a conditional embryonic lethal system in medfly; also based on the tet-off system for conditional expression. In this system a promoter/ enhancer (P/Es) element from cellularisation-specifically- expressed genes drive the expression of tTA. tTA activates the expression of the lethal gene *hidAla5* and leads to embryonic lethality in medfly.

Embryonic lethality has advantages for SIT since progeny dies early, therefore the damage to crops will be minimized. In embryonic lethality system the lethal gene is functional during spermatogenesis, therefore there is a risk if wild females are exposed to tetracycline, the strain's effectiveness will be affected (i.e. survival of progeny will be affected). Minimising the survival of progeny in the wild is an attractive feature for regulatory purposes. Therefore the generation of a lethal system that does not allow the formation of a viable zygote, for instance the pel system that is described here is considered even better. The female's ability to sense the motility and quality of sperm may compromise the effectiveness of SIT, if the released males produce sperm that is inferior compared to that of wild males (Kraaijeveld & Chapman 2004). We report here the development of a suitable system that provides genetic sterilisation in the sense that expression of the system in the male germline produces sperm that are not capable of forming a viable zygote when used for fertilisation. We also demonstrate that this system cleverly replicates the effects of irradiation in current SIT methods; it allows for the production of male sterile insects without killing the sperm or preventing its production so that it is capable of inducing refractoriness to re-mating in females. Male genetic sterility is linked to the "tet-off" system for conditional expression. Presence of tetracycline or analogues to the larval medium represses the sterility system, allowing simple mass-rearing of the strain. Combining a female specific lethal strain with a male-sterile insertion provides effective separation of the sexes allowing for a male only

release while males remain unable to give a viable zygote, but still capable of entering the egg in such a way as to exclude other sperm. For medfly the TSL strain exists for separation of sexes, but such a classical genetic strain doesn't exist for olive fly, therefore fsl (generated via transgenesis) can solve this problem.

9.1. The transgenic strategies for developing the Parental Effect Lethality strains

In a previous study *I-PpoI* (a nuclease effector) and *β2-tubulin* (testis-specific promoter) were used to develop male sterile strains in *An. gambiae* (Windbichler et al. 2008). Under the direct control of a *β2-tubulin* promoter and untranslated sequences, *I-PpoI* is expressed exclusively in testes. This protein targets the ribosomal RNA genes (rDNA) which are found on the X-chromosome, therefore leading to nucleolar fragmentation and cell death. Cleavage of the X-chromosome causes the X-chromosome bearing sperm to be inviable. Only spermatozoa carrying the Y-chromosome are viable and capable of fertilising the egg, this produces a strong bias towards males in the population and can eventually cause extinction of the targeted population. The mating competitiveness of *An. gambiae* carrying this design suggested that their mating ability was not impaired by the presence of the transgene, compared to wild-type *An. gambiae*. For full penetrance of the phenotype, the strain ideally should be made to homozygous for the transgene. In this design *I-PpoI* is under the direct control of the *β2-tubulin* promoter, therefore it produces an unconditional effect. As a result males of this strain are always sterile. The strain is propagated through continuous crossing of transgenic females to wild type males. The same mechanism can be incorporated into a tet-off system as has been described elsewhere (Fu et al. 2007). For the purposes of this thesis I altered this design so that *β2-tubulin* controls expression of tTAV, which induces expression of the effector under the control of *tetO*. This indirect system allows for a conditional expression, facilitating maintenance of the strain in the lab (or a breeding facility), and allowing us to potentially generate and maintain a strain homozygous for the insertion. Furthermore, I extended the analysis to other nucleases besides *I-PpoI*, with broader specificities, specifically *ZnF, FokI* and *EcoRI*. For the purpose of this project I am looking for endonucleases that have a short recognition sequence therefore high frequency of the target sites, which will result in DNA damage at multiple locations. Another important factor in nuclease selection is dimerisation of

the protein. A monomeric enzyme will have activity even at very low concentrations while a dimeric enzyme has a non-linear function response to the concentration of the enzyme. As a result, at low concentrations of an enzyme the probability of two molecules binding is low, and so they may not form a dimer to cut the targeted DNA sequence. At high concentrations the probability of dimer formation is much higher. The non-linear response to the concentration provides protection against the leaky expression of the effectors in un-targeted tissues. An effector such as *ProtamineFokI* is a dimeric enzyme therefore it is an ideal nuclease for this type of studies (Bitinaite et al., 1998).

To widen the chances for development of an ideal pel technology, I also tested whether the use of an alternative promoter (*topi*) could increase the efficiency of the system. *topi* is a testes specific gene, originally identified in *D. melanogaster*, and it expresses in early primary spermatocytes (Perezgasga et al. 2004). We determined that, of all the combinations tested, *β2-tubulin-tTAV* driving *tetO-ProtamineFokI* generates the highest pel in medfly. This is due to a key feature of *Protamine* and *FokI* in such a strain. Protamine in such a strain preserves its key property when expressed in medfly, binding to sperm DNA, while the FokI nuclease at the C-terminal cleaves the medfly genomic DNA (Durai & Mani 2005). This DNA damage in the male germline decreases the egg viability of wild-type females mated to such males. Furthermore use of the *β2-tubulin-tTAV* driving *tetO-ProtamineFokI* design of pel, creates infertility of all spermatozoa (X or Y-bearing chromosome) unlike *β2-tubulin* driving *I-PpoI* which produces a male biased population. This means no progeny will be produced post-insect release, thus minimizing crop damage.

9.2. Promoter considerations

The ideal properties for a promoter driving an one-part unconditional system such as that used by Windbichler et al. 2010, are the timing of transcription and translation of the promoter+ 5'UTR combination, and expression level in the testes. The timing of transcription and translation, and the stability of RNA and protein products, are crucial in generation of the bipartite, conditional, pel strain. Ideally the promoter that drives the expression of tTAV should act in early-mid primary spermatocytes and should utilise UTRs that promote efficient translation in primary spermatocytes. The tTAV would then activate the expression of the tetO effector gene cassette. The effector protein however should not be translated until the spermatid stages, to limit possibilities for repair of the damage, and reduce the potential for sperm morphology defects arising that would mean the sperm have reduced motility or potential for insemination. Low sperm quality could increase the chance of females re-mating another male, with potentially considerable implications to an operational programme. A promoter and 5'UTR of a pre-meiotically expressed gene is ideal to drive the expression of the *tetO*-effector cassette at the right time. This will result in the initiation of the nuclease transcription in spermatocytes. A delay of translation to spematids is desirable, so that enough double stranded DNA will be damaged to produce infertile sperm. A promoter of a post-meiotically gene doesn't act early enough for the *tetO* to be translated sufficiently, the late expression of the promoter may lead to inadequate tTAV protein binding to the tetO sequence for activation of the effector. Hence not enough DNA damage will occur for producing infertile sperm. On the other hand, if the activation of the promoter occurs too early in the spermatogenesis it will result in complete sperm damage or loss. Previous studies by Bonizzoni et al 2002 and Kraaijeveld & Chapman 2004 showed that complete loss of sperm in male medfly, increase the frequency of re-mating in females. The expression of *topi* is early in spermatogenesis but the level of expression is relatively low. On the other hand, the

expression of *β2-tubulin* is abundant but its normal translation timing is relatively late in primary spermatocytes. Jin 2011 tested a *β2-tubulin* promoter with two different 5'-UTRs (*β2-tubulin* 5'-UTR and *topi* 5'-UTR). She demonstrated that neither of the aforementioned combinations resulted in early expression. However the same *β2-tubulin* promoter fused to the *hsp83* 5′UTR from *D. melanogaster* gave the right temporal expression. The shift in the timing of the 5' UTR controlling the tTAV was important for the expression of the nuclease mRNA prior to meiosis in the male germline. It would be ideal to find other genes that have high expression in testes and express early in spermatogenesis. A wider range of testes-specific promoters would provide us with the opportunity of generating a wide range of pel systems and would improve our chances of developing strains with high-quality characteristics (e.g. fitness, mating competitiveness). To identify such promoters we used a candidate gene approach, using data from *D. melanogaster* as a guide. An alternative approach, started more recently, and for which analysis is currently ongoing (Helen White-Cooper, pers. comm.) was to directly screen for genes with the desired expression pattern by RNA-sequencing from early primary spermatocytes and late primary spermatocytes from medfly. This involved purifying the relevant populations of cells, isolating the RNA and sequencing. Reassuringly the genes used in this project all fit in the desired profile of early transcription in these data sets. Other candidate genes are being identified.

The candidate gene we selected for further study was the *hsp83* gene from medfly, which expresses in all stages of medfly development. The hsp83 protein is a heat shock-induced protein in somatic tissue and is constitutive in the male germline (Theodoraki & Mintzas 2006). The data from *D. melanogaster* suggests a variety of alternative transcription start sites, one of which is testis-specific. Based on this, Dr. Dafa'alla, head of molecular biology team at Oxitec, looked for, and found, testis-specific transcription start sites of the medfly *hsp83* gene. I tested a fragment with the region surrounding a germline –specific transcription start site in

the*hsp83*promoter, drivingDsRed2 (fluorescence protein); the expected phenotype would be expression of DsRed2 in male germline. RT-PCR results presented here (Figure 6.3) showed that the expression of this potentially male germline specific *hsp83* promoter fragment is capable of driving DsRed2 (fluorescence protein) in testes but the promoter + UTR combination did not result in the expression of the fluorescent protein in spermatids (Figures 6.1). We tried three alternative promoter and upstream regulatory sequences. More specifically, In the OX4324 construct; the *cchsp83* promoter (1584 bp) that was used contains the 5'UTR from the somatic transcription start site of the *cchsp83* (122 bp; note the intron in the 5'UTR just before the start-codon is not included). The OX4642 construct utilised the *cchsp83* promoter with the germline specific 5'UTR of the *cchsp83* (promoter = 2599 bp, germline 5'UTR = 554 bp, intron = 281 bp). The OX4656 construct contained the *cchsp83* promoter and the germline specific 5'UTR (promoter = 1153pb, 5'UTR = 552 bp, intron = 281 bp and part of exon 2 =134 bp). Although different-sized versions of the *hsp83* promoter and their upstream regulatory sequences were used in each design; the possibility still exists that the putative promoter sequences that were used were incomplete. Use of a longer sequence or a different fragment upstream the *hsp83* coding sequence may resolve this problem. Li Jin, 2011 showed that a long sequence upstream of *topi* cDNA was adequate for driving the expression of a fluorescent protein gene in medfly testes.

9.3. Combination of the Paternal Effect Lethality with the Female Specific Lethal in Medfly

A disadvantage of the pel strain to female specific lethal strains is the lack of a sexing mechanism. A sexing mechanism is very useful in an insect control programmes. The male-only release will be very beneficial for field experiments, meaning that the sterile males mate more eagerly to wild females in the absence of sterile females (McInnis et al. 2004). Furthermore it will make it easier to gain regulatory acceptance as there won't be any transgenic females to damage the crops further.

This can be achieved by the generation of a transgenic strain carrying both the pel and female specific lethal modules. The "promoter and effector" all in one strain of medfly (*β2-tubulin-tTAV* and *tetO-ProtamineFokI*) generated infertile males in the absence of tetracycline; the results were similar to the result from having them in two different constructs (Bilski 2012). This shows that promoter and effector don't need two separate insertions to be effective. According to this result it was assumed that fsl trait and pel trait should also be effective in one construct. Having generated strains that have high penetrance of the pel, the next step is to combine this with the fsl traits. However, injection of construct OX4751 demonstrated that combination of the two systems in one construct does not produce strains with the desired phenotype. OX4751 males were "sperm-less", it is possible that the presence of two tTAV sequences in one insertion, creates too much toxicity in medfly males. In the other hand, when the flies from pel and fsl strains were crossed, the flies that carried both transgenes had the desired phenotype. Therefore, development of a double homozygous strain that contains both pel and fsl systems is considered to be the most practical way forward. The fsl strains are very well characterised in both medfly and olive fly (Fu et al. 2007; Ant et al. 2011), and they can be used for the sex separation method for both flies. Therefore any potential pel strain can be crossed to fsl lead strains to develop both systems in one strain of flies.

In addition, in OX4353 strains, the leaky expression of the lethal component to the somatic tissue appeared to contribute to reduced fitness of the males and females this strain, to varying degrees depending upon the line. This variation between lines is likely a result of the positional effect of the transgene. Therefore generation of several lines helps to choose the best final strain, one that shows a more tightly-regulated expression of the lethal effect.

9.4. Unconditional sex-separation strain

A significant feature of the medfly SIT technology is the ability to separate sexes for a male-only release (Rendon et al. 2004). As females damage the crops by ovipositing into flesh of fruit or vegetables, the male-only release reduces damage to crops and greatly increases the per-male efficiency of SIT. Furthermore, it minimizes manual handling of the flies and would be very useful in quality control of the strain. For instance to increase the egg production we can place more females in a production cage. For medfly a male-only release was achieved by using a genetic sexing strain (TSL: Temperature-sensitive lethal mutation strain of medfly) (Rendon et al. 2004). TSL is based on a mutation that is used as a selectable marker for separation of the sexes and the Y-autosome translocation to link the inheritance of the wild-type allele to sex (Willhoeft & Franz 1996; Morrison et al. 2010). The same strain also benefits from a mutation that affects pupal colour, *white pupae* (*wp*). The mutation has been mapped to the right arm of chromosome 5 (Willhoeft & Franz 1996; Kerremans & Franz 1995) and is very closely linked to the *temperature sensitive mutation* (*tsl*). Females of this strain have white pupae whereas males retain the normal brown pupal colour. Neither the tsl nor the wp mutations have been identified todate for olive fly. Here the development of unconditional sexing strain for olive fly by recombination genetics was studied. I utilized the *tra*-specific female splicing of olive fly in combination with two different fluorescent markers; DsRed2 and ZsGreen (Clontech). Males transformed with construct OX4676 should show only DsRed fluorescence, whereas females should express both markers. Ant et al. 2012 described a tetracycline repressible sexing strain of olive fly, which is based on the *tra* gene from the olive fly. Here we used the first intron of the *tra* gene of olive fly to design a construct which should differentially label males and females in olive fly, however, olive fly OX4676 strains were variable in their success; the expression of DsRed2 marker was weak in some lines and wasn't expressed at all in the other lines. This was potentially because the

transcript produced in males was unstable due to inefficiencies in translation caused by the location of a stop codon in this design. To avoid the potential obstruction with splicing, no prior stop codon (no stop codon after DsRed, it was after ZsGreen) was introduced into the intron sequence. In this construct, an exogenous polyadenylation signal was located in the intron sequence. PolyA sequences act as a transcription stop (Gatfield et al. 2003). The DsRed2 protein in the males of this strain has extra amino acids at the C-terminus. The extra amino acids could possibly inhibit the proper folding of the DsRed2 protein and so weaken the fluorescence expression. If this is the case it could be improved by introduction of an earlier stop codon (e.g. immediately adjacent after the canonical GT at the 5' end of the intron). To improve the design of next strain, we could have the two fluorescent proteins expressing as two independent cassettes. One of them will be interrupted by a female-specific intron so that it express only in females.

9.5. Sperm marking in olive fly

In the pel system of olive fly that is presented here, one important feature was marking the sperm. Presence of a functional fluorescent sperm marker will provide improved monitoring system for a RIDL programme, allowing better assessment of the mating success of the released males. The current method for assessment of the mating success of the released males is to check the progeny of wild females (Katsoyannos et al. 1999). It is also possible to measure the length of the sperm heads of the mated wild female, since irradiated males have shorter sperm heads (Barry et al. 2003). In some cases it is technically challenging to assess the progeny developing in some species of fruits (Katsoyannos et al. 1999). Both of these methods are laborious and costly, therefore an undemanding and cost-effective method can be advantageous. For the reasons mentioned above, the development of a pel strain with fluorescent sperm was attempted.

In construct OX4705, mCherry is located between *Protamine* and *FokI*. The strain showed no fluorescent sperm marking. In the second attempt (OX4801 construct) an SG4 linker was used to help the bi-functional domains of a fusion protein. Although sperm head was marked successfully, the expression level of fluorescence protein was low. The presence of the linkers also negatively affected the function of *ProtamineFokI* and reduced the effectiveness of the pel strain (Figure 5.6). In the design of OX4705 or OX4801, mCherry protein was located in the middle of the fusion protein. Patterson, 2004 suggests that mCherry is appropriate for fusions with other proteins however, the mCherry protein was usually positioned either at the C-terminus or N-terminus of a fusion protein (Patterson 2004).

One way to overcome this problem would be the relocation of the mCherry coding sequence to the C-terminus or N-terminus of the fusion protein, which would most likely result in higher fluorescence of the mCherry in sperm heads. However this design would mean that the FokI

component of the effector cassette would be in the middle of the arrangement with possible implications to the activity of the nuclease. Possibly an independent arrangement of the sperm marker and the tetO-protamine-Fok1 cassette would be preferable. Bilski 2012 showed the successful marking of sperm head in *Aedes aegypti*, where a similar combination of fusion protein was used (Protamine-fluorescent protein-FokI). The difference was in the choice of fluorescent protein. He used TurboGFP which is a variant version of GFP. Although it is hard to say with certainty whether the same construct have similar efficacy in different insect taxa, this might indicate that the choice of a fluorescent protein is particularly important in the context of a fusion protein that has the dual purpose of both marking sperm and disabling it.

9.6. Monitoring method for field trials

A current method of evaluating the mating success of released males in an fsl RIDL programme, is to score the progeny for the expression of a fluorescent marker in crops. This method can also be used in a traditional SIT programme, to check for egg viability which is done by assessment of the proportion of eggs that hatch to eggs that don't hatch. The eggs assayed are either in infested fruits (McInnis et al. 2002; IAEA 1999) or those laid in special oviposition traps (Katsoyanos et al. 1999). This method is laborious and time-consuming, and also it is complicated to assess egg viability in some species of fruits, making the assay less accurate.

A more direct method of mating success determination in a SIT programme is to measure the length of the sperm head dissected from the mated trapped females, as irradiated males have sperm with shorter head than those of non-irradiated males (Barry et al. 2003). This is not possible for a RIDL programme as RIDL strain males produce sperm with heads the same size as those produced by wild-type males.

Andrés et al. 2007 and Juan-Blasco et al. 2012 studied the use of molecular markers and PCR in the "Vienna 8" strain of medfly (V8) to detect mating success of irradiated medfly males in the field. Both studies used the "ccYsp" primers to detect the presence of the Y chromosomes, and the "ccmt" primers to discriminate between wild-type and "Vienna 8" strain males (See (Juan-Blasco et al. 2012) for more details on the primers). The authors developed their method based on dissection of spermathecae, which contain the stored sperm.

Based on those studies, I investigated the use of PCR to develop a method of monitoring mating success of males of a transgenic strain of medfly males with wild-type females. To develop an easier technique, I extracted DNA from the whole abdomen of mated females. The PCR showed that wild females mated to strain OX3864Acan be distinguished from

unmated counterparts. My results proved that the use of PCR in sperm detection of wild-type females mated with female specific lethal strain males is comparable to the results from PCR of the wild-type female mated to “Vienna 8” strain males.

The method is cost-effective and quick compared to the previous study, as here DNA was extracted from female abdomen and not from spermathecae. The dissection of spermathecae, is time consuming and needs a trained specialist and a stereo microscope, on the other hand cutting abdomens is quick and easy, and can even be done without a microscope – a magnifying glass or hand lens would give sufficient magnification. The advantage of dissection of spermathecae as it is a long-term sperm storage organ (Twig & Yuval 2005), and false negative results are less likely compared to assays contaminated with the other parts of the body (e.g. whole female body, abdomen).

9.7. Future improvements to current project

Future work in the development of the pel strains would involve improving sperm marking and producing double homozygous lines that also contain a repressible female specific lethal trait. Sperm marking is very important for monitoring of mating success of this strain. Using PCR to detect mated wild female with this strain can be an alternative option. Both methods need further optimizations and modifications.

To minimise damage to crops from female medfly sting a male-only release has been proposed. It is important to develop a strain which has both pel and a sex-separation mechanism. Unconditional sex-separation is laborious and involves a lot of manual handling. Its advantages are that it doesn't produce toxicity in flies and it provides a high-quality method for quality control of strains. On the other hand, the female specific lethal strain can be toxic to flies, although it minimises the manual handling and labour. Further tests need to be done to identify which method is more effective. Post-mating of wild females with pel strain, the rate of their egg viability will be reduced. This will be advancement on strains exhibit the female specific lethal trait alone. Female specific lethal strains combined with a pel strain would avoid damage caused by male larvae. This is an advantage to achieve regulatory and public acceptance of such a strain (genetic-derivative of the SIT)

The ultimate tests for effectiveness of pel strain are assessing the production capacity and the amount of sperm produced by the males. Tephritid females have the ability to mate more than once and are capable of fertilising eggs using sperm from different males. In SIT and RIDL, fertilisation of eggs has to be achieved by sterile sperm therefore sperm competition might be an issue for sterile flies. In irradiated male flies, sperm competitiveness has been shown to be compromised compared to non-irradiated flies (Kraaijeveld & Chapman 2004). This results in post-copulatory competitiveness costs for radiation-sterilised flies. In RIDL,

the molecular method of sterilization will hopefully reduce the costs associated with radiation-induced deficits in sperm fitness. The sperm production will have an effect on sperm competitiveness, mating competitiveness of flies and also can be an indication of fitness of flies. To assess sperm production, longevity and rearing productivity should be completely studied. For example, it is desirable to assess whether lower sperm production would affect mass-rearing efficiency, and if there is an effect is it sufficiently severe to prevent uptake of the technology. The performance of pel strains in the field is really important, therefore longevity and mating competitiveness should be assessed, initially in caged trials, but finally in field trials. It is critical that males used in a population control programme can live long enough in a field to be able to mate enough wild females in order for the SIT programme to be effective.

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