Genetic control of the olive fruit fly, *Bactrocera oleae*

Candidate: Thomas Ant

Department of Zoology

St. Catherine's College

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

January, 2013

Thesis Supervisors:

Dr Luke Alphey
Dr Sebastian Shimeld

Table of Contents

Abstract

The olive fruit fly, *Bactrocera oleae*, (Rossi) (Diptera:Tephritidae), is a key pest of olive crops. The sterile insect technique (SIT) is an environmentally benign and species-specific method of pest control, aiming to reduce the reproductive potential of a wild population through the mass-release of sterile insects. Previous olive fly SIT trials, involving the release of gamma-ray sterilised mixed-sex populations, achieved limited success. Key problems included altered diurnal mating rhythms of the laboratory-reared insects, leading to assortative mating between released and wild populations, and low competitiveness of the radiation-sterilised mass-reared flies. Consequently, the production of competitive, male-only release cohorts is seen as essential.

The RIDL (Release of Insects carrying a Dominant Lethal) system is a transgene-based derivative of SIT, one version of which involves the mass release of insects carrying a female-specific lethal transgene (fsRIDL). This thesis describes: 1) the development of fsRIDL olive fly strains and the molecular analysis of transgene insertion and function; 2) the analysis of strain life-history parameters; 3) studies into sexual selection and mating compatibility; 4) a caged proof-of-principle population suppression trial; and, 5) selection dynamics on the fsRIDL trait in caged populations.

Olive fly fsRIDL strains were developed with full female-lethal penetrance and repressibility. The lead strain displayed similar life-history and sexual competitiveness traits to those of the wild-type strain from which they were derived. In addition, transgenic males showed photoperiod compatibility and strong sexual competitiveness with field-collected wild olive flies. The feasibility of the fsRIDL approach was demonstrated when repeated male releases caused eradication of caged olive fly populations. Although needing field confirmation, these results suggest that fsRIDL olive fly strains may help to mitigate key problems experienced in previous olive fly SIT trials, and could help form the basis of a renewed effort towards olive fly SIT control.

Acknowledgements

There are many people who have contributed either directly or indirectly to this thesis. Foremost, I would like to thank my academic supervisors Luke Alphey and Sebastian Shimeld, and my team-leader and mentor at Oxitec, Martha Koukidou. Their guidance through every stage of my DPhil has been truly invaluable.

The fruit fly room at Oxitec has always been a delightfully friendly and fun place to work, and for that I would like to thank Romisa Asadi, Zoe Lokhon, Martha Koukidou, Michal Bilski, Lucy Finnegan, Sian Morgan, Sara Martins, Nerys Humphrey-Jones, Clare Brand and Li Jin. The aforementioned people (especially Romisa Asadi and Zoe Lokhon) have also contributed immensely to the rearing and maintenance of the olive fly (that recalcitrant beast).

Many thanks to Caroline Phillips, Andrea Miles, Sarah Scaife, and Tarig Dafa'alla for their advice and molecular biology wizardry over the last four years. Thanks also to Timothy Harvey-Samuel for many helpful discussions, and Michael Bonsall and Hong-fei Gong for instruction on mathematical modelling. My thanks extends to everyone at Oxitec for providing a friendly, dynamic and stimulating work environment.

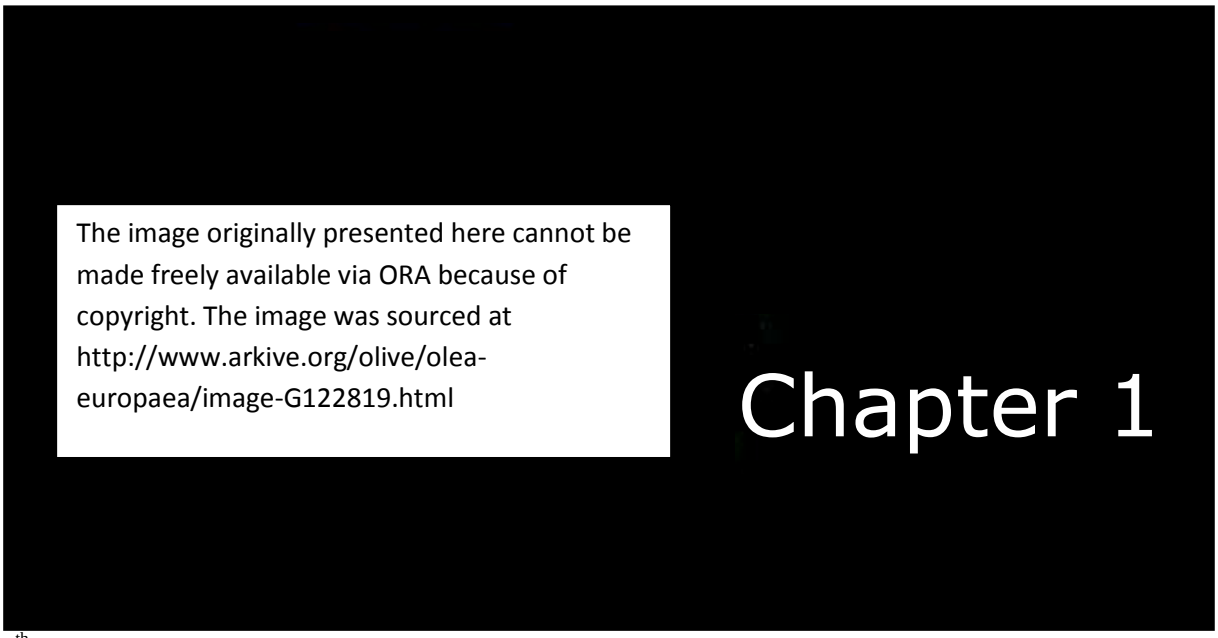
A special thanks to John Vontas and Aris Economopoulos for providing space and guidance during cage trials at the University of Crete.

Thanks also to the Biotechnology and Biological Sciences Research Council and (by extension) the British tax-payer for funding; I hope it was worth it!

Finally I would like to thank my partner Samantha Playford for providing unfettered support, patient proof-reading, and a constant flow of hot drinks during the writing of this thesis. I owe you one.

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 University of Oxford or another institute of higher education.



4th instar olive fly larva with olive, showing tunneling and fruit damage.

Chapter 1 - Overview.

1.1. The olive and the olive fly.

1.1.1. The olive

The fruit produced by the small evergreen tree, *Olea europaea* (here-after olive tree), is highly prized for its historic influence, symbolic significance, and economic importance. The olive tree has been cultivated since the early days of human civilisation (Liphschitz et al. 1991), with evidence suggesting the ancient Egyptians were well acquainted with its products even in predynastic times (Levinson, 1984). Present day olive production is a major agricultural industry. As well as being a staple dietary component of many cultures, the products of olive fruit are used widely for medicinal and cosmetic purposes. The global annual production of olive oil in 2010 was estimated at over three million tonnes; with Spain, Italy and Greece being the three largest producers respectively [International Olive Oil Council (IOOC) 2011]. As a commercial commodity the olive is highly susceptible to variations in quality. Buyers of table olives have a near zero tolerance for visible blemishes, and olive oil quality and therefore value is greatly reduced by increases in acidity resulting from fruit damage.

1.1.2. The olive fly

A range of phytophagous insects are known to attack the olive tree with the dacine fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), here-after olive fly, being considered by far the most destructive. With adult traits that include high mobility and high fecundity, dacine fruit flies are well-documented arthropod invaders and rank high on international quarantine lists (Clarke et al. 2005). The olive fly (**Fig. 1.1**), originating in Africa (Nardi et al. 2005), is currently found throughout the Mediterranean basin, South

and Central Africa, the Canary Islands, the Near and Middle East, Central America, and recently also in California (Daane, 2010).

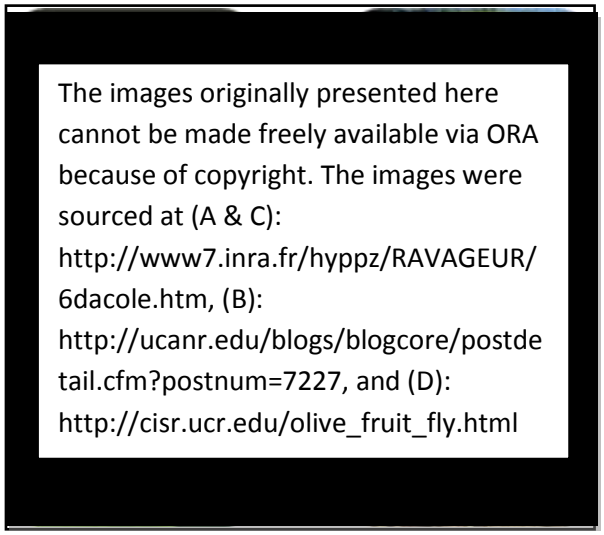


Figure 1.1 The life-cycle of the olive fly. (**A**) Olive with olive fly 'sting', containing egg deposited in olive mesocarp. A female olive fly will usually place a single egg per olive fruit. The neonate larva develops inside the egg, and emerges approximately 48-72 hours after oviposition. (**B**) Olive fly larva inside olive fruit. The growing larva feeds on the fleshy mesocarp of the olive, resulting in fruit damage and rotting. (**C**) Olive fly pupae in soil. Once the larva has achieved a sufficient size, it drops to the ground to pupate in the soil beneath the tree. Pupa to adult development lasts approximately 12 days (Tzanakakis, 2003). (**D**) Adult female olive fly ovipositing into an olive fruit. Wild adult flies require 12-14 days to reach sexual maturity (Zervas 1983). The entire duration of development, from egg to sexually mature adult, is approximately 35 days at 25ºC (Sime, Daane et al. 2006). (Development times vary greatly with temperature.)

Female olive fruit flies are oligamous, tending to mate 1-3 times during their lifetime (Benelli et al. 2012; Tzanakakis et al. 1968). Males are polygamous and will readily mate daily, provided receptive females are available (Zervas & Economopoulos, 1982). Mated females have a strong preference for laying a single egg per unattacked fruit (Gutierrez et al. 2009), are multivoltine (completing from two to five overlapping generations per olive growing season, depending on temperature) (Burrack et al., 2009; Mavragani-Tsipidou, 2002), and have a life-time egg laying capacity of between 200-500 eggs (Daane et al. 2005). Once hatched the neonate and developing larva tunnels through the olive, feeding on the fleshy mesocarp. Olive fly larvae are monophagous on all varieties and conditions

(ripe and unripe) of olive fruit (Estes et al., 2011). Adults feed on nectar, honeydew and other opportunistic sources of liquid or semi-liquid food. In regions with cool and cold winters, olive fly are able to overwinter in a facultative reproductive dormancy (Tzanakakis, 2003). Dormancy may also be induced during the summer when fruits are in short supply and during periods of particularly high summer temperatures (Bigler & Delucchi, 1981). If left uncontrolled, fruit infestation levels can reach 80-100% (Daane & Johnson, 2010). It has been estimated that the olive fly costs the global olive industry around 800 million US dollars in lost revenue per annum (Montiel-Bueno & Jones, 2002); improved control measures are therefore considered a major priority.

1.1.3. Control of the olive fly

Olive fly control has traditionally been based on the intensive use of insecticides in both cover-spray and bait forms. The organophosphorus insecticide dimethoate has been used most widely, although there has been a recent shift towards pyrethroids and spinosad, a macrocyclic lactone (Hawkes et al., 2005). However, intensive insecticide use has lead to the selection and proliferation of alleles associated with resistance (Vontas et al., 2002). As early as 1970, laboratory studies suggested that the olive fly had the potential to evolve resistance to dimethoate (Tsakas & Krimbas, 1975). Further research has shown (to varying degrees) the development of resistance in wild populations to: organophosphates (Skouras et al., 2007), pyrethroids (Margaritopoulos et al., 2008) and spinosad (Kakani et al., 2010). Proof of the development and possible spread of resistance associated mutations, coupled with environmental pollution, unintended effects on local ecosystems, the presence of insecticide residues in food and water and their possible implications for human health, and the increasing range of olive fly invasion due to climate warming (Gutierrez et al., 2009) has led to calls for the development of more effective, species-

specific, and environmentally-friendly olive fly control techniques and strategies (Mavragani-Tsipidou, 2002).

Several non-insecticidal alternatives have been suggested for olive fly control, including: mass-trapping, biological control using natural enemies, the use of hydrophobic particle film barriers (that render fruit visually or tactilely unrecognisable as a host) (Saour & Makee 2004), and the sterile insect technique (SIT). The introduction of natural enemies, perhaps the most widely implemented of these, was considered appealing when the relatively minor economic impact of the olive fly in the Republic of South Africa was attributed to the action of native natural enemies (Mkizea et al., 2008). However, it has been found that imported natural enemies do not provide reliable control in Europe, possibly because African parasitoids cannot establish on olive fly in fruit of the fleshier European cultivars as their short ovipositors are adapted for foraging in smaller wild African olives (Daane et al., 2008). Of the remaining options it has long been clear that SIT holds a strong potential for successful application to olive fly control, provided certain technical difficulties can be overcome (Estes et al., 2011; Economopoulos, 2002).

SIT has previously proved effective in controlling a variety of herbivorous Tephritid insect pests including the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Hendrichs et al., 2002), the Mexican fruit fly (mexfly), *Anastrepha ludens* (Reyes et al., 2000), and the melon fly, *Bactrocera cucurbitae* (Dhillon et al., 2005). Although previous pilot trials of olive fly SIT produced poor results, recent reports from prominent olive fly specialists, some of whom were involved in the original trials, have suggested that the development of an effective olive fly SIT programme has not been fully explored, and should be re-examined in light of new information on olive fly basic biology, advances in rearing and release techniques (Rempoulakis & Nestel, 2011), and the possibility of introducing novel phenotypes offered by genetic engineering (Estes et al., 2011; Koukidou et al., 2006).

1.2. The sterile insect technique.

1.2.1. Introduction

First proposed by Edward F. Knipling in 1955, the sterile insect technique is an insect control strategy that involves the mass-rearing of large numbers of a target species, sterilisation of one or both sexes (usually by irradiation), and the subsequent release into the wild of large enough numbers to adequately outnumber the wild population in infested areas. The mate-competition between wild and sterile males results in a decrease in total matings between fertile wild males and females, leading to lower numbers of viable offspring and a decline in the overall population size in the next generation. If releases of sufficient numbers of sterile insects are maintained for sufficient time, a target insect population may be suppressed and eventually eradicated. Moreover, sterile insect releases become more effective with time; if the initial rate of release of sterile insects remains constant, the ratio of sterile to fertile insects increases as the wild population declines (Knipling 1959) (**Table 1.1**).

Table 1.1 A basic theoretical prediction illustrating the population trend of a wild insect species that has been subjected to 5 rounds of sterile insect releases. The model considers a natural population of 1 million insects that has a capacity to increase 5-fold each generation. Releases of 9 million sterile insects are made each generation. Assuming equal competitiveness, the number of fertile insects remaining after the initial release is 100,000. This is subject to a 5-fold natural increase resulting in 500,000 fertile insects in the next generation. Because the population has halved, and the sterile release numbers are maintained, the ratio of sterile to wild insects doubles by the second release (i.e. 9:1 to 18:1). Sustained releases causes extinction of the wild population. Adapted from (Knipling 1959) and (Vreysen 2001).

Generation	No. of insects	No. of sterile insects released	The sterile to wild insect ratio	Percent sterility	No. of insects remaining fertile	Natural population growth rate
P	1 000 000	9 000 000	9 : 1	90,0	100 000	Fivefold
F1	500 000	9 000 000	18 : 1	94,7	26 316	Fivefold
F2	131 579	9 000 000	68 : 1	98,6	1 896	Fivefold
F3	9 480	9 000 000	949 : 1	99,9	10	Fivefold
F4	50	9 000 000	180 000 : 1	99,99	1	Fivefold

Aside from the potential power of the technique to control pest insects, SIT is an attractive control option for a variety of reasons. Firstly, released sterile insects should only mate with members of their own species. Control is therefore entirely conspecific, and effects on non-target organisms should be minimal. Secondly, insect release is relatively environmentally benign; an SIT control strategy used on its own should not result in the introduction of harmful chemical agents into the environment. Finally, SIT is compatible with a range of other biological control methods and can be used synergistically with integrated pest management (IPM) strategies. Indeed, because the impact of a sterile release is expected to be inversely density dependent (i.e. it is more effective at higher sterile to wild insect ratios), other tactics that can help reduce the wild population density prior to sterile insect release should be beneficial (Gould & Schliekelman, 2004).

In the 1950's, pilot trials successfully confirmed the field application of SIT. Sterile releases of a Dipteran livestock pest, the New World Screwworm (*Cochliomyia hominivorax*), were used to eradicate a wild population in an island off the coast of Venezuela (Diamant, 1963). Following this success, screwworm SIT was implemented across the Americas, with eradication from the United States finally achieved in 1966. Since 1991, several countries in Central America including Mexico, Belize, Guatemala, and El Salvador have been declared screwworm free (Wyss, 2000).

Arguably the most successful application of SIT against a Tephritid pest has been the eradication of the medfly from several highly infested areas of South America. The first large scale medfly SIT program was initiated in Tapacula, southern Mexico in 1977, with the construction of a 500 million sterile fly per week mass rearing facility. The program was initially aimed at eradicating medfly from southern Mexico, a task it accomplished within five years (Hendrichs et al., 1983). Since then an effective sterile fly barrier zone

has been maintained to assure the fly-free status of Mexico, the U.S.A. and a large part of Guatemala. SIT eradication of medfly was also achieved in northern Chile and large parts of Argentina (Hendrichs et al., 2002). The SIT strategy was also politically, environmentally and economically successful (costing on average less than half that of the previous recurrent strategies) (Hendrichs, 2000). The successes and expanding use of medfly SIT encouraged similar control efforts against other Tephritid fruit flies of economic importance, namely the *Anastrepha* and *Bactrocera* species (Hendrichs et al., 2002).

The olive fly was first considered as a candidate species for SIT in the 1960's. It was shown that exposure of pupae to 80-120 Grays (Gy) of γ-rays induced permanent dominant lethality in the sperm of males, and sterility in females (Tzanakakis et al., 1966). Ensuing field trials were organized during the early 1970's in an olive plantation in Northern Greece (Economopoulos et al., 1977), involving repeated releases of mixed-sex irradiated flies (Economopoulos, 1972). At the point when sterile releases were initiated, the olive fruit in the release area were not yet suitable for larval development and the sterilized flies succeeded in delaying high infestation levels by approximately a month relative to control areas (Estes, et al., 2011; Economopoulos, 1972). However, when environmental conditions became favourable for olive fly reproduction, the sterile flies were unable to reduce infestation, and both treatment and control plots showed high levels of infestation by the end of the season (Estes et al., 2011; Economopoulos, 1972). As a result funding was cut and the trials were abandoned (Estes et al., 2011; Economopoulos et al., 1977). Shortcomings were blamed on a variety of issues, including: (1) a significant reduction in mating competitiveness of sterilised males compared with wild males; (2) poor mass rearing methods and larval diets; (3) a differential timing of peak mating activity between

mass-reared and wild flies; and (4) the lack of any means of separating males from females (sexing) (Estes et al., 2011; Augustinos et al., 2008).

The ability of released sterile insects to successfully mate and transfer sterile sperm to their wild counterparts is the *modus operandi* of SIT. Bateman's principle (Bateman, 1948), states that in anisogamous species, such as fruit flies, where the female has the greatest reproductive investment, intra-specific competition will exist among males. Therefore, surviving release into the field and having successfully located a wild female, the released sterile males must compete with rival wild males, performing courtship behaviours with sufficient aptitude to satisfy the potentially highly discriminating female, mate successfully, and defend his paternity share against rival males that might mate with the same female in the future.

The effects of laboratory colonisation, artificial rearing, sterilisation procedures, and other handling methods can cause mating or field survival behaviours to differ greatly between released-sterile and wild flies, leading inevitably to reductions in sterile fly lifetime sexual competitiveness and strain quality (McInnis et al., 2002). If wild females are able to detect differences between released and wild males, they may reject the released males in favour of more attractive wild ones. Furthermore, if only slight differences exist, a selection pressure may favour females that have more acutely discriminating abilities or behaviour, further compounding the effect. If competitiveness is significantly reduced, the number of insects released must be correspondingly increased in order to attain the over-flooding ratio necessary to produce the desired downward trend in target population number (Hooper, 1972). Mathematical modelling of SIT releases by has suggested that a number of possible causes may combine to contribute to the failure of an SIT programme (Ito & Kawamoto, 1979). These include: (1) a shortage of released sterile insects; (2) weakened sexual competitiveness or a change in the sexual behaviour of

adults; (3) weakened sperm competitiveness if the females of a target species mate more than once; and (4) the immigration of wild flies into the control area. While the first three points can be mitigated by releasing more sterile males, there are economical and practical maximum threshold limits on release numbers above which an SIT programme may not be feasible.

1.2.2. Mating competitiveness

There are numerous factors that may reduce the mating competitiveness of released sterile insects for use in classical SIT. Of these, three major considerations are: (1) fitness costs due to the effects of radiation sterilisation; (2) colonisation effects on the genetic diversity of the colony i.e. phenomena such as founder effect, genetic drift and inbreeding depression; and, (3) the deleterious effects associated with mass-rearing, the addition of marking dyes, and the release procedure itself.

Radiation sterilisation forms the foundation of traditional SIT (**Fig. 1.2A**), usually relying on exposure of the late pharate stage of the pupa-bound adult to a source of ionising radiation [the late pharate stage is used as the adult tissues, with the exception of the gonads, have already completed development and are therefore less vulnerable to the effects of DNA damage (Hallman &Thomas, 2011)]. The extent of sterility may be increased by increases in the absorbed radiation dose, although because ionising radiation not only induces dominant lethal mutations in the germ cells but also causes mutations in somatic tissue (Sakurai et al., 2000), other biological functions are also affected (Kumano et al., 2010). Levels of exposure and sterility are therefore negatively correlated with sexual competitiveness (Parker& Mehta, 2007). The reduction in mating competitiveness of irradiated medfly strains has been estimated at 4-10 fold (Lance et al., 2000; Shelly et al., 1994).

Radiation sterilisation may also impact on post-copulatory sexual competitiveness. Mating decreases female receptivity to remating in many insects (Chapman et al., 1998), however, it has been found that irradiated male medflies have an impaired ability to reduce receptivity in mates compared to non-irradiated controls (Kraaijeveld& Chapman, 2004). This is possibly due to a reduction in irradiated male sperm load (Taylor et al., 2001). The problem of increased remating rates observed in females mated to irradiated males may be further compounded by effects of sperm competition. Tephritid females are capable of mating multiple times, storing and simultaneously maintaining viable ejaculates from several males. As SIT relies on the transfer and fertilisation of eggs by sterile sperm, reduced sperm competition may be a major factor concerning overall sterile strain efficacy. The sperm competitiveness of irradiated male olive fly has been found to be significantly compromised relative to non-irradiated controls (Cavalloro& Delrio, 1974). The combination of reduced ability to induce refractoriness to female remating, and lower sperm competitiveness suggests potentially high post-copulatory competitiveness costs for radiation sterilised insects.

Effects of losses in genetic diversity from laboratory colonisation may also affect strain quality. Founder effects may result if the colony was developed from only a few initial wild insects. Furthermore, the wild caught insects are brought into artificial caged conditions and exposed to artificial diets and high density cage environments; consequently, they may encounter a range of pathogens, toxins, and stresses not frequently found in the wild. A strong selective pressure is therefore exerted and many colonised insects may not survive the first generations. This genetic "bottlenecking" can further reduce the genetic diversity, and contribute to inbreeding depression caused by the increased genetic homozygosity of individuals. This can be through either an increased homozygosity for partially recessive detrimental mutations and/or increased homozygosity

at loci with a heterozygote advantage (overdominant alleles) (Charlesworth& Willis, 2009). Encouraging a healthy genetic diversity is therefore of considerable importance, with colonies often benefiting from regular introgression with wild caught insects.

Prior to their release, sterile insects are shipped and handled, emerged from their puparia, matured and are loaded into delivery vehicles for aerial or ground release; these are specialised procedures and minor variations in rearing procedures can have significant impacts on the quality of mass-reared flies (FAO/IAEA, 2007). For reasons of technical necessity, economic efficiency, or merely simplicity and convenience, these methods may not produce the highest quality insects possible. Negative effects can be minimised by careful planning and execution, however, a certain degree of reduction in quality at this stage may be unavoidable (Lux et al., 2002).

Various procedures have been developed in an attempt to maximise the competitiveness of sterile flies in the field. Studies (Parker & Mehta, 2007; Toledo et al., 2004) suggest that using a lower radiation dose with less sterility but increased competitiveness can be more efficient at achieving control. Although, in preventative release programs 100% sterile flies are often required in order to avoid the novel introduction of an insect pest (Schetelig et al., 2009). Aromatherapy has also been proven to increase the mating competitiveness of some Tephritid species. The exposure of medfly males to ginger root oil (GRO), for example, was found to double their competitiveness in field cage studies (Shelly et al., 2010). Interestingly, this effect is probably not due to a behavioural change in the males; rather, results suggest that the GRO interacts with the male exoskeleton and produces a scent attractive to females (Shelly 2006). Unfortunately, no similar chemicals have been found that increase male olive fly attractiveness, and are unlikely to ever be; the olive fly is somewhat unique among the Tephritids, being one of

the only species in which females produce the sex-pheromone in order to attract male mates (Wicker-Thomas, 2007).

1.2.3. Mating compatibility

During the course of laboratory adaptation, drastic differences in rearing conditions may cause the selection pressures that operate in nature to become relaxed (Krafsur et al., 1987), and may be replaced by novel selective pressures from artificial rearing. Behavioural changes may then be inadvertently selected for as the strain adapts to the laboratory environment. This results in a strain that is better adapted to the artificial mass-rearing environment, but may be less likely to survive in the field and may also have developed a certain degree of mating incompatibility with the target population.

One notable difference between the conditions under which flies mate in the wild and in mass-rearing facilities is the dense crowding in mass-rearing cages. It has been shown for mass-reared medfly strains that over-crowding can lead to more than half of all courtship displays being interrupted by other flies (Briceno et al., 1996), a far greater rate than observed in nature (Hendrichs& Hendrichs, 1990). Studies show that selection has favoured significantly reduced durations in courtship displays for mass-reared medfly strains (Briceno& Eberhard, 1998). Longer courtship displays are favoured by wild females; shorter displays by released males may therefore reduce mating compatibility with wild females and therefore compromise competitiveness in the field (Briceno& Eberhard, 1998).

Another perhaps more extreme example (at least for effects on SIT efficacy) is the development of diurnal mating asynchrony between mass-reared and wild olive fly. In the previously unsuccessful olive fly SIT trials, peak mating activity of the released laboratory males and females was found to occur one to two hours prior to that of the wild population

(Economopoulos, 1972; Zervas, 1981); wild olive fly began courtship activity at the on-set of scotophase, while laboratory flies initiated mating significantly earlier. Although not empirically confirmed, one possible explaination for this is the existence of a predatory constraint causing selection to favour mating after dark in the wild. Such a constraint would clearly not exist in the laboratory, and insects with a shift towards earlier mating activity may gain a fitness advantage. Whatever the cause may be, however the resulting assortative mating has led to a high degree of mating incompatibility and was doubtless a major contributing factor to the failure of the pilot olive fly SIT trials (Estes et al., 2011).

The laboratory strain used for the olive fly trials was colonised from Democritus, Greece in the 1970's. The differences in behaviour and quality between the Democritus strain and the wild insects prompted a series of behavioural and genetic studies in order to better characterise the differences between strains. Electrophoresis studies on the gene frequencies of certain enzymes showed extensive differences between the laboratory and wild insects; when wild insects from different localities (even very distant ones) were compared, the differences were not as big as those between the Democritus strain and wild flies (Economopoulos & Zervas, 1982). These changes were found to occur with extreme rapidity following colonisation (only 2-3 generations) (Tsakas& Zouros, 1980; Bush& Kitto, 1979). Furthermore, a significant divergence from natural mating photoperiod was found in laboratory insects that had been colonised for only several generations (Economopoulos, 1972). These findings have suggested that problems associated with colony adaptation may be too quick to develop to be overcome with introgression with wild insects.

Studies with laboratory reared wild-type and wild olive flies clearly demonstrated the assortative mating between laboratory-reared and wild olive flies (Zervas& Economopoulos, 1982). However, when laboratory females were not included in field cage

mating trials, laboratory males were far more effective in mating with wild females; the laboratory males persisted in their courtship activity until the wild females became receptive (Zervas& Economopoulos, 1982). It has therefore been suggested that some of the problems of olive fly SIT could be overcome by the removal of females from sterile releases (Estes et al., 2011; Economopoulos, 2002). Thus, the development of an effective olive fly sexing method is widely considered crucial to the future success of olive fly SIT (Estes et al., 2011).

1.2.4. Sexing for male-only release.

Sterile release theory focuses on the importance of the number of males released, whereas the number of sterile females released is considered a neutral factor on the basis that there is an excess of male mating potential in a population (Gould& Schliekelman, 2004). Indeed, in some cases the presence of female insects in the release population may even be harmful to an SIT programme (Morrison et al., 2010). Studies documenting the suppressive capacity of male-only versus bi-sex releases of medfly (McInnis et al., 1994; Rendon et al., 2004) have found that sexing insects can be highly beneficial for an SIT programme. It was found that male-only releases can produce a three to five-fold greater population suppression than equivalent numbers of males in mixed-sex releases. It has been suggested that the major benefit comes from reduced sterile female distraction of co-released males, resulting in heightened mobility in the open field and/or increased male searching behaviour for wild females (Rendon et al., 2004).

Removal of sterilised females may be desirable as such females may still be able to cause crop damage or, if a disease vector, transmit etiological agents from person to person. For mosquitoes, where the female is an obligatory blood feeder, their removal is considered essential (Lance& McInnis, 2005). Sterile female fruit flies may cause damage

via "sterile stings"; the piercing through fruit surface with her ovipositor and the laying of an unfertilised egg into the flesh of the fruit, resulting in increased chances of viral or fungal infection. Even if females are neutral to SIT effectiveness in the field and have no other negative consequences, they can significantly add to rearing, handling and distribution costs, thereby undermining the general efficacy of an operational SIT programme (Morrison et al., 2010).

Sexing for mosquito SIT can be achieved relatively easily thanks to exploitable morphological and behavioural differences between males and females; for example, in the mosquito species *Aedes aegypti*, size sorting is possible at the pupal stage (female pupae are significantly larger than males), or insecticide may be added to the adult female's blood-meal (Lowe et al., 1981). Unfortunately, no such dimorphism exists for most Tephritid or Calliphorid (the family that includes blowflies) species. A great deal of effort has therefore gone into developing strains and techniques by which females can be removed from sterile insect release populations.

(Whitten, 1969) developed a means of so-called 'genetic sexing' in the Australian sheep blowfly, *Lucilia cuprina* using a selectable male-linked chromosomal translocation (Robinson, 2002) (**Fig. 1.2B**). Using classical genetic methods a strain was generated whereby the segment of an autosome bearing a dominant wild-type allele for a selectable gene was translocated to the Y chromosome. Using such a translocation strain Whitten and colleagues found that males and females could be separated mechanically on a large scale. This encouraged the generation of similar strains in the medfly. Two sexing systems based on male-linked translocations have been successfully utilised for medfly SIT programmes, one using a pupal colour mutation, *white pupae* (*wp*), and the second using a temperature sensitive lethal mutation (*tsl*) (Robinson, 2002). However, sexing strains developed in this way have proven to possess certain drawbacks and limitations. Firstly, translocation-based

strains often have reduced fertility and developmental viability that can impact negatively on strain mass-rearing. For example, the medfly *tsl* strain was initially envisioned to be cheaper to rear due to an early acting female-specific lethality; however, due to reduced viability, the adult colony in a rearing facility is usually required to be about three times larger than that of an equivalent wild-type colony (Caceres, 2002). Secondly, the translocations tend to be unstable and reversions, although rare on a laboratory-scale, may be a serious problem when large populations of insects are generated for area-wide release. Finally, each of these sexing strains must be developed anew for each species (Alphey& Andreasen, 2002); as the genetic sexing strains described depend on specific mutations and chromosome translocations in a individual species, they cannot be transferred to others.

1.3. Genetic enhancements to SIT

1.3.1. Introduction

Advances in molecular biology and genetic engineering present new opportunities for insect population control. Whilst the avenues open to the transgenic control of disease vectors may include population replacement strategies (i.e. where genetic traits conferring refractoriness to disease transmission are driven through a population), control of agricultural pest insects tends to remain within the sterile insect control paradigm. Leading molecular approaches attempt to achieve genetic sterility through the incorporation of a dominant lethal genetic trait that is passed from parent to offspring and causes developmental inviability. This approach, termed 'release of insects carrying a dominant lethal gene' or (RIDL), differs from traditional SIT in a number of key respects.

Firstly, transgene-based lethality eliminates the requirement for sterilisation using irradiation. This may mitigate at least part of the relative fitness cost carried by the released sterile insects, and would totally remove safety concerns surrounding the use of

radioactive isotopes and the difficulties associated with production, transportation and eventual disposal of isotopic emitters. However, transgenesis may itself result in some fitness costs resulting from either negative effects of the transgene products, or through insertional mutagenesis after a transposition event (Marrelli et al., 2006). The selection of transgenic strains where these effects are minimal will therefore form a key part of the strain development and evaluation process. Although, it is likely that a small fitness penalty to carrying the transgene will be desirable from a regulatory perspective; natural selection in the field will thereby ensure that the transgene is highly unlikely to persist once transgenic insect releases have ceased.

Secondly, such lethal transgenes can be expressed sex-specifically, presenting an option for genetic sexing (Fu et al., 2007) (**Fig. 1.2C**). As previously discussed, a sexing mechanism may be highly advantageous in a control programme by increasing the dispersal and wild-mate seeking activity of released males, removing the sex that causes crop damage, and potentially reducing the costs of rearing (should the female-lethal trait express sufficiently early). Although female-specific lethal transgenic stains could potentially be used purely for genetic sexing, with the irradiation of males forming a downstream part of the rearing and release process, female-specific lethality can be considered a sterility mechanism in itself. As females are almost always a limiting reproductive resource for a species, female-specific elimination should have similar effects on reproductive capacity and therefore population size as would the elimination of both sexes. Furthermore, if males homozygous for a dominant female-specific lethal transgene are released, all the male F1 progeny resulting from a transgenic male/wild female mating will inherit a single copy of the transgene, which will, in turn, be passed on to half of his sons and daughters and a quarter of their sons' daughters, and so on, giving an additional dimension to the control (Alphey& Andreasen, 2002).

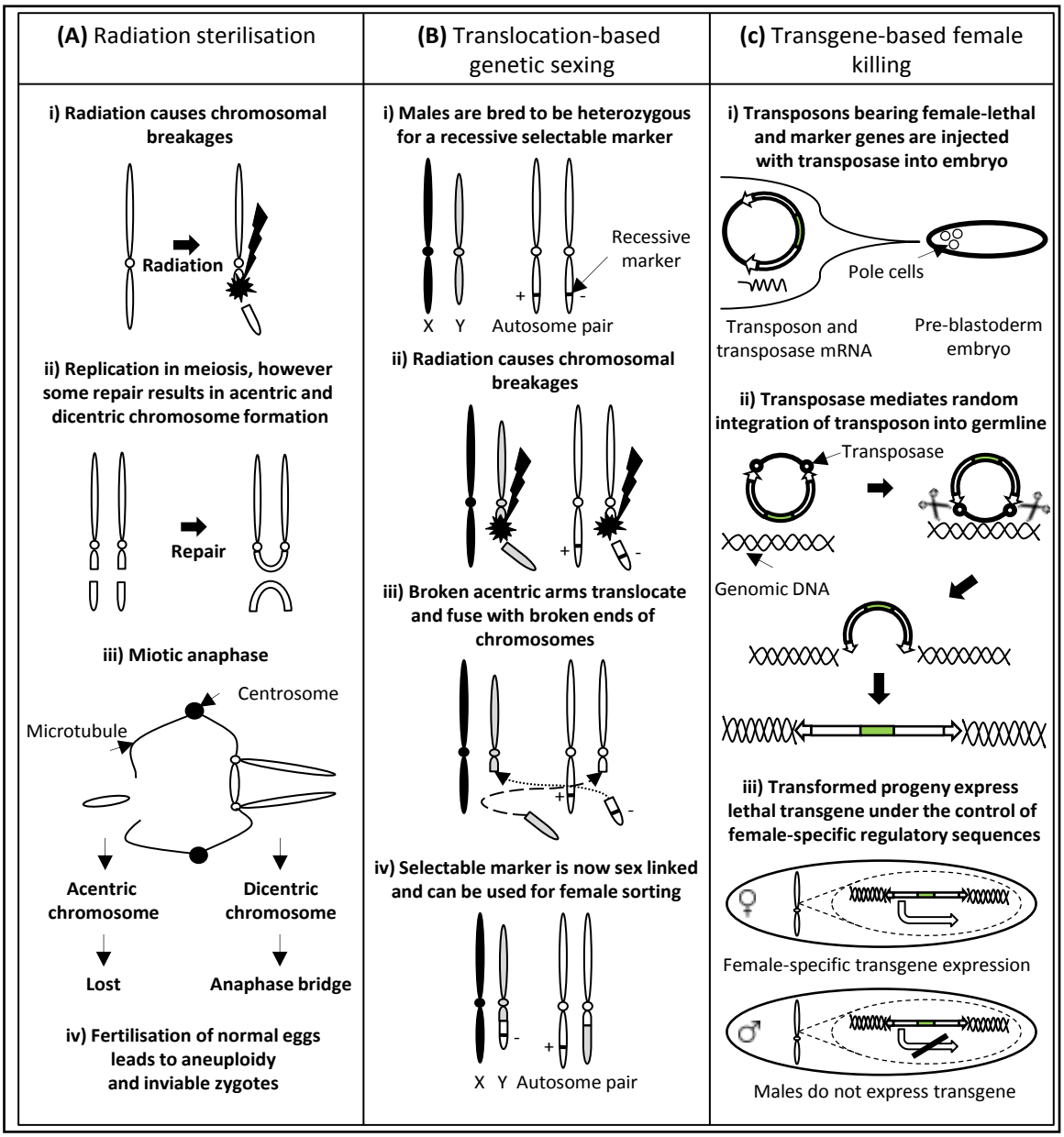


Figure 1.2. (A) Radiation sterilisation causes induction of dominant lethal mutations in insect germ cells. **(i)** Exposure to ionising radiation causes breakage of chromosomes. **(ii)** Two broken chromosomes, each with a spindle fibre attachment site, may adhere to form a "dicentric" chromosome with two centrosomes. Alternatively, two chromosomal fragments lacking a centrosome may adhere forming an acentric chromosomal fragment. **(iii)** During meiosis, the dicentric chromosome may be pulled toward opposite poles of the cell, breaking or causing failure of division. Acentric chromosomes cannot attach to a spindle and will be lost from one of the daughter cells. **(iv)** Each daughter cell will receive a deficiency or duplication of a particular piece of genetic information. These and other chromosomal events culminate in the death of an embryo fertilised by an irradiated male (Van der Vloedt, Klassen). **(B)** Translocations can cause sex-linkage of selectable traits. **(i)** Males are bred to be heterozygous (+,-) for a selectable trait e.g. a mutation causing a white pupal phenotype. **(ii)** Heterozygous males are irradiated, causing random chromosomal breakages. **(iii)** A small proportion of the resulting fragments rejoin as reciprocal translocations, with the dominant wild-type copy of the allele (e.g. brown-pupal phenotype) fusing to the Y chromosome. **(iv)** This results in wt male pupae, while females are homozygous for the recessive white pupa mutation. Sorting machines can be used to sort males on a large-scale. **(C)** Transgenic methods combine sexing and sterility. **(i)** A plasmid bearing a female-lethal expression cassette between transposon inverted terminal repeats (TIRs) is co-injected with transposase mRNA into a pre-blastoderm stage embryo, targeting the forming pole cells which develop into the insects germ-line. **(ii)** Transposase binds to the TIRs, liberating the transposon and inserting it into a random transposon recognition sequence e.g. TTAA for the *piggyBac* transposon. **(iii)** Progeny from a germ-line transformed individual are fully transformed. Female-specific regulatory sequences cause female, but not male lethality.

Finally, unlike SIT methods that are based on the sex-linked translocation of lethal mutations, once useful transgenes and regulatory sequences are identified, the same or orthologous sequences might be easily transferred to a range of related species. For example, Oxitec's female-specific lethal transgene construct OX3097 was designed for use in the medfly (*Ceratitis capitata*), and was indeed successful in this species (Fu et al., 2007). However, later transformation using the same unmodified construct in another tephritid pest of a different genus, the Mexican fruit fly (*Anastrepha ludens*), resulted in essentially the same functional phenotype (Condon et al., unpublished data). It is possible that this construct will be functional in a range of tephritid species, including the olive fly.

1.3.2. Transposable genetic elements and insect transgenesis

The genetic modification of a target organism is usually achieved through the genomic integration of modifying genes via a DNA transposon (transposable element). Transposable elements include a diverse collection of genetic elements that share the ability to promote recombination reactions, resulting in the movement of the element from one location in the genome to another (O'Brochta& Atkinson, 1996). Realising the potential for exploiting their mobility, classic experiments by (Spradling& Rubin, 1982) involving the *P*-element, a transposable element found to have recently invaded the *Drosophila melanogaster* genome, were responsible for the development of the first transposon-based gene vector system allowing heritable germline transformation in drosophilids (Handler& James, 2000). It was later found, however, that specific host factor requirements restrict *P*-element mobility, and therefore biotechnological functionality to *D. melanogaster*, and closely related species. Since then a series of other DNA-mediated transposable elements have been discovered that are independent of host factors, and are active in a wide variety of organisms (Horn& Wimmer, 2000). Multiple gene vectors are

now available for the purpose of transgene insertion and expression in virtually any insect species (Fraser, 2012).

Transposable elements are astonishingly widespread in nature, and have been found in the genomes of all organisms in which they have been sought (Quesneville& Anxolabehere, 1997), [although the *Plasmodium falciparum* genome may be an exception to this rule (Wicker et al., 2007)]. For use in insect transgenesis, transposable DNA elements have the properties of practical frequencies of successful transgenesis, relative randomness in their genomic integration, and in some cases controlled remobilisation (Fraser, 2012). The best characterised and most commonly utilised of these are the Class II transposable elements, of which four are used routinely to create non-drosophilid transgenic insects: *Hermes*, *Mos1* (*mariner*), *Minos*, and *piggyBac*. Class II transposable elements are distinguished by their movement in the absence of an RNA intermediate. Class II elements are further divided into two subclasses. Subclass 1 comprises elements that move via a 'cut and paste' mechanism (**Fig. 1.2C**); that is, the element is capable of precisely excising itself from its existing locus and reinserts itself elsewhere in a process that is completely independent of replication (O'Brochta et al., 2003). Subclass 2 elements undergo a transposition process that entails replication without double strand cleavage (Wicker et al., 2007).

piggyBac is a class II subclass 1 transposable element. It is approximately 2.5kb long, containing terminal inverted repeats (TIR) with asymmetrically distributed, 19bp internal inverted repeats (Cary et al., 1989). *piggyBac* has a preference for inserting in AT-rich genomic regions and exhibits extreme preference for TTAA target sites (Cary et al., 1989). As with other class II transposable elements, excision and insertion can be performed in the absence of DNA synthesis. The binding of the excised *piggyBac* transposon occurs by a direct nucleophilic attack of the 3' OH ends of the transposon to

staggered positions at the 5' ends of the TTAA target sequence. The TTAA overhangs from the excised transposon are then able to base-pair with the target DNA, which can be sealed by ligation (Mitra et al., 2008).

The development of transposon-based gene vector systems, such as *piggyBac*, with a wide host-range and high mobility has allowed the genetic transformation of a range of insect species. As a result, the generation of transgenic insects strains is now a relatively routine procedure, with a growing list of insect species (from a range of orders including Dipterans, Hymenopterans, Lepidopterans, and Coleopterans), having been proven amenable to genetic transformation. The first successful transformation of the olive fly was reported by Koukidou et al. (2006) with researchers using a *Minos*-based transposon vector carrying EGFP expressed under the control of the Tet-Off conditional gene regulation system.

1.3.3. Site-specific recombinases and transposon stability

Site-specific transgene integration systems, such the PhiC31 system from a *Streptomyces* bacteriophage, have been used to precisely integrate exogenous DNA sequences into pre-integrated docking sites in a variety of insect species, including *Drosophila* (Groth et al., 2003), the medfly (Schetelig et al., 2009), and the malaria mosquito *Anopheles gambiae* (Meredith et al., 2011). Site-specific integration systems are proving to be a useful tool for transgene introduction and can be utilised for a number of practical purposes.

The integration site of a transposon-mediated transgene is essentially a lottery, with the transgene inserting into a random place in the genome. The resulting transgene expression pattern is therefore strongly susceptible to variations in the chromatin state of the surrounding DNA. Furthermore, the random nature of the integration event may lead to

the disruption of some functional genomic sequence, potentially resulting in a fitness reduction or lethality for the transgenic strain. A large number of transgene integration events are therefore likely to be unattractive, necessitating the generation of several transgenic lines before one with favourable characteristics is found. Once a transgenic strain has been generated that has been found to give a suitable expression pattern with minimal fitness costs, and the insertions-site has been well characterised, it might be useful to take advantage of such an innocuous site to replace existing transgenes, or to introduce additional ones into the same position (Schetelig et al., 2009).

The PhiC31 integrase is capable of catalysing a recombination reaction between an acceptor (*attP*) site and donor (*attB*) site, causing the integration of an *attB*-bearing plasmid into the *attP* locus. The recombination reaction creates two hybrid junctions, known as the *attL* and *attR* sites (**Fig. 1.3**), that are no longer recognised by the integrase, and are therefore considered immune to remobilisation (Thorpe& Smith, 1998).

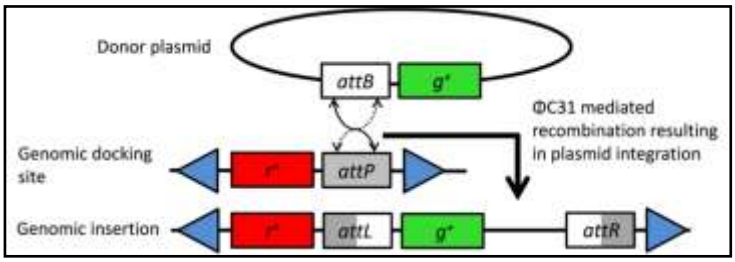


Figure 1.3. A plasmid containing an *attB* donor site and a dominant marker (g^+) integrates at an *attP* docking site that has been pre-integrated in to the genome of the target organism. The recombination reaction between *attB* and *attP* sites results in the formation of *attL* and *attR* hybrid sites.

In contrast, the existence of intact transposable element TIR sequences post integration leaves the element and transgenes contained within susceptible to remobilisation. Remobilisation of nonautonomous transposon insertions may occur through re-exposure to a suitable transposase source; though individual lab strains or wild populations can be tested for such transposases, other populations in the wild might

theoretically contain them, or an exogenous transposase gene might hypothetically be introduced, for example, by a virus (Dafa'alla et al., 2006). A method for stabilising a *piggyBac* insertion (**Fig. 1.4A**) using the PhiC31 site-specific integration system was devised by (Schetelig et al., 2009). The experimenters introduced an additional 3' *piggyBac* TIR to a site within an pre-integrated *piggyBac* transposon, creating a second, smaller, transposable element within the first. Re-exposure to a *piggyBac* transposase source, through back crossing of the transgenic insects to a jumpstarter strain (a line expressing an exogenous *piggyBac* transposase gene), caused the remobilisation of the smaller transposon, leaving behind the desired transgene upstream of a 3' *piggyBac* end. As the transposase requires both 5' and 3' TIR sequences, the removal of one drastically reduces the likelihood of transgene remobilisation.

Another method of stabilising *piggyBac* insertions was developed earlier by (Dafa'alla et al., 2006) (**Fig. 1.4B**), and involved the introduction of a large 'composite' *piggyBac* element containing four different potential transposable elements. Once the large composite plasmid was successfully integrated, the smaller 'flanking' transposons were removed through back-crossing to a jumpstarter strain. This is aided by a higher transposition frequency of smaller transposons (Berg& Spradling, 1991). Transgenes are arguably rendered more stable using this system as all flanking *piggyBac* TIR sequences are removed.

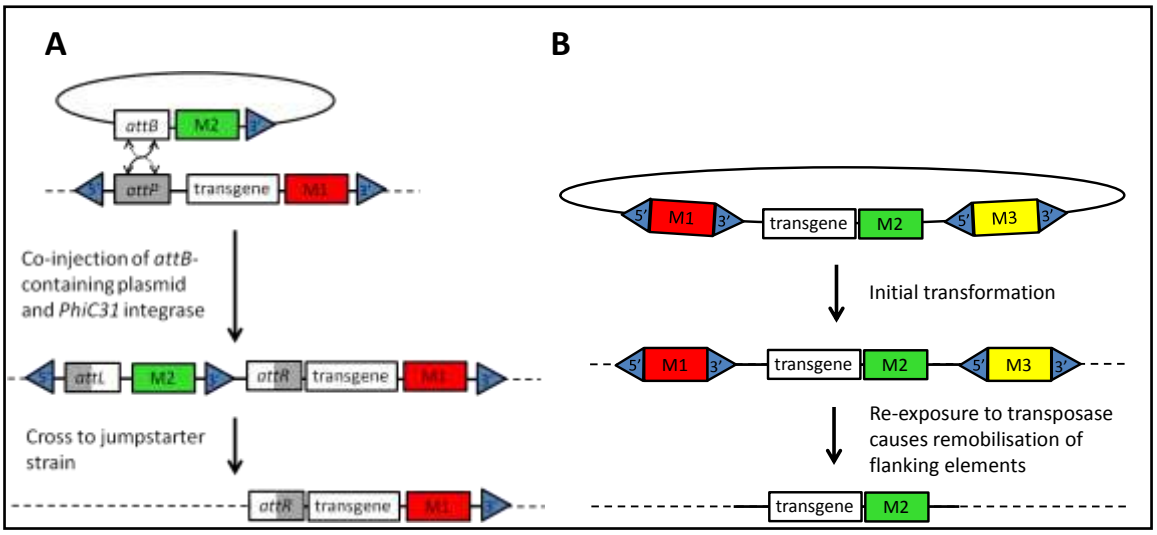


Figure 1.4. (**A**) Transgene stabilisation through the use of site-specific integration. The original transposon-mediated randomly integrated insert carries one pair of *piggyBac* TIRs (5' and 3'), an *attP* site, a transgene and a fluorescent marker gene (M1). The co-injection of *PhiC31* integrase with a plasmid carrying a single 3' *piggyBac* TIR, an *attB* site, and a different fluorescent marker gene (M2), leads to site-specific integration of the *attB* bearing plasmid into the pre-integrated *attP* site. The genomic insertion now carries two potential *piggyBac* transposons, one with the both the M1 and M2 markers, and one with the M2 marker only. Exposure to a transposase source by crossing to a jumpstarter strain causes the excision of the smaller *piggyBac* cassette, leaving one remaining 3' *piggyBac* TIR, an *attR* site, and the transgene (adapted from Schetelig et al., 2009). (**B**) The initial transformation uses a plasmid carrying a composite transposon with four potential transposon combinations. The integration of the entire intact transposon results in an insertion with three different fluorescent marker genes (M1, M2 and M3), with M1 and M3 isolated within their own smaller transgene-flanking transposon ends. The flanking transposon ends can be remobilised by crossing to a jumpstarter strain. The final insertion contains no *piggyBac* TIRs and is therefore stable.

1.3.4. Conditional gene expression systems

An optimally regulated transgene is one that is silent, or nearly silent, under basal conditions and strongly induced under activating conditions (Markstein et al., 2008); that is, it has a high signal-to-noise ratio. Furthermore, it is useful for a researcher to be able to determine in which cell types expression is active; the most useful gene expression systems therefore allow for controlled regulation within the dimensions of time and space (McGuire et al., 2004). Heat-shock promoters have proved useful in providing temporal transgene regulation, but are expressed in essentially all cells. The use of defined promoters (promoters whose expression is restricted to a specific tissue type) or the bipartite GAL4/UAS system, can provide spatially restricted gene expression, however, their expression cannot be easily regulated in time. Currently, several gene expression systems have been developed that are capable of providing both spatially and temporally

controlled expression, including systems employing the yeast FLP recombinase gene and FRT sites, steroid hormone responsive transcription factors (GeneSwitch and ER-GAL4), temperature-sensitive repressors of the classical GAL4-UAS system (TARGET), and tetracycline-responsive transcriptional factors (tet-On and tet-Off) (McGuire et al., 2004).

The tet-off system comprises a transcriptional activator called the tetracycline transactivator (tTA) [a hybrid transactivator combining a DNA binding domain (TetR), with the C-terminal transcriptional activation domain of virion protein 16 (VP16) of the herpes simplex virus], and a DNA response element [known as the *tet* operator sequence (tetO)]. Tetracycline binds tightly the TetR domain of tTA, with a nanomolar dissociation constant, inducing conformational changes that reduce the binding constant of TetR and DNA by 6-10 orders of magnitude (Aleksandrov et al., 2008). Therefore, minimal promoters linked to tetO can be virtually silenced in the presence of tetracycline, whilst in its absence the VP16 domain can activate transcription of downstream genes ~1000-fold (Gingrich& Roder, 1998) (**Fig. 1.5**).

(Thomas et al., 2000) showed that the tet-off system can be used for effectively regulating a dominant female-lethal gene system in *D. melanogaster*. 100% female lethality was observed in transgenic strains carrying a female-lethal gene downstream of a tetracycline regulated promoter when the insects were reared in the absence of tetracycline. Tetracycline supplied as a dietary supplement at concentrations of 0.1µg/ml, however, was sufficient to fully suppress female-lethality.

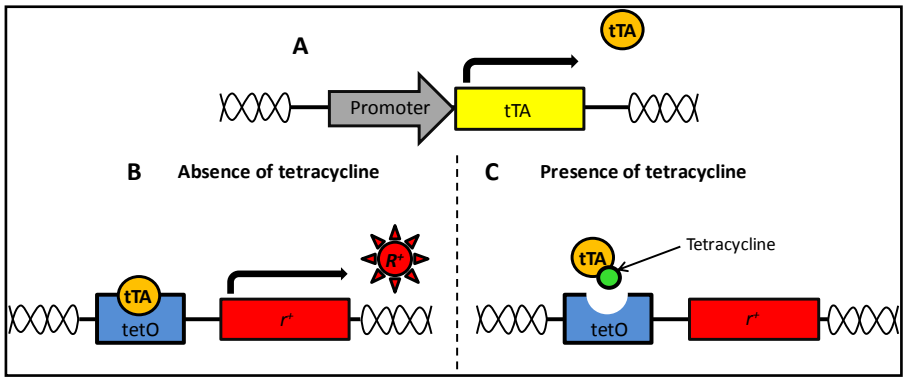


Figure 1.5. The Tet-Off system provides tightly-regulated conditional gene expression. (**A**) Expression of the tetracycline transactivator (tTA) is driven by a promoter. (**B**) In the absence of tetracycline (or the often used tetracycline analogue, doxycycline), tTA binds to the tetracycline operator (tetO) sequence, activating expression of the downstream gene (in this case r^+). (**C**) tTA becomes bound in the presence of tetracycline or doxycycline, and transcription of the downstream gene is silenced.

The tet-off system has been further utilised to not only provide a means of regulating lethality, but also as the lethal mechanism itself. A system, developed by (Gong et al., 2005), uses tTA as both a transactivator, and an effector. Although thought to be innocuous in low quantities, sufficient levels of tTA can be highly toxic to a cell. This is possibly due to the fact that the introduction of a potent transcriptional activator into a cell can cause a general down-regulation of essential genes, a mechanism known as transcriptional squelching. This is thought to result from the titration of one or more general transcription factors, causing a deficit and limiting the function of essential genes (Natesan et al., 1997). (Gong et al., 2005) showed that the tet-off system could be constructed in a positive feedback configuration; in the absence of tetracycline, initial basal expression of tTA leads to greater tTA expression and eventually cell death (**Fig. 1.6**). This 'all-in-one' configuration has the advantage of a compact design, minimizing the target for spontaneous mutation, and increasing transformation efficiency. The utility of the positive-feedback tTA configuration was demonstrated in transgenic lines of the medfly, with (Gong et al., 2005) generating strains that provided fully repressible dominant lethality. Furthermore the authors speculate that because the transgene construct contained no medfly specific DNA, the technology should be transferable to a range of

species [a hypothesis that was later validated by the successful implementation of the tTA positive feedback lethal system in *Aedes aegypti* (Phuc et al., 2007), the mexfly (Condon et al., unpublished data), and the pink bollworm, *Pectinophora gossypiella* (Morrison et al., unpublished data)].

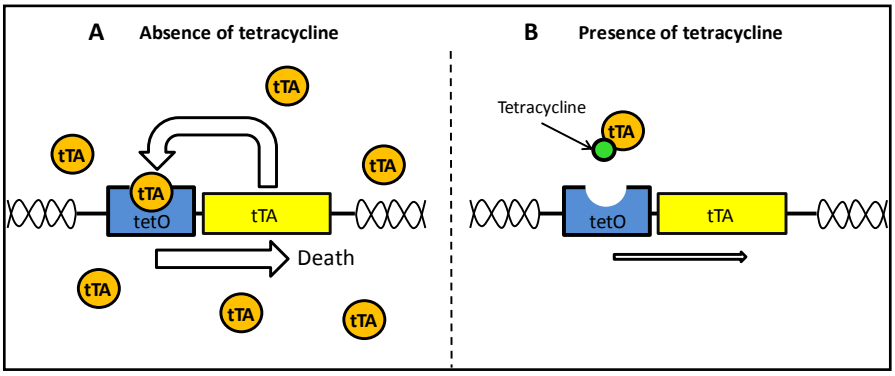


Figure 1.6. (A) In the absence of tetracycline, the tet-off system in a positive feedback configuration results in the accumulation of the tetracycline transactivator (tTA), resulting in cell death. **(B)** In the presence of tetracycline, tTA is inactivated, and is expressed only at basal levels (Gong et al., 2005).

1.3.5. Sex-specific transgene expression

For species where no easily exploitable sexual dimorphism is available, the introduction of a dimorphism through genetic means is necessary to achieve sexing. The generation of such strains using classical genetics methods has already been described. The classical methods are however somewhat limited by their non-transferability between species. In contrast, one of the major benefits of transgenic technology is the possibility of cross-compatibility of molecular components, and potentially entire transgene constructs between species.

Sexing could be achieved through the sex-specific expression of a marker that can be recognised by an automated sorting machine, or through the inducible expression of a female-specific lethal gene. Both approaches require the sex-limited expression of an inserted trait. Several strategies have been successfully utilised to achieve sex-specific

transgene expression, including: linkage to sex chromosomes; sex-specific promoter sequences; and, sex-specific alternative splicing systems.

(Condon et al., 2007) have shown that sex-specific expression of a fluorescent protein marker can be achieved through insertion of a transposable element into the Y chromosome. Experiments in the medfly have demonstrated that despite a highly heterochromatic Y chromosome, bright levels of expression of a fluorescent marker in males could be achieved with no expression in females. The authors speculate that this method might allow for accurate and automated sorting. Furthermore, male-specific transgene expression was visible at early larval stages potentially allowing for sorting at an early developmental stage which could cut down on the required space and materials, making rearing more economical. However, this method relies on the random insertion of a transposon-bound transgene into the Y chromosome, which can be relatively rare and therefore labour-intensive. It has been suggested that inserting a docking-site for a site-specific recombinase into the Y chromosome may allow for target integration and therefore much greater efficiency (Condon et al., 2007).

A number of sex-specific promoters have been characterised, cloned, and used to drive sex-specific gene expression in insects. Transgenic sexing strains can be constructed by using male-specific promoters that drive identifiable markers, or that encode resistance factors to environmental additives that would otherwise be lethal. (Christophides et al., 2001) describe the transformation of the medfly with *Drosophila* alcohol dehydrogenase (ADH) under the control of regulatory region (α2PS) from *D. melanogaster*, that is capable of conferring male fat body specific transcriptional activity. Although relative increases in male-specific ADH were observed by the investigators, expression levels were not sufficient for use as a robust sexing method. The use of female-specific promoters to drive transgenes has also been developed, and has been demonstrated to work effectively

in *D. melanogaster* (Thomas et al., 2000; Heinrich& Scott, 2000). Numerous genes have been characterised that are capable of conferring female-specific expression including yolk protein genes, chorion genes, and ceratotoxin genes. Effective and repressible female-specific lethality was independently demonstrated in *D. melanogaster* by (Heinrich& Scott, 2000) and (Thomas et al., 2000), where expression of the tetracycline transactivator (tTA) was under the control of the female- and fat-body-specific transcriptional enhancers from the *yolk protein 1* (*yp1*) and *yolk protein 3* (*yp3*) genes, respectively. The tTA-regulated tetO sequences were linked to various toxic genes. Both groups reported viability of males and females when reared in the presence of tetracycline, but male-only generations when reared in its absence. However, since most sex-specific promoters are active relatively late in development, being associated with sexual differentiation and function in the adult, very early sex specific expression may be difficult to achieve using this method (Condon et al., 2007).

The use of alternative intron splice sites can provide a mechanism by which a single gene can produce multiple protein variants. Genes of the sexual differentiation cascade are regulated by sex-specific splicing and therefore present a possible mechanism for sex-specific expression. The sexual differentiation gene cascade in the medfly has been well characterised (Pane et al., 2002), and relies on an initial signal from a Y-linked maleness factor (the 'M' factor) to lead to male development, with the absence of this factor leading to female development (Willhoeft& Franz, 1996). This is in contrast to *Drosophila* where the ratio of autosomes to X chromosomes determines sex. The presence or absence of the 'M' factor in medfly regulates sexual differentiation cascades, causing sex-specific splice variants of the *transformer* (*tra*) and *doublesex* (*dsx*) genes. In female embryos, a maternal *tra* mRNA provides full-length TRA protein that initiates a positive feedback loop, driving female-specific splicing of zygotically transcribed *tra* pre-mRNA, and producing new

TRA protein. This maintains production of more TRA (via autoregulation), and initiates female-specific splicing of *dsx* pre-mRNA, leading to a female-specific DSX isoform (DSX^F) and a female mode of development. In male embryos *tra* autoregulation is impaired by the M factor, resulting in the TRA protein not being produced and the production of DSX^M, which induces male development (Pane et al., 2002).

The *tra* gene is composed of five exons. The first, fourth, and fifth exons are included in the mature transcripts of both sexes, while the second and the third are male-specific. The female *tra* mRNA contains a long open reading frame (ORF), while the male mRNA contains stop codons that result in the truncated, non-functioning TRA protein. (Fu et al., 2007) have utilised the sex-specific splicing of the *tra* pre-mRNA to generate female-lethal strains of the medfly. By placing the first intron of the *Ceratitis capitata transformer* intron (*Cctra* intron) into the coding sequence for tTA. Since the full splicing of this intron is confined to females, a system was set-up whereby females spliced the intronic sequence, producing a functional lethal protein, whereas males recognise alternative splice sites, producing a longer transcripts containing an in-frame stop codon, and leading to the generation of a truncated tTA. With this method in conjunction with the tTA positive feedback-lethal configuration described in **Fig. 1.6**, the investigators were able to achieve efficient female-lethality in medfly. High levels of sequence conservation in sex determination genes between medfly and olive fly (Lagos et al., 2007) suggests that a similar approach, and possibly the same genetic construct, may be functional in the olive fly.

With the possibility of next-generation high-throughput sequencing of transcriptomes offered by developing technologies such as RNA-Seq (Wang et al., 2009), it is likely that new promoter regions and sex-specific alternatively spliced introns will become available to researchers, providing access to a vast repertoire of potential

regulatory sequences. These may also include micro-RNAs and other non-coding RNAs used in post-transcriptional regulation.

1.3.6. Genetic markers for transformation and field monitoring

The need to reliably identify released insects is paramount in sterile insect control. In classical SIT tephritid insects are marked before release using fluorescent powders such as Calco Blue dye. The pupae are marked by tumbling a known quantity of pupae with a known quantity of dye powder. Dye adhering to the pupa is contacted by the ptilinum on adult eclosion and the material is retracted into the head of the fly. Powder adhering to other parts of the flies' body are usually lost through the continual cleaning activity of the insect. The heads of captured insects are crushed, and the powder, if present, can be visualised under a UV light (Schroeder& Mitchell, 1981). However, the use of fluorescent powder dyes presents a few drawbacks. There is evidence to suggest that the application of too much powder can kill the insects, or produce adverse behavioural effects, and that reducing the quantity of powder used can significantly increase the survival and quality of fruit flies (Hagler& Jackson, 2001). Furthermore, powder dyes require an additional processing step and the dye powder itself may pose a health hazard to employees (Parker, 2005). There is also some evidence to suggest that a fraction of released sterile insects may lose some or all of the powder (Hagler& Jackson, 2001; Hagler& Miller, 2002), resulting in their false identification as wild insects in traps. This may be of low concern in SIT releases where the wild insect is prevalent, but could elicit an unnecessary, and potentially drastic response in areas where releases are acting as a buffer to reinvasion.

In *D. melanogaster* eye colour genetic mutants have been widely used as a marker. However, field monitoring using naturally occurring mutations may not be practical and

homologues would potentially be required for each new species. Moreover, most easily identifiable mutations will likely have some deleterious effect on the fitness of the insect.

Genetic marking through the expression of easily identifiable dominant transgene products provides another option for insect marking. Transgenic insect strains carrying transgenes encoding fluorescent protein markers have been developed in a range of tephritid species, including the olive fly (Koukidou et al., 2006). Various fluorescent proteins have been identified, cloned, and sequenced, and offer a range of absorbance and emittance spectra, meaning that a transgenic insect can be transformed with more than one fluorescent protein marker that can be visualised separately.

Tagging insects with fluorescent protein markers offers several potential advantages over more conventional marking procedures. Depending on the availability of suitable regulatory sequences controlling expression, a fluorescent protein marker can be permanently expressed throughout an insect's lifecycle. Fluorescent proteins such as the green fluorescent protein illuminate independently of cofactors (Chalfie et al., 1994), and can therefore be excited by mere exposure of the surface of the insect to the relevant wavelength of light. Moreover, PCR-based identification of the transgene sequences can be used as a back-up identification method, should there be any ambiguity. Fluorescent proteins expressed in the sperm, may also allow more rapid and accurate classification of the mating status of trapped females than current methods (Alphey et al., 2006).

1.4. Aims of the thesis

Although the olive fly was first proposed as a candidate species for SIT over 50 years ago, the lack of a sexing strain has inhibited progress towards effective field application; this thesis presents an attempt to address this. The primary aim was to produce a sexing strain utilising the female-specific RIDL (fsRIDL) transgenic technology, and to show that such a strain was capable of overcoming the key problems encountered in the previous olive fly SIT attempts.

The objectives of the project can be split into three rough parts. Firstly, to develop a transgenic olive fly strain capable of providing as close to the optimal fsRIDL characteristics as possible. This involved generating a range of candidate strains, the selection and further development of those that provide the most promising phenotypes, and an analysis of transgene exression using molecular techniques. Secondly, to test whether the engineered fsRIDL olive fly strain would be more sexually compatable and competitive, and therefore a greater potential for control application than strains used in the traditional bi-sex release technique, while also showing that the engineering proccess itself did not themselves cause any detrimental effects that might restict the application of the strain. This involved characterising the putative strain for a rangle of life-history parameters relevant to mass-rearing and a study of the mating competitiveness and mating compatability of males of the strain with field collected wild adults. Finally, the aim was to assess the capability of the fsRIDL approach in suppressing target insect population through proof-of-principle trials using repeated releases of fsRIDL olive fly males into stable populations of wild-type insects.

Transgenic (left) and wild-type (right) 4th instar olive fly larvae. Transgenic larva shows expression of the DsRed2 fluorescent protein marker.

Chapter 2 - Olive fly transformation and fsRIDL strain development

2.1. Introduction

Two key problems encountered in the previous unsuccessful olive fly SIT trials were the assortative mating between the sterile and wild olive populations, and a low quality of released insects, caused in part by the fitness reducing effects of radiation sterilisation (Esteset al., 2011; Economopoulos, 1972). It was therefore decided to attempt olive fly transformation with OX3097 (**Fig. 2.1**), a female-lethal transgene construct that can provide genetic sexing [encouraging random mating (Rendon et al., 2004)] (see *Section 1.2.4.*), and genetic sterility (removing the requirement for radiation sterilisation). OX3097 combines the positive feedback tTAV lethality mechanism described in **Fig. 1.6** with the female-specific splicing of the medfly *transformer* (*Cctra*) intron (*Section 1.3.5*). The positive feedback tTAV component of OX3097 has previously proved highly effective in inducing repressible lethality in medfly strains (Gong et al., 2005), and the *Cctra* intron has proven capable of providing female-specific lethality in transgenic medfly (Fu, et al., 2007) and mexfly (Kirsty Stainton, personal communication). Moreover, Schetelig & Handler (2012) have used a medfly *tra* intron to provide female-specific tTAV expression in the Caribbean fruit fly, *Anastrepha suspensa*, and Fu et al. (2007) showed that a transgene construct based on the splicing of the *Cctra* intron was capable of producing female-specific lethality in *D. melanogaster*, despite the differences in *transformer* alternative splicing regulation (Pane et al., 2002) brought about by some 120-150 million years of evolutionary divergence (Gaunt& Miles, 2002).

The olive fly *transformer* (*Botra*) presents remarkable structural and functional similarity to its medfly orthologue (Lagos et al., 2007), and as no other components of the OX3097 construct were medfly derived, it was inferred that the construct would have a high probability of similar functionality in the olive fly without modification.

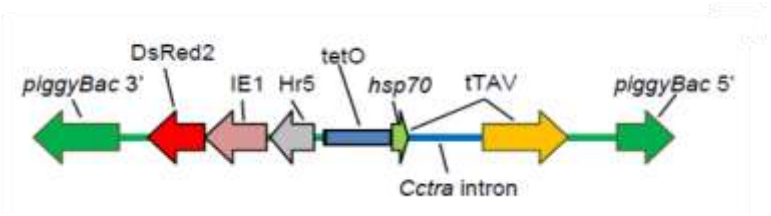


Figure 2.1. Schematic representation of the OX3097 transposon consisting of nuclear-localised fluorescent transformation marker DsRed2 and the tTAV expression cassette. Transcription of the fluorescent marker is regulated by the ubiquitous IE1-Hr5 promoter-enhancer. The tTAV expression cassette includes tTAV coding region with *Cctra* intron placed within the open reading frame. Initiation of tTAV transcription from the *Dmhsp70* minimal promoter and female-specific RNA processing leads to production of functional tTAV in females only. (The minimal *Dmhsp70* promoter includes the proximal promoter fragment only, including the transcription start site and a short leader sequence; the promoter therefore, is not expected to respond to heatshock.) tTAV binds to the upstream 21 canonical repeats of tetO resulting in increased transcription from the adjacent minimal promoter and greater expression of tTAV in a positive feedback mechanism (see **Fig 1.6**). High levels of tTAV are lethal as the transcriptional activation domain (VP16) acts as a transcriptional squelcher in high concentrations (Gong et al., 2005) and topoisomerase II inhibitor (Asami et al., 2002)].

Insect germline transformation is routinely accomplished through the microinjection of a DNA plasmid containing a transposon of interest and an accompanying transposase source (usually provided via a helper plasmid or as messenger RNA) into a partially desiccated pre-blastoderm stage embryo, while the embryo is still a syncytium. A fraction of transposition events that occur will insert into chromosomes of the presumptive germline, potentially resulting in heritable transformation. The transgene can then be passed to offspring, with progeny containing the transgene insertion in all diploid cells. A larger portion of injected embryos will experience no transposition, or transposition of the transgene into chromosome structures destined to be incorporated into somatic tissue; these somatically transformed individuals do not pass the transgene onto their progeny. Transient transgene expression in injected individuals indicates the presence of the injected plasmid in the insect, but does not necessarily imply either somatic or germline transformation.

For these studies the *piggyBac* transposon system was used (see *Section 1.3.2.*). If precise transposition is achieved, which is typical but not universal for *piggyBac*, only the portion of the vector plasmid contained within the *piggyBac* TIRs should integrate into the

host genome. The other vector plasmid sequences, including a selectable antibiotic resistance marker, and the helper plasmid containing a gene coding for the transposase, should be lost during subsequent insect development (Hoy, 2003).

The pseudo-random nature of *piggyBac* insertion leaves the expression of the contained transgene vulnerable to the positioning effects of surrounding genomic DNA sequences; that is, the level, spatial and/or temporal pattern of transgene expression can vary among individual transformants with different insertion sites, that have the same transgene copy number (van Leeuwen et al., 2001). The majority of position effects reflect the action of proximal silencer or enhancer elements (Wilson et al., 1990). Development of a successful transgenic strain therefore usually involves an analysis of transgene expression from several insertion lines, and the selection of lines that are most appropriate for purpose.

In this chapter I describe the transformation of the olive fly with OX3097, and the development of the first sexing strain for the olive fly. Furthermore, I describe the testing of transgenic strains for penetrance and repressibility of the OX3097 female-lethal phenotype, molecular analysis of sex-specific expression, sequencing of transgene-flanking genomic DNA, candidate strain homozygosis, and the RFLP-PCR screening of the homozygous strain for an organophosphate-resistance associated point mutation.

2.2. Results and discussion

2.2.1. Transformation with OX3097 and strain assessment

The OX3097 construct (Fu et al., 2007) was micro-injected into 4,500 pre-blastoderm stage olive fly embryos of the Democritus wild-type strain (Athens, Greece). 2,500 of the embryos were injected with the OX3097 plasmid and *piggyBac* mRNA, and 2,000 were injected with the OX3097 plasmid and OX3022, a *piggyBac* DNA helper plasmid. Of 2,000 embryos injected with OX3097 and *piggyBac* mRNA, approximately 550 survived to the L1 larval stage. This resulted in 113 pupae and a total of 67 adults were recovered (44 males and 23 females) (3.4% egg to adult survival). Approximately 17% of these pupae displayed some level of transient DsRed2 expression, confirming the presence of the OX3097 plasmid in those individuals. Of 2,500 embryos injected with OX3097 and the OX3022 helper plasmid, approximately 450 survived to the L1 larval stage, resulting in 90 pupae and a total of 71 adults (37 males and 32 females) (2.8% egg to adult survival). Of these pupae, approximately 11% displayed some level of transient DsRed2 expression (**Table 2.1**).

Injection survivors were back-crossed to wild-type flies in small pools, and the G_1 progeny were screened for expression of the DsRed2 fluorescent marker. Four independent genomic insertion lines (transgenic lines OX3097A-Bol, OX3097B-Bol, OX3097C-Bol, and OX3097D-Bol) were recovered from injections using *piggyBac* mRNA (a transformation efficiency of 6%) and two independent genomic insertions (transgenic lines OX3097E-Bol and OX3097F-Bol) were recovered from injections with the *piggyBac* helper plasmid (a transformation efficiency of 2.8%) (transformation efficiency is defined as the number of independent lines obtained per G_0 adult).

The Democritus olive fly strain was used for the initial transformation with OX3097, as a high level of laboratory adaptation has given it favourable characteristics for the labour intensive process of insect microinjection (e.g. high fecundity and good larval

survival on artificial diets). However, this strain also suffers from some drawbacks of laboratory adaptation (discussed in *Section 1.2.3.*), potentially compromising its application to field release. With the availability of the more recently developed Argov hybrid strain (Israel, 2007), introgression of the OX3097 insertion lines into the Argov wild-type genetic background was performed. Males and females of the OX3097 olive fly insertion lines were out-crossed to Argov males and females for five generations ensuring introgression of the transgene into the nuclear and mitochondrial genomes of the Argov background. Five generations of out-crossing were performed constituting a theoretical >95% introgression. Refreshing the genetic diversity of laboratory/mass-rearing colonies is an important part of maintaining strain viability for SIT release, and is used routinely in the control programs of Tephritid species (Briceno& Eberhard, 1998; Carey et al., 2008). All subsequent discussions of transgenic lines and wild-type flies will refer to lines post-introgression to the Argov wild-type background, unless otherwise stated.

In order to assess OX3097 copy number in each insertion line, heterozygous males and females from each line were crossed to wild-type insects, and the progeny were collected in the presence of tetracycline (100µg/ml). Pupae were screened and scored for the presence or absence of the fluorescent protein marker (**Table 2.1**).

Table 2.1. Results from the microinjection of wild-type olive fly embryos with the transgene construct OX3097 either with *piggyBac* provided as mRNA or on a DNA helper plasmid. Table shows the percentage survival to adulthood of the injected embryos, the number of stable germ-line transformations, and the numbers of wild-type and transgenic OX3097 progeny resulting from back-crosses to wild-type with the progeny collected in the presence of tetracycline (100µg/ml). The *P* values are shown of a Pearson's Chi Squared test comparing the observed ratio with a theoretical 1:1 ratio expected from the segregation of a single insertion of the OX3097 transgene. An asterisk next to a *P* value indicates a statistically significant difference.

***piggyBac* source (number of embryos injected)**	**% embryo to adult survival**	**Number of OX3097 lines (% transformation efficiency)**	**OX3097 Lines**	**Number of wild-type progeny G1+G2 (%)**	**Number of transgenic progeny G1+G2 (%)**	**Pearson's Chi Square Test (1 d.f.)**
mRNA (2,000)	3.4	4 (6)	OX3097A	101 (43)	135 (57)	0.225
			OX3097B	185 (53)	164 (47)	0.595
			OX3097C	226 (51)	218 (49)	0.871
			OX3097D	204 (52)	187 (48)	0.698
DNA helper (2,500)	2.8	2 (2.8)	OX3097E	100 (33)	203 (67)	0.002**
			OX3097F	227 (55)	185 (45)	0.359

Crossing of transgenic and wild-type insects produced transgenic to wt progeny ratios not significantly differing from a 1:1 ratio expected from the segregation of single transgene insertion in five of the lines. However, line OX3097E-Bol produced 67% transgenic progeny, showing a statistically significant deviation from a 1:1 ratio (P = 0.002, 1 d.f., Chi Square), suggesting the presence of multiple transgene insertions.

Single insertion lines were assessed for penetrance and repressibility of the female-specific lethal phenotype by crossing heterozygous transgenic males to wild-type females, and rearing the progeny in the presence (on-tet) or absence (off-tet) of the transgene repressor, tetracycline (added as a supplement to the larval diet). Insects were reared, allowed to pupate, and screened approximately 48 hours post-pupation. Upon eclosion the transgenic adults were sexed, and the numbers of males and females were scored (**Fig. 2.2**) (**Table 2.2**).

Table 2.2. Strains OX3097A-D & F are five insertion lines of OX3097 in olive fly. From their history and inheritance pattern the OX3097D insertion lines appear to represent independent autosomal insertions of the OX3097 construct. Transgene penetrance and repressibility were assessed by crossing heterozygous males of each strain to virgin wild-type (WT) females. The sex ratio of adult progeny expressing the DsRed2 fluorescent marker is shown for each strain compared with wild-type (WT) progeny. Table also shows *P* values from a Pearson's Chi Square test comparing the observed sex ratio with the relevant (on or off-tet) sex ratio observed in the WT strain. An asterisk next to a *P* value indicates a statistically significant difference.

Strain	On-tet			Off -tet		
	♂ Adults (%)	♀ Adults (%)	Pearson's Chi Square Test (1 d.f.)	♂ Adults (%)	♀ Adults (%)	Pearson's Chi Square Test (1 d.f.)
OX3097A	157 (60)	105 (40)	0.008**	172 (100)	0 (0)	$2.2e^{-16}$ ***
OX3097B	108 (57)	81 (43)	0.064	155 (74)	54 (26)	$6.4e^{-5}$ ***
OX3097C	162 (52)	150 (48)	0.371	146 (100)	0 (0)	$2.2e^{-16}$ ***
OX3097D	198 (49)	206 (51)	0.811	123 (100)	0 (0)	$2.2e^{-16}$ ***
OX3097F	172 (54)	147 (46)	0.174	238 (74)	83 (26)	$5.3e^{-10}$ ***
WT	109 (48)	118 (52)		237 (52)	218 (48)	

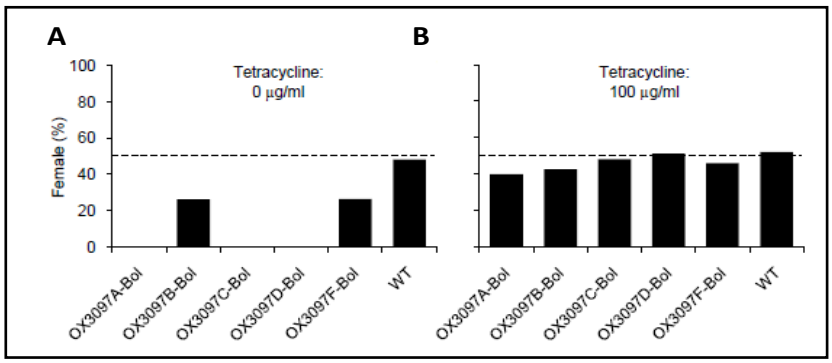


Figure 2.2. (**A**) Penetrance and (**B**) tetracycline repressibility of female lethality in the five OX3097 olive fly lines. Penetrance and repressibility of female-specific lethality was assessed by crossing heterozygous males of each strain to virgin wild-type (WT) females, and collecting eggs on filter paper saturated with water containing either 0µg/ml tetracycline or 100µg/ml tetracycline, and rearing on larval diet produced with water containing either 0µg/ml tetracycline or 100µg/ml tetracycline.

The five independent insertion lines of the OX3097 transgene in olive fly displayed a range of phenotypes suggesting different levels of expression of the transgene. As transgene copy number appears to be the same in each line, the differences in expression strength are likely to be due to chromosomal position effects. As seen here, chromosomal position can significantly affect the engineered phenotypes resulting from an inserted transgene. The OX3097 construct is relatively short and does not contain insulating elements; the gene promoters contained within are therefore vulnerable to influence from any genomic regulatory elements acting at the insertion site. While all OX3097 insertion lines gave statistically significant levels of female-lethality compared to a wild type control when reared in the absence of tetracycline, only lines OX3097A-Bol, OX3097C-Bol, and OX3097D-Bol showed fully penetrant female specific lethality; that is, they produced no female progeny off-tet in this assay. Conversely, lines OX3097B-Bol and OX3097F-Bol, achieved only partial female-lethality when reared off-tet. It is possible that 100% female lethality could be obtained in these lines when female progeny are homozygous for the OX3097D-Bol insertion, which would make them candidates for a sexing strain (with radiation sterilisation forming part of the downstream processing). However, as all progeny in the field mating with a released fsRIDL male would be heterozygous for the transgene, fsRIDL lines require as close to 100% dominant female lethality as possible.

Only insertion line OX3097A-Bol showed a statistically significant deviation from the sex ratio of the wild-type control when reared in the presence of tetracycline (P = 0.008, d.f. 1, Chi Square), suggesting that the female-lethal trait was not fully repressed in this line at this concentration of tetracycline. Full repressability of the female-lethal trait is important as large numbers of females will be required in a mass-rearing facility to produce sufficient males for release. Any problems associated with female survival could

seriously compromise mass-rearing efficiency and also potentially lead to selection for loss of the trait. Consistent with transgenic strains using the OX3097 construct in medfly (Fu, et al., 2007) and mexfly (Kirst Stainton, personal communication), female-specific lethality in the olive fly OX3097 lines occurred at the late larval/early pupal stage.

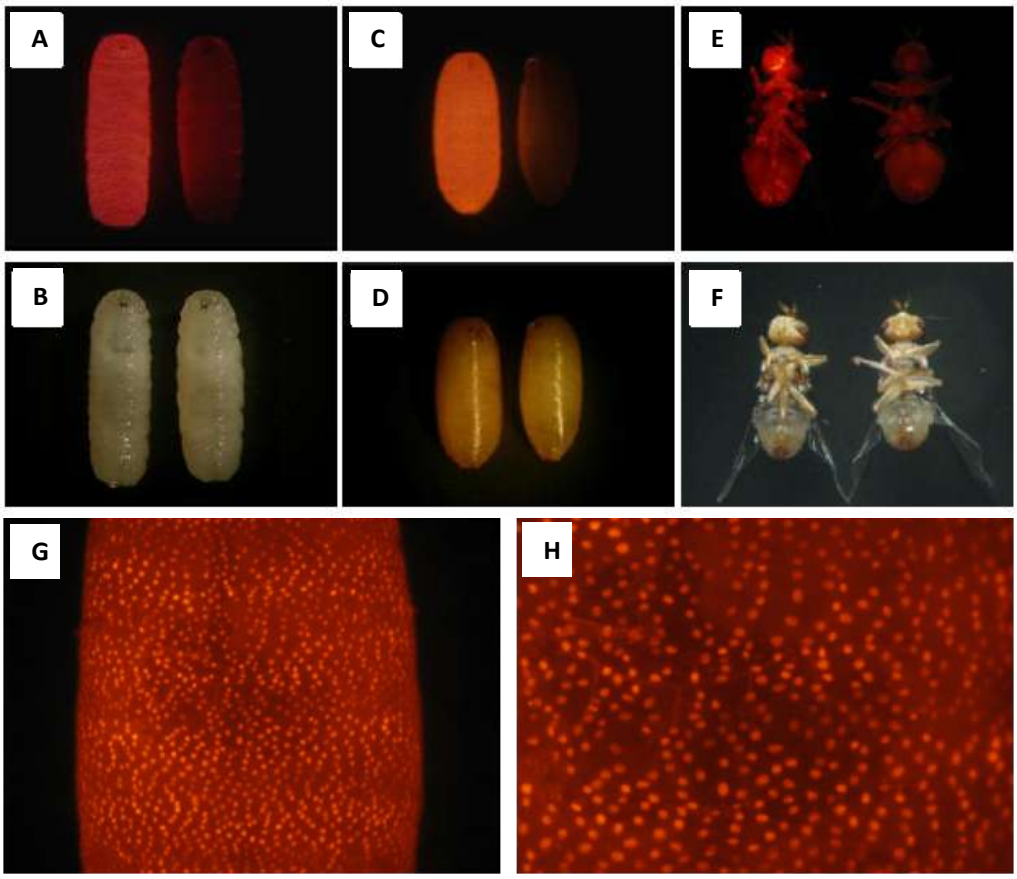


Figure 2.3. Fluorescence microscopy allows discrimination of OX3097D-Bol from wild type at larval, pupal, and adult stages. Photomicrographs of OX3097D-Bol and wild-type olive flies under (upper panels) fluorescence and (lower panels) bright-field illumination. Each panel shows OX3097D-Bol to the left and wild-type to the right: OX3097D-Bol and wild-type (**A**,**B**) larvae, (**C**,**D**) pupae, and (**E**,**F**) adults are shown. Expression of DsRed2 is clearly visible all over the OX3097D-Bol larvae and pupae, and in areas of less opaque cuticle (for example, the labellum, upper thorax, leg joints, and anus) of OX3097D-Bol adults. (**G**, **H**) Nuclear localisation of the DsRed2 fluorescent protein resulting from nuclear localisation sequences attached to the 5' and 3' ends of the fluorescent protein DNA sequence. This localisation causes fluorescent DsRed2 protein to accumulate in the nucleus of each expressing cell, producing a distinctive dotted fluorescence phenotype which helps distinguish fluorescent protein expression from background auto-fluorescence that is sometime visible.

All lines expressed the nuclear localised DsRed2 fluorescent protein marker throughout all life stages. Expression was bright and transgenic individuals were easily

distinguishable from wild-type siblings (**Fig 2.3**), although in adult flies, an investigator is required to search for fluorescence in the regions where the fly's cuticle is less opaque (i.e. the joints of the legs, the labellum, the upper thorax and the anus) (**Fig. 2.3E**). However, the expression intensity differed between different insertion lines. This is not surprising as it has been found that while the expression levels of transgenes at different chromosomal locations may vary greatly, the expression pattern consistently resembles the expression pattern of its endogenous counterpart; that is, the quantity of transcripts in normally expressing tissues may vary considerably, but the expression pattern usually does not show qualitative changes (Wilson et al., 1990).

The intensity of the DsRed2 fluorescence was found to correlate positively with levels of female-lethality penetrance: OX3097A-Bol gave very strong expression of the fluorescent marker and showed incomplete repressibility of the female-lethal trait, while OX3097F-Bol and OX3097B-Bol showed relatively weak expression of DsRed2 and gave incomplete penetrance of the female-lethal trait. Consistent with this trend, OX3097C-Bol and OX3097D-Bol showed intermediary levels of DsRed2 expression, and female-lethal penetrance and repressibility traits that suggest intermediate levels of tTAV production. However, of these two insertion lines, OX3097D-Bol displayed stronger expression of the DsRed2 marker. This suggests that OX3097D-Bol females reared off-tet may also be expressing tTAV at higher levels than OX3097C-Bol females reared off-tet; female-lethality in the OX3097D-Bol insertion line may therefore be more robust.

Both OX3097D-Bol and OX3097C-Bol gave 100% female-specific lethality off-tet and full repression of the lethal trait on-tet, optimal for the development of an fsRIDL strain. These two insertion lines were therefore chosen for further analysis and development.

2.2.2. Transgene-flanking genomic DNA sequencing

Obtaining transgene-flanking genomic DNA sequences can be useful and informative for several reasons. Firstly, flanking sequences can be used to design DNA primers for genotyping PCR procedures that can be used in conjunction with internal transgene primers to determine the zygosity of a transgenic insects for the transgene insert. This is useful in the development of homozygous transgenic strains (see *Section 2.2.3.*), and for monitoring insects in the field. Secondly, sequencing across the junction between inserted and genomic DNA can help determine whether transgene integration was indeed a canonical *piggyBac*-mediated event. Finally, flanking sequences can be used to search for homology with the sequenced genomes of related species, which may provide information on: the expected chromatin state of proximal sequence, flanking sequence variability between individuals (an important consideration when designing primers from flanking sequences), and the functionality of sequences that may have been disrupted by transposition.

Transgene flanking genomic sequences from both the OX3097C-Bol and OX3097D-Bol insertion lines were obtained by adaptor-mediated-PCR (see *Appendix 2* for full sequences). Flanking sequence accuracy was confirmed by PCR, using primers designed from transgene-flanking sequences in conjunction with internal transgene primers, with amplicon lengths matching predictions. 5' and 3' flanking sequences from both OX3097C-Bol and OX3097D-Bol insertion lines showed TTAA target site duplication, strongly suggesting *piggyBac* vector-mediated transposition, as opposed to random recombination events (**Fig. 2.4**). *piggyBac* vector integrations have been shown to be stable in a variety of tephritid species (Condon et al., 2007; Handler et al., 1998; Handler& Harrell, 2001; Handler& McCombs, 2000).

OX3097D-Bol and OX3097C-Bol flanking sequences were web-BLAST searched against the *D. melanogaster* genome (the insect with the shortest evolutionary distance from *B. oleae* with an available genome sequence); however, no significant similarities were found, perhaps suggesting insertions are in variable genomic regions.

OX3097C	*TTCTTGCC***TTAA<INSERT>TTAA***CCGTCCTA*
OX3097D	*CATTTCTG***TTAA<INSERT>TTAA***ATAACAGC*

Figure 2.4. Immediate transgene-flanking genomic DNA sequences obtained via adaptor-mediated-PCR for lines OX3097C and OX3097D in olive fly, showing duplication of *piggyBac* TTAA target site. (Full flanking sequences are given in Appendices.)

2.2.3. Strain selection and homozygosis

To ensure the development of a pure-breeding OX3097 olive fly line, PCR-based genotyping methods were used to identify homozygous OX3097 progeny resulting from double heterozygous crosses. It is important to be confident that a fully homozygous strain is obtained as a wild-type genotype, even at low initial frequencies, will be likely to spread through a population. The speed of wild-type allele spread through a transgenic colony will be proportionate to the relative fitness costs of the transgenic genotype compared to the wild-type genotype, but even relatively minor costs may become significant when a strain is reared over many generations. This problem is somewhat compounded by the fact that the strains carry a dominant fluorescent marker, meaning that a wild-type allele may increase in frequency to significant levels before homozygous wild-type individuals are noticed through routine screening for the fluorescent marker. This could result in the release of heterozygous or wild-type flies in a release programme and could therefore compromise a control effort. The release of heterozygous insects can be costly to a RIDL release programme, as half of the daughters from a heterozygous male x wild female mating would be fully viable (this issue is discussed in greater detail in *Chapter 6*).

It is possible that the levels of transgene repressibility observed in heterozygous females of a given line may not be retained when a strain is made homozygous, and is producing transgene product from two genomic loci. Additional homozygous lethality could result from (i) an increased dosage of any unrepressed and deleterious transgene products, (ii) through the homozygosis of recessive-lethal mutations brought about by the disruption of endogenous sequences by insertion of the transgene, or (iii) via the homozygosis of transgene linked overdominant genes. As the production of a homozygous strain is a relatively time-consuming and labour-intensive process, the heterozygous lines were initially tested to determine whether any additional non-repressible lethality was observable in homozygotes. Heterozygous transgenic males and females from each insertion line were crossed, eggs were collected and larvae were reared on-tet. The ratio of emerging transgenic to wild-type adults were compared (**Table 2.3**). Any homozygous inviability would manifest as a greater proportion of wild-type than the 1:3 (wild-type to transgenic) expected from the segregation pattern of an innocuous dominant marker.

Table 2.3. Homozygous viability in 5 OX3097 olive fly single insertion lines. Flies were crossed in pools of 5 heterozygous males with 10 heterozygous females. Eggs were collected and reared on-tet. Adults were screened for fluorescence 48 hours post-eclosion. Numbers and percentages of emerged wild-type and transgenic flies are shown. The ratios between the numbers of transgenic and wild-type adults were compared against a theoretical 3:1 segregation pattern expected for a dominant phenotype with no effects on developmental viability using a Pearson's Chi Square Test (1 d.f.). An asterisk next to a *P* value indicates a statistically significant difference.

Line	Transgenic (%)	Wild-type (%)	*P* value from Pearson's Chi Square Test (1 d.f.)
OX3097A	251 (58)	182 (42)	0.002**
OX3097B	219 (70)	94 (30)	0.333
OX3097C	201 (73)	74 (27)	0.711
OX3097D	383 (74)	135 (26)	0.824
OX3097F	200 (74)	70 (26)	0.856

Insertion line OX3097A-Bol produced ratios of transgenic to wild-type adults that differed significantly from the expected 3:1 ratio ($P = 0.002$, d.f. 1, Chi Square). This was expected as non-repressibility of the transgene was observed even in on-tet heterozygous

females (see *Section 2.2.1*). All other insertion lines produced ratios consistent with full homozygous repressibility of the transgene.

The main selection criteria used to determine which insertion line to develop to homozygosis are shown in the Venn diagram in (**Fig. 2.5**). As position effects can significantly affect transgene expression, several insertion lines of OX3097 were initially tested, with the expectation that some lines would give suboptimal expression levels. As OX3097C-Bol and OX3097D-Bol both satisfied the three major selection criteria, and of these two insertion lines OX3097D-Bol showed the clearest expression of the DsRed2 fluorescent marker, it was favoured for homozygosis and further analysis.

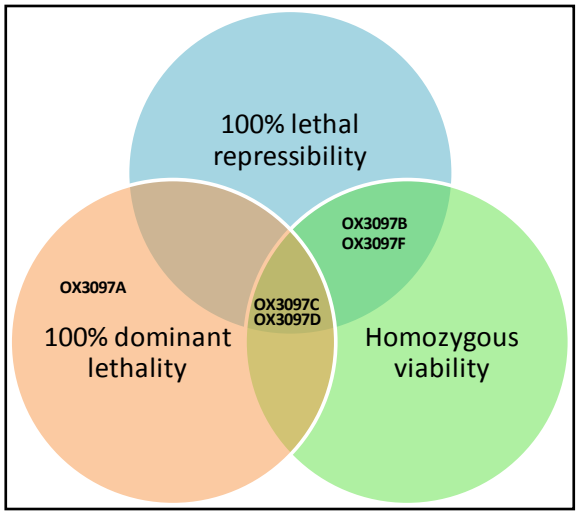


Figure 2.5. Venn diagram illustrating considerations taken into account when considering which OX3097 insertion line would be chosen for further development. OX3097A provided 100% female-lethality off-tet, however, this was not fully repressible. OX3097B and OX3097F both had full homozygous viability and tet-repressibility, but did not provide fully penetrant dominant lethality off-tet. OX3097C and OX3097D satisfied all three criteria.

To make the OX3097D-Bol insertion line homozygous, a genotyping PCR was used based on primers designed from transgene-flanking genomic DNA sequences. Primers were designed such that a forward primer from the upstream genomic sequence would amplify a fragment of a given length (*x*) with a reverse primer specific for a sequence internal to the transgene construct, and a fragment of length (*y*) with a reverse primer specific to the downstream genomic sequence (**Fig. 2.6A** & **2.6B**). Therefore, if both

primer combinations were used in a multiplex PCR, a homozygous individual (with a transgene insert on homologous chromosomes) produced amplicons of length *x* only, a heterozygous individual with one transgenic and one wild-type chromosome produced two amplicons of lengths *x* and *y*, and a wild-type individual produced amplicons of length *y* only (**Fig. 2.6C**).

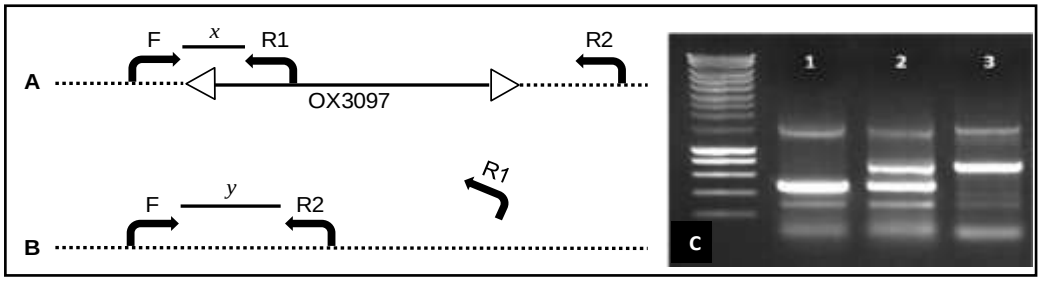


Figure 2.6. (**A**) Schematic representation of a chromosome (dotted line) containing an OX3097 transgene insert. The forward genomic primer (F) amplifies a fragment of length *x* (450bp) with reverse primer 1 (R1 internal construct primer). Distance between F and R2 is too great to yield an amplification product. (**B**) Schematic representation of a wild-type chromosome (dotted line). The forward primer amplifies a fragment of length *y* (750bp) with reverse primer 2 (R2). R1 is unable to bind as it is specific for an internal transgene sequence. (**C**) Agarose gel photo from a multiplex PCR of (**1**) homozygous OX3097D olive fly genomic DNA amplifying a band of length *x*; (**2**) heterozygous OX3097D olive fly genomic DNA amplifying bands of lengths *x* and *y*; (**3**) wild-type olive fly genomic DNA amplifying a band of length *y*. Left-most lane shows DNA size standards. Sizes are as follows: from bottom, 200bp increasing in 200bp increments until 1000bp, then: 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, and 10000bp (Eurogentec Smartladder).

Over 100 male and 100 female heterozygous OX3097D-Bol individuals were crossed in a single pool and the resulting transgenic progeny were reared on-tet. A leg non-essential to mating (see *Section 2.4.10*) was removed from each resulting adult and was genotyped in a multiplex PCR as described in (**Fig. 2.6**). 28 female and 22 male olive fly were identified as putative homozygous candidates, and single crosses of males and females (18 cages of 1:1 male to female and 4 cages of 1:2 male to female) were set-up. Due to mortality or inability to mate/oviposit only 13 of the cages produced viable eggs. Eggs collected from each cage were crossed only with siblings for three successive generations. After three generations no cages had produced any wild-type individuals and

the third generation offspring were pooled. The homozygous OX3097D-Bol strain was thereby generated from a founding 13 males and 15 females.

A re-testing of lethality traits in the homozygous OX3097D-Bol strain showed no significant differences in female-lethal penetrance ($P > 0.9$, d.f. 1. Chi Square) and repressibility ($P = 0.228$, d.f. 1. Chi Square) to those observed for the heterozygous line (**Table 2.4.**), confirming the homozygous viability of the strain.

Table 2.4. Comparing female-lethal repressibility (on-tet) and penetrance (off-tet) of the OX3097D-Bol insertion in heterozygous (Het) and homozygous (Hom) lines. Homozygous results are from crosses of homozygous OX3097D-Bol males crossed to homozygous OX3097D-Bol females. Heterozygous results are from crosses between homozygous OX097D-Bol males and wild-type females. Percentages are shown in brackets. The ratios of heterozygous and homozygous males and females are compared statistically both on and off-tet using a Pearson's Chi Square test (1 d.f.).

	On-tet		Off-tet	
	Males	**Females**	**Males**	**Females**
OX3097D-Bol Het	198 (49)	206 (51)	123 (100)	0 (0)
OX3097D-Bol Hom	272 (53)	241 (47)	212 (100)	0 (0)
***P* value (Chi Square)**	0.228		>0.9	

A sufficient level of genetic diversity in a strain is important to ensure the effects of inbreeding depression are minimised. It is likely that laboratory populations of wild-type insects already have a limited genetic diversity due to bottlenecking effects during colonisation and the development of a homozygous strain with a low number of founding individuals may further compound this issue. It is unclear how many founders are sufficient to provide enough genetic diversity so that inbreeding depression will not be a concern in the homozygous OX3097D-Bol line. This depends on factors including the genetic diversity of the original colony from which the line was derived, and the degree of relatedness as well as number of the founder individuals. Analysis of the life-history characteristics and mating competitiveness of the strain should give an indication of how well the strain is likely to perform in rearing and release, and so can be used to judge whether the genetic diversity is adequate. Investigation of inbreeding depression by

(Saccheri et al., 1999) in the butterfly *Bicyclus anynana*, suggested that a founder population of at least ten unrelated pairs should be sufficient to avoid complications in that species. More than ten pairs were used to initiate the homozygous OX3097D-Bol strain, however, the degree of relatedness of the founder individuals of the homozygous OX3097D-Bol line is unknown, as indeed, is the level of genetic diversity the Argov wild-type colony.

2.2.4. *Cctra* alternative splicing and tTAV lethality in olive fly

The female-specific lethality observed off-tet in the OX3097 olive fly lines strongly suggests appropriate splicing of the *Cctra* intron (i.e. the production of a female-specific tTAV transcript corresponding to complete splicing of the *Cctra* intron). This was expected due to high sequence and exon/intron boundary similarity observed for the endogenous *Bactrocera oleae transformer* (*Botra*) gene (Lagos et al., 2007). To confirm that the use of male and female-specific alternative splice sites were as expected, the amplification of male and female spliced tTAV transcripts across the *Cctra* intron region was performed by RT-PCR. Female medfly splice the *Cctra* intron to produce three splice variants (F1, M1 and M2), males however, produce only the M1 and M2 variants (Pane et al., 2002; Gabrieli et al., 2011) (**Fig. 2.7A**).

Males and females of the OX3097D-Bol strain were initially reared on-tet until the adult stage. At this point all sources of tetracycline were removed, allowing the over-expression and cellular accumulation of tTAV transcripts (in females). An RT-PCR was performed on OX3097D-Bol using primers amplifying across the *Cctra* intron region. Results showed that the *Cctra* intron spliced to generate three different transcripts of tTAV in olive fly, one of which was only produced in females (F1) (**Fig. 2.7C**). Sequencing the

transcripts revealed that the F1 transcript was the only variant encoding tTAV without some intervening *Cctra* intronic sequence.

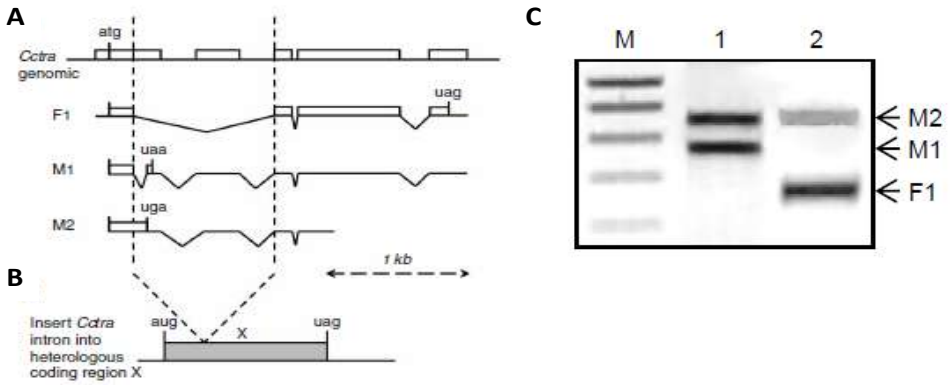


Figure 2.7. (**A**) Schematic of alternative splicing of medfly *transformer* gene. Three splice variants (F1, M1 and M2) of the intron produced by female medfly. Males, however, produce only M1 and M2 variants. The F1 variant corresponds to a complete splicing of the intron and a reconstitution of the open reading frame. M1 and M2 variants incorporate in-frame transcription termination codons (indicated). (**B**) The intron was inserted into the tTAV coding region for the purpose of sex-specific gene regulation. (Diagram from Fu et al., 2007). (**C**) Products of alternative splicing of Cctra:tTAV in (lane 1) male and (lane 2) female OX3097D-Bol olive flies. Three splice variants were detected, corresponding to Cctra transcripts M1, M2 and F1 (identity confirmed by sequencing). Only females produce the F1 splice variant, corresponding to the reconstitution of the tTAV open reading frame and leading to production of functional tTAV. Lane M shows DNA size standards: 200-1,000 bp in 200-bp increments (Eurogentec Smartladder).

Only female olive fly were found to produce the F1 transcript; males produced two longer transcripts (M1 and M2), incorporating in-frame transcription termination codons. The bands were isolated and sequenced, and confirmed that intron splicing was as predicted.

Analysis of the olive fly OX3097D-Bol transgene-derived transcripts indicated that the alternative splicing pattern of the *Cctra* intron is equivalent to that in native medfly context (Pane et al., 2002), and suggests that this *tra* intron is functionally highly conserved. To the my knowledge, the olive fly is now the fifth dipteran species in which a medfly *tra* intron has been successful in driving female-specific expression of tTAV; the others being the medfly (Fu et al,. 2007), *D. melanogaster* (Fu et al., 2007), the Caribbean

fruit fly, *Anastrepha suspensa* (Schetelig& Handler, 2012), and the mexfly (Kirsty Stainton, personal communication).

The lethal effects of the OX3097 construct in olive fly are not observed until the late larval/early pupal stage of development, meaning that the developing larvae are able to survive for almost 2 weeks with no external supply of tetracycline before succumbing to lethal levels of tTAV. This was also observed in OX3097 lines of the medfly (Fu et al., 2007) and mexfly (Kirsty Stainton, personal communication). It is desirable, however, for lethality to act as early as possible. Moribund female larvae resulting from a transgenic male/wild female matings may still cause crop damage in the field if the lethal trait acts after (or during) the larval stage. Moreover, during mass-rearing early female lethality would allow the rearing of more males for release in a given space, with a given amount of material resource. A question of interest therefore concerns the rate limiting factor of the lethal mechanism in the OX3097 construct. The following are a series of potential explanations for the late-acting larval lethality seen in the OX3097 lines.

Firstly, the persistence of maternally contributed tetracycline in the developing embryo/larva suppressing the accumulation of tTAV. During normal colony rearing, OX3097 olive fly adults are maintained on a water source containing 100µg/ml tetracycline in order to suppress tTAV throughout their life-time. It is therefore possible that parentally contributed tetracycline could cause a delay in tTAV-induced lethality in their progeny. However, results from our laboratory suggest that a wide range of concentrations of tetracycline (as low as 1µg/ml) provided to OX3097D-Bol adults have no noticeable temporal effects on the action of the transgene in their progeny; that is, at this low parental dose of tetracycline death of progeny is still observed at the late larval/early pupal stage. It is therefore unlikely that sufficient parentally derived

tetracycline is transferred to the offspring to significantly delay the action of lethality in the OX3097 transgene at 100µg/ml.

Secondly, late initiation of *Cctra* intron splicing delays the initiation of functional tTAV expression in females. Male and female secondary sexual characteristics are not visible until the adult stage in tephritid, it therefore seems plausible that sexual differentiation gene cascades may not initiated until the pupal stage, when holometabolism results in the formation of the distinctive male and female forms. However, the presence of maternally derived *Cctra* transcripts have been described in medfly embryos, and it is believed that the resulting proteins drives female-specific splicing of zygotically transcribed pre-mRNAs in XX embryos; (in XY embryos *Cctra* autoregulation is impaired by a hypothesised male determining '*M*' factor on the Y chromosome) (Pane et al., 2002). Moreover, recent papers by (Schetelig& Handler, 2012) and (Ogaugwu et al., 2013), describe the development of embryonic lethal strains of the Caribbean fruit fly, *Anastrepha suspensa* and medfly using a *Cctra* intron, respectively.

Thirdly, there is a tTAV build-up lag. If this is the case, and there is some critical threshold of tTAV below which developing transgenic insects are able to survive, and above which they are not, it may be possible to include two more tTAV elements in a construct, allowing the reaching of this threshold more quickly. However, evidence from the olive fly OX3097 insertion lines presented here suggests that this interpretation may be overly simplistic. The OX3097A-Bol insertion line displayed very strong expression of the DsRed2 marker and non-repressible fully dominant female lethality, while OX3097B-Bol and OX3097F-Bol displayed weak expression of the fluorescent marker and incomplete female lethal penetrance. This suggests that transgene expression strength is varying considerably between insertion lines. Nonetheless, lethality in all lines analysed was found to be temporally similar (at the larval/pupal transition), regardless of apparent expression

strength. Therefore, expression strength does not seem to affect time-of-death, but does impact strongly on lethality penetrance and repressibility; increases in expression may reduce repressibility to an impractical level before causing earlier acting female lethality.

Finally, lethality occurs at the larval/pupal transition as this stage is more susceptible to the effects transcriptional squelching. This may be due to a greater general requirement for basal transcriptional machinery during the stages of holometabolism, which are sequestered by the positive feedback mechanism of tTAV (Natesan et al., 1997).

It therefore seems possible that OX3097 insertion lines produce sex-specific tTAV at an early stage, and could be crossed to transgenic lines carrying a tetracycline operator (tetO) sequence linked to a strong effector. (Schetelig& Handler, 2012) recently used *Cctra* alternative splicing in *A. suspensa* to produce sex-specific tTAV which was used to drive a strong pro-apoptotic effector gene (hid^{Ala5}), and thereby generating a embryonic lethal sexing strain.

2.2.5. RT-PCR of *doublesex* in OX3097D-Bol

The sex-specific effects of the build-up of tTAV in OX3097D-Bol females can be visualised in the insect in two ways. Firstly, through the unique pattern of tissue necrosis observable at the larval/pupal transition. This is clearly visible in approximately 50% of the OX3097D-Bol larvae prior to pupation when reared off-tet. Secondly, through the concomitant over-expression of the *tTAV*-adjacent DsRed2 marker. Lethality caused by tTAV over-expression also results in a pinkish hue in the dead/moribund larvae, possibly a result of the over-expression of the DsRed2 fluorescent marker in these individuals.

Interestingly, female larvae raised off-tet can be easily distinguished from male larvae of the same batch even before onset of tissue necrosis (as early as the 3rd larval instar). The intensity of expression of the DsRed2 fluorescent transformation marker is

highly upregulated in the developing female larvae. (In all other respects they appear morphologically and behaviourally normal.) TetO is a bi-directional promoter; positive feedback of tTAV causes activation of tetO, resulting in activation of both upstream and downstream genes (in this case DsRed2 and tTAV, respectively). The over-expression of the fluorescent marker, and the distinct pattern of tissue necrosis is hypothesised to be a result of tTAV accumulation and therefore should be associated with female insects only.

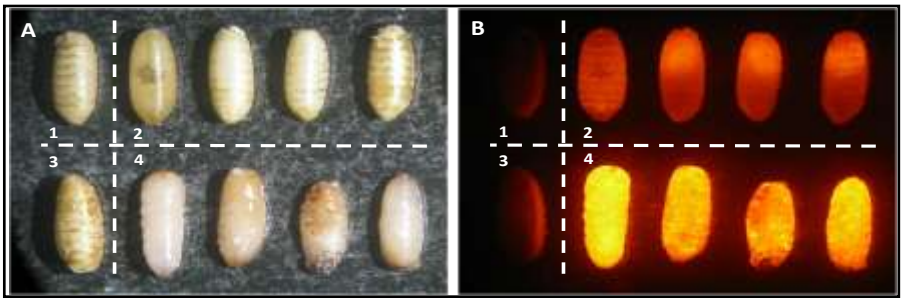


Figure 2.8. Photomicrographs of OX3097D and wild-type olive fly pupae raised off-tet under (**A**) visible light, and (**B**) DsRed2 excitation light. (**1**) Shows a typical wild-type male pupa. (**2**) shows typical off-tet male OX3097D pupae. (**3**) shows a typical wild-type female pupa. (**4**) shows typical off-tet female OX3097D pupae. The sex of female pupae were confirmed by an RT-PCR analysing sex-specifically spliced *dsx* transcripts. The sex of the male pupae were confirmed by eye upon adult eclosion. Tissue necrosis is clearly visible in the female off-tet pupae. Upregulation of the DsRed2 fluorescent marker is also expected in females when raised off-tet. This is due to the bi-directionality of the tetO promoter, upregulation of which during positive feedback accumulation of tTAV also causes over-expression of the adjacent downstream DsRed2 gene. These photomicrographs may suggest that the increased fluorescence of the OX3097D female pupae is due to the increased auto-fluorescence of necrosed tissue; however, similarly increased DsRed2 fluorescence is also clearly visible in healthy-looking, early-stage female OX3097D larvae.

The *doublesex* (*dsx*) gene occupies a key position at the bottom of the sexual-differentiation sexual differentiation gene cascade and is alternatively spliced in males and females (**Fig. 2.9**) (Lagos et al., 2007; Burtis& Baker, 1989). In order to confirm that the observed tissue necrosis and DsRed2 over expression in OX3097D-Bol are associated with females only, a selection of OX3097D-Bol pupae reared off-tet, and displaying this phenotype were analysed by RT-PCR to determine which sex-specific mode of splicing the olive fly *doublesex* (*Bodsx*) gene was following in these pupae.

The *Bodsx* gene contains six exons that are spliced together to form two sex-specific variants. Primers were designed from the olive fly *dsx* sequence [GenBank ID: 46019688

(male) and 46019686 (female)] so that PCR amplicons would cross alternatively spliced intronic regions.

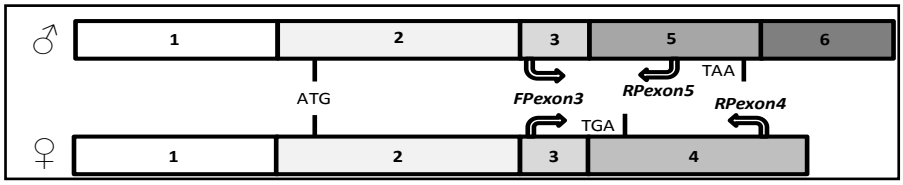


Figure 2.9. Schematic diagram of olive fly male and female *doublesex* mRNA exon configurations. Male mRNAs incorporate exons 1,2,3,5 and 6; while, female mRNAs incorporate exons 1,2,3 and 4. For RT-PCR analysis, both male and female cDNAs bind the *FPexon3* forward primer; while, only males bind the exon 5 specific '*RPexon 5'* reverse primer; and, only females bind the exon 4 specific '*RPexon4*' reverse primer. This RT-PCR can therefore be used to differentiate between genotypic males and females. Diagram also shows male and female ATG start codon and female-specific TGA and male specific TAA stop codons. Adapted from Lagos et al., (2005).

RNA was extracted from one wild-type adult male, one wild-type adult female, and five pupae displaying the distinct lethal phenotype from the OX3097D-Bol line. cDNAs were generated and the primers specific to *Bodsx* exons 3, 4 and 5 were used to determine the sexes of the sampled pupae. Resulting bands from the RT-PCR from OX3097D-Bol cDNAs (**Fig. 2.10**) indicate this phenotype is associated with a female-specific *Bodsx* splicing pattern. A PCR product corresponding to a male-specific splice variant is expected to be 400bp in length; a female-specific splice variant is expected to be 560bp; and a fragment amplified from the genomic sequence is expected to be 700bp long. The male and female-specific bands were sequenced and matched the predicted exonic patterns for sex-specific *Bodsx* mRNA fragments. Since *dsx* is the major determining factor for sexual differentiation, it can be concluded that all the pupae displaying the suspected tTAV lethal phenotype were following a female mode of sexual differentiation.

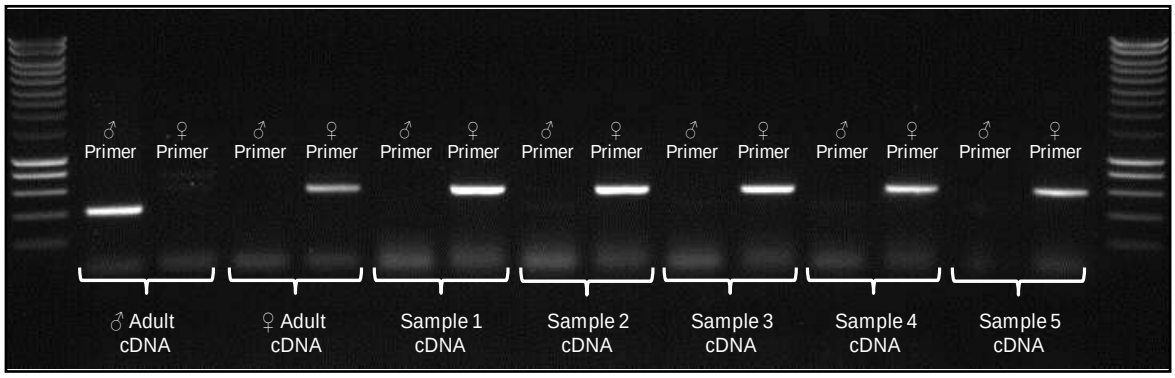


Figure 2.10. Gel photo showing RT-PCR of *Bodsx* mRNAs. For each sample a PCR has been carried out with both male and female specific primer sets. Both adult male and adult female samples gave bands of expected sizes with their respective sex specific primers (the two left-most samples), and no products with the primers of the opposite sex. The five unknown-sex pupae (samples 1-5) each displayed female-specific splicing patterns. No-RT controls were run for each sample but produced no product suggesting no genomic DNA contamination. Left and right-most lanes shows DNA size standards. Sizes are as follows: from bottom, 200bp increasing in 200bp increments until 1000bp, then: 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, and 10000bp (Eurogentec Smartladder).

2.2.6. Testing for organophosphate resistance

Acetylcholinesterase (AChE) is a key enzyme of the nervous system, and is the primary target for organophosphate insecticides which inhibit activity by covalently phosphorylating the serine residue within the enzymes active site gorge (Corbett, 1974). The enzyme is therefore under high selective pressure in areas of organophosphate insecticide use, and point mutations that cause a modification to the kinetic parameters of acetylcholine hydrolysis have been identified as being responsible for insecticide resistance in olive fly (Vontas et al., 2002). The G488S resistance-associated point mutation has been identified in the olive fly AChE gene (*boache*), the frequency of which was found to be high in geographic areas where organophosphates had been used with particular intensity (Hawkes et al., 2005).

As fsRIDL involves the release of insects that are not fully sterile (male offspring are viable), the introgression of alleles from a transgenic strain into a wild population will occur. Most genes from a laboratory background are likely to be at most selectively neutral. However, a resistance-associated mutation introduced into a population under

insecticide spraying is likely to have a strong selective advantage. This could have negative population control consequences, and be unpopular with those that rely on insecticide control.

Conversely, in an area where organophosphates are used widely and the prevalence of the resistance-associated mutations is correspondingly high, a fsRIDL strain that has been shown to contain only the susceptible version of an insecticide target allele could cause the introduction of susceptibility into a wild population through gene flow; mathematical modelling has shown that fsRIDL strategies can form an effective component of a resistance management strategy (Alphey et al., 2007).

The OX3097D-Bol olive fly strain was tested for the presence of the G488S point mutation by a RFLP-PCR method devised by (Hawkes et al., 2005), shown in (**Fig. 2.11**).

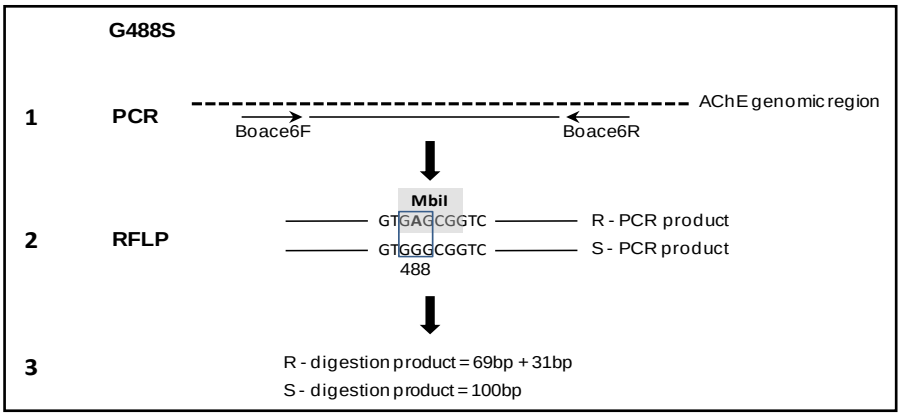


Figure 2.11. Genotyping for an organophosphate resistance-associated point mutation in the AChE gene of OX3079D-Bol using an PCR-RFLP assay. (**1**) A PCR is performed using primers Boace6F and Boace6R, crossing the AChE genomic region containing the 488th codon. (**2**) PCR products of 100bp are shown for resistant (R) allele with a G to A point mutation in the second base of the 488 codon forming an MbiI restriction site; and susceptible (S) allele. (**3**) Digestion of the PCR products with the MbiI restriction enzyme yields products of 69bp and 31bp for the resistant allele, and 100bp only for the susceptible allele. (Figure adapted from Hawkes et al., 2005).

The G488S RFLP-PCR was performed on 40 individuals randomly selected from a pool of pupae produced from the OX3097D-Bol colony (parental colony size of >5,000 adult insects), constituting a screening of 80 alleles from the strain.

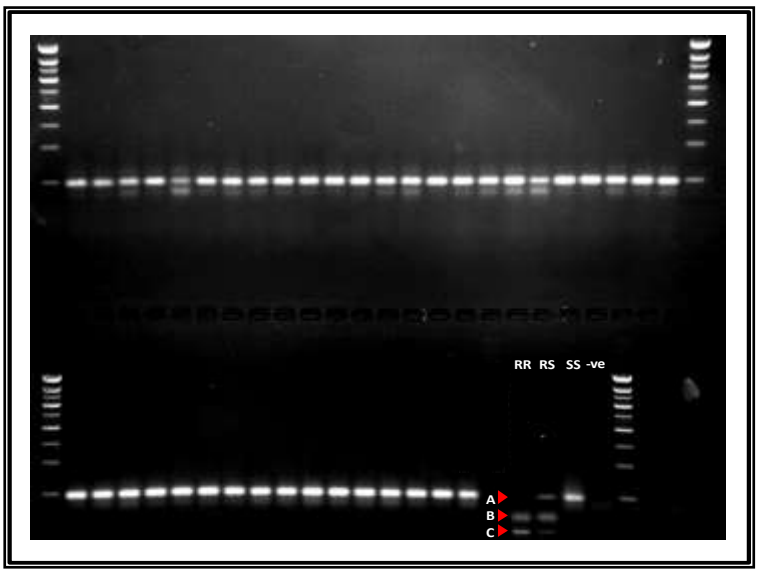


Figure 2.12. Agarose gel showing products from the G488S PCR-RFLP of OX3097D-Bol. 40 randomly selected OX3097D-Bol pupae were used. (SS), (RS), (RR) and (-ve) are controls. (SS) is the product from a known homozygous susceptible individual; (RS) is the product from a known individual heterozygous for the resistant allele; (RR) is the product from a known homozygous resistant individual; (-ve) is a no-DNA control. Bands were sequenced to confirm identity. Arrow (A) indicates a fragment of 100bp corresponding to the wild-type allele. Arrows (B) and (C) indicate fragments of sizes 69bp and 31bp corresponding to samples containing the G488S resistance mutation. Some smaller fragments in sample lanes were observed (~80bp), however, sequencing suggested these bands were primer dimers. DNA samples for RR, RS and SS controls were kindly provided by Dr Jon Vontas (University of Crete). Outermost lanes show DNA size standards. Sizes are as follows: from bottom, 100bp increasing in 100bp increments until 800bp, then 1000bp (Eurogentec SF Smartladder).

Results (**Fig. 2.12**) show the production of the 100bp amplicon from the OX3097D-Bol individuals screened, with no digestion products indicating cleavage of the DNA fragments by the MBi1 restriction enzyme at the site associated with the G488S point mutation. This suggests that the point mutation is not present in the 80 alleles of the OX3097D-Bol strain sampled, strongly indicating that the strain does not contain the resistant allele. This is not surprising as even if the mutation was present in the wild-type population when first colonised, according to the theoretical framework of adaptive changes [e.g. (Lande, 1983)] the resistance mutation is expected to impose a selective

disadvantage relative to the susceptible wild-type alleles in the absence of the organophosphate insecticide.

The extensive and long-term use of insecticides for the control of the olive fly has lead to the development of a considerable degree of resistance in certain countries, largely in the eastern Mediterranean (Skouras et al., 2007). Hawkes et al. (2005) have reported detecting a surprisingly high incidence of the G488S mutation in olive fly samples surveyed from different European sites. The OX3097D-Bol strain was found to contain only susceptible AChE alleles; the introgression of susceptibility from the strain into a wild population could therefore provide a source for the flow of organophosphate susceptibility. This could provide significant benefits from the perspective of insecticide resistance management (IRM).

2.3. Summary and conclusions

An effective non-chemical approach to olive fly management is highly desirable, and SIT could achieve this provided certain technical issues can be overcome. This chapter presents the transformation of the olive fly with OX3097, a female-specific lethal transgene construct that gave varying degrees of female-lethality in a range of olive fly insertion lines. For further development, we chose an insertion line, OX3097D-Bol, that is capable of providing dominant female-specific lethality when reared in the absence of tetracycline, equal sex ratios when reared in its presence, and bright expression of the DsRed2 dominant fluorescent marker. OX3097D-Bol transgene flanking genomic DNA regions were sequenced showing the expected duplication of *piggyBac* TTAA target site. PCR primers designed from flanking sequences were used to identify individuals homozygous for the transgene insert, and a pure-breeding homozygous strain was developed from 28 founder individuals.

Splicing of the *Cctra* intron in the OX3097 olive fly lines was found to be the same as that found in native medfly context (Pane et al., 2002), with only females producing the F1 mRNA transcript corresponding to the splicing of the complete *Cctra* intron. A distinct phenotype of DsRed2 over-expression and tissue necrosis at the late larval/early pupal developmental stage was associated with construct lethality, and was found to be female-specific through examination of sex-specific splice variants of *Bodsx*, a key gene in the sexual-differentiation gene cascade.

A RFLP-PCR screen of 40 individuals of the homozygous OX3097D-Bol strain for the organophosphate-resistance associated G488S acetylcholine esterase allele produced no alleles positive for resistance. The introgression of susceptibility through the female line may therefore provide an organophosphate-resistance management option (Alphey et al., 2007; Alphey et al., 2009; Alphey et al., 2011).

The OX3097D-Bol olive fly strain developed here was designed to directly address several of the weaknesses found in the previous olive fly SIT trials. Inbuilt genetic sterility should help mitigate the fitness-associated problems of radiation sterilisation, while genetic sexing should encourage random mating with wild insects, while providing established performance benefits (Rendon et al., 2004). With high levels of transgene penetrance and repressibility, OX3097D-Bol displays traits that are ideal for application to fsRIDL control. The following chapter focuses on quantifying potential fitness costs the transgene imposes on the life-history characteristics of the homozygous OX3097D-Bol fsRIDL strain.

2.4. Materials and methods

2.4.1. Olive fly strains and rearing

Flies used for micro-injections originated from the Democritus laboratory *B. oleae* stocks (Athens, Greece). Later out-crossing of OX3097 insertion lines occurred with *B. oleae* from the Argov hybrid strain (Israel) (described further in Argov et al., 2011) and were supplied by Biofly (Bio-bee biological systems Ltd, Israel). This colony is referred to in subsequent experiments as the Argov wild-type strain.

Adult fly colonies were kept at 25°C, 30-50% relative humidity, and a 13hr light 11 hr dark cycle, and were fed on a diet of: 100g yeast hydrolysate, 400g powdered sugar, and 30g egg yolk powder. Flies with a Democritus genetic background, were supplemented with 250mg streptomycin per 500g of adult diet. Cages were provided with a wet cotton-wick as a hydration source.

To collect eggs, a ceresin wax cone was placed inside a cage through which female olive fly oviposit (**Fig. 2.13**). Eggs were then washed from the cone with distilled water containing 0.05% propionic acid into a glass beaker. Recovered eggs were pipetted onto damp filter paper saturated with either 0µg/ml tetracycline (off-tet) or 100µg/ml tetracycline (on-tet) and left for 48-72 hours to develop into 1st instar larvae. Neonate larvae were washed off the filter paper using a 0.05% propionic acid solution and were pipetted onto a petri-dish containing larval diet at a density of ~1 larva per 0.8g of diet. The petri-dish of larvae and diet was placed inside a small Tupperware-box with a small mesh-covered opening in the top, and sand covering the floor on the inside of the box.

1Kg of larval diet consists of: 550ml distilled water, 30g soy hydrolysate, 0.5g potassium sorbate, 2g Nipagin, 20g sugar, 75g brewer's yeast, 30ml concentrated HCl, 275g cellulose powder, 20ml olive oil, and 7.5ml Tween-80.

Larval development time is highly dependent on temperature, but 1st instar development to pupation is usually complete within 9-days at 25°C. Once larvae begin to emerge from the diet, the petri-dish lid is removed and larvae are allowed to drop onto the floor of the box. The larvae pupate in the sand, and are collected by sieving. Pupae were kept between 18-25°C (depending on time and experimental requirements), until adult emergence.

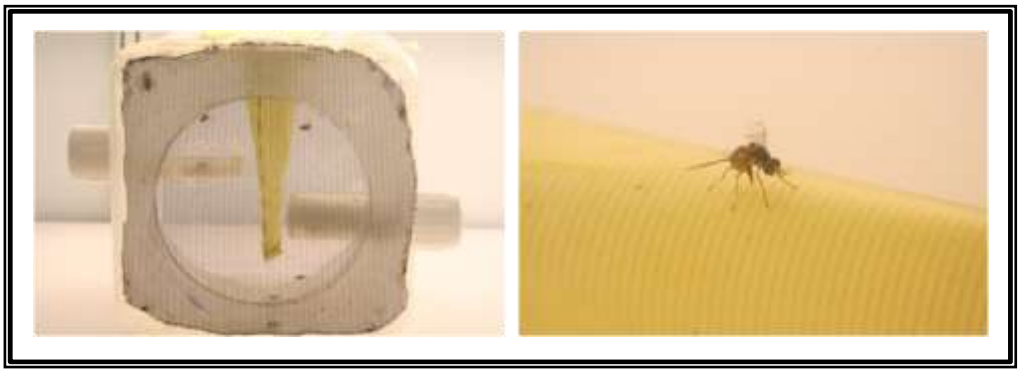


Figure 2.13. (Left) Small rearing cage containing adult olive flies with yellow kerosene wax cone. Insert on left-hand-side of cage contains adult diet, and insert on right-hand-side contains wet cotton-wick. (Right) Female olive fly ovipositing across ceresin wax membrane.

2.4.2. Transformation with OX3097 and outcrossing

Approximately 4,500 pre-blastoderm stage olive fly embryos of the Democritus laboratory (Greece) B. oleae strain were micro-injected with the OX3097 plasmid and *piggyBac* mRNA as described by (Koukidou et al., 2006).

Micro-injection procedure was as follows: (1) Pre-blastoderm embryos (between 0-3 hours old) were collected from a large wild-type stock cage. (2) Embryos were decorionated for 1 minute in a solution of 5% sodium hypochlorite. (3) Eggs were lined-up on a glass slide containing an adhesive strip with the posterior end oriented towards a specific end of the slide. (4) Embryos were desiccated in an oven at 37ºC for 2-5 minutes, and were immediately covered with halocarbon oil upon removal. (5) Embryos were

micro-injected with transposon plasmid OX3097 (400ng/µl) and either *piggyBac* mRNA (600ng/µl) or a *piggyBac* DNA helper plasmid (400ng/µl). (6) After 48 hours, 1st instar larvae surviving micro-injection were picked out of halocarbon oil using a blunt needle, and were placed on larval diet containing tetracycline.

G_0 injection survivors were back-crossed to their wild-type parental strain in batches of: 5 G_0 males with 10 virgin wild-type females, and 5 G_0 females with 5 wild-type males. G_1 eggs were collected daily until egg production ceased. G_1 pupae were screened for expression of the DsRed2 fluorescent marker.

2.4.3. Assessing copy number in OX3097 insertion lines

Males and females of each OX3097 insertion line were crossed to wild-type adults of the opposite sex separately, and progeny were reared in the presence of tetracycline. Pupae were screened and the ratio of wild-type to transgenic was calculated.

2.4.4. Assessing OX3097 penetrance and repressibility

Virgin heterozygous transgenic males and females from OX3097 insertion lines were crossed to virgin wild-type adults, and eggs were collected. Eggs were left for 48-72 hours on wet filter paper saturated with distilled water containing either 100µg/µl tetracycline or 0µg/µl tetracycline to measure repressibility or penetrance of lethality, respectively. Resulting 1st instar larvae were placed on larval diet seeded with water containing 100µg/µl tetracycline or 0µg/µl tetracycline. Resulting pupae were collected and screened for the presence of the transgene. Transgene-positive pupae were separated and were stored until adult eclosion. Upon eclosion, adults were sexed and the numbers of males and females recorded.

2.4.5. Genomic DNA extractions from whole insect samples

DNA extractions were performed on insect material using the Fermentas "GeneJet Genomic DNA Purification Kit (# K0722)" according to manufacturer's instructions.

2.4.6. Genomic DNA extractions from adult legs

A buffer solution ("squishing buffer") was prepared for the shearing of adult leg tissue and contained: 10mM Tris HCl; 1mM EDTA; 25mM NaCl. When ready to perform an extraction, 2µl of Proteinase K was added per 100µl of squishing buffer, and 15µl of the buffer and Proteinase K mixture was added to each well of a PCR plate, using as many wells as leg samples. A single leg was extracted from each adult insect, on ice, and using sharp tweezers. Legs were chosen such that the remaining five legs should allow successful copulation and oviposition (a hind male, and middle female leg were used). Single legs were placed in individual PCR tubes containing the buffer and enzyme solution, and the legs were broken-up using a sterile pipette tip. PCR tubes were then placed on a PCR heating block under the following programme: 37ºC - 30mins; 95ºC - 10mins; 4ºC - pause. Extracted DNA was then ready for use in a PCR (4µl was used in a 20µl PCR mix).

2.4.7. RNA extractions

Olive fly adults were homogenised in individual micro-centrifuge tubes in 200µl of TRI reagent (Life Technologies). 100µl of chloroform was added to each tube, and was shaken thoroughly. Tubes were centrifuged for 10 minutes at 4ºC and the RNA-containing aqueous phase was pipetted off and transferred to a fresh tube. 100µl of isopropanol was added, and tubes were vortexed. Samples were then centrifuged for 15 minutes at 4ºC. The resulting RNA pellets were washed in 75% ethanol and resuspended in RNAse free water.

RNA samples were then incubated for 30 minutes at 36°C with DNAse. All RNA samples were subsequently stored at -80°C.

2.4.8. cDNA synthesis

Extracted RNA was run on a clean (freshly prepared) agarose gel to check quality. 1µl of RNA was placed in an micro-centrifuge tube (although 2µl was used in some cases, depending on the intensity of RNA signal from the agarose gel), to which was added: 1.25µl oligo DT (10mM) and 1.25µl dNTPs (10mM). Reaction was brought to a total volume of 10µl using RNAse-free water. Samples were incubated at 70°C for 5mins and then cooled on ice for 1min. A reaction master mix was made using the following reagents: 5 x RT buffer (5µl), 0.1M DTT (2.5µl), RNAse free water (5µl), RNAse-out (0.5µl), and Reverse transcriptase (0.5µl). Samples were placed in a thermo-cycler and run on the following program: 25°C - 10mins; 48°C - 45mins; 95°C - 5mins. Reagents used were from the commercial kit "Superscript II" (Life Technologies).

2.4.9. Obtaining transgene flanking sequences (adaptor-mediated PCR)

To obtain transgene flanking genomic sequences, genomic DNA was extracted from whole adults according to (*Section 2.4.5.*). Purified genomic DNA was digested with restriction enzymes: MspI, BclI, ClaI, Nar1, BglII and BstBI according to manufacturer's (NEB) instructions.

Overhang specific adaptors were ligated to complementary digestion products as follows: Msp adaptor - MspI, ClaI, NarI, BstBI; Dpn adaptor - BglII, BclI. Each reaction contained: 2µl - 10x ligation buffer; 2.5µl - adaptor; 0.5µ - T4 ligase. Ligation reactions were left overnight at room-temperature.

Primers specific to ligated adaptor and internal 3' and 5' *piggyBac* sequences were then used to amplify fragments. Each PCR mix contained: 2µl (10mMol) - 5' or 3' construct-internal primer; 1µl - adaptor primer; 0.5µl - (10mMol) dNTPs; 2.5µl - TaqPol Buff; 0.2µ - TaqPol; 1µl - ligation reaction DNA. The PCR was then run on the following programme: 94ºC - 2mins; 94ºC - 20secs; 55ºC - 45secs; 68ºC - 90secs *(back to step 2 for 34 cycles); 68ºC - 9mins.

PCR products were then diluted (1µl of product in 199µl dH_2O) and an additional PCR was performed using nested primers, the same quantities of reagents, and the same PCR programme as described above. DNA fragments were cloned into a pJET plasmid (Fermentas) according to manufacturer's instructions, and were sent to GATC-Biotech (Konstanz, Germany) for sequencing, and were later analysed using VectorNTi (Life Technologies).

2.4.10. OX3097D-Bol strain homozygosis

To develop a homozygous strain, a pool of 96 homozygous and heterozygous OX3097D-Bol adults was generated by crossing heterozygouss male and female OX3097D-Bol. Genomic DNA from legs of putative homozygous offspring was extracted as described in (*Section 2.4.6.*). Genomic DNA was analysed by PCR using primers *Bo*-10 and *Bo*-28 designed from sequenced transgene flanking genomic regions (see *Section 2.4.5.*). Homozygous individuals were identified as described in (**Fig. 2.06**). Individuals identified as heterozygous were discarded and 50 homozygous individuals were crossed in 18 "founder" cages. 13 founder cages produced viable eggs. Progeny were crossed only with siblings from the same founder cage for 3 generations. No cages produced any wild-type progeny during this time and on the third subsequent generation the pupae were

pooled. The homozygous strain was thereby initiated from the 13 founder cages containing 15 female and 13 male homozygous OX3097D-Bol individuals.

2.4.11. RT-PCR for *Cctra* splicing

Total RNA was extracted as described in (*Section 2.4.7.*), and cDNAs were synthesised as described in (*Section 2.4.8.*). A PCR to amplify *Cctra* transcripts was set-up as follows: 0.5µl - Hsp (forward primer) (10mMol); 0.5µl - tetRr (reverse primer) (10mMol); 2.5µl - 10x TaqPol Buff; 0.5µl - dNTPs (10mMol); 0.2µl - TaqPol; 1µl - cDNA template; 19.3µl - H_20. The PCR was run on the following programme: 94ºC - 90secs; 94ºC - 15secs; 57ºC - 45secs; 68ºC - 1min *(back to step 2 for 32 cycles); 68ºC - 7mins; 4ºC - pause. The major RT-PCR products were sent for sequencing to GATC-Biotech (Konstanz, Germany) and were analysed using VectorNTi (Life Technologies).

2.4.12. RT-PCR for *dsx* splicing

Total RNA was extracted as described in (*Section 2.4.7.*), and cDNAs were synthesised as described in (*Section 2.4.8.*). A PCR to amplify male and female *dsx* transcripts was set-up as follows: 0.5µl - dsxM (male-specific forward primer); 0.5 - dsxF (female-specific forward primer); dsxexon3 (reverse primer) (10mMol); 2µl - 10x TaqPol Buff; 0.5µl - dNTPs (10mMol); 0.2µl - TaqPol; 1µl - cDNA template; 15µl - H_20. The PCR was run on the following programme: 94ºC - 2mins; 94ºC - 30secs; 60ºC - 30secs; 72ºC - 20secs *back to step 2 for 34 cycles; 72ºC - 7mins; 4ºC - pause. The major RT-PCR products were sent for sequencing to GATC-Biotech (Konstanz, Germany), and were analysed using VectorNTi (Life Technologies).

2.4.13. RFLP-PCR for G488S mutation in *BoACHE*

DNA was extracted from 40 randomly selected OX3097D-Bol pupae as described in (*Section 2.4.5.*). RFLP-PCR was conducted on each sample according to the protocol described by Margaritopoulos et al. (2008). Samples for positive and negative controls were kindly provided by the laboratory of Dr John Vontas (Heraklion, Crete). Digestion products were sent to GATC-Biotech (Konstanz, Germany) for sequencing, and were analysed using VectorNTi (Life Technologies).

2.4.14. Primer sequences

OX3097D-Bol flanking primers:

Bo-10: 5'-CCTGCGTTTGGAGATGACGAAATC-3'

Bo-28: 5'-CTTACATATAGAGCAGTGCGCTCACAT-3'

Plasmid backbone primers:

Forward: 5'-ACGTCAGGTGGCACTTTTCG-3'

Reverse: 5'- CGTGAGTTTTCGTTCCACTG-3'

***Cctra* RT-PCR primers (designed by G. Fu):**

Hsp: 5'-CAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTA-3'

TetRr: 5'-GCGGAACGACTTGGCGTTATTGCG-3'

***Bodsx* RT-PCR primers:**

dsxFemale: 5'-AAACTGTAGTGTGCCCGGAT-3'

dsxMale: 5'-CGGATGACGTCAAGCTTGT-3'

dsxexon3: 5'-CAAAAACTATTGGAGAAATTTCGA-3'

***BoACHE* primers:**

D6F: 5'-TATTATACACACCAGCTGGGTTGTAATCCG-3'

D6R: 5'-ATATTTATAAGGGCAGGTGAAGAAITGACCGC-3'

Female olive fly ovipositing through an artificial wax membrane used in laboratory rearing.

Chapter 3 - Analysis of OX3097D-Bol life-history traits

3.1. Introduction

The mass-rearing and release of many millions of sterile insects per week may be required for area-wide population control, and as sterile releases frequently do not achieve total eradication (due to migration of fertile insects from surrounding areas), the release of large numbers must often be continuously maintained to prevent reinvasion. A good understanding of insect basic biology, life-cycle parameters, and efficient methods for mass-rearing is therefore crucial for the success of a sterile release programme. Advances in olive fly mass-rearing, largely through efforts of researchers at the Democritus Nuclear Research Centre (Athens, Greece) and the IAEA (Seibersdorf, Austria), now allows for large-scale artificial rearing with reasonable economic efficiency (Tsitsipis, 1977).

The ultimate goal of insect mass-rearing is to produce the maximum number of high quality target insects with minimal man-hours and space, in as short a time and as inexpensively as possible (Finney& Fisher, 1964). Favourable strain life history traits of high fecundity and high developmental viability are therefore of key importance if a strain is to be produced efficiently. Strains used for mass-rearing have typically been laboratory colonised for some time, and have therefore been selected for adaptations to artificially high density culture. This can be beneficial from a rearing perspective [i.e. selection pressure is directed towards parameters that are good for productivity, e.g. high fecundity and rapid development (Sorensen et al., 2012)]. However, this does not necessarily translate to optimal performance of a strain in the field; for example, (Briceno& Eberhard, 2002) describe selection pressures in medfly mass-rearing leading to shorter courtship displays, and resulting in reduced mating compatibility with wild insects (discussed further in *Chapter 4*).

The processes involved in transgenic strain development can sometimes result in lower egg productivity and strain developmental viability [e.g. studies on *Aedes aegypti* by Irvin et al. (2004), who showed major fitness costs in a range of transgenic lines], and can be caused by a variety of potential factors, including: the disruption of endogenous genomic DNA sequences due to transposon insertion, the production of foreign and potentially deleterious transgene proteins, inbreeding effects, and the homozygosis of transgene-linked overdominant alleles. Quantifying these costs therefore forms an important part of strain evaluation; there are minimum thresholds in insect developmental viability below which strain mass-rearing may not be economically practicable.

In this chapter I sought to assess the homozygous fsRIDL olive fly strain, OX3097D-Bol, for a variety of life history traits relevant to mass-rearing. As a comparison we used two wild-type colonies: the Democritus strain (Democritus, Greece) colonised over 30 years ago and the Argov hybrid strain (Israel) developed within the last 5 years from repeated outcrossings of the Democritus strain with wild olive flies caught in Israel, constituting a hypothesised 93.75% 'Israeli genome' (Argov et al., 2012). The OX3097D-Bol strain was initially transformed using embryos of the Democritus strain, however, before homozygosis, OX3097D-Bol was introgressed for five generations into the Argov background (see *Section 2.2.1.*). Theoretically, OX3097D-Bol shares greater than 95% genetic similarity with the Argov background. It is therefore of interest to directly compare the Argov and OX3097D-Bol strains; any significant differences are likely to result from the effects of OX3097D-Bol transgenesis and homozygosis. It is also of interest to compare life-history parameters of Democritus and Argov - two wild-type olive fly strains with more than 25 years difference in laboratory-colonisation duration.

Another important consideration is the density at which the mass-reared insect colonies are kept. Maximising egg production will inevitably involve using high density

rearing, however, merely increasing density will subject egg production to diminishing returns due to stresses resulting from competition for space and the increased build-up of waste materials. I therefore sought to examine the effects of rearing density on OX3097D-Bol life-history traits relevant to maximising egg production.

Here I present an examination of: (1) female fecundity between strains at high density, but also examining OX3097D-Bol fecundity at a lower ('medium') rearing density; (2) variation in egg hatch with parental colony age; (3) larval survival to pupation; (4) the rate of successful adult eclosion from the puparium; (5) adult female longevity (at medium and high rearing densities); and, (6) male longevity under stress.

3.2. Results and discussion

Some of the experiments presented in the following chapter were performed at two different rearing densities - termed 'medium' and 'high'. **Table 3.1** provides details on the levels of crowding used at each density. The high density scenario shown represents an estimate of the likely levels of insect crowding to be found in high density olive fly rearing, and was provided by BioFly (BioBee Ltd, personal communication), a company with some olive fly mass-rearing experience.

Table 3.1. Densities used for measuring the effects of crowding on various OX3097D-Bol life-history traits. Experimental cages used had the following dimensions: (H: 12cm, W: 12cm, L: 15cm). Females were provided with an ovipositional surface equalling 90cm^2.

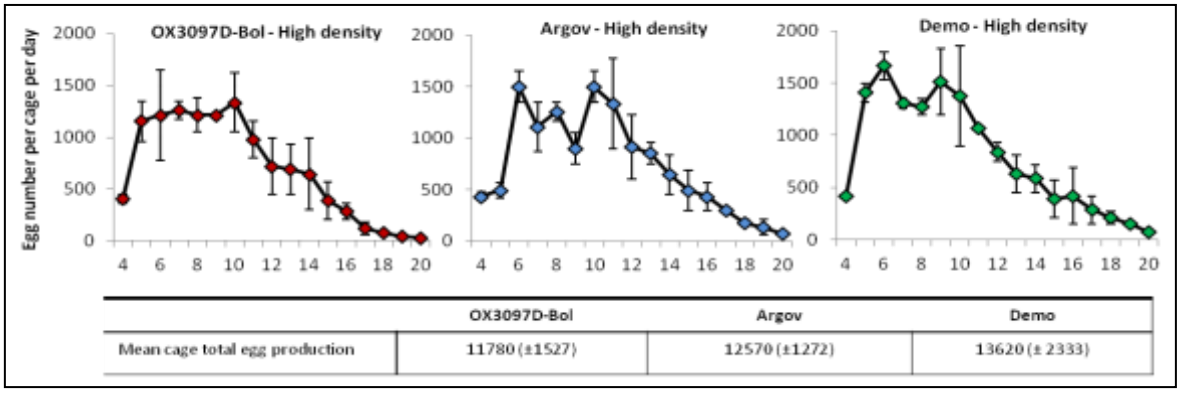
	Number of adult males	Number of adult females	Absolute density (flies per cm^3)	Flies per cm^2 resting surface	Females per cm^2 of ovipositional surface
Medium density	25	25	0.025	0.050	0.280
High density	50	50	0.050	0.100	0.560

Tetracycline was provided in larval diets and adult water sources for all strains (transgenic and wild-type), except when stated otherwise. The aim of the experiments was to quantify effects of the transgenesis alone, we therefore wanted to eliminate non-transgene related effects of tetracycline as a potential confounding factor.

3.2.1. A comparison of realised fecundity

Rate of egg production from 50 females caged with 50 males of the OX3097D-Bol, Argov, and Democritus strains was measured at a high rearing density between days 4 and 20 post adult eclosion (PE). Fecundity was also measured in the OX3097D-Bol strain at a medium rearing density. Three replicates were performed for each strain at each density, and all cages were given a water source containing tetracycline (100µg/ml). Total egg production and the total number of surviving females for each cage were measured daily. **Fig. 3.1** shows the pattern of mean total cage egg production per strain per day. The table

within the figure summarises the combined total egg outputs per cage (±SD) over days 4 to 20 PE.



	OX3097D-Bol	Argov	Demo
Mean cage total egg production	11780 (±1527)	12570 (±1272)	13620 (± 2333)

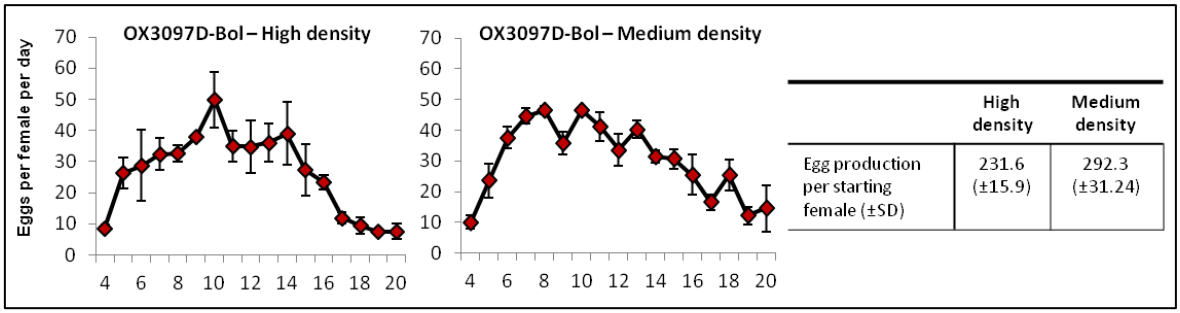
Figure 3.1. Graphs show mean number of eggs produced by cages containing 50 females and 50 males of the OX3097D-Bol, Argov, and Demo (Democritus) strains per day PE reared at a high density. Error bars show the standard error from three repeats. Table shows the mean total egg production (±SD) for each strain over the 16-day period.

Cages containing 50 males and 50 females of the OX3097D-Bol, Argov and Democritus strains reared at high density produced a mean total of 11,780 (±1,527 SD), 12,570 (±1,272 SD), and 13,620 (±2,331 SD) eggs between days 4 and 20 PE, respectively. Cage egg production totals for each strain passed a Shapiro-Wilk normality test ($P > 0.7$, Shapiro-Wilk). A 1-way analysis of variance (ANOVA) was therefore used to compare mean total egg outputs, and suggested that differences between the OX3097D-Bol and Argov strains were not statistically significant ($P = 0.29$, 1 d.f., 1-way ANOVA), indicating no measurable effects of transgenesis or homozygosis on OX3097D-Bol fecundity at this rearing density. Differences between the Argov and Democritus strains were also not found to be significant ($P = 0.39$, 1 d.f., 1-way ANOVA).

The three strains showed a similar pattern of egg production over time, with a peak egg-laying period between approximately days-5 and 12, suggesting that mass-reared insects should be replaced with fresh colonies after this period to maintain maximum continuous egg production. The decline in productivity after approximately day-10 is

likely a result of increasing female mortality in the cages and a reduction in individual female productivity with time (see **Fig. 3.2**).

Egg production rates were also measured at a medium rearing density for OX3097D-Bol. In order to compare productivity between densities, production was normalised for female number. **Fig 3.2** shows egg production rate per female per day for OX3097D-Bol females reared at medium and high densities. Rates per female were calculated by dividing total cage egg production per day, by the total number of surviving females on that day. The curves show the egg laying pattern of a female that survived to the end of the experiment; this does not represent the expected egg laying pattern of an average starting female (the large majority of females do not last until day 20) (see *Section 3.2.5.*). The expected mean total fecundity per starting female (±SD) at both densities is provided in the table within the figure.



	High density	Medium density
Egg production per starting female (±SD)	231.6 (±15.9)	292.3 (±31.24)

Figure 3.2. Graphs show mean number of eggs per OX3097D-Bol female per day PE when reared at either high or medium densities. For each data point, the total cage egg production per day was divided by total number of remaining females. Error bars show the standard error of the mean from three repeats. Table shows the average expected fecundity of a starting female at each density (total average cage egg production over three repeats divided by the total initial female number).

The data for medium-density total egg production per female across the repeats were found to be normally-distributed (P = 0.52, Shapiro-Wilk). Lowering the OX3097D-Bol rearing density from high to medium had a statistically significant impact on egg productivity per female (P = 0.038, d.f. =1, 1-way ANOVA). A female in a medium density cage laid an average of 292.3 eggs, 61 (~26%) more eggs on average than a female

reared at high density. This is likely to be due to a combination of reduced environmental stresses resulting from lower waste product accumulation, and less competition for resting and ovipositional surface. Increasing adult density results in the build-up of larger quantities of waste-products, such as dead and decomposing adult flies, excretory products, and requires the presence of a greater amount of adult diet. Consequently, bacterial and fungal growth are expected to be higher within a high density cage. Competition for optimal ovipositional substrate and resting surface are also expected to increase. It is likely that all these factors combine to cause elevated levels of stress on females as density increases, and reduce female life-time productivity.

Although egg production per female is significantly higher when rearing at a medium density, the advantages of high density rearing (in this case providing twice the number of females per unit area than at medium density), are clear. High density rearing provided 58% more eggs per unit of cage resting area than at medium density, and suggests that the rearing density of OX3097D-Bol could be further increased beyond the highest used here. However, significant reductions in productivity per female indicate that a density optimum is approaching, over which returns in egg production may start to diminish. Further experiments looking at egg return per starting female at a range of higher densities, will help to identify the exact point at which increasing density leads to lower overall productivity, setting an upper density limit, and helping to maximise production.

3.2.2. Egg hatch rates

Cages were set-up containing 20 males and 20 females of either the OX3097D-Bol, Argov or Democritus strains, and were maintained on a water source containing tetracycline (100µg/ml). Eggs were collected every three days between days 5 and 20 PE and hatch rates were measured. Proportional egg hatch was assessed by comparing the

total egg number in each sample (100<n<200) with the resulting number of non-hatched. Cages were run in triplicate for each strain. Rates of egg hatch over time for the three strains are shown in **Fig. 3.3**.

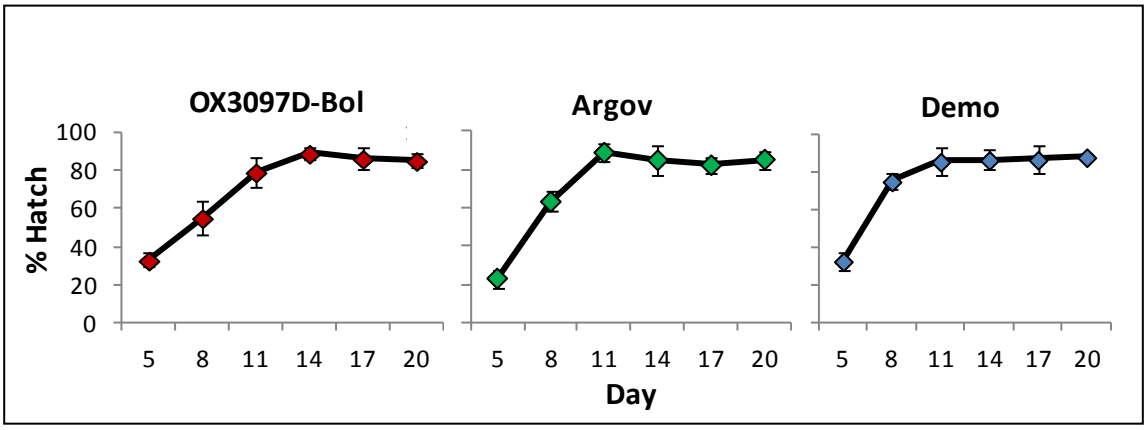
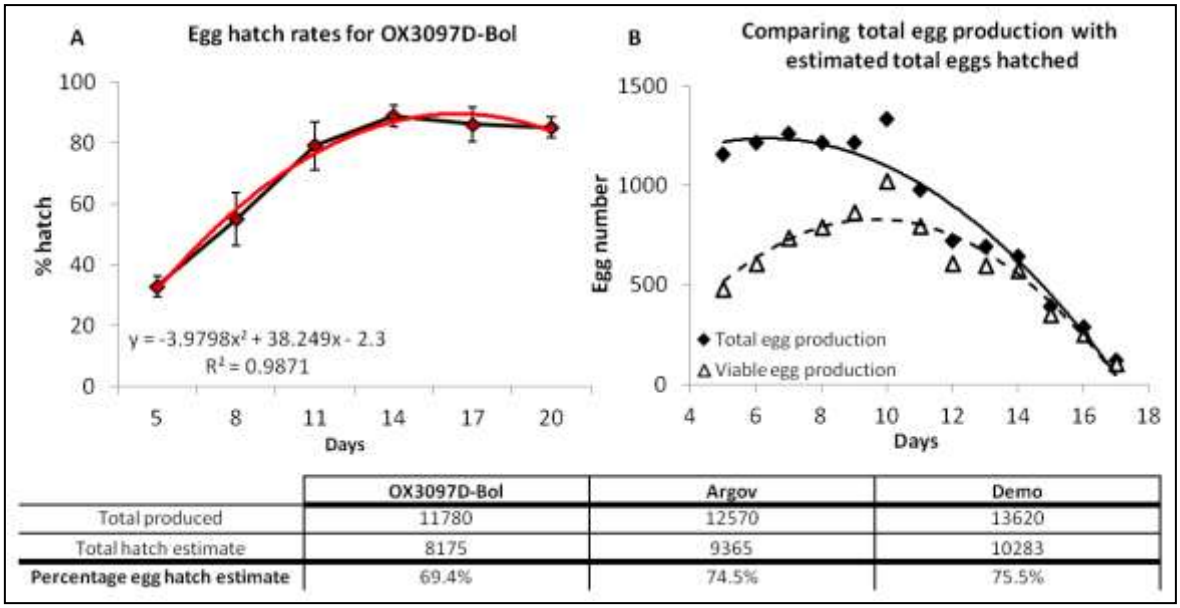


Figure 3.3. Graphs show changing percentage egg hatch rates for OX3097D-Bol, Argov and Democritus (Demo) strains over days 5 to 20 PE. Error bars show standard error over three replicates.

The three strains showed relatively low initial egg hatch rates (less than 40% of total production on day 5 PE) with a steady increase until approximately 11-days PE, after which rates stabilised at greater than 80% for all strains. However, the rate of increase to plateau was measured to be somewhat slower in the case of OX3097D-Bol, averaging at 67% between days 8 and 11, compared to 76% and 81% for Argov and Democritus, respectively.

The rates of egg production (**Fig. 3.1**) and egg hatch (**Fig. 3.3**) have been shown to vary greatly with time. Therefore, in order to gain a more accurate estimate of how the egg production rates and egg hatch rates translate to the production of total hatching eggs, the data from the two experiments were combined for each strain. The method used for these calculations is described for OX3097D-Bol in **Fig. 3.4**., and contains a table providing estimates of total eggs hatched from the total produced from the high density rearing cages for each strain (production cages are those described in *Section 3.2.1.*).



	OX3097D-Bol	Argov	Demo
Total produced	11780	12570	13620
Total hatch estimate	8175	9365	10283
Percentage egg hatch estimate	69.4%	74.5%	75.5%

Figure 3.4. (**A**) Egg hatch rates for OX3097D-Bol (data points and black line) with trend-curve (smooth-red curve). The equation for the trend-line was used to calculate estimates of percentage hatch rates for days where no data were available. (**B**) Curves showing OX3097D-Bol high rearing density female total egg production as measured in *Section 3.2.1.* (black diamond markers with smooth black trend line), and an estimate of viable (hatching) egg production (opaque triangle markers with dashed trend line), calculated by multiplying egg production for a given day by the estimation of proportional hatch rate as calculated from trend-line equation (from **A**). Table shows a combination of production data with egg hatch data for each strain independently, providing an estimate of total egg hatch rate for eggs produced between days 5 and 20 PE.

The proportion of eggs produced to those hatching for the OX3097D-Bol and Argov strains was compared using a Pearson's Chi-Squared test and suggested a statistically significant difference (P = 0.004, 1 d.f., Chi-Squared). Thus, although females of the Argov and OX3097D-Bol strains were found to be equally fecund, the percentage of those eggs that successfully hatched to give L1 larvae was found to be significantly different; Argov strain females laid significantly more viable (hatching) eggs than OX3097D-Bol females. The differences between the Argov and Democritus strains were not found to be statistically significant (P = 0.54, 1 d.f., Chi-Squared).

It is somewhat surprising that egg hatch rates are so low between days 5 and 11, as the fecundity curves for total cage (**Fig. 3.1**) and individual female egg production (**Fig. 3.2**) show that by the time the egg hatch rates reach their maximum, the females will have already laid approximately half of their eggs. Based on differences in area underneath the trend curves in **Fig. 3.4B**, nearly one third (30.6%) of the eggs laid by mass-reared

OX3097D-Bol olive fly females do not develop to L1 larvae. This is largely due to low hatch rates between days 5-10 PE. Based on the strong visual similarity of the curves for the three strains in egg production (**Fig. 3.2**) and hatch rates (**Fig. 3.3**), it is reasonable to say that the phenomenon is found in all the olive fly strains, and is not unique to OX3097D-Bol.

As eggs are presumably quite costly for female olive fly to produce, this seems unduly wasteful and suggests a potential inadvertent effect of artificial rearing. It is difficult to say what this might be, although it has been observed that the vast majority of non-viable eggs laid between days 5 and 10 show no signs of development (arresting larval development in the egg is usually clearly visible), therefore indicating that they may be unfertilised. This is consistent with our observations that females will frequently lay unfertilised eggs if they have not copulated. Moreover, the continuous increase in hatch rate with time can be explained by the increasing likelihood that any given female will have mated as time progresses, suggesting that low initial hatch rates may be a result of the failure of some females to copulate until after they have started egg laying.

The inadvertent strong directional selection to decrease the age of first reproduction is a recognised phenomenon in medfly mass-rearing [e.g. (McCombs& Saul, 1992)]. It is possible that the olive fly females have been selected to develop sexual maturity more quickly in laboratory mass-reared strains (despite the costs of some infertile egg production - which may be reduced in an artificial rearing scenario where high quality nutritional resources are abundant), and may mature on average more quickly than males. If this is correct, a process of using older males with younger females may produce a greater fertilised egg yield (although without a method of automated pupal sexing this would be problematic).

3.2.3. Larval survival to pupation

Larval survival to pupation in the OX3097D-Bol, Argov and Democritus strains was measured by placing 50 1st instar larvae onto 40g of larval diet seeded with tetracycline (0.8g larval diet per larva), and assessing the resulting pupation rate. Five replicates were performed for each strain (giving a total of 250 1st instar larvae assessed for each). The mean percentage (±SD) yields in pupae from neonate larvae are given in **Fig. 3.5**.

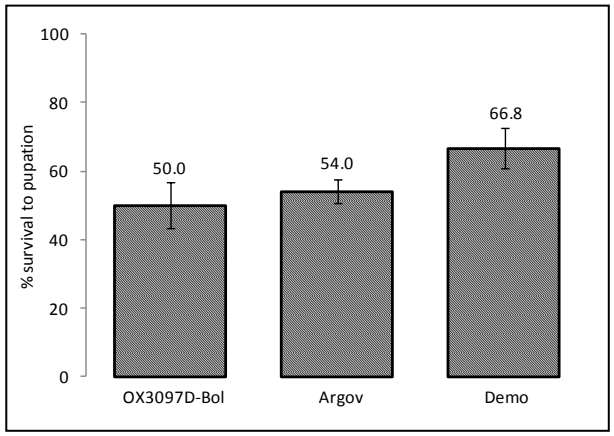


Figure 3.5. Mean larval survival to pupation from 5 replicates of 50 newly hatched L1 larvae from the OX3097D-Bol, Argov and Democritus (Demo) strains (for each repeat, n = 50). Error bars show the standard error.

An average of 50% of the OX3097D-Bol 1st instar larvae survived to pupate, compared with 54% and 66.8% for Argov and Democritus, respectively. Values of percentage survival for each strain were found to be normally distributed ($P > 0.20$, Shapiro-Wilk). A one-way ANOVA suggested that there were no statistically significant differences between rates of larval survival to pupation between the OX3097D-Bol and Argov strains ($P = 0.47$, 1 D.F., 1-way ANOVA). A 1-way ANOVA comparing the Argov and Democritus strains, however, found a significantly higher rate of survival for the Democritus larvae ($P = 0.02$, 1 d.f., 1-way ANOVA). The lack of a significant difference between OX3097D-Bol and the genetically similar Argov strains, suggests a limited influence of transgenesis or transgene expression when larvae are reared on-tet.

3.2.4. Rate of adult eclosion

The rate of adult emergence from the pupal case was measured from 192 randomly selected pupae from each strain reared on-tet at a density of 0.8g of larval diet per larva. Emerging adults were scored visually as: emerged normal (emerged adult flies that could fly and had no obvious physical defects); emerged abnormal (emerged adult flies that could not fly and possessed physical defects such as curly wings); and non-emerged (including partial emergence but inability to escape the pupal case). The results are shown in **Fig. 3.6** and give the percentages of each category obtained for the three strains.

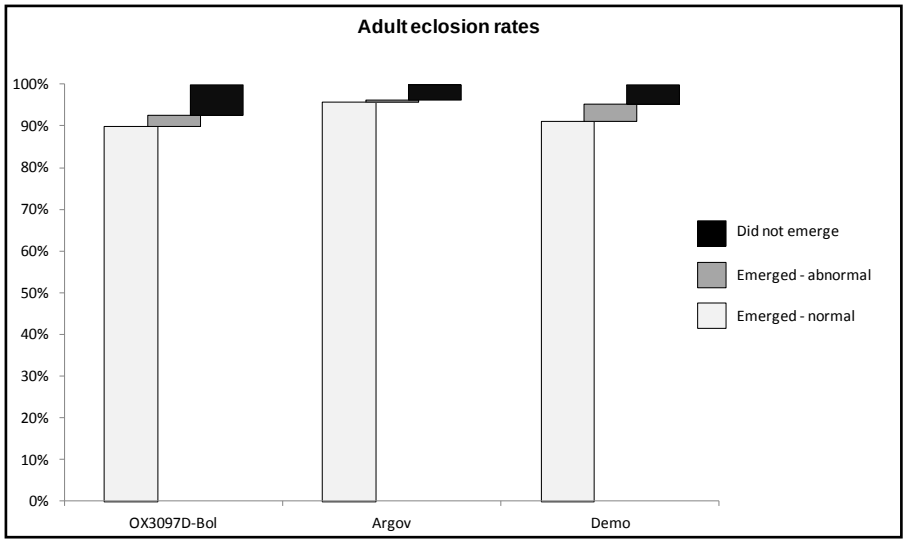


Figure 3.6. Percentage adult eclosion rates for OX3097D-Bol, Argov, and Democritus (Demo) pupae. Larvae for the experiment were reared on a diet seeded with 100µg/ml of tetracycline. (n = 192 for each strain)

Greater than 90% of pupae from each strain successfully eclosed to give adult flies that were capable of flight and had no obvious visible physical abnormalities. A small percentage of adults obtained from each strain (2.6%, 0.5%, and 4.2% for OX3097D-Bol, Argov, and Democritus, respectively) possessed a curly wing or other damaged wing phenotype and were therefore incapable of flight. A slightly larger proportion from each strain (7.3%, 3.6%, and 4.7% for OX3097D-Bol, Argov, and Democritus, respectively) had either died as a pupa or were malformed to such an extent that escape from the pupal

casing was not possible. A Pearson's Chi-squared test comparing the OX3097D-Bol and Argov strains for the proportions of emerged-normal with defective individuals (combining the emerged-abnormal and non-emerged categories) was performed, and found no significant difference (P = 0.08, 1 d.f., Chi-Squared test). Furthermore, no statistically significant differences were found between the Argov and Democritus wild-type strains from the pupae sampled (P = 0.15, 1 d.f., Chi-Squared test).

3.2.5. OX3097D-Bol and Argov female longevity on-tet

The longevities of 50 OX3097D-Bol females and 50 Argov females when caged with 50 males were measured at a high rearing density and in the presence of tetracycline. As females are the egg source and therefore the major production limiting factor in mass-rearing only female mortality was monitored. Survival curves are shown in **Fig. 3.7** (experiments were run in triplicate).

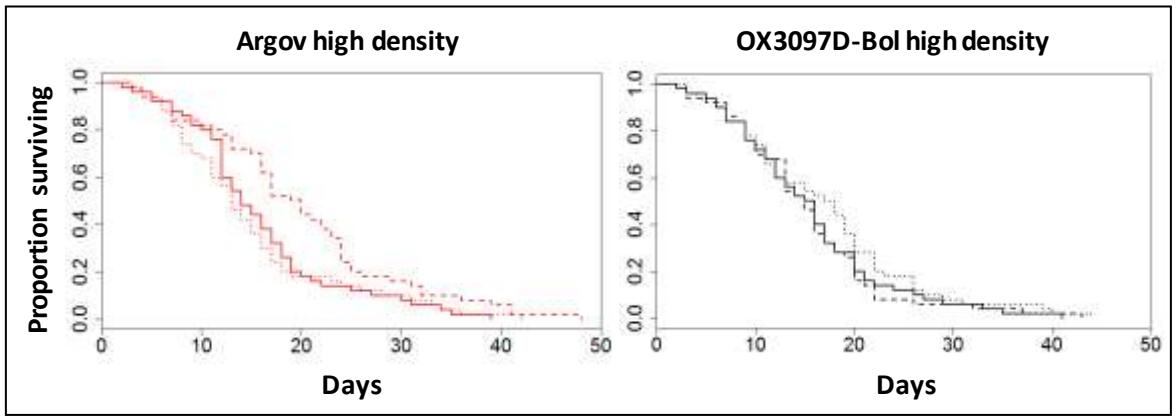


Figure 3.7. Kaplan-Meier survival curves for Argov females (left panel) and OX3097D-Bol females (right panel) reared at a high density on-tet. All experiments started with an initial 50 females, data for each of three replicate experiments is shown).

Argov females were found to have a median survival time of 15.5 days and a mean of 16.9 days. OX3097D-Bol females were found to have a median survival time of 14.9

days and a mean of 16 days. A Logrank test using the Kaplan-Meier survival estimate was performed to compare survivorships. Differences between survival times of females of the OX3097D-Bol and Argov strains were found not to be statistically significant (P = 0.43, 1 d.f., ChiSq = 0.6, Logrank test), suggesting that transformation and homozygosis in the OX3097D-Bol strain does not cause females to have a measurably reduced life-span compared to Argov females when reared at high density.

3.2.6. OX3097D-Bol female longevity at medium and high densities

The longevities of OX3097D-Bol females were also measured when reared at a medium density. Survival curves of the females are shown in (**Fig. 3.8**).

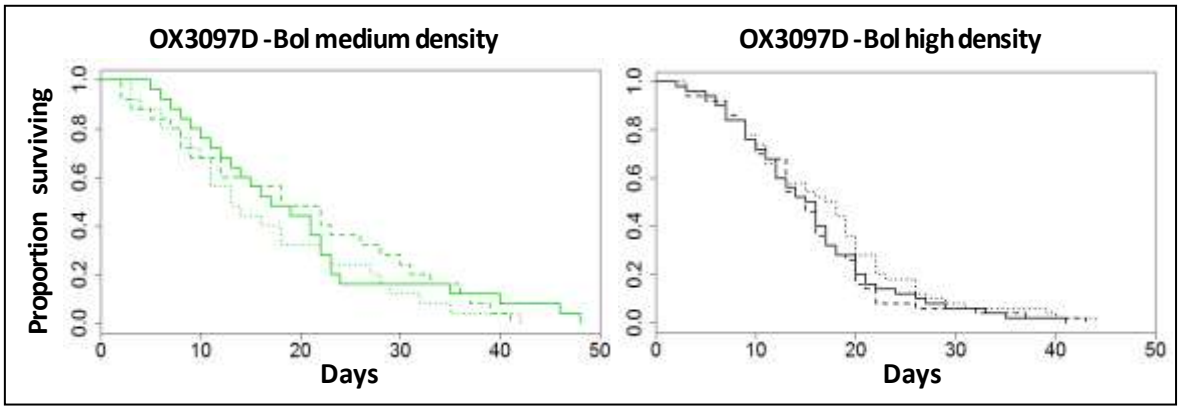


Figure 3.8. Kaplan-Meier survival curves for OX3097D-Bol females reared at medium (green lines) and high (black lines) densities on-tet. High density experiments started with cages containing an initial 50 females and 50 males, while medium density experiments started with an initial 25 females and 25 males.

OX3097D-Bol females reared at a medium density were found to have a median survival time of 16-days and a mean of 18.5-days, while those reared at high density were found to have a median survival time of 14.9-days and a mean of 16-days. A Logrank test was performed to compare the survival times. Reducing rearing density was found to significantly increase female longevity (P = 0.027, d.f. = 1, ChiSq = 4.9, Logrank test). A

similar median but a higher mean longevity in the medium density rearing cages suggests that differences are mainly due to the presence of a greater number of relatively long-lived females at medium density. However, as colonies are likely to be replaced after the peak egg laying period (approximately between days-5 and 12 PE) it is unlikely that this increase in mean longevity alone will have much impact on egg production in mass-rearing.

3.2.7. OX3097D-Bol and Argov male longevity off-tet and under stress

The duration of OX3097D-Bol and Argov male survival without a source of food and water was measured, in order to test the relative nutrient reserves available to the adult fly at the time of emergence from the pupa (teneral reserves); the test is often used as a measure of fly quality in tephritid mass-rearing (IAEA/USDA/FAO, 2003). OX3097D-Bol and Argov larvae were reared at a density of 0.8g of larval diet per larva in the absence of tetracycline. Resulting pupae were eclosed in petri-dishes and 35 eclosing late-pharate adults were separated into each cage (performed in triplicate for each strain). Survival curves for the OX3097D-Bol and Argov strains are shown in **Fig. 3.9**.

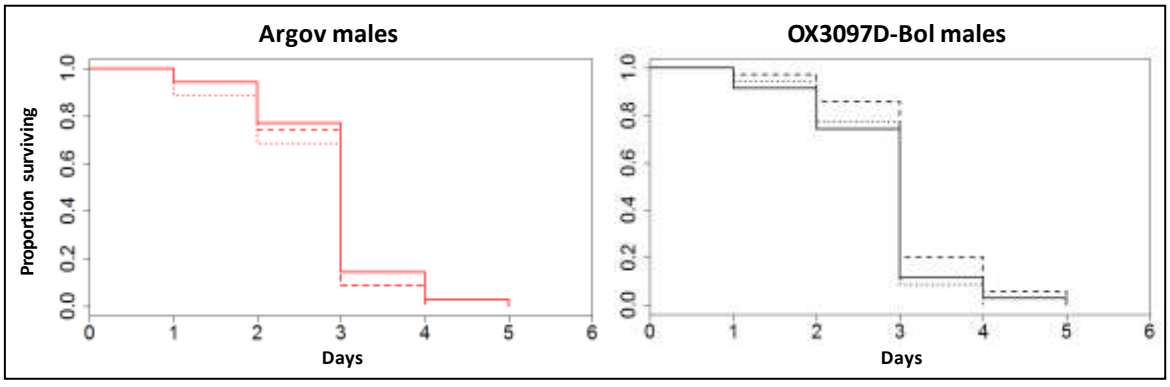


Figure 3.9. Kaplan-Meier survival curves of Argov (red lines) and OX3097D-Bol males (black lines) separated at the late-pharate stage and placed in cages 'under-stress' i.e. containing no food or water source. Three repeats were performed for both strains. For each repeat (n=35).

The median survival time for both male genotypes when reared under stress was 3 days, with mean times of 2.9 days and 2.7 days for OX3097D-Bol and Argov males respectively. A Logrank test using the Kaplan Meier survival estimate was performed, and suggested that there were no statistically significant differences in the life-expectancy of newly emerged males of the OX3097D-Bol and Argov strains (P = 0.284, 1 d.f., ChiSq = 1.1, Logrank test). The results of the stress-test give an indication of the ability of the insects of both strains to store fat reserves through the larval and pupal stages, and thus sustain longevity of the adult fly. The lack of difference between OX3097D-Bol and Argov males suggests that there is no measurable effect of transgenesis on the storage of teneral energy reserves in OX3097D-Bol males. Guidelines developed by the IAEA, USDA, and FAO (IAEA/USDA/FAO, 2003), give advised minimum survival estimates for such experiments for several species. Although the olive fly is not among these species, the guidelines state that there is a cause for concern if less than 50% of adults of the medfly *tsl* strain are able to survive more than 48-hours under stress. Here we show that greater than 80% of OX3097D-Bol males were able to survive past the 48-hour mark in all replicates performed.

3.2.8. Combining the life-history data

As the life-history data presented here includes measurements of fecundity and an estimate of viability for each of the stages of olive fly development for the OX3097D-Bol, Argov, and Democritus strains, the collective outcomes of various fitness impacts can provide an estimate of the net reproductive rate in terms of flight capable adult offspring per rearing cage under high density.

A summary of the life-history data is given in **Table. 3.3**, and includes a cumulative assessment of the losses expected during each developmental stage. The starting point for

the collective assessment of output is the mean total egg yield measured in the high density rearing cages (containing 50 males and 50 females as measured in *Section 3.2.1.*), and follows the expected returns on those eggs. The results show that we expect a cage of 50 OX3097D-Bol females reared at high density to produce on average approximately 3,679 flight capable adult offspring from eggs collected between days-4 and 20 PE. This compares to 4,845 and 6,257 for the Argov and Democritus wild-type strains, respectively.

The reproductive output (in terms of egg to adult survivorship) was measured to be approximately 31% for OX3097D-Bol, compared to 39% for the Argov strain. Transgenesis-related fitness costs could be due to one or more of the following effects associated with homozygous transgenic strains: (1) strain development from a limited number of founding individuals, potentially resulting in inbreeding effects; (2) transposon insertional mutagenesis causing gene disruption; and, (3) expression of potentially deleterious exogenous transgene products. It is difficult to predict how significant this reduction will be (in practical terms) when applied to mass-rearing, however, the widely used medfly temperature sensitive lethal (*tsl*) genetic sexing strain has been measured as possessing a three-fold reduction in viability compared to traditional wild-type strains, requiring mass-rearing *tsl* colonies to be up to three times larger (Caceres, 2002). The benefits that the *tsl* sexing-strain provides in the field [a 3-5 fold increase in effectiveness has been demonstrated for medfly sexing strains over mixed-sex release (McInnis et al., 1994; Rendon et al., 2004; Nitzan et al., 1993)] are considered to easily compensate for any reductions in production efficiency (Caceres, 2002). The relatively modest reduction in OX3097D-Bol developmental viability measured here is therefore not expected to be problematic. Indeed, one argument for using genetic transformation in insects is that large benefits can be achieved with only small and reasonably specific genetic changes, unlike the previous techniques used on the medfly *tsl* strain that rely on chromosomal

translocations, with consequent dramatic declines in fitness (Irvin et al., 2004). Furthermore, there are a variety of further processing steps required for wild-type strain release preparation (e.g. radiation-sterilisation and the application of fluorescent powder dyes) that introduce a variety of additional handling steps into sterile fly production that are not required for OX3097D-Bol. These additional steps are likely to cause reductions in net productive output that have not been taken into account here. Gamma-radiation of the closely-related *Bactrocera invadens* (African invader fly), for example, was found to significantly reduce pupal eclosion rates (between 10-20% lower emergence) when fully-sterilising gamma-ray doses (75Gy) were applied (Ogaugwu et al., 2012). There are also major economic advantages to using a sexing strain that come into effect post-production, when the removal of females results in packaging, transport, and release costs being essentially halved (Caceres, 2002).

The Democritus wild-type strain was found to have a higher developmental viability in a high density mass-rearing scenario than the Argov wild-type strain. Democritus has been in laboratory colonisation for a far greater length of time than the Argov strain; these results therefore seem to be consistent with the hypothesis that Democritus has a greater level of laboratory adaptation to artificial culture compared with Argov. While this may be the case, it is also possible that the Argov strain suffers from a greater degree of inbreeding depression or another genetic problem [(although promising field competitive results with this strain suggest that this is probably incorrect (Rempoulakis & Nestel, Personal communication)]. From the life-history parameters measured, it may seem that the Democritus strain would provide a preferable genetic background for OX3097D-Bol. However, it is often the case that changes in one desirable trait are associated with decreased values in another desirable trait; adaptation parameters may be positive or negative under different environments (Stearns, 1989). Indeed, no single genotype is

expected to provide high trait values in every environment, and a high quality and productive insect in a mass-rearing facility could easily be a poor performer in the field (Sorensen et al., 2012). While this fact does not necessarily condemn Democritus individuals to a fate of poor field performance, studies as early as 1982 (Zervas& Economopoulos, 1982), (some 10 years after first colonisation of the Democritus strain) have documented mating incompatibility effects and low Democritus male competitiveness with wild insects. Conversely, the more developed Argov hybrid strain has exhibited very promising mating competitiveness and compatibility parameters with wild olive flies (Rempoulakis & Nestel, Personal communication). Most mass-rearing protocols follow the principle of regular replacement or refreshment of strains, helping to manage loss of field quality (and avoid genetic problems, e.g. the build-up of deleterious mutations and inbreeding depression) (IAEA/USDA/FAO, 2003). The advantages in life-history traits exhibited by the Democritus strain over the Argov strain under the high density mass-rearing conditions are marginal, and it is highly unlikely that mass-rearing the OX3097D-Bol strain with an Argov background will be problematic. The Argov genetic background was therefore considered a more suitable choice of genetic background for field application than the Democritus background.

Table 3.2. The 'Experimental measurement' columns show a summary of the mean (median for longevities) experimental measurements of the fsRIDL olive fly strain OX3097D-Bol and the two wild-type olive fly strains - Argov, and Democritus, for a variety of life-history traits. The 'Expected returns' columns show an estimate of cumulative loss through each developmental stage from the initial starting egg number (an average of 16-days of egg collections from 50 females caged with 50 males reared at high density).

	OX3097D-Bol		**Argov wild-type**		**Democritus wild-type**	
	Experimental measurement	Net reproductive rate	Experimental measurement	Net reproductive rate	Experimental measurement	Net reproductive rate
Fecundity (high density cage with 50 females)	11,780 (±1,527)	11,780	12,570 (±1,272)	12,570	13,620 (±2,333)	13,620
Egg hatch rate to 1st instar lava	69.4%	8,175	74.5%	9,365	75.4%	10,283
1st instar larval survival to pupation	50.0%	4,088	54.0%	5,057	66.8%	6,869
Pupal eclosion to visually normal adult	90.0%	3,679	95.8%	4,845	91.1%	6,257
% egg to adult survivorship	31%		39%		46%	
Median female survival (days)	14.9	N/A	15.5	N/A	N/A	N/A
Median male survival under stress (days)	3	N/A	3	N/A	N/A	N/A

3.3. Summary and conclusions

Some key issues involved in the mass-release of sterile insects relate to strain quality, including the ability to economically rear large numbers of robust insects (Knipling, 1959). Transgenic fsRIDL strains offer the potential for vast gains in field performance, especially for the olive fly, where conventional wild-type strains have been shown to be ineffective for sterile control (Estes et al., 2011; Economopoulos, 1972; Economopoulos et al., 1978). However, transgenic strains may suffer from deleterious effects of transgene insertion, unrepressed expression of toxic products, and/or effects of reduced genetic diversity that can potentially affect overall developmental viability; strain analysis for mass-rearing potential therefore forms a critical component of strain evaluation.

The present study has examined and compared life-history characteristics of the OX3097D-Bol fsRIDL olive fly strain with the genetically similar Argov wild-type strain, and the Argov strain with the highly laboratory adapted Democritus wild-type strain. OX3097D-Bol was found to have a reduction in developmental viability relative to the Argov strain, producing on average 24% less viable adult offspring per female. However, reductions in fitness are expected when constructing a genetically modified strain (Irvin, 2003), and the benefits provided by OX3097D-Bol, including genetic-sexing, genetic sterility, and endogenous fluorescent marking are likely to far outweigh this reduction in net reproductive output.

Females of the Democritus wild-type strain were found to slightly outperform Argov females in terms of reproductive output. Although it is not possible to determine the exact cause of this, one possibility is a greater degree of laboratory adaptation in the Democritus strain. However, the poor track-record of Democritus male field performance with wild olive flies (Economopoulos, 1972; Economopoulos& Zervas, 1982), and promising new

field performance data for the Argov strain (Rempoulakis & Nestel, Personal communication) suggests that the Argov background is likely to provide field performance benefits.

A low initial egg hatch rate (between days 5-11 post adult eclosion) was found for all strains. Reasons for this are unclear, although it appears that the vast majority of non-hatching eggs are unfertilised.

Increasing the rearing density of OX3097D-Bol was found to cause an overall reduction in egg production per female. This is likely a result of the increased stresses of greater competition for space and a higher rate of waste-product build up with insect overcrowding. Nonetheless, the highest density used here (**Table 3.1**) was found to have the greatest egg production rate per unit of rearing area, suggesting that higher densities should be assessed in order to establish an upper limit and optimise egg production.

It is important to take into consideration the concept of statistical power when planning experiments and drawing conclusions from experimental data. That is, whether a sample size (*N*) is sufficiently large to detect (as significant), an effect of a given minimum size (where effect size is considered to be the discrepancy between a null and alternative hypothesis). The effect size that you may wish to detect may differ from experiment to experiment. For example, a high level of statistical power may be desirable when assessing deleterious effects of transgene influence on life-history traits relevant to mass-rearing. Very slight differences between rival strains may exist, and may be important when considering the costs of scaling-up production for mass-rearing. Detecting a small effect requires a larger sample size; if *N* for a given experiment is too small, statistical testing may lead to non-significant results - even if an effect exists that is relevant to your conclusion (a type II statistical error).

On the other hand, it may also be of interest to consider how much statistical power is really necessary for your particular needs; this may be defined as the difference between statistical significance and practical significance. The inference that a statistically significant difference exists indicates only that the likelihood of an effect of given magnitude occurring is lower than the set critical limit. It does not tell you that the effect is large, important or significant in practical terms. For example, it may be of little practical relevance to detect a small difference in egg hatch rate between a transgenic and a wild-type strain if the transgenic strain, through genetic-sexing, is theoretically able to produce nearly twice as many males per unit resource. In this instance you may only wish to know if transgenic egg hatch rates were compromised to such a high degree as to cause the wild-type strain to be more productive; testing would then be interested in detecting only a large effect and the sample size could be correspondingly decreased, allowing experimental resources to be better invested elsewhere.

Although not performed in this chapter, there are statistical methods that can be utilised to determine the appropriate minimum sample size required to detect an effect of a given magnitude. Ideally an analysis of statistical power would be performed before an experiment is carried out. A number of *a priori* power analyses are available and can provide an investigator with a proposed minimum sample size. *Post-hoc* power analyses also exist and can provide an investigator with an indication of how confident they can be about conclusions drawn from their data.

Although a reduction in net reproductive output was measured for OX3097D-Bol, suggesting some costs to transgenesis, the differences are not severe compared to other sexing strains used in tephritid SIT, and do not give a significant concern for mass-rearing. Life-history comprises just one of the major components of strain fitness that are important for SIT application. The following chapter focuses on assessing the competitiveness of

OX3097D-Bol for a variety of pre and post-copulatory sexual-selection traits with both the Argov strain, and field-collected wild olive flies.

3.4. Materials and methods

3.4.1. Assessing fecundity

Pupae were reared at a larval density of 1 larva/0.8g of larval medium (as described in *Section 2.4.1.*). On pupal eclosion, standard rearing cages of dimensions 12x12x15cm were set-up containing 50 males and 50 females (for high density) or 25 males and 25 females (for medium density) (see **Table 3.1**). Cages were supplied with fresh wax cones daily for female oviposition. Cages were also supplied with tetracycline water (100µg/ml), and ~2g of olive fly adult diet (as described in *Section 2.4.1.*). Eggs were collected between days 4 and 20 post adult eclosion (PE), pipetted into a volumetric measuring tube, and left for 5 minutes to settle on the bottom. The egg volume was recorded. Conversions of egg volume to egg number used a rate of 10µl = 300 eggs. (Estimated from three repeats of 10µl of eggs placed onto filter paper and counted under a dissection microscope, giving a mean ± SD of 303 ± 36 eggs.) Numbers of dead females in each cage were assessed daily. Egg production per female was assessed by dividing egg number by number of remaining females for any given day.

3.4.2. Assessing egg hatch rates over time

Adults from the OX3097D-Bol, Argov and Democritus strains were reared at a high density in standard rearing cages (three cages for each strain). Eggs were collected at 3-day intervals between days 5 and 20 PE. Three samples of between 100 and 200 eggs were taken from each cage, and pipetted onto damp filter paper containing tetracycline (100µg/ml). Eggs were counted and left to develop for 72-hours at 23ºC, where-upon the numbers of non-hatched eggs were counted (at this temperature, 48-hours is sufficient for hatching), and percentage hatch rates were calculated. A mean hatch rate value was

calculated across the three egg samplings from each cage. A mean of these means was then calculated across the three cage-repeats for each strain.

3.4.3. Assessing larval survival to pupation

Eggs were collected from adults of each strain at day-6 PE, and reared at a high density in standard rearing cages. Eggs were left on damp filter paper containing tetracycline (100µg/ml) in a sealed Tupperware box at 23ºC for 48 hours to develop. For each repeat 50 L1 larvae were individually placed onto petri-dishes containing 40g of larval dietary medium (0.8g larval medium per larva). Petri dishes containing larvae and diet were placed in individual sealed Tupperware boxes with a large ventilation hole in the lid. Sand was spread on the floor of each box, and boxes were left at 23°C for 12 days. 5 repeats were performed for each strain. Pupae were collected by sieving the sand, and picking out individuals that had pupated in the larval diet, and were counted. Percentage survival to pupation was calculated.

3.4.4. Assessing adult emergence rate

Larvae of the OX3097D-Bol, Democritus and Argov strains were reared at a density of 0.8g of larval diet per larva, with larval diet seeded with tetracycline (100µg/ml) and resulting pupae were collected. A selection of pupae from each strain were placed individually into the wells of a 96-well plate and left at 23°C to develop. To avoid bias in pupal selection, the tests were performed blind. Emerging adults were scored as emerged-normal, emerged abnormal (including fully emerged but with obvious deformities) or non-emerged. Two 96-well plates totalling 192 pupae were analysed for each strain. Percentages of each category for each strain were calculated.

3.4.5. OX3097D-Bol and Argov female longevity

Larvae from the OX3097D and Argov strains were reared at a density of 0.8g of larval diet per larva in the presence of tetracycline. Resulting pupae were collected and were left at 23ºC until adult fly eclosion. Upon eclosion, flies were sexed and standard rearing cages were set-up with 50 males and 50 females from each strain (high density). Each cage was supplied with fresh adult diet, water (containing 100μg/ml of tetracycline), and egg laying substrate every 3 days. Cages were checked once every 24 hours for deceased females, and the numbers were recorded. Observations continued until all flies were deceased.

3.4.6. OX3097D-Bol female longevity at medium and high rearing densities

Experimental protocol was as in *Section 3.4.5.*, however only the OX3097D-Bol strain was used and standard rearing cages were set-up containing 25 males and 25 females for the medium rearing density.

3.4.7. OX3097D-Bol and Argov male longevity under stress

Larvae from the OX3097D and Argov strains were reared at a density of 0.8g of larval diet per larva. Resulting pupae were kept at 23ºC until adult fly eclosion, whereupon three cages for each treatment were set-up containing 35 late-pharate stage males in each. The cages were provided with no food or water source. Observations for dead males were made daily, and dead flies removed from each cage daily to avoid double-counting. Observations and counting continued until all flies were dead.

3.4.8. Statistical analysis

For the statistical analysis, the genetically similar OX3097D-Bol and Argov strains are compared separately, with any significant differences being attributed to OX3097D-Bol transgenesis and homozygosis. Similarly, the Argov and Democritus strains both posses a purely wild-type background and were compared separately. When using a parametric analysis, data were first subject to a test of normality using a Shapiro-Wilk test. All statistical tests were performed using 'R', with the 'Survival' package used for Kaplan-Meier plots and Logrank tests. All tests assumed a standard 95% confidence level.

Olive fly pair *in copula.*

Chapter 4 - Sexual selection of OX3097D-Bol males

4.1. Introduction

Mating competitiveness is regarded as the most important indicator of sterile male quality (Hendrichs et al., 2002), and the field-cage mating test is the standard method for determining the relative mating competitiveness of a strain (IAEA/USDA/FAO, 2003). Mating competition tests are comparative studies, usually involving equal numbers of males from a sample strain and a reference strain competing for copulations with a limited number of females. The most robust version of the test is performed using wild-caught insects as a reference, in as realistic semi-field conditions as can be achieved. This often involves the use of cage-contained host trees and natural light cycles.

Mating tests can provide an investigator with a host of useful information on male quality including data on ability to competitively attract females, peak mating activity, and average copulation duration. Estimating the peak mating behaviour is an especially important parameter for the olive fly, as one of the envisioned advantages for a genetic sexing strain was that female removal would encourage males to synchronise with the wild female mating photoperiod [a critical problem concerning olive fly SIT in the past (Estes et al., 2011; Economopoulos& Zervas, 1982)]. Significant reductions in copulation duration have been found for mass-reared strains of *Anastrepha obliqua* (Rull et al., 2012), and medfly (Briceno et al., 1996; Field& Yuval, 1999). It has been suggested that this may be an indicator of low post-copulatory male competitiveness, and may suggest reduced levels of sperm transfer (or other substances in the ejaculate e.g. accessory gland products) which could result in increased propensity for female remating (Rull et al., 2012). If remating does occur, reduced sperm transfer may lead to lower sperm competitiveness and lower fertilisation success for the sterile males (potentially affecting efficacy of the strain

in sterile insect control). We therefore also looked for effects of male genotype on copulation duration and ability to induce refractoriness to female remating.

The sexual selection tests presented in this chapter fall roughly into two categories. Firstly, tests were performed with OX3097D-Bol males and the Argov wild-type strain in a glasshouse in Oxford. This was a preliminary study to determine whether any obvious impacts of transgenesis and strain homozygosis were affecting male sexual competitiveness. Any significant reductions in competitiveness with the genetically similar Argov wild-type strain would give serious concerns as to the field applicability, and raise doubts over the value of investing further time and resources in strain development. Females of the Argov strain were also used for sperm competition studies, requiring the rearing and analysis of progeny. Unfortunately, wild insects have impractically low levels of larval survival in artificial culture and so were not used for sperm competition assessment. Secondly, a series of mating competition tests were performed with OX3097D-Bol males and wild-collected olive flies in glasshouses in Crete (glasshouse space was provided by the Laboratory of Dr John Vontas, Laboratory of Applied Entomology, The University of Crete in Heraklion). This was a more robust estimation of the likely mating competition of OX3097D-Bol males in the field, and allowed us to investigate photoperiod compatibility between the OX3097D-Bol males and wild females.

When the sexual competitiveness of a strain is discussed in relation to the sterile insect technique, it is often assumed to mean an estimation of the copulatory ability of males. However, a more encompassing definition includes a consideration of parameters concerning both pre and post-copulatory sexual selection. Sperm from male tephritids are transferred to females and are stored in specialised sperm storage organs called spermathecae; in theory therefore a female need only mate once in order to fertilise all the eggs she will lay in her life-time. However, whether due to reasons of sperm senescence,

sperm depletion, male inadequacy, increasing the genetic heterogeneity of offspring, or insurance against male sterility, female remating (polyandry) often appears to be a sensible reproductive strategy [the work of Neilson & McAllan (1965), for example, reports that increasing mating frequency in the tephritid apple maggot, *Rhagoletis pomonella*, can increase female fertility and therefore lifetime reproductive success], and female insects will often mate multiple times [reviewed for *Drosophila* in (Singh et al., 2002)].

Because sperm from a previous mating is stored, the sperm of successive matings can coexist in sperm storage organs simultaneously, creating the potential for competition between the sperm for the fertilisation of eggs (Parker, 1970), and the possibility of differential non-random usage of sperm by a female (cryptic female choice) (Eberhard, 1996). If the sperm from a released sterile or transgenic male is somehow impaired or of low quality, the sperm from a previously or subsequently mated wild male may gain a correspondingly increased paternity share. We therefore set out to determine the competitiveness of OX3097D-Bol male sperm. We consider both sperm defence and offence ability; that is, the proportion of offspring fathered when a target male is the first of two to mate, and the paternity share when the mating of a target male proceeds that of a rival, respectively (Boorman& Parker, 1976).

Female remating is clearly in conflict with the interests of the first male mate, and after mating, female insects often show reduced receptivity to further matings, a process that is, at least in part, a male adaptation (Wolfner, 1997). In *Drosophila melanogaster* female post-mating responses are elicited by male pheromones, sperm, and peptides found in the ejaculate. The male accessory glands have been implicated in the production of the sex-peptide, a component of the seminal fluid that has been found to reduce female receptivity to mating, and stimulate egg production (Chapman, 2001). The deleterious processes involved in irradiation or transgenic male strain generation may affect the ability

of a male to induce female post-mating responses, possibly causing a reduction in male post-copulatory competitiveness. The irradiation of medfly, for example, has been shown not only to reduce the ability of males to compete for mates, but also to increase the likelihood that mated females will subsequently remate (Kraaijeveld& Chapman, 2004). Furthermore, the second mate is also preferentially wild rather than sterile. The ability of transgenic males to induce refractoriness to female remating is therefore an important consideration for sterile insect control, and is a further parameter considered in this chapter.

4.2. Results and discussion

4.2.1. Mating competition tests

The OX3097D-Bol fsRIDL olive fly strain was assessed to determine the ability of males to compete with Argov wild-type males for copulations with Argov wild-type females. The experiments were carried out according to standardised guidelines for mate competition tests (IAEA/USDA/FAO, 2003). Tests were performed in small field cages containing olive trees situated in a glasshouse at the Department of Zoology, the University of Oxford. Each experiment contained 50 OX3097D-Bol males, 50 Argov males, and 50 Argov females. Eight replicates were performed with a total of 235 female mate-choices analysed.

Temperature in the glasshouses could not be regulated and so was recorded using a HOBO® data logger (see *Section 4.4.1.*). In accordance with guidelines (IAEA/USDA/FAO, 2003), a single experiment was only considered valid if the mating propensity was greater than 0.2 (that is, only if more than 20% of the females copulated). A lower propensity is considered to be an indication of low general quality of the insects or a problem with the experimental conditions. Copulation initiation times and durations were also recorded for each couple. The genotype of successfully mated males were checked by fluorescence microscopy.

The results from mating competitiveness experiments are usually presented as a parameter known as the 'relative sterility index' or (RSI). The RSI is the proportion of the total matings that the sample strain gained in a given mating test. Results are summarised in **Fig. 4.1**.

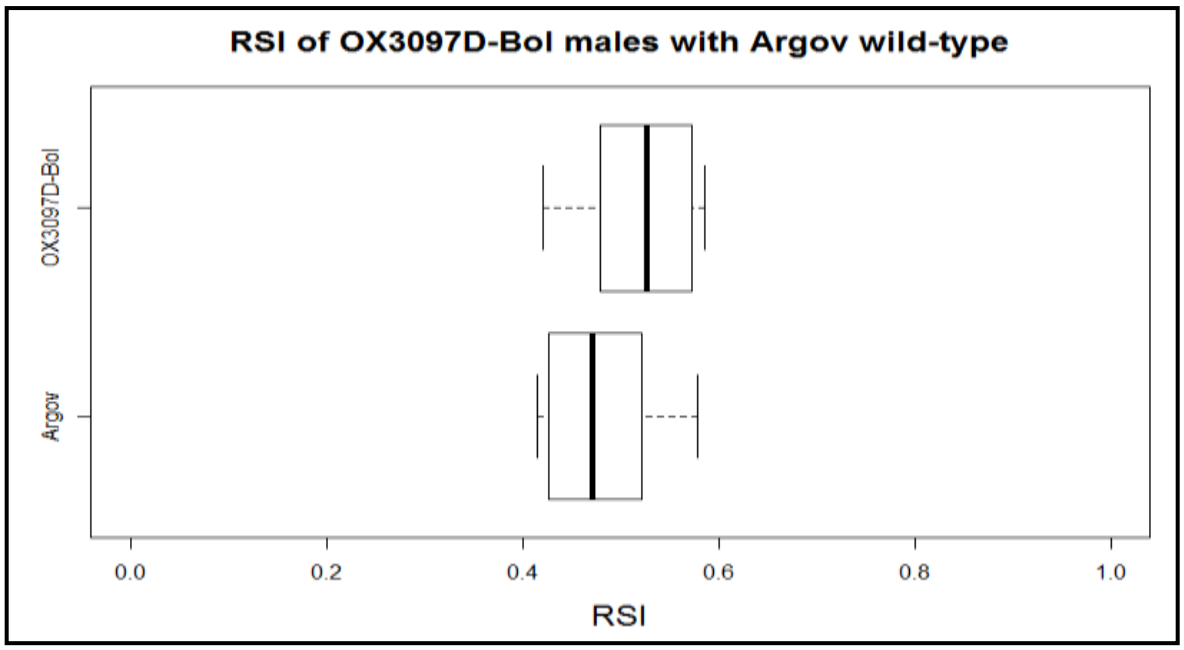


Figure 4.1. Box and whisker plots showing range of RSI values for OX3097D-Bol males and Argov males when competing for copulations with Argov females. Eight replicates were performed (n=8).

The OX3097D-Bol males gained an average of 52% of the matings when competing with Argov males for copulations with Argov females (RSI = 0.52 ± 0.06 SD). Differences between the RSI values for the OX3097D-Bol and Argov males were not found to be statistically significant (P = 0.38, 1 d.f., likelihood ratio test for goodness of fit), suggesting that OX3097D-Bol has no reduction in mating competitiveness relative to Argov males when competing for Argov females in glasshouse-based field cage studies.

Mating competition tests were also performed in a glasshouse at the University of Crete with wild adults recovered from pupae in infested olive fruit collected around Heraklion, Crete. Each experiment used 50 OX3097D-Bol males, 50 wild males, and 50 wild females. In total, 15 replicates were performed, culminating in the analysis of the mating choices of 406 wild females. Results are summarised in **Fig. 4.2**.

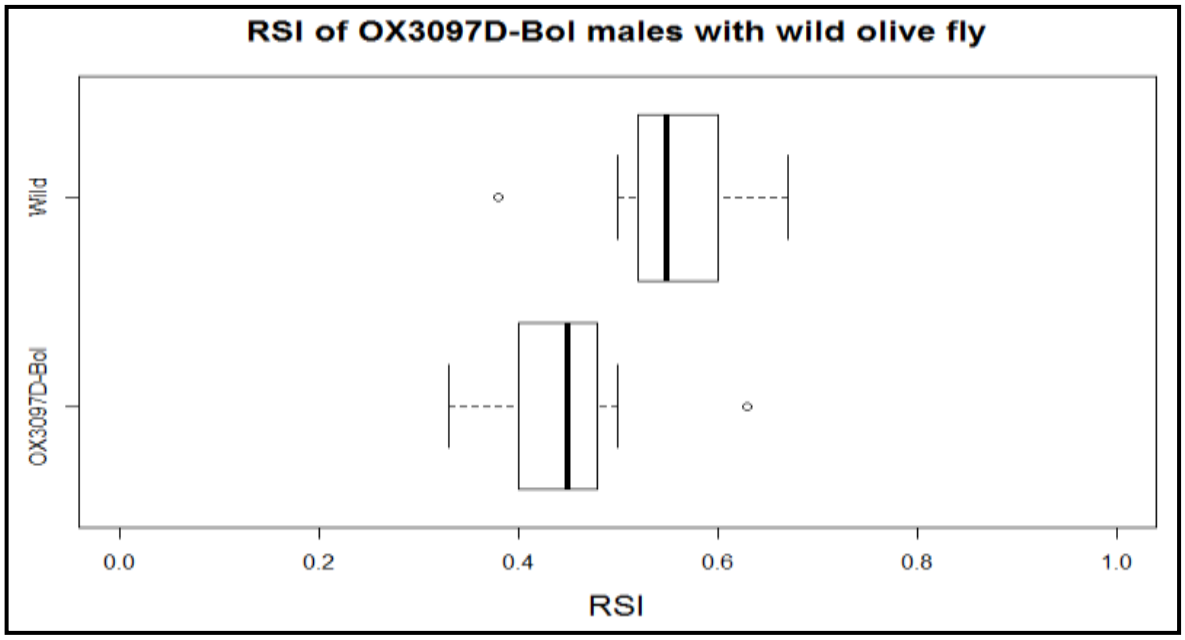


Figure 4.2. Box and whisker plots showing ranges of RSI values obtained for OX3097D-Bol males and wild males when competing for copulations with wild females (n=15).

Wild males slightly outperformed the OX3097D-Bol males, gaining an average of 56% of the total matings (RSI values of 0.56 and 0.44 for wild and OX3097D-Bol males, respectively). Differences in mating success were found to be statistically significant (P = 0.012, 1 d.f., likelihood ratio test for goodness of fit).

OX3097D-Bol males showed a slight but significant reduction in competitiveness when competing against wild-caught olive flies. Although this difference represents a clear reduction in relative competitiveness with wild males, an RSI of 0.44 is exceptionally high for a sterile strain and far exceeds the established minimal thresholds usually applied to SIT. For medfly, standardised guidelines suggest that an acceptable level of sterile male competitiveness with wild insects in caged mate competition studies is an RSI of over 0.2 (IAEA/USDA/FAO, 2003).

Past experiments performed with olive fly have shown that radiation sterilisation can have a strong negative impact on male mating competitiveness, with irradiated wild males raised on artificial diets becoming less sexually active by a factor of 3 or 4

(Economopoulos, 1972). As no reduction in competitiveness was measured between the OX3097D-Bol males and the Argov males, it reasonable to infer that the differences in mate competition observed between OX3987D-Bol males and wild males are largely a result of laboratory colonisation and adaptation common to the Argov wild-type background, and not a result of transgenesis.

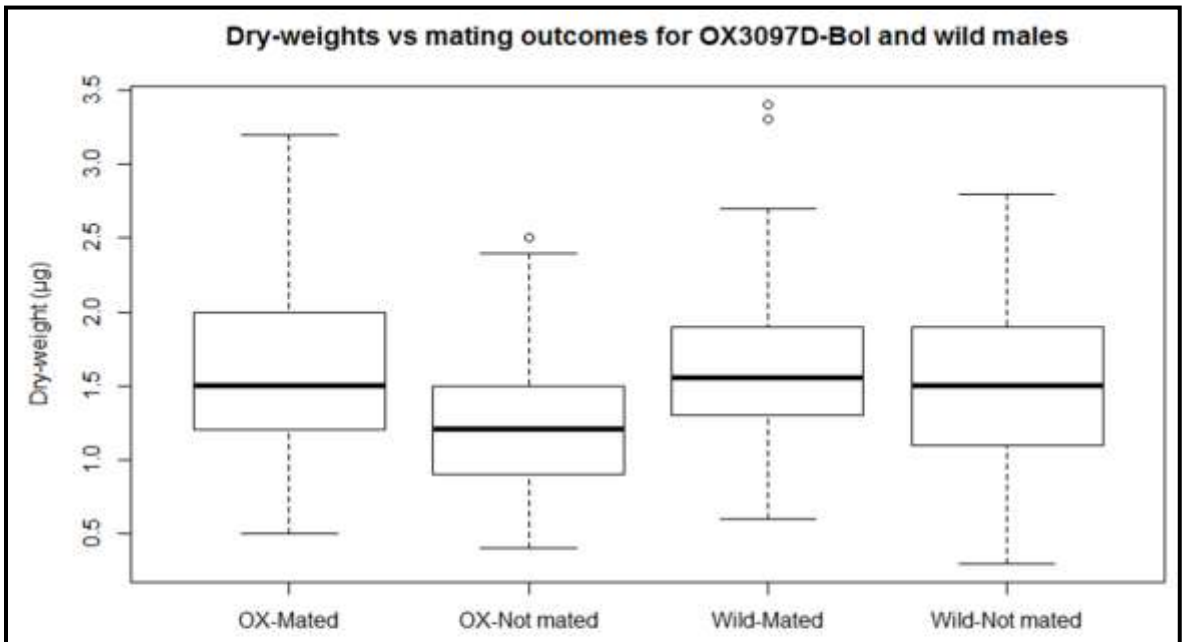
The results presented here agree well with preliminary studies by Rempoulakis and Nestel (personal communication), who found an RSI value of 0.4 for Argov males manually-sexed, and competing with wild olive flies collected in Israel. They found however, that when the Argov males were irradiated (exposed to 100Gy of gamma rays) the RSI value fell to less than 0.2. This suggests that the inbuilt genetic sterility of the OX3097D-Bol strain may provide a clear mating competitiveness advantage over traditional wild-type olive fly strains that require radiation sterilisation.

4.2.2. The effects of size on male mating competitiveness

Male size has been found to be an important determining factor in reproductive success for a variety of insect species [including *Drosophila melanogaster* (Partridge& Farquhar, 1983); *Drosophila pseudoobscura* (Partridge et al., 1987); and the medfly (Kaspi et al., 2000), reviewed in (Thornhill& Alcock, 1983)]. Interestingly, the body size of a male mate has also been correlated with female life-time reproductive success in the seed beetle, *Stator limbatus* (Fox et al., 1995).

The dry-weight of insects has often been used as a convenient relative measure of body size (see Honěk, 1993; Banno, 1990; Ochieng-Odero, 1990), and other measures of size like body-length have been correlated with weight (e.g. Rogers et al., 1976; Jarosik, 1989). The dry-weights of wild and OX3097D-Bol males that were either successful or

unsuccessful in copulating with a wild female during the mating competition studies were measured. The dry-weights for the respective cohorts are given in **Fig. 4.3**.



	OX3097D-Bol mated	OX3097D-Bol not mated	Wild mated	Wild not mated
Mean dry weight (µg)	1.63±0.67	1.25±0.56	1.62±0.49	1.47±0.56
Median dry weight (µg)	1.5	1.2	1.6	1.5
OX3097D-Bol Mated (*P*)				
OX3097D-Bol Not mated (*P*)	$4.4x10^{-5}$			
Wild Mated (*P*)	0.96	$1.1x10^{-5}$		
Wild Not mated (*P*)	0.12	0.02	0.17	

Figure 4.3. Box and whisker plots showing distributions of dry-weights (µg) of OX3097D-Bol (OX) and wild males that were either successful (mated) or unsuccessful (not mated) in copulating with wild females in mate competition tests. Table (upper) shows mean (±SD) and median dry weights for each cohort. A 1-way ANOVA was performed followed by a Tukey HSD multiple pair-wise comparison test. Table (lower) shows the resulting *P* values. For OX3097D-Bol mated n=177, not mated n=232. For wild mated n=227, not mated n=272.

The dry-weights for each cohort passed a test for normality ($P > 0.1$, Shapiro-Wilk). A 1-way ANOVA was performed followed by a Tukey HSD multiple pair-wise comparison test. The mean dry weight for a mated OX3097D-Bol male (1.63µg ± 0.67 SD) was found to be significantly higher than that of an un-mated OX3097D-Bol male (1.25µg ± 0.56 SD) ($P = 4.4x10^{-5}$, 1 d.f. 1-way ANOVA followed by Tukey HSD).

However, the mean dry weight for a mated wild male (1.62µg ± 0.49 SD) was not found to be significantly higher than that of an unmated wild male (1.47µg ± 0.56 SD) (P = 0.178, 1 d.f., 1-way ANOVA followed by Tukey HSD).

Significant differences were also not observed between mated OX3097D-Bol males or mated wild males (P > 0.9, 1 d.f., 1-way ANOVA followed by Tukey HSD). However, the mean dry weights of an unmated OX3097D-Bol male was found to be significantly lower than that of an unmated wild male (P = 0.018, 1 d.f., 1-way ANOVA followed by Tukey HSD).

The size data for mated and unmated males of both genotypes were pooled. A comparison of the overall means sizes of the OX3097D-Bol males and wild males used in the mate competition study is shown in **Fig. 4.4**.

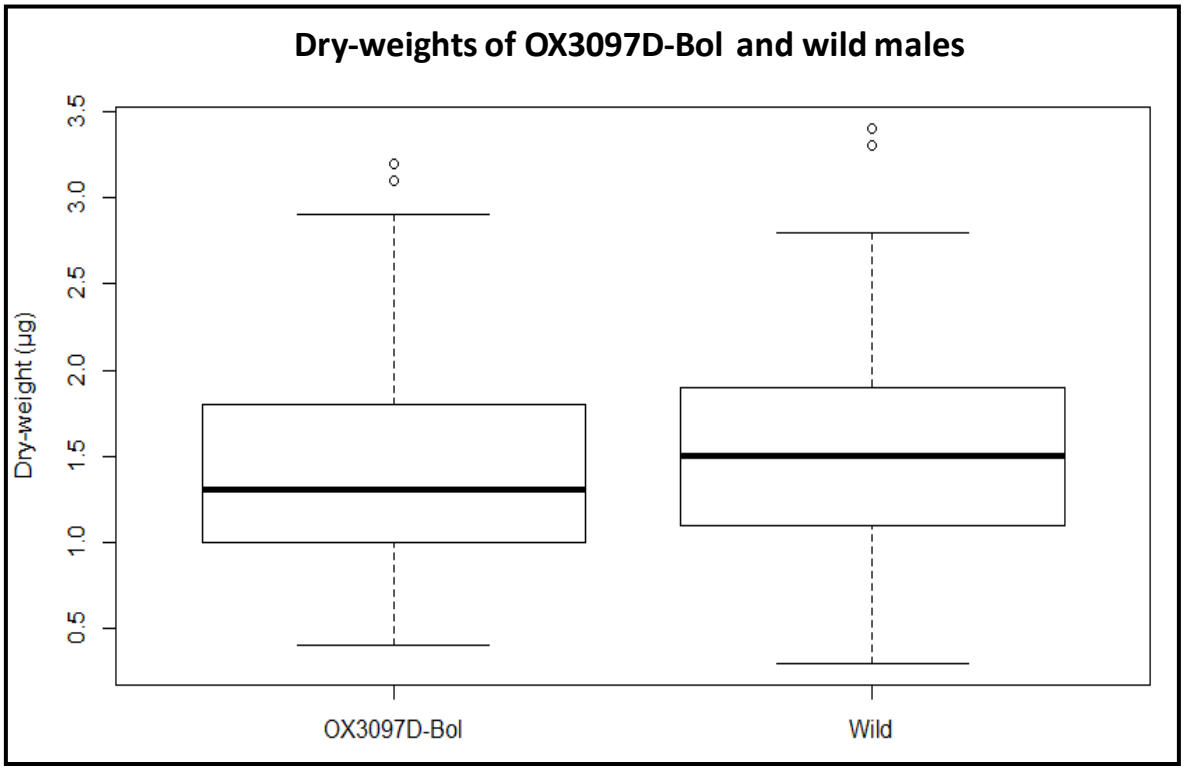


Figure 4.4. Box and whisker plots showing medians and distributions of dry-weights (µg) of OX3097D-Bol and wild males used in mate competition tests. The median weight of an OX3097D-Bol male was found to be 1.30µg (mean of 1.33µg ± 0.62 SD), and the median weight of a wild male was found to be 1.5µg (mean of 1.544µg ± 0.53 SD). For wild males n=498; for OX3097D-Bol males n=509.

A Shapiro-Wilk test suggested that the data were normally distributed ($P > 0.1$, Shapiro-Wilk). A 1-way ANOVA was used and suggested that the dry-weight of OX3097D-Bol males was significantly lower than the dry-weights of wild males ($P = 0.005$, 1 d.f., 1-way ANOVA). A coefficient of variation was calculated to normalise the data distributions of the two cohorts by size, and was found to be 0.46 for OX3097D-Bol males and 0.34 for wild males, suggesting that the variation in size between the OX3097D-Bol male cohort was greater than that found within the wild male cohort.

We found that OX3097D-Bol males that successfully gained copulations with females were significantly larger on average than unsuccessful males, suggesting that male size may be a contributing factor or is covariable with another factor important in determining mating success. However, size was not found to have a significant influence on wild male mating success in the experiments presented here. The lower mean male dry-weight recorded for OX3097D-Bol compared to wild males (1.33µg for OX3097D-Bol and 1.54µg for wild), and the greater coefficient of variation found for the sizes of OX3097D-Bol males compared to wild males, suggests the presence of a greater number of relatively small males in the OX3097D-Bol cohort. It is possible that the wild females were discriminating against the relatively smaller males in the mating study (or indeed, that smaller males were less good at vying for optimal territories). Producing larger OX3097D-Bol males (or males with lower size variability) may therefore help to increase strain sexual competitiveness, although this is clearly in a trade-off with production volume. In Drosophilids, a number of environmental factors are known to influence adult size, including: temperature, larval crowding, larval nutrition, and genetic factors (Partridge et al., 1987). Improving rearing or artificially selecting for larger males may therefore allow for a more uniformly large male population to be produced at the same rearing density.

4.2.3. Copulation initiation times

The copulation initiation times of males of both genotypes were recorded during the mating competition tests. During courtship, olive fly males vibrate their wings and stridulate by rubbing the wing microtichia against the abdominal pecten (Benelli et al., 2012), creating a mating stimulus for females that may serve to attract and test their willingness to mate. In attempting to copulate, the male jumps onto a female, trying to align himself along her body axis; if receptive, the female remains still, allowing male's intromission. In the following experiment, copulation initiation is defined as the point at which male-female intromission occurs.Initiation times shown are relative to scotophase (the dark period in a light/dark cycle). As experiments were performed in a glasshouse under natural light, the onset of scotophase has been taken to be sun-down on a given day [exact time provided by meteorological data (www.metoffice.gov.uk for tests in Oxford, and www.poseidon.hcmr.gr for tests in Crete)]. The initiation of mating was recorded as the point of female mounting by a male. Results for mating initiation times for OX3097D-Bol males and Argov males with Argov females are shown in **Fig. 4.5**.

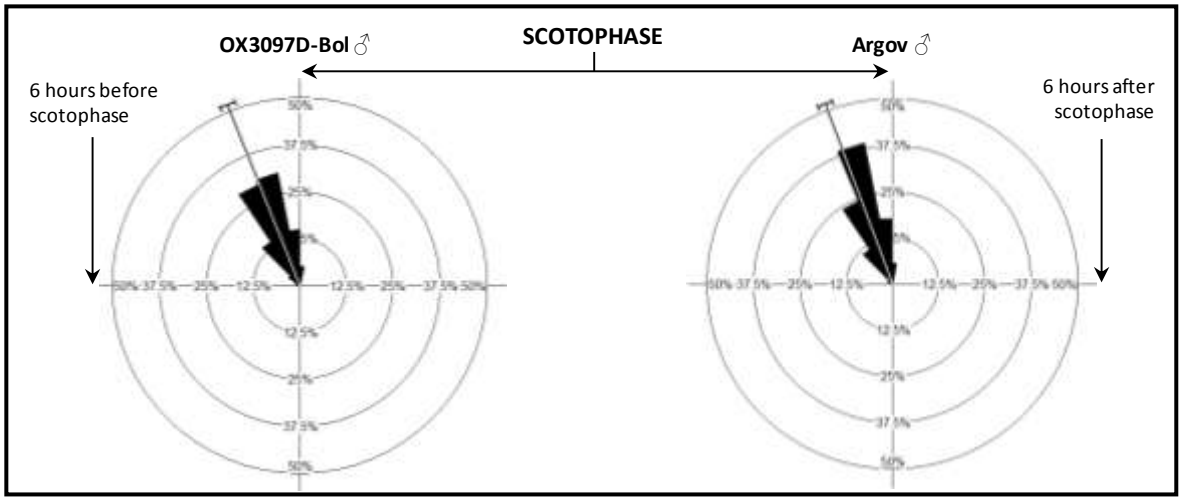


Figure 4.5. Circular histograms showing mating initiation times for Argov females with OX3097D-Bol males (left circle, n = 122), and Argov males (right circle, n = 113). Scotophase is the dark phase of a light/dark cycle. Each 'wedge' on the circular graphic represents a time interval of 45 minutes; the radial length of the wedge indicates the proportion of total matings of that type that occurred in each time segment. Mean copulation initiation time for Argov females and either OX3097D-Bol or Argov males was 82 and 87 minutes prior to scotophase respectively. Peak mating activity times were not significantly different between the two types of male (*P* = 0.43, 1 d.f., circular statistics Watson-Williams F-test).

The mean copulation initiation time for OX3097D-Bol males with Argov females was found to be 87.4 minutes prior to scotophase (±56.5mins SD), and the mean copulation initiation time for Argov males with Argov females was found to be 82.4 minutes prior to scotophase (±48mins SD). A Watson-Williams circular statistics F-test was performed and suggested no significant differences between the copulation initiation times of Argov females with either OX3097D-Bol males or Argov males (P = 0.432, 1 d.f, Watson-Williams circular statistics F-test).

The copulation initiation times were also recorded for experiments in Crete involving couples containing wild females and either OX3097D-Bol or wild males. Results are shown in the circular histograms in **Fig. 4.6**.

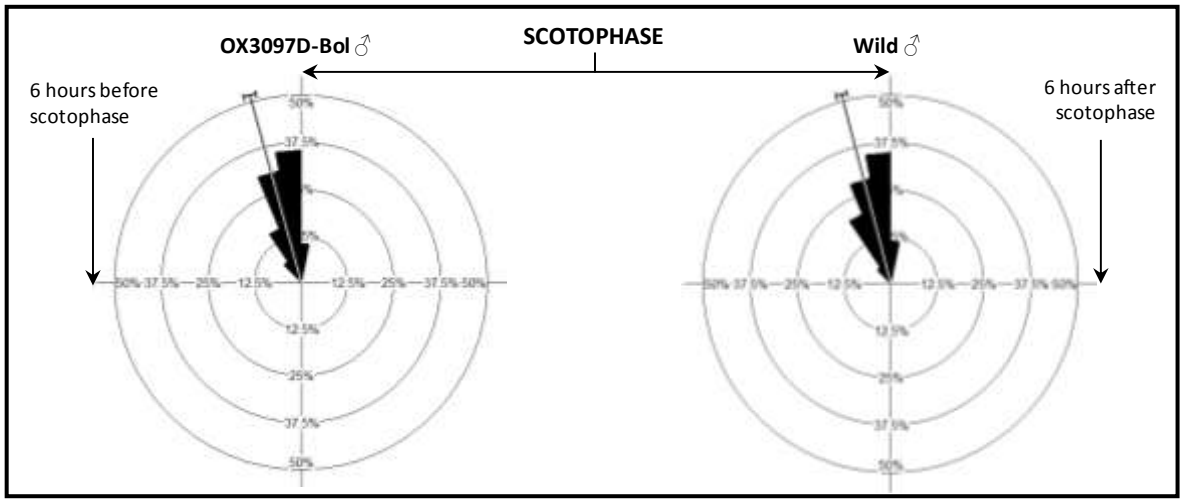


Figure 4.6. Copulation initiation times were similar for OX3097D-Bol males and wild males. Copulation initiation times were recorded for all mating pairs; each pair contained a wild female and either an OX3097D-Bol male (left circle, n = 216) or a wild male (right circle, n = 161). Scotophase is the dark phase of a light/dark cycle. Each 'wedge' on the circular graphic represents a time interval of 45 minutes; the radial length of the wedge indicates the proportion of total matings of that type that occurred in each time segment. Mean copulation initiation time for wild females and either OX3097D-Bol or wild males was 63 and 66 minutes before scotophase respectively. Peak mating activity times were not significantly different between the two types of male (P = 0.45, 1 d.f., circular statistics Watson-Williams F-test).

The mean copulation initiation time was found to be 63 minutes (± 43.5 mins SD) prior to scotophase for OX3097D-Bol males and wild females, and 66 minutes (± 53 mins SD) prior to scotophase for wild males and wild females. A circular statistics Watson-

Williams F-test suggested that there were no significant differences in mating initiation times between OX3097D-Bol males and wild males competing for wild females (P = 0.45, 1 d.f., Watson-Williams circular statistics F-test).

Mating asynchrony is a major concern in the application of SIT to olive fly control (Zervas& Economopoulos, 1982; Estes et al., 2011; Economopoulos& Zervas, 1982). It was found that when mixed-sex olive flies of the Democritus strain were released into populations of wild insects, the two types of females tended to mate with their own males. This was hypothesised to be the result of an inadvertent artificially selected shift away from the natural mating photoperiodicity in the highly laboratory-adapted strain. The assortative mating, however, was not observed when Democritus females were removed from the field cages (Estes et al., 2011; Economopoulos& Zervas, 1982), leading to the suggestion that a sexing strain would be highly beneficial for olive fly SIT.

Results presented here suggest that sex sorted OX3097D-Bol males do not have a significant difference in peak mating activity when competing against Argov wild-type males for copulation with Argov wild-type females. This was expected as both share the same genetic background. Moreover, and more significantly, the mating initiation times recorded with OX3097D-Bol competing against wild males for copulations with wild females also suggests no difference in peak mating activity for the sex sorted males.

The previous studies of (Economopoulos& Zervas, 1982) using the Democritus strain and wild flies found that Democritus strain couples initiated the majority of copulations approximately 120 minutes before that of the wild couples (that is, approximately 240-300 minutes prior to scotophase); far earlier than we report here for Argov couples (an average of 87 minutes prior to scotophase). This is presumably caused by a greater degree of laboratory adaptation in the Democritus strain, and raises the possibility that the Argov strain would not require sexing for successful SIT application.

However, even slight levels of assortative mating is likely to be greatly exaggerated in the field as females are dispersed and can more easily escape copulation attempts from undesired males (Estes et al., 2011), and unless active steps were taken, the development of further photoperiod asynchrony would be a continuing concern with the Argov strain.

Factors affecting sexual competitiveness, while important, may not be pivotal to the success of an SIT programme. After all, these effects can be mitigated by increasing the number of released sterile males. Mathematical modelling has shown that changes in mating synchrony, however, can cause complete or almost complete failure of mating under field conditions (Ito& Kawamoto, 1979). The finding of fully synchronous mating between OX3097D-Bol and wild female olive flies is therefore highly significant for olive fly SIT.

4.2.4. Copulation duration

The copulation durations of couples were also measured for the experiments involving OX3097D-Bol males and Argov males with Argov females. The results are shown in **Fig. 4.7**.

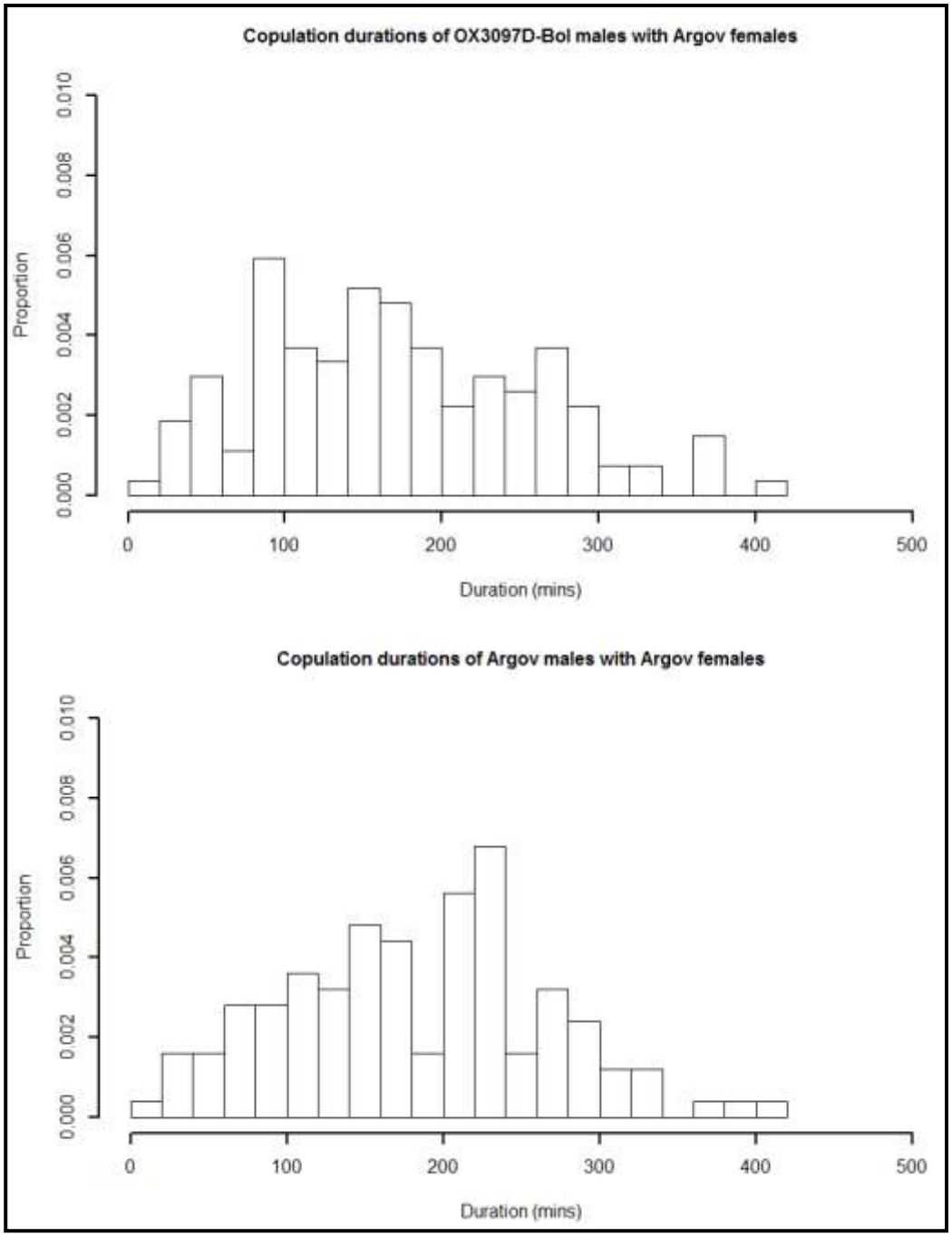


Figure 4.7. Histograms showing mating durations (mins) for Argov females with (**A**) OX3097D-Bol males (n= 122), and (**B**) Argov males (n=113).

The mean copulation duration for OX3097D-Bol males and Argov females was found to be 171 minutes (± 67.8 mins SD), and 183.2 minutes (± 82.9 mins SD) for Argov males and Argov females. The durations for each male type passed a test for normality (*P* = 0.179, Shapiro-Wilk) and a 1-way ANOVA was performed. Statistical testing suggested

no significant differences between the copulation durations of Argov females with either OX3097D-Bol males or Argov males (P = 0.211, 1 d.f., 1-way ANOVA).

Copulation durations were also recorded for experiments involving OX3097D-Bol males and wild males with wild females. The results are shown in **Fig. 4.8**.

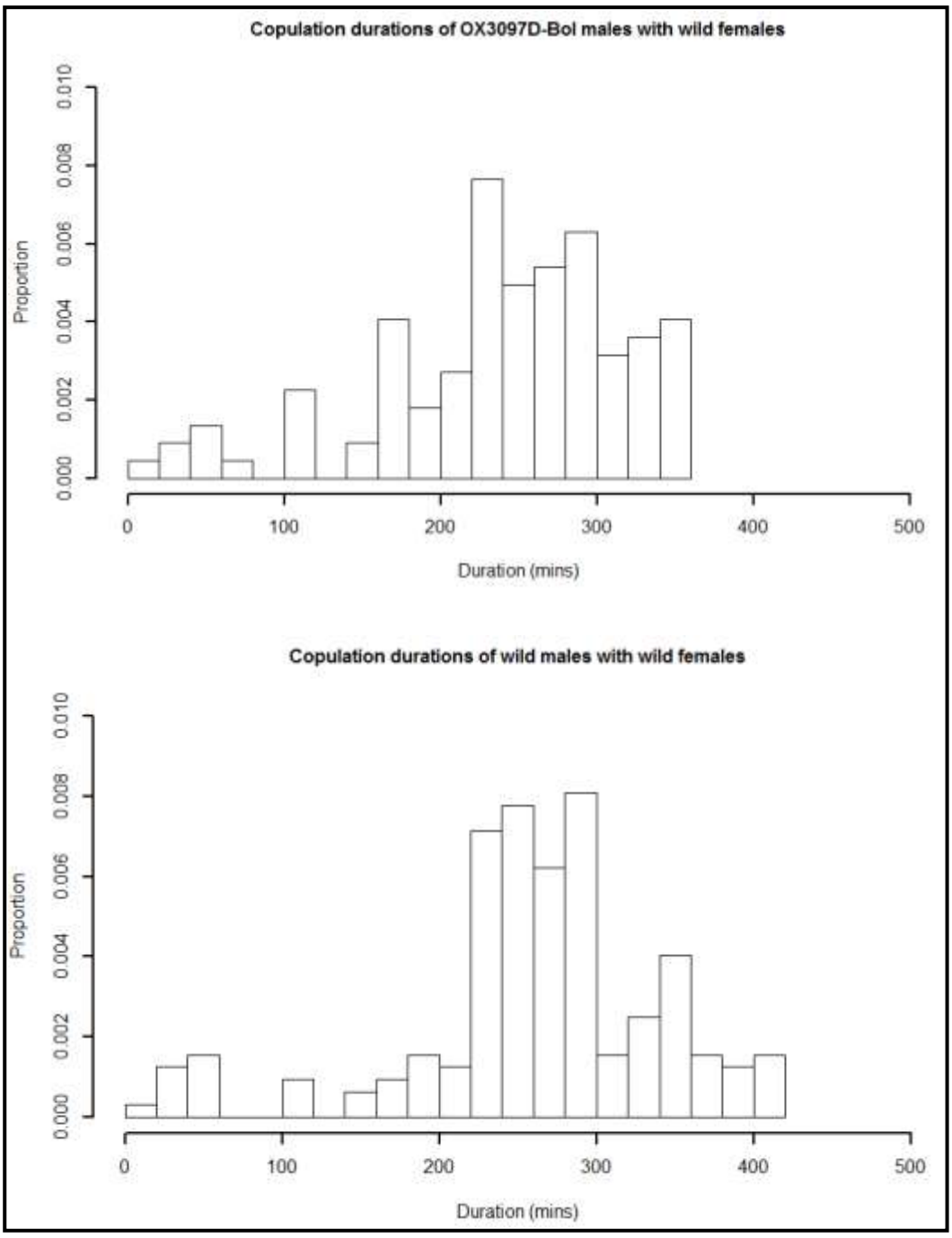


Figure 4.8. Histograms showing mating durations (mins) for wild females with (**A**) OX3097D-Bol males (n=179), and (**B**) wild males (n=225).

The mean copulation duration was found to be 242 (± 87.9 mins SD) minutes for a wild female and an OX3097D-Bol male, and 263 (± 77.8 mins SD) minutes for a wild male and a wild female. The data were not found to be sufficiently normally distributed to perform a means-based parametric statistical analysis ($P<0.01$, Shapiro-Wilk). Therefore, a non-parametric Wilcoxon rank sum test was performed, which suggested that copulations containing an OX3097D-Bol male and a wild female had a significantly shorter duration than those containing a wild male and wild female ($P = 0.02$, W=7528.5, Wilcoxon rank sum test).

The length of time spent *in copula* can be an indication of the degree of laboratory adaptation of a strain and can be related to transfer of sperm and accessory gland fluid to the female fly; mass reared males tend to have reduced copulation times when compared with wild males which is associated with increased tendency of females to remate (McInnis et al., 2002, IAEA/USDA/FAO, 2003).

Longer copulation durations have been shown to increase a male's fertilisation success for a variety of insect species [reviewed in Alcock (1994)], including the medfly (Field& Yuval, 1999). Increased copulation duration may represent a type of mate-guarding by males, thereby reducing the likelihood that females will immediately remate a subsequent male. Long-duration copulation could also reduce competition with previous ejaculates if males insert more sperm, or spend longer removing their rival's sperm (Singh et al., 2002). As the subsequent re-mating of a mated female is detrimental to the reproductive success of the first male, but provides potential benefits to the female (Arnqvist& Nilsson, 2000; Mazzi et al., 2009), remating is likely to represent an area of sexual conflict between males and females.

We measured a slight but significant difference between the mating duration of couples containing either a wild female and an OX3097D-Bol male or a couple containing

a wild female and a wild male. This appears to be a difference related to the Argov wild-type genetic background; the difference in average copulation duration between Argov couples and wild couples was found to be highly significant (mating durations for Argov couples were on average 80 minutes shorter). This observation is consistent with reports of reduced copulation durations in laboratory medfly strains (McInnis et al., 2002). As the copulation duration of the OX3097D-Bol males varied widely with the genotype of the female (copulations were on average over 70 minutes longer with a wild female), it appears as though females have the majority of control over the copulation duration [consistent with findings in the medfly (Field& Yuval, 1999) and in *Drosophila montana* (Mazzi, Kesaniemi et al. 2009), although this is not always the case, see (MacBean& Parsons, 1967; Parsons& Kaul, 1966)], and in artificial culture selection has favoured shorter matings.

It is unclear why laboratory-rearing would select for shorter copulation durations; however, it appears to correlate with increased levels of remating (McInnis et al., 2002; IAEA/USDA/FAO, 2003). It is possible that couples reared at high density are more likely to be interrupted by wandering males, and that selection will therefore favour those males that are able to deliver a full sperm load (thereby filling a spermatheca) in a shorter time. Correspondingly, overcrowding may increase female remating rates as mating may be less costly than the consistent rejection of large numbers of persistent and aggressive males. Whatever the causes, however, a possible consequence of increased female remating may be an increased importance of post-copulatory selection in mass-rearing high-density culture. It is possible that increasing sperm competition will favour a highly polyandrous female mating strategy; by obtaining a mixture of paternal sperm genotypes, a female can ensure that her eggs are fertilised by the most highly competitive sperm, thereby increasing her indirect fitness [i.e. via the 'sexy-sperm hypothesis' of Hoenigsberg (1999)]. Although

this increase in the level of post-copulatory sexual selection could be beneficial for SIT, it could be accompanied by reductions in the relative importance of pre-copulatory sexual selection.

4.2.5. Sperm competition

Sperm competition results in the evolution of both offensive and defensive male sperm competition traits (Singh et al., 2002). Sperm competitiveness of OX3097D-Bol males was measured against that of Argov males by performing a series of alternate matings using Argov females with either an OX3097D-Bol male as the first mate and an Argov male as the second mate [providing an estimate of the OX3097D-Bol paternity defence ability or (P1)]; or with an Argov male as the first mate and an OX3097D-Bol male as the second mate [giving an estimate of the OX3097D-Bol paternity offence ability or (P2)]. The resulting paternity shares were determined by rearing progeny on-tet and screening for the presence or absence of the DsRed2 fluorescent marker. The results for OX3097D-Bol and Argov male paternity defence abilities (P1) are shown in **Fig. 4.9**.

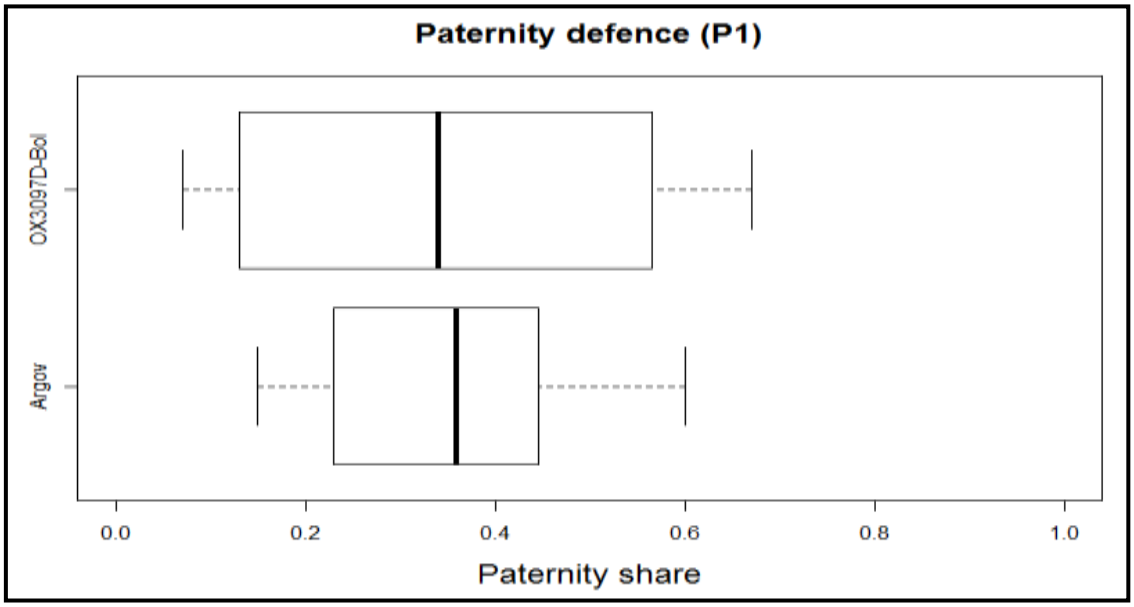


Figure 4.9. Box and whisker plot showing range of paternity shares obtained when males of the OX3097D-Bol (n=12) and Argov (n=17) strains were the first of two males to mate with an Argov female.

The mean P1 values obtained for OX3097D-Bol and Argov males were found to be 0.34 (± 0.22 SD) and 0.36 (± 0.15 SD), respectively (**Fig. 32**). The P1 values for each strain passed a test of normality (P = 0.238, Shapiro-Wilk); a 1-way ANOVA was performed and suggested no significant differences in the ability of OX3097D-Bol and Argov males to defend paternity against second males (P = 0.794, 1 d.f., 1-Way ANOVA). The P2 values were also measured and are given in **Fig. 4.10**.

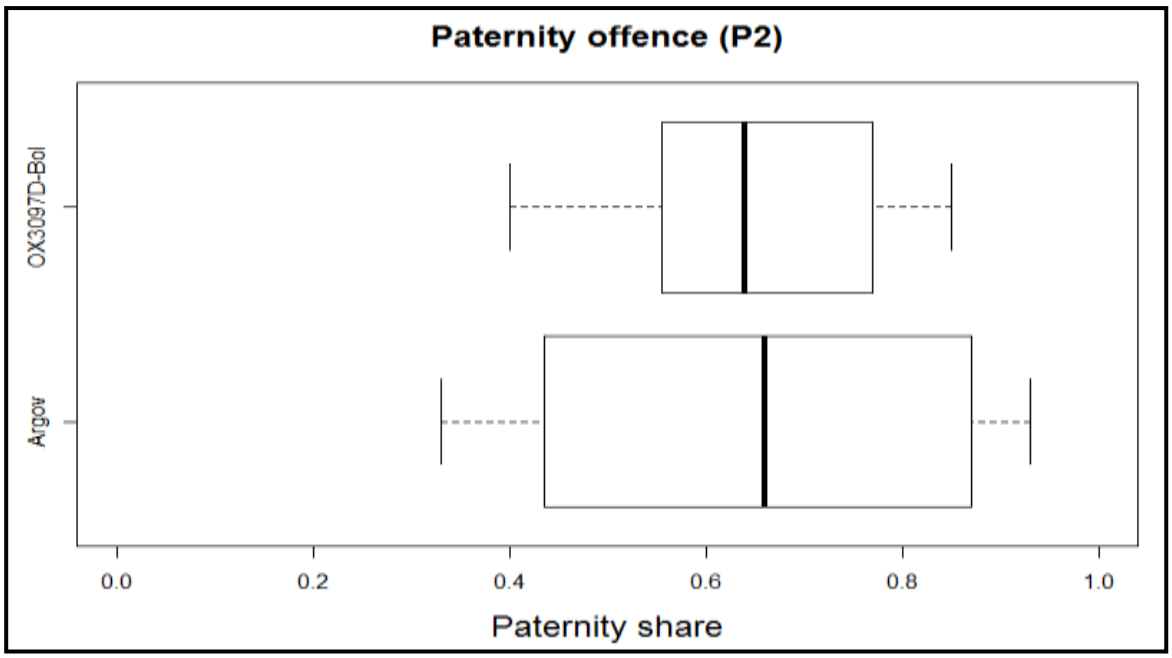


Figure 4.10. Box and whisker plot showing range of paternity shares obtained when males of the OX3097D-Bol (n=17) and Argov (n=12) strains were the second of two males to mate with an Argov female.

The mean P2 values obtained for OX3097D-Bol and Argov males were found to be 0.64 (± 0.147 SD) and 0.66 (± 0.216 SD), respectively (**Fig. 33**). The data for the P2 values passed a test of normality (P = 0.238, W = 0.947, Shapiro-Wilk); a 1-way ANOVA was performed and suggested no significant differences between the OX3097D-Bol and Argov males in ability to steal paternity from previously mated males (P = 0.794, 1 d.f., 1-Way ANOVA). Similar P1 and P2 values for OX3097D-Bol and Argov males suggests that OX3097D-Bol male sperm competitiveness has not been measurably reduced.

The OX3097D-Bol and Argov P1 and P2 values were pooled and used to estimate sperm precedence in olive fly. Results are given in **Fig 4.11**.

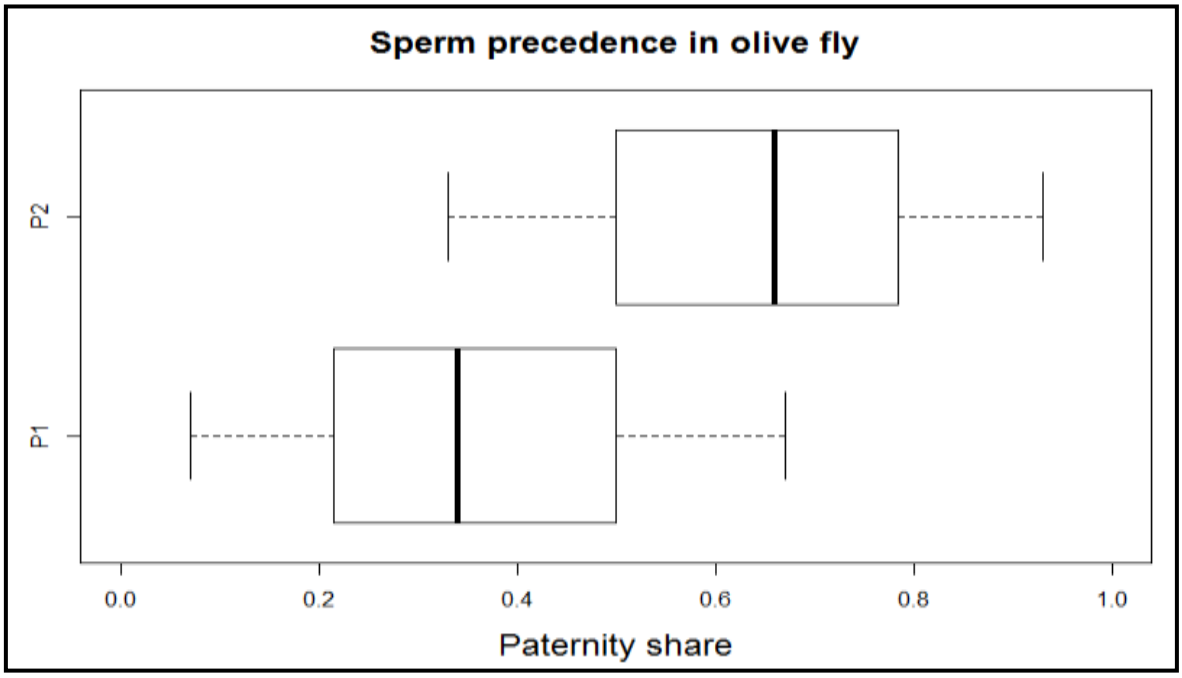


Figure 4.11. Box and whisker plot showing range of paternity shares obtained by males that were either the first (P1) or second (P2) of two males to mate with a female (n=29).

The mean paternity share for olive fly males that were the first of two to copulate with a female (P1) was 0.35 (± 0.182 SD), and the mean paternity share for males that were the second of two males to copulate with a female (P2) was 0.65 (± 0.182 SD). The pooled data passed a test for normality (P = 0.262, Shapiro-Wilk); a 1-way ANOVA was performed comparing the means and distributions, and suggested a significant shift towards P2 in these experiments ($P = 8.36e^{-07}$, 1 d.f., 1-way ANOVA). The mean of the P2 values significantly exceeded that of the P1 values, strongly suggesting second male precedence in olive fly, with the second male of two gaining on average almost two thirds of the paternity share. The box and whisker plot shows quite a large variation in the P1 and P2 values recorded. This may suggest that sperm competitiveness, or ejaculate size, varied considerably between males. An even higher second male precedence has been found in the medfly, with (Lee et al., 2003) describing average P2 values of 0.85.

Although it was not possible to carry-out these tests with wild olive flies, the lower dry-weight (and by implication body size) of the OX3097D-Bol males relative to wild males may have some bearing on sperm competition. *Drosophila melanogaster* male body size, testes size, and therefore ejaculate size have been found to be positively correlated (Pitnick& Markow, 1994). Larger males have a greater paternity share than their smaller rivals; this is possibly because the larger males fill the spermathecae of the females more extensively than the smaller males, thereby leaving less volume for smaller males (Singh et al., 2002; Simmons& Siva-Jothy, 1998). If these traits hold true for olive fly, small sized OX3097D-Bol males may have a further post-copulatory selection cost.

4.2.6. Re-mating tests

We investigated the effect of first-male genotype on wild female re-mating propensity, and whether the genotype of the first male influenced the females second mate genotype choice. Wild females who had initially mated either an OX3097DBol male (n = 188) or a wild male (n = 296) were caged and allowed to mate with equal proportions of OX3097D-Bol and wild males over 15 days. Remating females were removed and the genotype of the second male was assessed. Results are shown in **Fig. 4.12**.

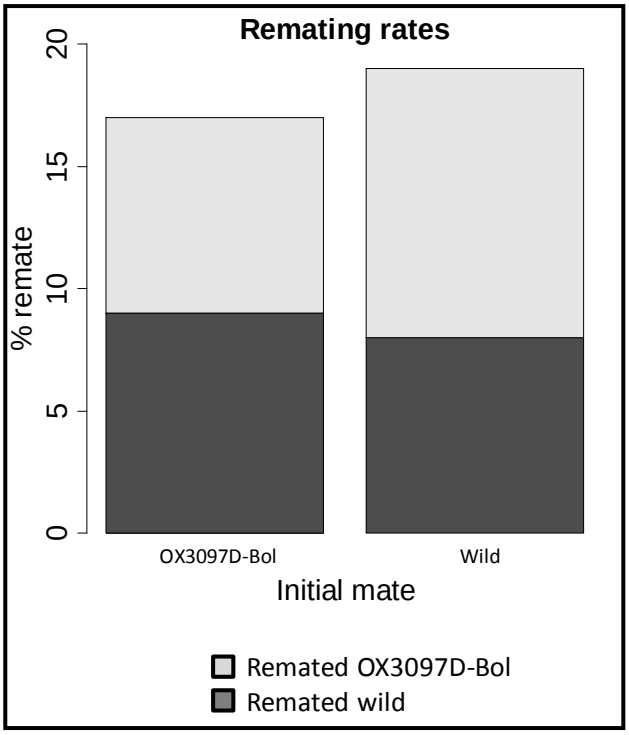


Figure 4.12. Genotype of first mate (OX3097D-Bol or wild) did not affect female re-mating frequency or genotype of second mate. Of 188 females initially mated to OX3097D-Bol males 32 (17%) re-mated, of which 17 (9%) re-mated to wild males (open portion of left bar), and 13 (8%) to OX3097D-Bol males (solid portion of left bar). Of 296 females initially mated to wild males 55 (19%) re-mated, of which 23 (8%) re-mated to wild males (open portion of right bar) and 32 (11%) to OX3097D-Bol males (solid portion of left bar). Re-mating propensity of wild females initially mated with either an OX3097D-Bol male or a wild male were not significantly different (P = 0.7, 1 d.f., Chi-Square test). Furthermore, the re-mating preference of the wild females was not found to differ significantly depending on first-mate choice (P = 0.38, 1 d.f., Chi-Square test).

Of the wild females that initially mated an OX3097D-Bol male, 17% went on to remate (9% to wild males, and 8% to OX3097D-Bol males). Of the wild females that initially mated a wild male, 19% went on to remate (8% to wild males and 11% to OX3097D-Bol males). Statistical analysis revealed no significant differences in remating rate (P = 0.7, 1 d.f., Chi-Square test), or choice in second-mate genotype (P = 0.38, 1 d.f., Chi-Square test).

Sterile males should ideally be able to keep wild females from remating or, if remating does occur, possess seminal products that compete on equal terms with those of wild males (McInnis et al., 2002). The reduced mating duration of OX3097D-Bol gave concerns regarding potential higher remating rates for wild females initially mated to an

OX3097D-Bol male. Results presented here, however, suggest that wild females previously mated to OX3097D-Bol males do not have an increased propensity to remate (17% remated) than those initially mated to wild males (19% remated), with no significant differences found in female choice of the second male genotype. Similar experiments in medfly have found that females mated to radiation sterilised males are more likely to remate than females mated to fertile males (Kraaijeveld& Chapman, 2004). Increased remating can have negative impacts on the efficacy of an SIT programme; it has been found, in contrast, that females mated to irradiated males were able to significantly recover fertility when subsequently remated to a non-irradiated male, this is facilitated in a large part to strong second male sperm precedence in medfly (Lee et al., 2003). Therefore, second male precedence in olive fly may give OX3097D-Bol an indirect advantage over traditional wild-type strains; that is, the negative effects of irradiation on wild female remating rates means that second male precedence would be an active disadvantage for traditional strains that is not necessarily applicable to OX3097D-Bol.

4.3. Summary and conclusions

This chapter presents an analysis of OX3097D-Bol males for a range of pre- and post-copulatory sexual competitiveness and compatibility parameters with laboratory reared wild-type and (where possible) wild field-caught olive flies.

OX3097D-Bol has shown excellent mating characteristics over a range of male pre- and post-copulatory sexual selection and mating compatibility parameters. When compared against the genetically similar Argov wild-type strain, OX3097D-Bol males showed no measurable loss in pre or post-copulatory competitiveness. However, OX3097D-Bol males showed a slight reduction in copulation success with wild insects (RSI = 0.44), but showed full photoperiod synchronicity. Although a significant difference in copulation duration was observed between OX3097D-Bol and wild males mating with wild females, no reduction in ability to stimulate remating refractoriness in wild females was found. Small relative male size was found to correlate with low reproductive success in OX3097D-Bol; this may have some significance on mass-rearing strategy.

An RSI value of 0.44 recorded with wild insects is strikingly high for a sexing strain. However, one of the benefits of modern transgenic technologies is that desired phenotypes can be achieved with small and specific genetic changes (unlike the previous techniques used that relied on chromosomal translocations, with consequent dramatic declines in fitness) (Irvin et al., 2004). Moreover, the mating system of the olive fly may be less discriminating to lower-quality males. Following the predictions of (Burk, 1981), monophagous species such as the olive fly will mate directly on the plant which provides the ovipositional substrate, and will have relatively simple mating and signalling systems. It certainly appears to this observer that the olive fly courtship display is far simpler than that of the highly polyphagous medfly. Monophagous species, Burk argues, lack the requirement for long-distance attractants, exhibit male territoriality centred on the

oviposition source, and their courtship displays need only be simple, usually limited to species identifying wing waving. Olive fly males establish single-leaf territories underneath leaves during the period of peak sexual activity. However, medfly males and the males of many other polyphagous tephritid species, group into clusters of displaying males known as leks (Sivinski& Burk, 1989), providing exactly the sort of mating system where a high degree of sexual selection on males is expected. The olive fly mating system may therefore be more forgiving of small relative reductions seen in mating competitiveness than for similar strains in medfly. In this respect, the olive fly may be more conducive to SIT-based application.

The OX3097D-Bol olive fly strain has been shown to possess desirable life-history traits and excellent sexual selection characteristics. The following chapter considers a caged population suppression scenario, where fsRIDL control of wild-type olive fly populations is tested as a proof-of-principle with periodic releases of OX3097D-Bol males.

4.4. Materials and methods

4.4.1. Mating competitiveness with Argov wild-type

The protocol for mating competitiveness closely followed FAO/IAEA/USDA guidelines (FAO/IAEA/USDA 2003). These guidlines were developed as a set of standardised protocols for assessing strains for use in SIT programmes, with the aim of allowing comparisons of strain quality over time, and between rearing facilities and field trials. Adults were obtained from homozygous OX3097D-Bol and wild-type larvae reared at equal densities (1 larva/0.8g larval medium). OX3097D-Bol larvae were reared off-tet (so that only adult males would survive). Immediately after pupal eclosion, Argov wild-type flies were separated by sex and separate cages were made up of 50 Argov males, 50 OX3097D-Bol males, and 50 Argov females. Cages were supplied with adult diet and a water source, and flies were left at 25ºC (50% relative humidity) for 5-6 days to reach sexual maturity. Mating tests were performed in glasshouse facilities at the University of Oxford, Department of Zoology. Field cages were constructed inside the green-house (1.25m tall with a base of 0.75m x 0.75m) with small olive trees (~1m tall) placed inside. A data logger monitored the temperature and humidity in the greenhouse during the experimental mating period (which varied between 22˚C - 34˚C and 35% - 50%, respectively) (**Fig. 4.13**).

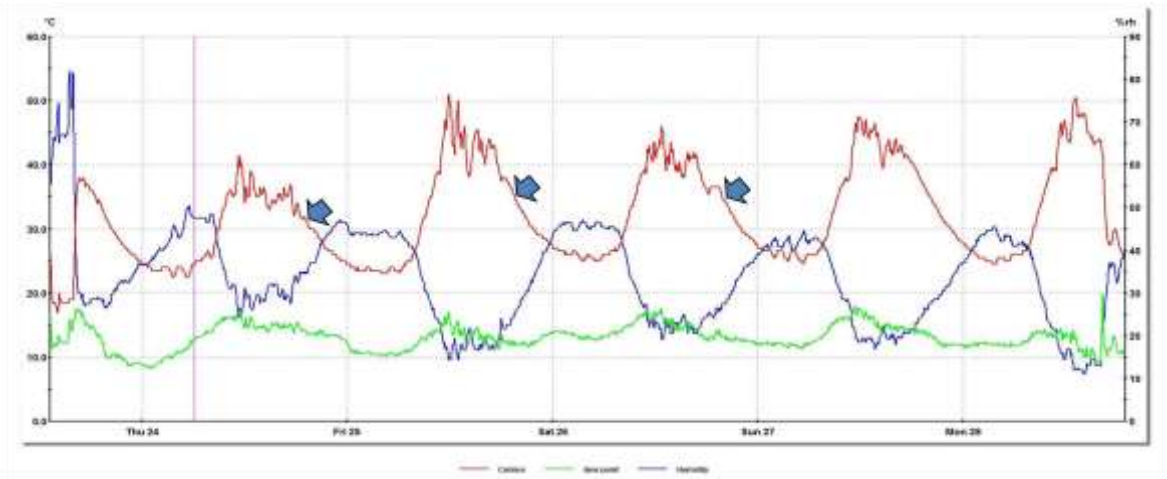


Figure 4.13. Temperature (ºC) (red line), relative humidity (%) (blue line), and dew point (green line) for glasshouse used during mate competition tests with Argov wild-type flies. Blue arrows show points at which flies were introduced into experimental cages.

Copulating pairs were removed from the experimental cages using a micro-centrifuge tube as shown in **Fig. 4.14**. The time of mating initiation for the couple was recorded, and the tube was labelled. Periodic checks (every 10 minutes) determined whether the couples were still mating. After all couples had ceased mating, mated males were cooled on ice and were examined for the presence or absence of the DsRed2 fluorescent marker under a fluorescence microscope.

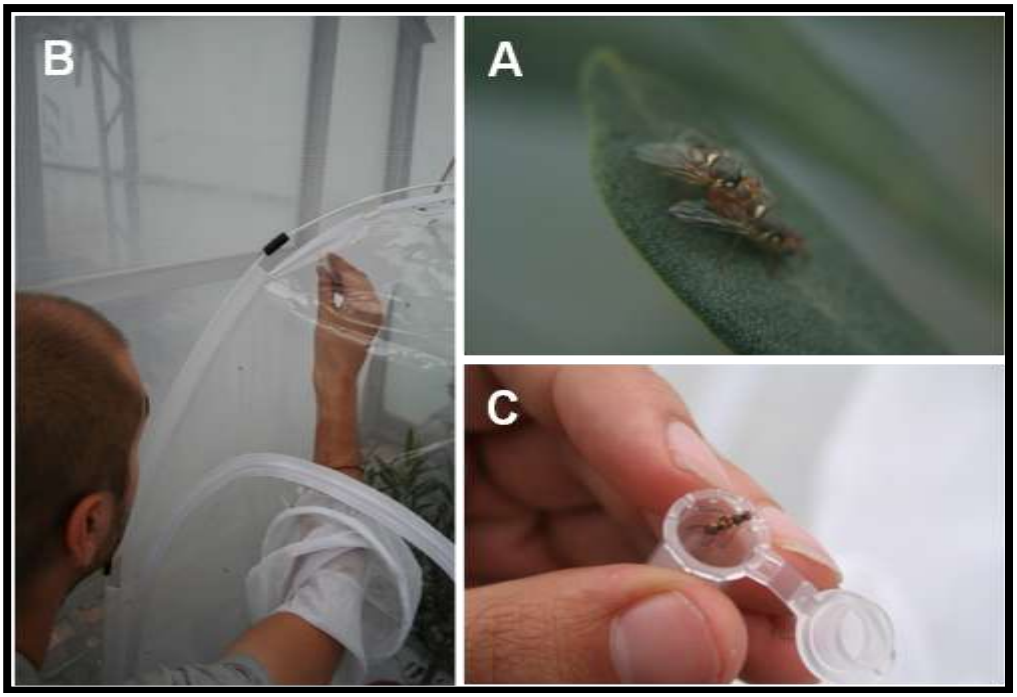


Figure 4.14. (**A**) A mating pair is spotted within the cage. (**B**) The experimenter reaches inside the cage and removes the couple in micro-centrifuge tube. (**C**) The micro-centrifuge tube is labelled with the initiation time and is stored with periodic (every 10 minutes) checks to assess mating duration.

4.4.2. Mate competition and wild field collected olive flies

Mating competitiveness tests with wild olive flies were also carried out in accordance with FAO/IAEA/USDA (2003) guidelines. Tests were performed in semi-natural caged conditions (cages were 1.25m high with a base of 0.25m^2 and contained a large olive branch) in glasshouse facilities at the University of Crete in Heraklion, under natural light. Glasshouses were temperature controlled and maintained at a constant 25ºC. Adult male OX3097D-Bol flies were obtained from larvae reared in the absence of tetracycline ("off-tet") at a density of 1 larva/0.8 g larval medium. Wild pupae were recovered from infested olives gathered from olive orchards near to the University of Crete (geographical co-ordinates: 35˚20'N 25˚8'E). Infested olives were picked and suspended in nets over buckets containing saw-dust in a temperature controlled room at 25ºC. Resulting pupae were recovered by sieving the saw-dust. Wild adults were eclosed and immediately sexed. Mating tests used 5-6 day old OX3097D-Bol males and 8-9 day old wild males and

females. Each mating test used 50 OX3097D-Bol males, 50 wild males, and 50 wild females. Mated males were scored for the presence of the DsRed2 fluorescent marker by epifluorescence microscopy. OX3097D-Bol mating competition tests with wild olive flies were performed in 15 replicates. Each experiment yielded a mating propensity of greater than 0.2.

4.4.3. Assessment of male dry-weights

After the mating tests described in *Section 4.4.2.* non-mated and mated males of both strains were frozen over-night at -20ºC. The following day the males were desiccated as follows: (1) males were individually placed in labelled micro-centrifuge tubes; (2) micro-centrifuge tubes were placed in 96 well racks with the lids open; (3) racks were placed on a large heating plate set to 50ºC and were left overnight (flies were desiccated for 15 hours). The following day the flies were weighed on a microbalance to the nearest tenth of a µg. Each sample was weighed three times, and a mean was taken.

4.4.4. Re-mating test

Mating cages for the first step of the re-mating tests contained 400 wild females and 400 OX3097D-Bol males or 400 wild females and 450 wild males. Mating couples were removed from cages. Mated females were grouped in accordance with the genotype of the first mate (OX3097D-Bol, n = 188 or wild-type, n = 296) and transferred the following day to new cages with sufficient fresh wild and OX3097D-Bol males to give a 1:1:1 ratio of mated females, wild males, and OX3097D-Bol males. Cages were checked daily for re-mating events over the following 15 days. Re-mating couples were removed, and the male genotype assessed by epifluorescence microscopy. When a male of a particular genotype was removed from a cage he was replaced to maintain equal ratios.

4.4.5. Sperm competition experiments

I tested the sperm competitive ability of OX3097D-Bol and Argov wild-type males by mating them over two consecutive days either as the first or second mate of Argov females, with an individual of the opposite genotype as a rival male. Two cages were set up with over 400 OX3097D-Bol males and Argov females in one, and over 400 Argov males and Argov females in the other. Mating pairs were removed and left to complete mating. The following morning mated females were caged with an abundance of males of the opposite genotype. Mating pairs were removed and left to complete mating with females subsequently caged individually. Eggs were collected from individual females for a period of two weeks, or until the female died (whichever came first). Eggs were placed on larval diet seeded with tetracycline (100µg/ml) and the resulting pupae were screened for the presence or absence of the DsRed2 fluorescent marker. Paternity shares were calculated from this ratio. P1 values were recorded for OX3097D-Bol and Argov males when they were the first male to mate with a female (who subsequently mated a male of opposite genotype 24 hours later). P2 values were recorded for OX3097D-Bol and Argov males when they were the second male to mate with a female (who had mated a male of opposite genotype 24 hours previously). P1 and P2 values were calculated from a total 29 double-mated Argov females (12 where an OX3097D-Bol male was the first to mate and 17 where an Argov male was the first to mate).

4.4.6. Statistical analysis

Comparison of mating competitiveness between OX3097D-Bol and wild males was performed using a likelihood ratio test for goodness of fit with numbers of successfully mated males from both genotypes pooled across experiments, and compared against a theoretical 1:1 genotype ratio expected in random mating. Differences in copulation

initiation time between the two possible mating combinations (OX3097D-Bol/wild female and wild male/wild female) were analysed using a circular statistics F-test with the statistical software Oriana for Windows (Kovach Computing Services, Anglesey, UK). The Pearson's Chi-Squared test was used to compare numbers of re-mated to non-re-mated females for both initial mating combinations. The same test was used to compare initial and second-mate choice. Chi-Squared tests were performed using SPSS Inc., Chicago IL USA. Male weights were compared using a 1-way ANOVA followed by a Tukey HSD multiple pair-wise comparison test. Sperm competition values were compared using a 1-Way ANOVA. Before an ANOVA was performed, data was checked for sufficient adherence to a normal distribution using a Shapiro-Wilk test. ANOVA, Tukey, and Shapiro-Wilk tests were performed using RStudio (Version 3). For all comparisons, significance was set at $P < 0.05$.

Inside of one of the large net cages (containing olive tree and resting surface) used in the population suppression experiments.

Chapter 5 - Caged population suppression

5.1. Introduction

Proof-of-principle testing is integral to strain evaluation, and occupies an important part of the graduated evaluation procedure used in the development of a RIDL strain. The caged suppression trial allows for a simulation of the effects of RIDL releases on a target population under controlled conditions. The methodology for the caged suppression trial described in this chapter was based on that introduced by (Wise de Valdez et al., 2011), where the OX3604C-*Aae* female-specific flightless strain of the yellow fever mosquito, *Aedes aegypti*, was shown to be capable of suppressing caged wild-type populations.

Here, we describe the establishment of Argov wild-type olive fly populations in four large field cages. Once populations were established, OX3097D-Bol males were released achieving an initial over-flooding ratio of approximately 16:1 RIDL to wild-type.

5.2. Results and discussion

Wild-type populations were established in each of four large ($8m^3$) cages in a glasshouse at the University of Crete. The first 12 weeks of the experiment constituted a wild-type population establishment and stabilisation period. 200 wild-type pupae were added to each cage each week. Based on death rates, the total male and female standing crop after population densities stabilised was approximately 400. Egg production from each cage was monitored daily. Weekly averages were found to be normally distributed ($P > 0.3$, Shapiro-Wilk), and did not deviate significantly between the cages over the first 12 weeks ($P = 0.73$, 3 d.f., 1-way ANOVA) (**Fig. 5.1A**).

At week 13 from first establishment, two of the four cages were assigned randomly for treatment with OX3097D-Bol (cages A and B). Approximately 1,600 OX3097D-Bol male pupae were introduced into each treatment cage per week. At this point, following (Wise de Valdez et al., 2011), the numbers of pupae returned to the treatment cages were adjusted to reflect any impact of OX3097D-Bol releases on egg production; the rate of return in the experimental cages was the same as that used for the control cages (with the return rate in the control cages maintained at a constant 200). This approach allowed for the return of equal numbers of pupae into each cage when egg production was the same in treatment and control cages. If egg densities were lower or higher in treated cages, the pupal return rates would be correspondingly lower or higher, respectively.

Population density was monitored by measuring egg production (**Fig. 5.1A**) and female mortality (**Fig. 5.1B**). To track mating outcomes, pupae taken from the cages were scored for fluorescence (**Fig. 5.1C**). Fluorescent pupae were detected at three weeks post-first release (PR), indicating successful matings between homozygous OX3097D-Bol males and wild-type females, and the proportion increased rapidly thereafter to 100% by week 10 PR. This indicates that the presence of OX3097D-Bol males began impacting the

adult population about three weeks after the initial release. Death of fluorescent (transgenic) female pupae as a result of expression of the lethal phenotype would reduce the number of egg-laying adult females; based on the fluorescence data, no wild-type females emerged (eclosed) in the treatment cages from week 10 PR. Egg production was lower in the treatment than in the control cages in week 6 PR, and declined rapidly thereafter.

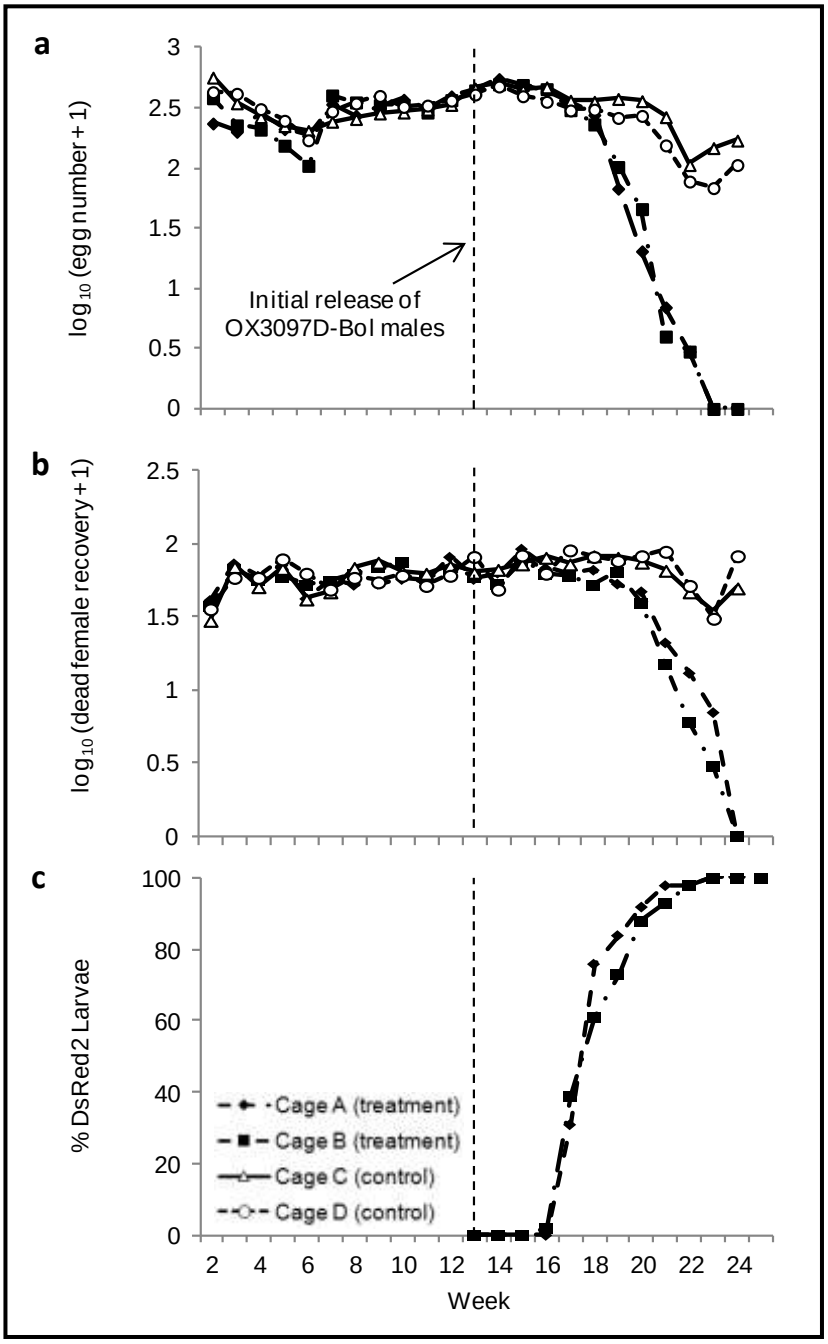


Figure 5.1. Population elimination by periodic release of OX3097D-Bol males. (**A**) The average daily egg production for each cage. Weeks 1 to 12 constituted the population stabilization period with 250 pupae added in the first week, and 200 pupae added to each cage per week thereafter. From week 13, 1,600 OX3097D-Bol pupae were added weekly into cages A and B. After week 13, weekly pupal return to the treatment cages was made proportional to the weekly egg production in the cage relative to the control cages. From 5 weeks after initiation of RIDL introductions, egg production in each treatment cage was consistently lower than in either control cage; the difference increased until eventual extinction of the wild-type population in both treatment cages by week 24 (12 weeks after the first RIDL release). Extinction was defined as 2 weeks of zero egg production. Egg numbers in control cages remained relatively stable. (**B**) Dead flies were removed from the cages weekly, and the numbers of dead females are shown. From 7 weeks after the initiation of RIDL release, increasingly fewer such females were recovered from the treatment cages than from the control cages. (**C**) Frequency of DsRed2 in treatment cages. Larvae selected for return were screened for presence of DsRed2 marker by fluorescence microscopy before being returned to the treatment cage (see *Section 5.4.2.*). The proportion of returning pupae carrying the OX3097D-Bol transgene reached 100% in both treatment cages by week 23 (10 weeks post-RIDL release). Olive fly females typically mate only once. Females start to lay eggs approximately 2 days after mating, and lay most of their eggs within the next 10 days. Egg to pupa development time was approximately 12 days. These pupae therefore indicate female mating choice of approximately 3 weeks before each measurement.

The numbers of dead females recovered from treatment cages started to decrease from around week 6 to 7 PR, presumably due to a decline in overall female numbers. Defining extinction as 2 weeks of zero egg production (Wise de Valdez et al., 2011), both treatment cage populations were extinct by week 12 PR.

The data show that periodic releases of OX3097D-Bol males can cause suppression and eradication of stable caged wild-type olive fly populations, while control cages not treated with OX3097D-Bol males continued to produce large quantities of eggs and maintained stable wild-type numbers. As expected, over-flooding a wild-type population with homozygous OX3097D-Bol males resulted in the flow of the OX3097D-Bol fsRIDL allele into the progeny of the wild-type population. There was a short delay of 3-4 weeks before the cages treated with OX3097D-Bol males started producing OX3097D-Bol positive progeny, reflecting a delay in sexual maturation of the released OX3097D-Bol males (added as pupae), pre-OX3097D-Bol male release matings of females to wild-type males, and development time of OX3097D-Bol progeny to the pupal (screening) stage. Increasing prevalence of the OX3097D-Bol transgene in subsequent generations resulted in increasing female-lethality, and a concomitant drop in egg production. In addition, the decreasing number of pupae returned to the treatment cages resulted in an increasing release ratio of OX3097D-Bol males to wild-type males, despite the actual release numbers of OX3097D-Bol males remaining the same.

This experimental approach makes some assumptions that may not be realistic under field conditions and should be taken into account when interpreting the times to extinction. (i) The experiment assumes that the reproductive success of a female (that is, the number of adults she sires for the next generation) is likely to be the same under population suppression conditions as it is under that of a stable population density. The fact that a population density is stable suggests the presence of a limiting resource that is stopping the

population from growing further. In the case of the olive fly, and other agricultural pests, this is likely to be intraspecific competition for host fruit. Under conditions of population suppression, however, intraspecific competition will be relaxed and a greater quantity of host fruit will be available per female; a given fertile female will then be expected to achieve a greater lifetime reproductive success (perhaps by several-fold depending on the level of suppression and the form and intensity of density dependence). Therefore, applying the same scenario of restricted population growth on the experimental cages (as was performed for the control cages) may over-estimate the likely suppressive effect under true field conditions. (ii) The experimental model assumes an entirely closed population of insects. Unless releasing in a totally isolated geographic area, an important factor affecting a sterile insect population suppression will be the immigration of wild flies into the control area. Wild females already fertilised by a wild male may enter from outside of the release zone; populations may be maintained by such immigration with complete eradiation impossible unless the control effort was spread to include all connected geographic regions. However, fsRIDL control of the olive fly is not likely to be used on its own. Control efficacy is inversely dependent on wild population density, and OX3097D-Bol would likely be used synergistically with a range of other IPM control strategies, for example, mass-trapping or bait spraying.

A recent paper by Robert et al. (2012) explores the design of the caged suppression experimental proposed for simulating *Aedes aegypti* by (Wise de Valdez et al., 2011) (and adapted for the olive fly in the present study), and attempts to expand on some of its conclusions using stochastic mathematical modelling. The assumptions that are made and parameters that are used in their modelling, although designed from *A. aegypti* data, are also relevant for olive fly. Modelling outputs suggest that release ratio, wild-type population size, density dependence, and immigration can significantly impact mean

extinction time among experiments. Variance in extinction time due to changes in release ratio was found to be far higher for lower release ratios, with differences in time to extinction becoming increasingly gradual with higher release ratios. They also found that factors of density dependence had a marked effect on extinction at lower release ratios (10:1 and 1:1 transgenic to wild-type), although increasing release ratios counteracted these effects, although the time to extinction would be greater than when the population was not regulated by density dependence. Immigration of insects from outside of the control area resulted in the wild-type population being maintained regardless of how many RIDL individuals were being introduced. For high release ratios (1:10 and 1:100, transgenic to wild-type), the wild population was maintained exclusively through immigrants. Furthermore, Robert et al. (2012) conclude that fitness costs associated with the transgenic genotypes are not detectable in field cage experiments that use high release ratios unless the fitness costs are greater than 0.8.

Simulating the effects of RIDL in an enclosed and controlled setting is challenging, and sacrifices of realism are inevitable. However, proof-of-principle testing represents an important part of the process of acquiring confidence in a technique or product; our data strongly supports the transition of the OX3097D-Bol olive fly strain towards more robust open-field trials.

5.3. Summary and conclusions

Although the results presented here need field confirmation, they provide proof-of-principle findings on the suppression and elimination of caged wild-type olive fly populations through OX3097D-Bol male release. This provides evidence for the fsRIDL approach as a viable method for olive fly control. The following chapter considers the dynamics of the OX3097D-Bol fsRIDL allele in olive fly populations.

5.4. Materials and methods

5.4.1. Population establishment

The protocol for the caged suppression experiment was based on that of (Wise de Valdez et al., 2011), with adaptations to accommodate various differences between olive fly and *Aedes aegypti*. Stable populations of wild-type (Argov) olive flies were established in four field cages (each $8m^3$ and containing an olive tree 1.5 m tall; all the cages were contained within a single large glasshouse) over a 12-week period by introducing a fixed number of pupae to each cage weekly (250 in week 1, and 200 from weeks 2 to 12). To assess egg production rates and to sustain caged olive fly populations, four ceresin- wax cones (combined surface area approximately $550cm^2$) were added to each cage daily for female oviposition. Pupal additions for the first 4 weeks during population establishment originated from a wild-type stock colony; thereafter, experimental cages were self-sufficient. Eggs were collected from the cages and counted daily. On week 12, the experimental cages were randomly divided into two RIDL-treatment and two no-treatment (control) cages.

5.4.2. OX3097D-Bol release and population maintenance

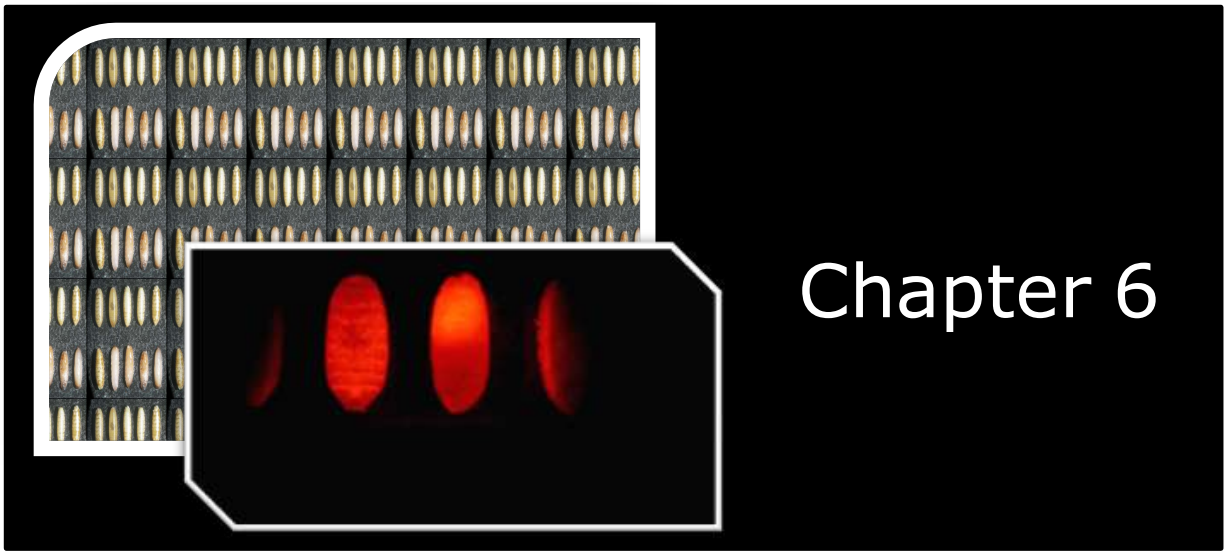
From week 13 onwards, RIDL-treatment cages received weekly additions of 1,600 OX3097D-Bol male pupae reared off-tet (an initial recruitment rate ratio of approximately 16 OX3097D-Bol males to 1 wild-type male). Once the OX3097D-Bol introductions began, pupal introductions to the RIDL-treatment cages were proportional to the cage's respective rate of egg production, with the control cages providing a coefficient of weekly egg production to pupal return. For example, if a control cage produced 1,000 eggs in a given week the return of 200 pupae would result in a return rate of 0.2 (200/1000).

Therefore, if a treatment cage produced 800 eggs, then 160 pupae would be returned (800 x 0.2).

Numbers of females in a cage were monitored by collecting and counting dead females. The ratio of RIDL heterozygous to wild-type pupae returning to RIDL-treatment cages was monitored by fluorescence microscopy (OX3097D-Bol heterozygous females reared off-tet pupate but fail to eclose). Larvae were screened only after the returning population was separated, to remove the possibility of bias in selecting individual flies for reintroduction to the cage populations.

5.4.3. Statistical analysis

All statistical tests were performed using RStudio (Version 3). For all comparisons, significance was set at $P < 0.05$.



Mixture of wild-type and OX3097D-Bol olive fly pupae under bright-field and fluorescence illumination.

Chapter 6 - Population genetics of the OX3097D-Bol fsRIDL insertion

6.1. Introduction

In bisex-lethal RIDL no progeny resulting from a released RIDL parent survives to transmit the transgene to subsequent generations (provided penetrance of the lethal trait is 100%); thus, the RIDL trait does not persist beyond the F_1 generation, and would be expected to disappear from a population with the death of the released insects and their F_1 progeny. Control over the persistence of the transgene in a wild population is therefore relatively direct and immediate. However, in female-specific (fsRIDL) the male progeny inheriting the transgene in the field are fully viable, and the transgene can pass through a population by gene flow; the trait may therefore persist in the wild for a number of generations after releases cease. While this provides an added dimension for population-control (females inheriting the transgene in subsequent generations will still be vulnerable to its lethal effects), control over transgene persistence in the wild may be seen as less direct and intuitive, and may encounter a certain amount of political and social resistance.

Two main selection mechanisms are expected to contribute to the eventual disappearance of the fsRIDL trait from a wild population once releases stop. Firstly, as the trait is female-lethal, half the progeny inheriting the trait in a given generation will not develop to reproduce. Therefore, even if the reproductive success of the transgenic males is equal to that of their wild counterparts, the frequency of the fsRIDL insertion would be expected to halve every generation until the trait is lost. Secondly, the presence of the fsRIDL insertion is expected to confer a selective disadvantage to male carriers through reduced field survivability and sexual competitiveness. The extent of this will vary from strain to strain and depends on factors including the level of coding sequence disruption at the genomic locus of transposition (insertional mutagenesis), and/or leakiness in the

expression of deleterious transgene products. Homozygous fsRIDL males of arthropod strains so far tested have shown slight but significant reductions in fitness relative to wild-type (Morrison et al., 2009; Bargielowski et al., 2011) and wild insects (Ant et al., 2012; Phillip Leftwich, Personal communication). Deleterious effects measured in homozygous individuals will likely be offset somewhat in males inheriting the fsRIDL trait in the field; deleterious recessive mutations caused by insertional mutagenesis or the homozygosis of transgene-linked overdominant alleles will clearly not be as significant in heterozygotes. Nonetheless, the presence of the transgene insertion in heterozygous males is expected to be deleterious relative to a wild-type male genotype, and the rate of negative selection against the transgene in a given strain is expected to form a function combining the two aforementioned parameters.

In this chapter, simple deterministic and stochastic (Monte Carlo) population-genetic modelling of the dynamics of the OX3097D-Bol fsRIDL allele in olive fly populations reared in the absence of the transgene repressor are presented. We supplement our modelling with results from laboratory-cage experiments that follow the change in frequency of the fsRIDL trait in caged OX3097D-Bol populations.

The dynamics of the fsRIDL insertion in populations containing the OX3097D-Bol transgene insertion reared in the presence of tetracycline are also considered. Although the presence of residues of tetracycline in the wild sufficient to suppress the female-lethal phenotype in olive fly is extremely unlikely (the tetracycline would actually have to be available to the developing larva inside the olive), it is useful from a regulatory perspective to show that transgene-related fitness costs will cause the eventual loss of the transgenic allele from a population. Moreover, experiments measuring the rate of wild-type allele spread through a population can provide information concerning potential loss of strain homozygosity through accidental contamination in a rearing facility. The integrity of a

homozygous transgenic strain is vulnerable to the accidental introduction of a wild-type allele into a laboratory population, which may be difficult to detect initially. The accidental inclusion of a wild-type fly in a homozygous OX3097D-Bol cage will result in the production of heterozygous progeny (provided mating occurs). As these progeny will express the dominant fluorescent marker and will be phenotypically similar to homozygous flies, the presence of heterozygosity in the population may go initially unnoticed and may accumulate before wild-type individuals are detected during routine fluorescence screening. The spread of the wild-type allele through a population on-tet will be driven by relative differences in genotype fitness, and measuring/modelling the likely spread of such an invasion will help to anticipate the dynamics of a contaminating wild-type allele in laboratory populations of OX3097D-Bol.

6.2. Results and discussion

6.2.1. Selection in the absence of tetracycline

6.2.1.1. Model development and rationale

Discrete generation stochastic and deterministic models were produced predicting dynamics of the fsRIDL trait in a population in the absence of tetracycline; that is, with 100% dominant female lethality. Both models assume that the population in question remains at a consistent size and that there is random mating. The deterministic model assumes that at every generation matings are exactly in proportion to the abundance of the relative male genotypes (i.e. if 25% of the males carry the fsRIDL gene, they will sire 25% of the offspring), and that each male will produce equal numbers of male and female progeny. The discrete generation stochastic model was run on Monte Carlo principles, and allowed for random variation around (i) the number matings by the different male genotypes, and (ii) the ratios of male and female offspring produced. That is, each hypothetical offspring in each generation is assigned two random numbers, one that will determine genotype (with probabilities proportional to the genotype frequencies of the parental generation) and one that will determine sex (the probability of a given insect being assigned a particular sex in any given any generation is 0.5).

6.2.1.2. Experiments

For comparison with modelling, the frequencies of the fsRIDL allele were followed in caged populations of 200 olive flies reared in the absence of tetracycline. Cages with an initial 100 heterozygous OX3097D-Bol males and 100 wild-type females were set-up [(initial fsRIDL allele frequency (f_A) = 0.25)], simulating a population of fsRIDL males one generation post release. For each subsequent generation a sample of 200 progeny were

blindly selected at the larval stage, screened, and were introduced into a fresh cage as pupae, making the population for the next generation. **Fig. 6.1** shows the changing proportions of transgene positive larvae over time in the three experimental cages. The figure also shows the deterministic curve, and the results from 25 iterations of the stochastic model.

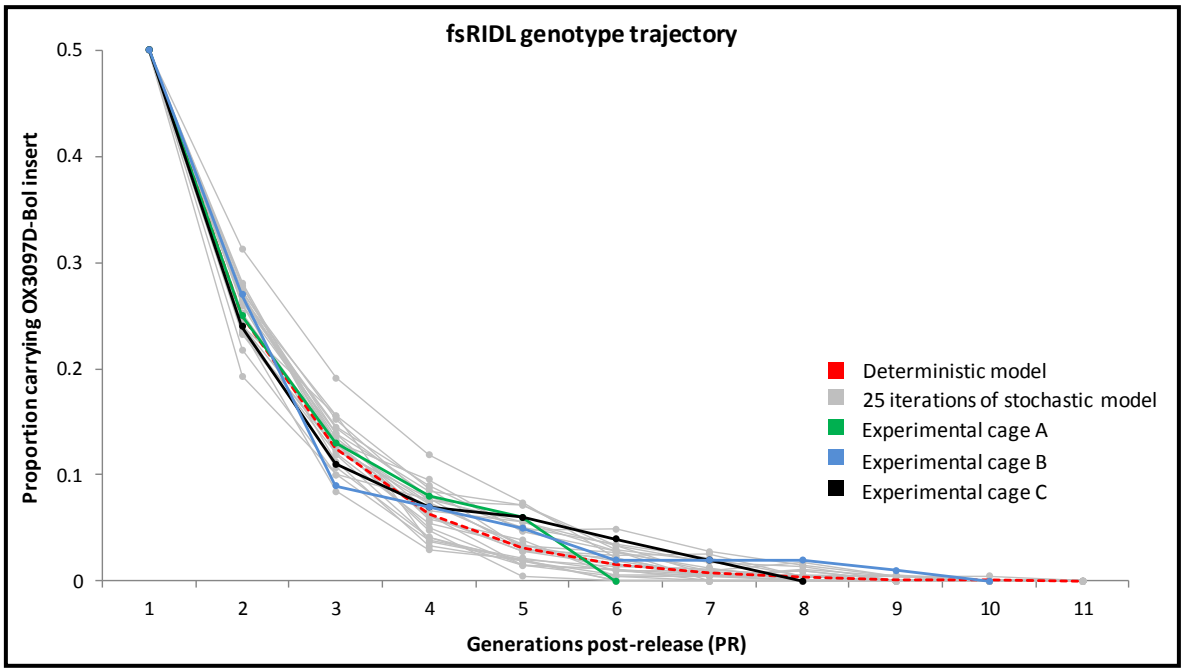


Figure 6.1. Temporal dynamics of OX3097D-Bol positive individuals in olive fly populations reared in the absence of the transgene repressor. Models and experimental data consider a population of 100 heterozygous OX3097D-Bol males and 100 wild-type (Argov) females. The experimental points in green, blue and black for cages A, B and C respectively, are overlaid onto predicted dynamics derived from a deterministic model (red dashed line) and 25 iterations of a stochastic model (grey lines).

The deterministic model predicts that for 100 heterozygous transgenic males with 100 wild-type females, the heterozygous genotype frequency will reach less than 0.005 (less than one individual in this instance), after 7 generations. A summary of outputs from 100 iterations of the stochastic model is shown in **Fig. 6.2**, and gives an indication of the distribution expected from a large number of repeats of the experiment.

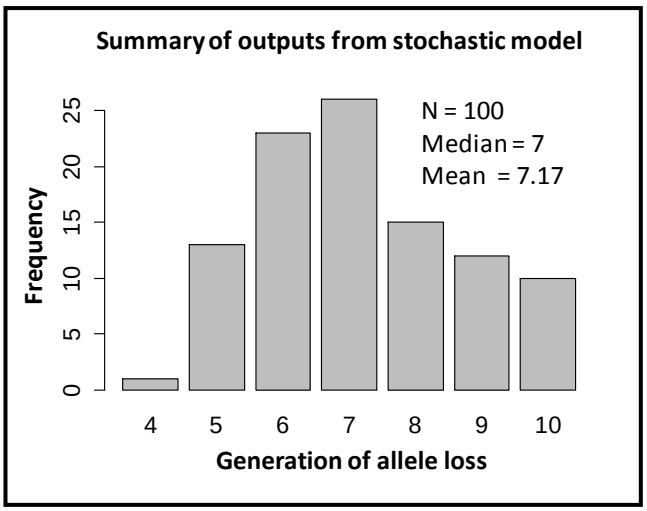


Figure 6.2. Summary of outputs from stochastic modelling of number of generations required for an fsRIDL gene to be lost from a population at constant density containing an initial 100 heterozygous fsRIDL males and 100 wild-type females in the absence of the transgene repressor.

In approximately 50% of the 100 iterations of the stochastic model, the transgene was lost from the population between generations 6 (1st quartile) and 8 (3rd quartile), with the 7th generation being the most frequent. The experimental cages produced no more transgenic progeny at generations 6, 10 and 8 for cages A, B, and C respectively, agreeing closely with modelling predictions.

The close correlation between modelling predictions and experimental results suggested that our assumptions about the rate of fsRIDL allele loss under these conditions were reasonable. We therefore used the model to extrapolate and predict the number of generations required for fsRIDL allele loss in larger populations; after all, fsRIDL is expected to involve the release of hundreds of thousands, or even millions of males.

Post-release, the majority of fsRIDL males are expected to die without mating; the number of generations taken for an allele to disappear should therefore consider not the initial release number, but the number of initial matings that successfully produce progeny. Equation 1 (*eq.1*), a simple expression based on the assumptions of the deterministic model describing numbers of generations until fsRIDL allele loss at different initial mating frequencies was derived.

$$n = \frac{\ln(x)}{\ln(2)} \quad (eq.1)$$

where:

n = the number of generations

x = the number of successful matings by heterozygous fsRIDL males in the first generation

Using (*eq.1*), we calculated the expected number of generations until allele loss for a range of initial mating frequencies (**Fig. 6.3**).

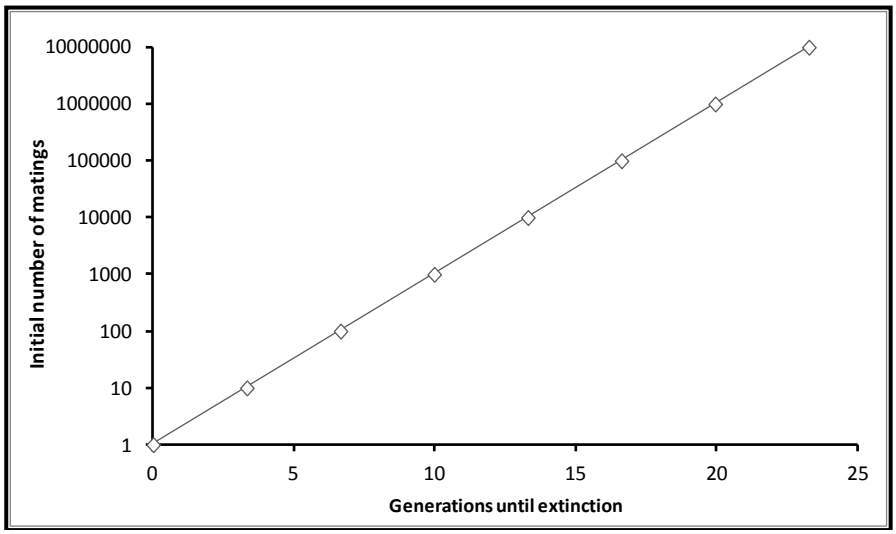


Figure 6.3. An estimation of the number of generations required for the reduction of the fsRIDL trait to one copy for a variety of initial mating frequencies using *eq.1* as a model.

This model makes a couple of basic assumptions. Firstly it assumes that the population in question maintains a constant population size (i.e. all mating pairs give rise on average to two progeny, one male and one female). Wild population density is likely to be considerably reduced post fsRIDL release, and would potentially be well below carrying capacity and may therefore be expected to pass through a recovery growth phase before stabilising. However, for olive fly this is complicated by variations in intrinsic seasonal growth rate [e.g. dependence on ambient temperatures, humidity and the

microclimate within the olive canopy (Daane& Johnson, 2010)] and variations in fruit availability [larval development is dependent on the availability and quality of olive fruit (Burrack et al., 2009)]. Furthermore, Allee effects [population shrinkage at very low densities due to a paucity of reproductive opportunities (Allee, 1939)] may be relevant if fsRIDL releases have caused the population density to decline below a certain critical limit. However, if the population does indeed pass through a growth phase, a successfully mating fsRIDL male would be expected to pass the trait on to more than the two surviving progeny assumed here, with generations to fsRIDL trait loss correspondingly increasing. We also make the assumption of random mating between wild and fsRIDL males, and equal survival of the two male genotypes. As we have seen in the sexual selection experiments (Chapter 4), OX3097D-Bol possesses sexual competitiveness costs relative to wild insects, and differences are likely to be exaggerated in the field and would act to decrease the numbers of generations to allele loss predicted here. However, the experimental data shows that rates of allele loss under laboratory conditions conform to predicted dynamics. Accurately modelling rates of selection against the allele in the field will be a complex task, and should take into account seasonality factors including variance in population growth rates, carrying capacity and mortality. Moreover, these parameters are likely to vary significantly with location. However, the predictions of **Fig. 6.3** provide a reasonable first approximation of the time of possible fsRIDL trait persistence in a wild population.

6.2.2. Selection in the presence of tetracycline

Measuring and modelling the dynamics of the OX3097D-Bol trait in the presence of tetracycline is more complex. Here we must consider three possible genotypes,

homozygous OX3097D-Bol, heterozygous, and homozygous wild-type, here after referred to as AA, Aa, and aa, respectively.

The rate of negative selection against the fsRIDL trait was measured in experimental cages of OX3097D-Bol in the presence of the transgene repressor. Initial populations of 100 heterozygous male and 100 heterozygous female OX3097D-Bol adults were set up, with populations of 200 randomly selected progeny replacing the parental population for ten subsequent generations. The genotypes of random samples of 96 adults removed from the cages once sufficient eggs had been collected, were determined by PCR, to provide estimates of changing genotype frequencies. **Fig. 6.4** shows the change in allele and genotype frequencies over ten generations for two experimental populations.

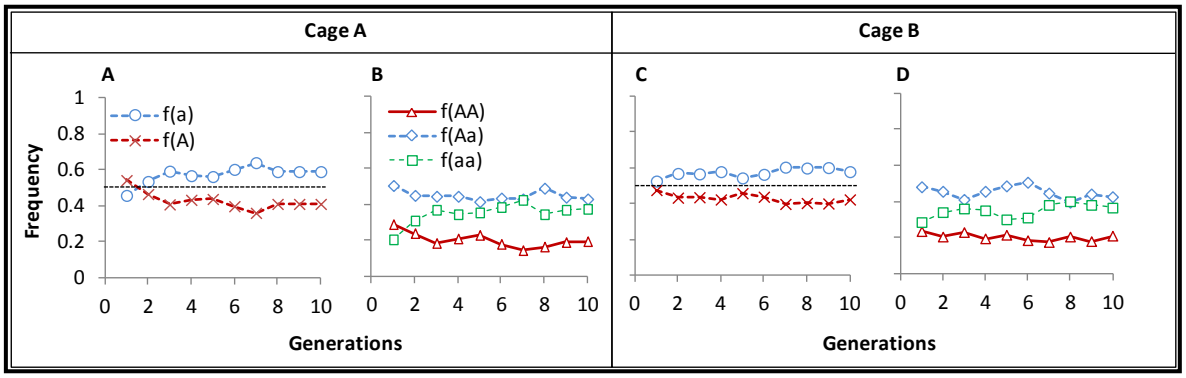


Figure 6.4. Line graphs showing changes in OX3097D-Bol [f(A)] and wt [f(a)] allele frequencies (**A** and **C**) and homozygous OX3097D-Bol [f(AA)], heterozygous [f(Aa)], and homozygous wild-type [f(aa)] genotype frequencies (**B** and **D**) over ten generations in two caged populations raised in the presence of tetracycline. Initial (generation 0) allele frequencies were equal and initial crosses involving 100 heterozygous males and 100 heterozygous females. Dotted lines in (**A**) and (**C**) show equal proportions.

Both cages showed a small but significant decline in the frequency of the OX3097D-Bol transgene after the first generation and tended to reduce over the course of the experiment, with proportional increases in the frequency of the wild-type allele. This suggests a case of natural selection favouring the wt allele. Cage A showed a reduction in OX3097D-Bol allele frequency from 0.54 in the first generation post-initial heterozygous

crosses, to 0.41 by F10 (**Fig 6.4A**). Cage B showed a reduction from 0.47 to 0.43 (**Fig 6.4C**). A Kwiatkowski-Phillips-Schmidt-Shin (KPSS) test for stationarity (Kwiatkowski et al., 1992) was performed on the changing allele frequencies to determine if the changes represented a significantly diverging trend. Results suggested that trends were significant in cages A (P = 0.0349, KPSS level = 0.5299, KPSS test) and B (P = 0.0335, KPSS level = 0.5364, KPSS test). Significance suggests that some factors other than random drift are responsible for the observed changes in allele frequencies (e.g. natural selection acting on the OX3097D-Bol insertion).

Genotype frequencies were close to the expected 1 : 2 : 1 (AA : Aa : aa) ratio in the first generation (G1). However, by G10, the relative frequencies of the homozygous genotypes had diverged in both populations; the frequency of the homozygous wild-type individuals numbering approximately double that of the homozygous OX3097D-Bol (**Fig. 6.4B** & **Fig. 6.4D**). Similarly the frequencies of heterozygous individuals showed a decrease in both cages by G10. Both cages showed a corresponding increase in the frequency of the homozygous wild-type genotype. A summary of the change in frequency of the three genotypes after ten generations of natural selection is given in **Fig. 6.5**. The similar relative fitness values obtained for the AA and Aa genotypes from the experimental populations suggest that the fitness costs associated with the OX3097D-Bol transgene may be largely dominant.

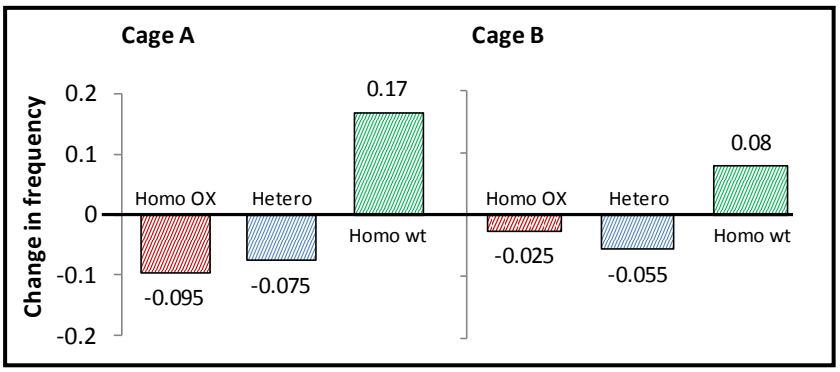


Figure 6.5. Changes in genotype frequencies over ten generations of natural selection in the presence of tetracycline from experimental cages A and B.

The ratio of genotype specific growth rates is called the relative fitness and is represented by the symbol *W* in models of natural selection with discrete generations (Hamilton, 2009). Relative fitness is a measure of the rate of increase or decrease of a given genotype, relative to a genotype picked as a standard of comparison (usually the genotype with highest absolute fitness, in this case homozygous wild-type). Once an estimate of relative fitness for each genotype is obtained, it is possible to predict a trajectory for each genotype in a population over time using the general selection model (*eq.2*) (Haldane, 1931). The general selection model is a model of natural selection that incorporates the relative fitness values for three genotypes produced by a single locus with two alleles, and the Hardy-Weinberg model of genotype frequencies.

$$p_{t+1} = \frac{w_{AA}p^2 + w_{Aa}pq}{w_{AA}p^2 + w_{Aa}2pq + w_{aa}q^2} \quad (eq.2)$$

Where:

w_{AA} is the relative fitness of the AA genotype;

w_{Aa} is the relative fitness of the Aa genotype;

w_{aa} is the relative fitness of the aa genotype;

p is the frequency of the A allele;

q is the frequency of the a allele.

The general selection model assumes an infinite population size and random mating between all individuals in a population; selection is therefore entirely viability based. The assumption of viability selection is reasonable when considering the OX3097D-Bol fsRIDL allele in a population of Argov wild-type insects; results from chapters 3 and 4 have suggested no difference in mating competitiveness between the genotypes, only differences in developmental viability. The changes in genotype frequencies observed in

the experimental cages and their resulting relative fitness calculations over ten generations are given in **Table 6.1**.

Table 6.1. Calculations of relative fitness values for genotypes (AA - homozygous OX3097D-Bol, Aa - heterozygous, and aa - homozygous wt) over ten generations in experimental cages A and B. ***f(G1)*** and ***f(G10)*** shows the frequencies of genotypes measured at generations 1 and 10, respectively. ***R*** shows the corrected rate of increase for each genotype (the ratio of actual frequency to the frequency expected in the absence of selection). ***W*** is the fitness of each genotype relative to the most fit genotype in the population (aa in both cages).

	Cage A			**Cage B**		
	AA	Aa	aa	AA	Aa	aa
f(G1)	0.290	0.505	0.205	0.235	0.480	0.285
f(G10)	0.195	0.430	0.375	0.210	0.425	0.365
R	0.663	0.866	1.792	0.931	0.852	1.324
W(over 10 generations)	0.370	0.484	1	0.702	0.643	1
W(per generation)	**0.937**	**0.948**	**1**	**0.9702**	**0.964**	**1**

A predicted genotype frequency trajectory was calculated using the general selection model (*eq.2*), the experimentally-measured relative fitness values, and the initial genotype frequencies used in the experimental populations (**Fig. 6.7**).

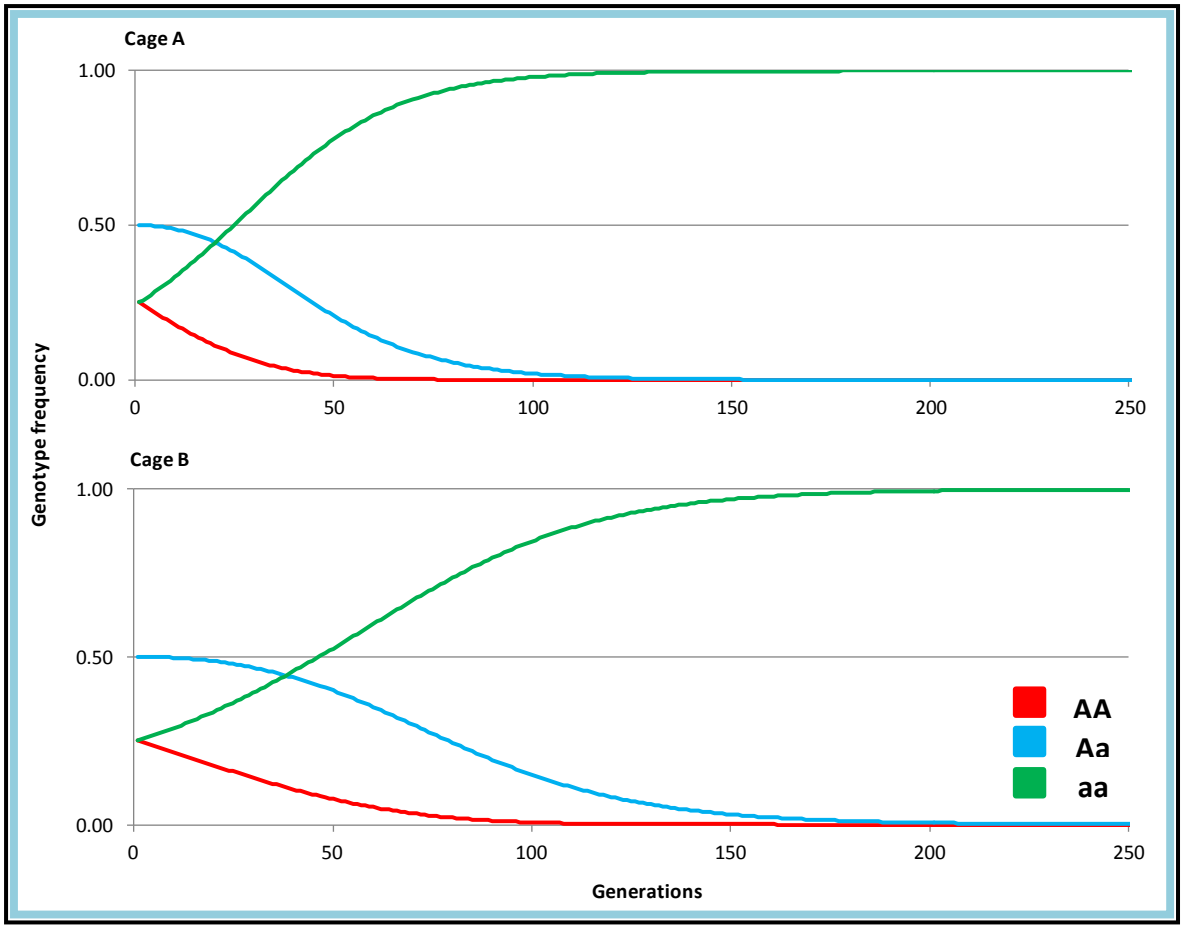


Figure 6.7. Genotype frequency trajectories for experimental cages A and B reared on-tet containing AA (homozygous OX3097D-Bol), Aa (heterozygous), and aa (homozygous wild-type) individuals at an initial 1:2:1 ratio using the general selection model (*eq.2*). For cage A the model assumes relative fitness values of 0.937 for AA, 0.947 for Aa, and 1 for aa; for cage B the model assumes relative fitness values of 0.970 for AA, 0.964 for Aa, and 1 for aa (as determined from the experimental populations).

If we consider an allele extinct once its frequency drops below 0.001, the OX3097D-Bol allele was extinct by generation 157 in cage A, and generation 217 in cage B. The relative fitness values of the genotypes varied quite substantially between the two cages; however, a trend of lower relative fitness (<1) was found for all transgenic genotypes in both cages. The rates of allele loss modelled here are not expected to represent rates in the field. Differences in relative fitness due to the deleterious effects of the transgene insertion are likely to be exaggerated in the field where environmental conditions are harsher and the average fitness of the wild population is higher. However, modelling predicts that even

under relatively transgene-permissive conditions, selection will cause the transgene to disappear from a population.

Part of the rationale for carrying out the selection experiment on-tet was to gain an insight into how quickly a wild-type allele is be likely to spread through a homozygous transgenic population at a low initial frequency (gaining an insight into dynamics if a population was contaminated by a wild-type insect). The values of relative fitness (*W*) were averaged for the genotypes over the two experimental cages giving mean relative values of 0.954 for AA, 0.956 for Aa, and 1 for aa. A frequency trajectory was calculated for each genotype for a population of homozygous OX3097D-Bol olive flies, that was contaminated with a wild-type insect at an initial genotype frequency of 0.05 (**Fig. 6.8**).

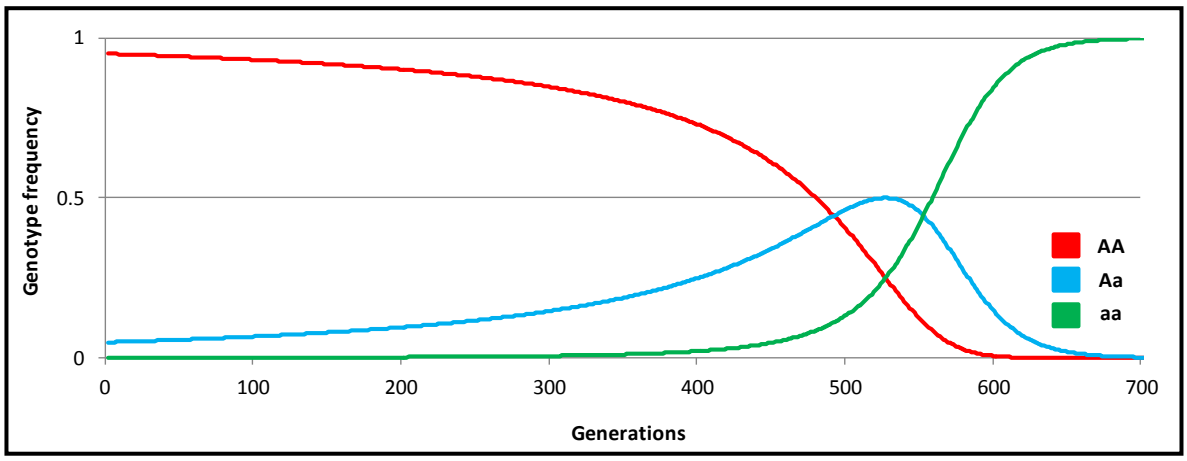


Figure 6.8. Genotype frequency trajectory for a population of homozygous OX3097D-Bol (AA), with a contamination of the wild-type (aa) genotype at an initial frequency of 0.05. The model assumes mean relative fitness values of 0.954 for AA, 0.956 for Aa, and 1 for aa.

The modelled trajectory predicts an initial steady increase in heterozygous genotype frequency. However, the frequency of the wild-type genotype in resulting generations does not exceed 0.01 until generation 280, by which time the heterozygous genotype frequency has reached a frequency of approximately 0.2 in the population. As the proportion of wild-

type individuals in the population increases, the increasing average fitness of the population causes the heterozygous genotype to become selected against, and we see a frequency decline starting at approximately generation 520. The wild-type allele eventually goes to fixation at around generation 700, although in practice, the contamination would presumably have been noticed and rectified long before this point.

The results of the model suggest that the number of OX3097D-Bol heterozygotes could accumulate quite considerably before the proportion of wild-type individuals becomes significant. The point at which the wild-type contamination is noticed will be dependent on the extent of contamination, the size of the stock colony, and the proportion of the flies that are routinely screened for fluorescence each generation. However, these results strongly suggest that careful screening of a significant proportion (if not all) of the individuals making up a rearing population should be performed each generation.

6.3. Summary and conclusions

Here I present an examination of the population genetics of the fsRIDL transgene insertion in the olive fly strain OX3097D-Bol. We consider selection on the transgene both in the absence and presence of the transgene repressor. In the absence of tetracycline we show that modelling predictions conform closely to laboratory-based caged population studies. The results indicate that in the absence of tetracycline, female-specific lethality is the main driver of negative selection against the transgene, with experimental results conforming closely to predictions based on a simple halving of allele frequency each generation.

Selection on the OX3097D-Bol insert with repression of female lethality, however, is due to inherent fitness costs associated with carrying the transgene. The production of viable female offspring resulting in unintended persistence of the transgene in a population could be due to a failure of the employed lethal trait due to the build-up of resistance mechanisms or through the presence of a transgene repressor in the wild. We provide estimates of the relative fitness values for the homozygous and heterozygous transgenic genotypes by following changes in genotype frequencies over several generations, and use modelling to show that the OX3097D-Bol transgene insert is expected to be lost from a population even when lethality is repressed.

The relative fitness of the OX3097D-Bol genotypes in the field are expected to be lower than those measured here (a result of a variety of factors including harsher environmental conditions and higher levels of mate competition). Conditions in the field are also expected to differ in regards to population growth, carrying capacity and mortality rates.

Risks of homozygous strain contamination with a wild-type allele at the genomic locus of OX3097 transposition in the OX3097D-Bol line are intensified by dominance of

the fluorescent protein transformation marker. Using genotype specific relative fitness values, we show that increase in the wild-type allele frequency is slow. This is contrasted with what is expected to be far lower relative fitness values for classical translocation-based sexing strains, where aneuploidy results in high fitness costs relative to wild-type. The wild-type genotype would therefore be expected to spread far more rapidly in these strains. Nonetheless, regular screening of OX3097D-Bol by fluorescent microscopy (to detect wild-type individuals) or by PCR (to detect heterozygotes) is strongly advised.

6.4. Materials and methods

6.4.1. Measuring fsRIDL trait dynamics off-tet

All experiments were conducted in temperature controlled rooms set at 25°C with a 16:8 light:dark cycle. Initial experimental populations required crossing 100 heterozygous OX3097D-Bol males to 100 Argov wild-type females. Heterozygous OX3097D-Bol males were initially obtained by crossing males of the stock homozygous OX3097D-Bol colony to wild-type females, with progeny reared in the absence of tetracycline. For the first generation 100 adult virgin heterozygous OX3097D-Bol males were added to each of three rearing cages (30x30x30cm), with 100 adult virgin Argov females (sexed by hand) added immediately afterwards. Colonies were maintained on distilled water and olive fly adult diet. All egg collections were made between days 7-10 post adult eclosion. Eggs were reared according to standard olive fly rearing techniques (in the absence of tetracycline), and larvae were obtained. A selection of 200 larvae were blindly selected, and were screened by fluorescence microscopy. The ratio of fluorescent to non-fluorescent larvae were recorded. As lethality occurs at the pupal stage, the ratio of wild-type to OX3097D-Bol represents the proportion of wild-type to OX3097D-Bol in the parental generation. The 200 screened larvae were added to fresh rearing cages, making the population for the next round of selection. This process was repeated until no insects were positive for the OX3097D-Bol transgene insertion.

The experimental data were compared to modelling predictions. The deterministic model assumed a constant halving of fsRIDL allele frequency each generation. The stochastic model was build around the Monte-Carlo method in Excel (Microsoft, California, USA), using a programme coded in Visual Basic (Microsoft, California, USA) to record outputs from generations of random numbers. Each insect was assigned two random numbers each generation, one determining sex (always at a 0.5 probability) and

one determining genotype (probability equal to the proportion of male transgenics in the parental generation divided by 2).

6.4.2. Measuring fsRIDL trait dynamics on-tet

Initial populations consisted of 100 heterozygous OX3097D-Bol males crossed to 100 heterozygous OX3097D-Bol females. Heterozygotes were obtained by crossing homozygous OX3097D-Bol males to Argov wild-type females. Adults in the initial generation were sexed by hand, and experiments were performed in duplicate. Colonies were maintained on tetracycline water (100µg/ml) and olive fly adult diet. All egg collections were made between days 7-10 post adult eclosion. Eggs were reared according to standard olive fly rearing techniques [(in the presence of tetracycline (100µg/ml)], and larvae were obtained. All resulting larvae were screened for presence of the OX3097D-Bol transgene insertion (the exact number varied from generation to generation but was always $300<n<500$), and a population of 200 of those pupae (representing the same ratio of wild-type to transgenic found in the screening of all larvae) was used to make up the following generation. After egg collections the adult population was frozen, and 96 adults were randomly selected. Genomic DNA was extracted from these adults, and a PCR was performed as described in (**Fig. 2.6**) to determine genotype. The experiment was run for 10 generations.

6.4.3. Statistics and modelling

The stochastic model was built using Microsoft Excel (Microsoft, California, USA), using a Monte Carlo design. The progeny resulting from parental crosses (200 each generation) were assigned two random numbers, one determining their sex, and the other their genotype. The probability of being a male was set at a constant 0.5 each generation,

and the probability of being assigned a fsRIDL genotype was dependent on the proportion of fsRIDL males in the parental generation. Multiple runs of the model were performed, with outputs of each iteration recorded using code programmed using Microsoft Visual Basic (Microsoft, California, USA).

Modelling genotype trajectories was performed using the Populus population biology simulation software (Version 5.4) considering selection on a diallelic autosomal locus. Significance testing for changing allele frequencies was performed using the Kwiatkowski-Phillips-Schmidt-Shin (KPSS) test for stationarity (Kwiatkowski et al., 1992), part of the 'tseries' package for RStudio (version 3). For all comparisons significance was set at $P < 0.05$.

Wild olive fly male on leaf.

Chapter 7 - General conclusions and future considerations

Tephritid fruit flies are responsible for major losses in yields of fruit and vegetable crops worldwide. They are often the target of intensive insecticide applications, and can affect international trade through fears of importing infested goods. The development of effective methods of control without causing harmful environmental side-effects has therefore become a global challenge.

The sterile insect technique is increasingly being utilised as part of integrated pest management strategies against tephritid insects. Its application has created numerous technical challenges, some of which are general and concern all species [for example, the exigencies of colonisation, mass-rearing, and the stress and damage incurred by sterilisation, shipping and release, usually produce insects that are of lower quality (Liimatainen et al., 1997; Cayol et al., 2002; Perez-Staples et al., 2012)], and some of which are more species specific. Previous olive fly SIT attempts, for example, were hampered by several technical problems including difficulties in mass-rearing [partially a result of insufficient knowledge of essential aspects of the fly's basic biology (including information on its interactions with bacteria,dietary requirements, and knowledge of the dynamcis of wild olive fly populations) (Economopoulos, 2002)], the production of low quality insects (Economopoulos, 1972, Economopoulos et al., 1978), and photoperiod mating incompatibility with members of the wild population (Economopoulos, 1972; Economopoulos et al., 1978).

It is widely recognised within the olive fly research community that the refinement of mass-rearing techniques and the development of a means of sexing (Economopoulos, 2002) to allow for male only releases will be necessary if these problems are to be overcome (Estes et al., 2011; Economopoulos, 2002). Although a great deal of work has been performed (and is ongoing) investigating olive fly basic biology and attempting to

address the major problems in mass-rearing (due largely to a co-ordinated Research Project supported by the FAO/IAEA) [reviewed in (Daane& Johnson, 2010; Estes et al., 2011)], the lack of a sexing strain has remained a critical issue; this thesis presents an attempt to address this.

We have developed OX3097D-Bol, an fsRIDL olive fly strain that incorporates engineered phenotypes, directly addressing problems encountered in previous unsuccessful olive fly SIT trials. Chapter 2 describes experiments showing that OX3097D-Bol provides 100% male-only release generations when reared in the absence of a dietary transgene repressor. Male-only releases encourage random mating between the wild and the released sterile populations, providing established performance benefits (Rendon et al., 2004). Female lethality renders redundant the requirement of performance-reducing and costly sterilisation using ionising radiation. Furthermore, the dominant fluorescent marker has the potential to improve field monitoring, and the introgression of insecticide susceptibility alleles through the female line provides a resistance management option (Alphey et al., 2007; Alphey et al., 2009; Alphey et al., 2011).

Chapter 3 provides an analysis of the life history traits of OX3097D-Bol relevant to mass-rearing. We hypothesise that although OX3097D-Bol shows a reduction in egg to adult developmental viability relative to the wild-type strain from which it was derived, the potential benefits offered by a fsRIDL approach to olive fly control far outweigh the potential costs. However, the mass-rearing protocol for olive fly requires further refinement; optimising the rearing and egg collection systems remains a priority. We describe some observations regarding egg collection and optimal rearing densities, but more work is needed on the basic aspects of mass-rearing, such as cage design. It is important in olive fly rearing that ovipositional wax membranes are easily accessible to technicians and can be designed into a space-efficient high density caging system whilst

maintaining the greatest possible surface area for female oviposition. It is also crucial that the wax membranes do not allow a high rate of egg desiccation [olive fly eggs are extremely sensitive to low relative humidity (Tsitsipis& Abatzis, 1980)]. The design of optimum mass-rearing should take into account lessons learned from advancements in medfly rearing [e.g. cage designs with internal compartments that allow for high levels of fly movement and air circulation and so preventing early mortality due to adult crowding at the top of the cage (Caceres, 2002)]. Furthermore, although significant progress has been made in producing an effective larval diet (e.g. Manoukas, 1972; Tzanakakis, 1989), the current formulation is less than ideal and it remains a target of future research to develop a larval diet that provides high yields, is less labour intensive to produce, and is above all cheap (current diets use expensive cellulose fibres as a bulking agent) (Estes et al., 2011).

Chapter 4 presents an analysis of the pre and post-copulatory sexual selection and sexual compatibility traits of OX3097D-Bol males with both a laboratory strain, and field-collected wild insects. OX3097D-Bol males displayed highly desirable mating characteristics in these studies, including high sexual competitiveness, photo-period compatibility, and efficient induction of remating refractoriness in wild females. Whilst the data obtained describing OX3097D-Bol copulation success with wild-flies gives an indication of possible male sexual competitiveness in the field, the tests were performed in small field cages with a significant amount of courtship activity taking place on the (sometimes crowded) surfaces of the cage. Although this is a valid and widely used method for evaluating the relative competitiveness of a strain (FAO/IAEA/USDA, 2003), restricted spatial aspects of the tests limit the inferences that can be drawn regarding field competitiveness. In this respect the environment was similar to the scenario in a rearing cage, and somewhat removed from the single leaf territories found in the wild. Moreover,

the tests presented in chapter 4 were restricted to a temperature controlled glasshouse. Exceptionally low (Avidov& Harpaz, 1969) or high (Rempoulakis& Nestel, 2011) temperatures, are known to have a negative impact on dispersal and survivability of artificially reared strains (although whether this affects mass-reared olive flies disproportionally compared to wild olive flies is not known). Further studies utilising large outdoor field cages with large olive trees may provide a more realistic mating environment.

Proof-of-principle experiments detailing the suppression and elimination of caged wild-type olive fly populations are discussed in Chapter 5, and provide evidence for the fsRIDL approach as a viable method for olive fly control. The results, however, are from testing in a contained facility, therefore some uncertainty remains as to whether the control approach will be fully efficient in open field environments. Extending this work would require progressing from standard glasshouse field cages to large field enclosures and eventually to the open field.

Chapter 6 presents an exploration of the dynamics of the fsRIDL allele in populations of olive fly both in the presence and absence of the transgene repressor, using both theoretical modelling and experimental data. We show that dynamics of the transgene in laboratory-caged populations reared in the absence of the transgene repressor adhere closely to predictions, showing that the requirement of tetracycline for female development mitigates risks of accidentally released transgenic populations establishing in the wild. Moreover, small fitness costs associated with the transgene insertion cause selection against the transgene, even if lethality is repressed. Therefore, if the average relative fitness of a population is higher than that of individuals carrying the OX3097D-Bol transgene insertion (which is highly likely due to inherent fitness costs associated with the transgene), the insert will not persist permanently in that population.

The generation of OX3097D-Bol represents a major technical advance towards a working application of sterile insect control for the olive fly. However, there are numerous research avenues that should be explored in order to refine and optimise protocols for olive fly SIT. For example, in this thesis it is hypothesised that using older males with younger females may significantly improve egg hatch rates and therefore mass-rearing outputs (see *Section 3.2.2.*). Further experiments could easily test this, and if confirmed, methods should be devised for using mixed-age populations in rearing cages. Moreover, research on the olive fly microbiota is producing very interesting results, and may have high significance for SIT application [e.g. (Capuzzo et al., 2005; Ben-Yosef al., 2010)]. The importance of symbiotic bacteria to the survival and reproduction of their hosts is becoming increasingly apparent (Douglas, 2007), and an understanding of the bacterial-host interactions may help to improve mass-rearing diets and the general quality of flies produced. 40 species of transiently associated bacteria (bacteria acquired while on the phylloplane) have been associated with wild olive flies, in contrast, only 25 species have been found in laboratory strains [7 of which are novel to the laboratory (Estes et al., 2011)]. Identifying the interactions between microbiota and the host fly could help to identify bacteria that could be used to supplement artificial diets, or may identify nutrients made available by the bacteria that are lacking in current diets. It should also be determined how tetracycline provided in the transgenic olive fly diet effects the gut microbiota. If bacteria that are important for providing nutritional sources are absent in OX3097D-Bol (or are replaced by pathogenic bacteria), reductions in fitness relative to wild flies who have a full complement of symbiotic bacteria may result. Probiotic diets have been successful in supplementing the diet of laboratory reared medflies, and have provided significant increases in overall quality (Niyazi et al., 2004), and may be particularly useful as a pre-release supplement for RIDL strains reared on tetracycline.

Another major challenge for the application of OX3097D-Bol for olive fly control concerns the current stance of many countries to genetic engineering technologies. The use of genetic technology in food production has been highly controversial in some countries, most notably within the European Union (EU) (the olive fly is endemic in Europe and present wherever olive trees are cultivated; this is largely in the Mediterranean basin, which is the anticipated maximum area of release). Two areas of concern are adverse effects associated with horizontal gene transfer (e.g. transmission of recombinant DNA traits which confer altered characteristics in receiving individuals to a non-target species) (discussed further in *Appendix 1*), and the presence of recombinant DNA or recombinant DNA products in human consumables (e.g. foodstuffs or cosmetics). It is possible that the use of genetically modified arthropods to protect the crop in a sustainable way, which leaves little or no residue in the food or cosmetic product (here olives or olive oil) might be more acceptable in the EU than food products that are produced directly from genetically modified organisms (Ant et al., 2012). For comparison, the Standing Committee on the Food Chain and Animal Health stated that foods and feeds produced by fermentation with genetically modified microorganisms that are not present in the final product are "excluded from the scope of regulations" [European Union Report (1829/2003)] (Ant et al., 2012). Additionally, it is recognized in EU legislation that the presence of technically unavoidable traces of genetically modified organisms in food and feed products should not trigger labeling requirements [European Union Regulation (1830/2003)], as long as appropriate steps to avoid the presence of the materials have been taken; this is also analogous to the situation regarding the presence of wild-type insect parts as contaminants in food products [European Union Regulation (543/2011) 2011] (Ant et al., 2012).

Although, 'it's tough to make predictions, especially about the future', it seems probable that regulatory approval for future OX3097D-Bol field release would occur first

in the United States, with California being the region with the highest infestation levels. The first permit for the release of a genetically modified arthropod species was a predatory mite in the US in 1996 (Hoy, 2000) and since then strains of the pink bollworm have also been tested (Beech et al., 2012). Releases of sterile insects usually take place when the wild population is at its lowest density; due to a natural seasonal variation and/or the application of other methods of control. Releasing at this time ensures the most favourable wild male to released male ratio, with a given release number. The olive fly has two major population peaks, the first in the middle of spring, and the second in early autumn, and so OX3097D-Bol male releases are most likely to begin just prior to these periods to attempt to halt the increase.

Genetic modification provides a powerful tool that could be employed to overcome many of the difficulties encountered in modern agricultural pest control. Although the number of agriculturally important arthropod species with available transgenic control strains is increasing every year, it remains to be seen how national decision-makers will react to the use of genetically modified arthropods in the food industry. Public perceptions for the control of arthropods may be seen as less crucial for agricultural pests, than for disease vectors. Policy makers may therefore be more risk-averse with regards to agriculture, and it is probable that significant precedence will first be set through the use of transgenic vector species, with agricultural pests following in the their footsteps. The likelihood of significant application of transgenic strains of olive fly, therefore, does not depend only on the generation of sophisticated strains, but also on the development of robust regulatory frameworks and ultimately public perception of the risks involved.

Appendix 1 - *piggyBac* remobilisation for post-integration transgene stabilisation

Introduction

During transgenic strain generation transposons bearing modifying genes are co-injected as part of a DNA plasmid into a host embryo with a transient source of transposase. This is usually supplied as a DNA helper plasmid (encoding the transposase gene), or in the form of messenger RNA (mRNA). In a limited number of individuals, the transposase catalyses transposon germline integration. However, neither the DNA helper plasmid nor the mRNA contain any transposase binding sequence, and are unable to integrate. In the absence of a transposase source, the integrated transposon is theoretically as stable as any other gene.

A regulatory concern, however, regards whether the integrated transposon might be re-exposed to a transposase that could mobilise the inserted transgene, and what the consequences of such an exposure might be (Beech et al., 2012). While the presence of such a transposase source in a wild insect population of a given insect species can be easily proved, its absence cannot.

There are numerous mechanisms of horizontal gene transfer that an autonomous transposon could theoretically utilise to invade a host species genome. A well-known case in point is the recent, rapid and almost ubiquitous spread of the *P* family of transposable elements into the *Drosophila melanogaster* species. Evidence suggests that the *P* element came from another *Drosophila* species, and was possibly transferred by parasitic mites (Engels, 1992). A variety of hypothetical mechanisms could result in horizontal gene transfer, e.g. uptake of transgene DNA released from genetically modified organisms upon decay or ingestion and integration into a microbial host (soil miocroorganisms or gut microflora), transposition into new host species by transfer through host parasite/parasitoid

interactions or transfer by insect viruses [Loreto et al., 2008; Gilbert et al., 2010; (EFSA Report, CT/EFSA/GMO/2009/03)]. Bacteria and viruses are frequently implicated in transposable element horizontal transfer because of their known propensity to transduce and recombine host DNA fragments and because they are often able to enter and exit eukaryotic cells (Schaack et al., 2010). Several active DNA transposable elements have been 'caught in the act' of a horizontal escape from eukaryotic host to viral genome in the laboratory (Schaack et al., 2010) [including *piggyBac* (Fraser et al., 1983)].

piggyBac, and elements with high sequence similarity to *piggyBac*, have been found in a range of Dipterans and Lepidopterans; its existence in both closely and distantly related species suggests that it has recently traversed orders by means of horizontal transmission (Handler, 2002). Although the presence of *piggyBac*-related sequences has not been reported in any wild olive fly populations, they have been identified in several related *Bactrocera* species, including the Oriental fruit fly *Bactrocera dorsalis* (Handler& McCombs, 2000), and the Queensland fruit fly, *Bactrocera tryoni* (Raphael et al., 2011). However, despite the presence of endogenous sequences, *piggyBac* was used to develop stably transformed insertion lines in both these species (Handler& McCombs, 2000; Raphael et al., 2011).

The precautionary principle is often applied to the risk management of modern biotechnology, and post-integration stabilisation methods have been developed to remove one (Handler et al., 2004) or both (Dafa'alla et al., 2006) of the transposon terminal inverted repeats (TIRs). Daffa'alla et al. (2006) report the post-integration elimination of all transgene flanking *piggyBac* transposon sequences in a transgenic strain of the medfly, thereby rendering the insertion inert to further transposase exposure. In this section we describe the transformation of the olive fly with OX3713, a female-specific lethal transgene construct, based on the OX3097 transposon described in Chapter 2 (**Fig. 2.1**).

However, unlike the single pair of 5' and 3' *piggyBac* TIR sequences used in OX3097, OX3713 carries the composite *piggyBac* transposon configuration described by Dafa'alla et al. (2006); that is, containing two pairs of opposed *piggyBac* ends. We also describe the transformation of the olive fly with OX3133, a second composite *piggyBac* construct containing a gene coding for *piggyBac* transposase. Insertion lines containing this gene can be used as an endogenous source of transposase, and can cause remobilisation of *piggyBac* elements of other independent insertions [i.e. as a transposon 'jumpstarter' (Dafa'alla et al., 2006)]. We crossed OX3713 and OX3133 insertion lines in an attempt to resolve (remobilise) the *piggyBac* ends in an olive fly fsRIDL strain (see *Section 1.3.3.* and **Fig. 1.4B**).

Results and discussion

Transformation of the olive fly with OX3713

The OX3713 construct (**Fig. 7.1**) was micro-injected into approximately 5,000 pre-blastoderm stage olive fly embryos of the Democritus wild-type strain (Athens, Greece). All of the embryos were injected with the OX3713 plasmid and *piggyBac* mRNA at a ratio of 400ng/µl of plasmid to 600ng/µl of mRNA. Of the 5,000 embryos injected approximately 700 survived to the L1 larval stage and were placed on larval medium containing tetracycline. This resulted in 184 pupae and a total of 96 adults were recovered (1.92% egg to adult survival). Approximately 11% of these pupae displayed some level of transient fluorescent marker expression, confirming the presence of the OX3713 plasmid in those individuals.

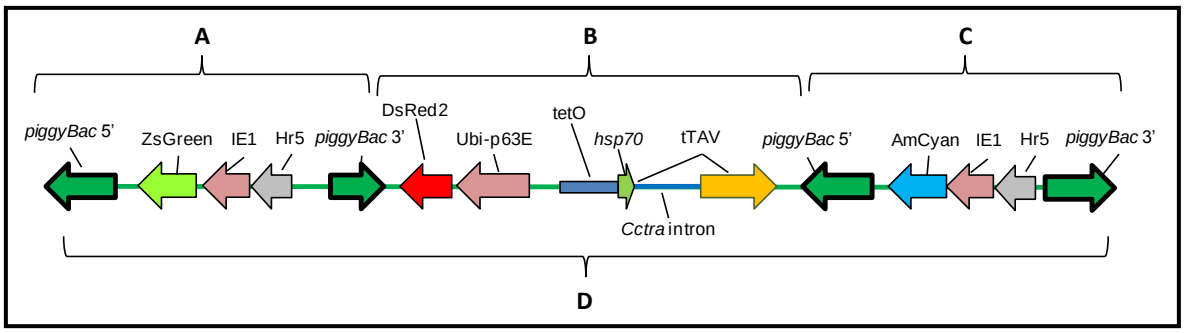


Figure 7.1. Schematic representation of the OX3713 composite transposon. OX3713 is a female-specific lethal construct based on the positive feedback tTAV and Cc*tra* splicing systems of OX3097 described previously (see *Section 2.1*). OX3713 has four potential transposons (A, B, C and D). The insertion of the desired D transposon can be identified by the fluorescence screening of the fluorescent protein markers contained within the three smaller transposons (ZsGreen in A; DsRed2 in B; and, AmCyan in C). In principle, the post-integration exposure of the insertion to transposase can remobilise both the flanking transposons, rendering the remaining central (B) segment trasposon-free and inert to transposase.

Injection survivors were back-crossed to wild-type flies in small pools, and the G_1 progeny were screened for expression of the fluorescence markers. A single genomic insertion line (OX3713A-Bol) was recovered (a transformation efficiency of ~1%), and contained all three fluorescent markers. This is a considerably lower transformation

efficiency than was described for the OX3097 insertion lines (see *Section 2.2.1.*). This may be due to random variation between injection sets, however, it may also represent the lower transformation efficiencies expected for larger transposons (Fu et al., 2008) (the size of the inter-transposon region of OX3713 is nearly double that of OX3097).

In order to assess copy number in OX3713A and to determine if the individual fluorescent markers were segregating together, heterozygous males and females from each line were crossed to wild-type insects, and the progeny were collected in the presence of tetracycline (100µg/ml). Pupae were screened and scored for the presence or absence of the three fluorescent markers (**Table 7.1**).

Table 7.1. Results from the microinjection of wild-type olive fly embryos with the transgene construct OX3713. Table shows the percentage survival to adulthood of the injected embryos and the percentage transformation efficiency. Table also gives the numbers (and percentages) of wild-type and transgenic OX3713 progeny resulting from back-crosses to wild-type with the progeny collected in the presence of tetracycline (100µg/ml). Numbers of transgenic progeny have been split into columns of those displaying all three fluorescent protein markers, and those displaying two or fewer. The *P* value from a Pearson's Chi Squared test comparing the observed ratio of transgenics to wild-type with a theoretical 1:1 ratio expected from the segregation of a single insertion of the OX3713 transgene is also given.

Line	Embryo to adult survival (%)	Transformation efficiency (%)	Number of wild-type progeny (%)	No. of transgenic progeny displaying all three markers (%)	No. of transgenic progeny displaying two or less markers (%)	*P* value: Pearson's Chi Square Test (1 d.f.)
OX3713 A-Bol	1.92	1.04	216 (55)	176 (45)	0 (0)	0.172

Crossing OX3713A-Bol to wild-type produced transgenic to wild-type progeny ratios that did not significantly differ from the 1:1 ratio expected from the segregation of single transgene insertion. All three fluorescent protein markers segregated together in all individuals (**Fig. 7.2**), suggesting that they represent an insertion event at a single genomic locus.

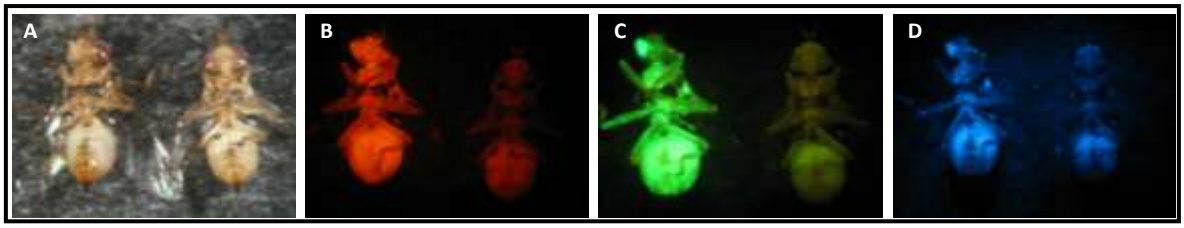
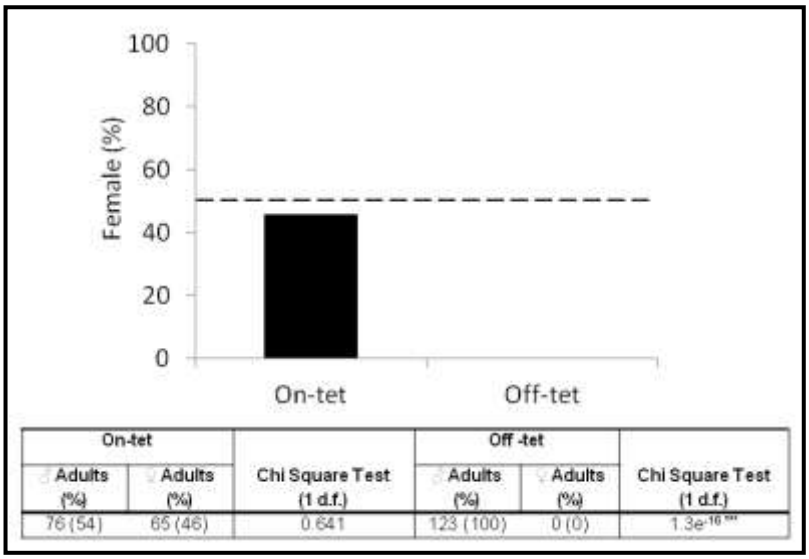


Figure 7.2. Photomicrographs of adult OX3713A-Bol (left) and wild-type (right) olive flies under bright-field (**A**) and fluorescence illumination. Panels show (**B**) DsRed, (**C**) ZsGreen, and (**D**) AmCyan fluorescence.

OX3713A-Bol was assessed for penetrance and repressibility of the female-specific lethal phenotype by crossing heterozygous transgenic males to wild-type females, and rearing the progeny in the presence (on-tet) or absence (off-tet) of the transgene repressor. Progeny were reared, allowed to pupate, and screened for fluorescence approximately 48 hours post-pupation. The transgenic adults were sexed, and the numbers of males and females were scored (**Fig. 7.3**).



On-tet			Off-tet		
Adults (%)	Adults (%)	Chi Square Test (1 d.f.)	Adults (%)	Adults (%)	Chi Square Test (1 d.f.)
76 (54)	65 (46)	0.641	123 (100)	0 (0)	$1.3e^{-16***}$

Figure 7.3. Penetrance (off-tet) and tetracycline repressibility (on-tet) of female lethality in the OX3713A-Bol olive fly insertion line. Penetrance and repressibility of female-specific lethality was assessed by crossing heterozygous males of each strain to virgin wild-type (WT) females, and collecting eggs on filter paper saturated with water containing either 0 µg/ml tetracycline or 100 µg/ml tetracycline for off-tet and on-tet, respectively. Neonate larvae were then reared in larval medium seeded with either 0 µg/ml tetracycline or 100 µg/ml tetracycline for off-tet and on-tet, respectively. Table also shows *P* values from a Pearson's Chi Square test comparing the observed sex ratio with the relevant (on or off-tet) sex ratio with a 1:1 ratio expected if the transgene was having no effect. An asterisk next to a *P* value indicates a statistically significant difference.

OX3713A-Bol gave 100% penetrant, dominant female-specific lethality when reared off-tet, and female lethal repressibility when reared on-tet [i.e. not differing significantly from a theoretical 1:1 sex ratio (P = 0.641, 1 .d.f., Chi-Square)]. Similar to the OX3097 olive fly insertion lines (also based on the female-specific positive feedback of tTAV), female lethality was found to occur at the late-larval/early pupal stage. It is possible that the levels of transgene repressibility observed in heterozygous females of a given line may not be retained when a strain is made homozygous. The homozygous viability of OX3713A-Bol was therefore assessed. Heterozygous transgenic males and females were crossed, eggs were collected, and larvae were reared on-tet. The ratio of emerging transgenic to wild-type adults was compared (**Table 7.2**). Any homozygous inviability would manifest as a greater proportion of wild-type than the 1:3 (wild-type to transgenic) expected from the Mendelian segregation pattern of an innocuous dominant marker.

Table 7.2. Homozygous viability in OX3713A-Bol. Flies were crossed in pools of 5 heterozygous males with 10 heterozygous females. Eggs were collected and reared on-tet. Adults were screened for fluorescence 48-hours post-eclosion. Numbers and percentages of emerged wild-type and transgenic flies, and the numbers and percentages of transgenic and wild-type females for progeny are given. The ratio of transgenic to wild-type adults was compared to a theoretical 3:1 segregation pattern using a Pearson's Chi Square Test (1 d.f.). The ratio of females to males in the transgenic and wild-type cohorts was also compared. An asterisk next to a *P* value indicates a statistically significant difference.

Transgenic		**Wild-type**		***P* value - ratio of transgenic to wild-type**	***P* value - proportion of females to males**
Total (%)	**Female (%)**	**Total (%)**	**Female (%)**		
142 (67)	61 (43)	70 (33)	36 (52)	0.02*	0.56

Heterozygous crosses of OX3713A-Bol produced ratios of transgenic to wild-type adults that differed significantly from the Mendelian 3:1 ratio (P = 0.02, d.f. 1, Chi Square). Statistical testing indicated no significant reduction in the proportion of OX3713A-Bol females produced compared to wild-type suggesting no additional unrepressed tTAV lethality specific to homozygous OX3713A-Bol females (P = 0.56, d.f. 1, Chi Square). This suggests that any additional homozygous lethality is affecting both

male and female OX3713A-Bol, and may possibly be the result of recessive insertional mutagensis of the OX3713 transposon. While a significant reduction in survival is undesirable for an fsRIDL strain (see *Section 2.2.3.*), this was the only OX3713 insertion line available, further experiments with *piggyBac* remobilisation therefore utilised OX3713A-Bol.

Transformation of the olive fly with OX3133

OX3133 is a composite *piggyBac* construct that carries a gene coding for *piggyBac* transposase (**Fig. 7.4**). Designed by Dafa'alla et al. (2006), OX3133 can provide transposase for: (i) the excision of its flanking transposons; and, (ii) the post-integration remobilisation of the *piggyBac* sequences of other transposon insertions (i.e. as a jumpstarter for other insertion lines) (Dafa'alla et al., 2006).

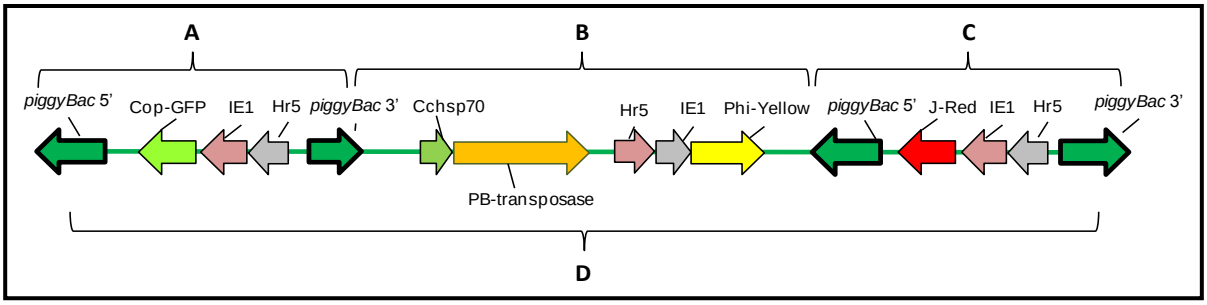


Figure 7.4. Schematic representation of the OX3133 inter-transposon region. OX3133 has four potential transposons (A, B, C and D). The insertion of the desired D transposon can be identified by the fluorescence screening of the fluorescent protein markers contained within the three smaller transposons (Cop-GFP in A; Phi-Yellow in B; and, J-Red in C). Expression of the *piggyBac* transposase gene in segment B should cause preferential excision of the smaller flanking transposons, and can be used as an endogenous transposase source for other composite *piggyBac* insertions.

OX3133 (**Fig. 7.4**) was micro-injected into 6,000 pre-blastoderm stage olive fly embryos of the Democritus wild-type strain. All of the embryos were injected with *piggyBac* mRNA at a ratio of 400ng/µl of plasmid to 600ng/µl of mRNA. Of the 5,000 G_0

embryos obtained, approximately 900 survived to the L1 larval stage and were placed on larval medium. This resulted in 476 pupae and a total of 296 adults were recovered (4.93% egg to adult survival). Approximately 21% of these pupae displayed some level of transient fluorescent marker expression.

Six insertion lines were obtained with G_1 progeny displaying the phenotypes shown in **Table 7.3.**

Table 7.3. Seven independent insertion lines were obtained from injections with construct OX3133. Numbers of G_1 individuals obtained from each cage, and fluorescent phenotypes are given. Table shows different possible marker combinations (i.e. GYR corresponds to an insertion line displaying Cop-GFP, Phi-Yellow, and J-Red, representing the transposon segments A, B, and C as defined in **Fig. 7.4**, respectively).

	Fluorescence phenotype of G_1 transgenic progeny						
Line	**GYR**	**GY**	**YR**	**GR**	**G**	**Y**	**R**
OX3133A	0	1	0	0	0	0	0
OX3133B	0	0	0	0	0	0	3
OX3133C	5	0	0	0	0	0	0
OX3133D	2	0	0	0	0	0	0
OX3133E	0	0	0	0	0	0	2
OX3133F	3	0	0	0	0	0	0
OX3133G	0	0	0	0	0	0	4

Once the G_1 progeny had been successfully backcrossed, individuals from the various insertion lines were frozen, and genomic DNA was extracted. The presence of transposon segments predicted from the fluorescence screening was confirmed by PCR (**Fig. 7.5**).

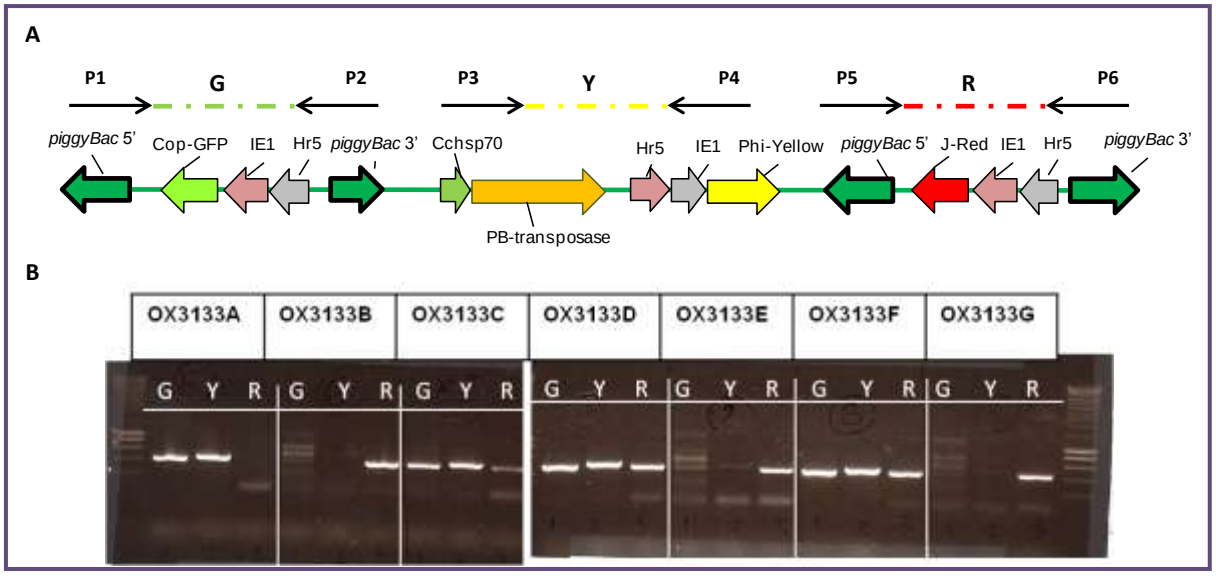


Figure 7.5. PCR results from analysis of G_1 individuals from OX3133 insertion lines. (**A**) Schematic diagram of OX3133 construct with screening primers and amplicons illustrated. 5' flanking transposon was identified using primers P1 and P2 producing amplicon 'G'; central segment was identified using primers P3 and P4 producing amplicon 'Y'; and, 3' flanking transposon was identified using primers P5 and P6 and produced amplicon 'R'. (**B**) PCR results for each insertion line with primers amplifying the G, Y and R amplicons. Lines producing amplicon 'Y' -corresponding to the central region- were retained for further analysis. Amplicons G, Y and R give bands of sizes: 435bp, 479bp, and 437bp, respectively. Outermost lanes show DNA size standards. Sizes are as follows: from bottom, 200bp increasing in 200bp increments until 1000bp, then: 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, and 10000bp (Eurogentec Smartladder).

The fluorescence screening and PCR results confirmed the presence of the central transposon region in insertion lines OX3133A, OX3133C-Bol, OX3133D-Bol, and OX3133F-Bol. OX3133A-Bol already showed remobilisation of the 3' flanking *piggyBac* transposon.

Insertion lines OX3133B-Bol, OX3133E-Bol and OX3133G-Bol contained the insertion of one of the flanking transposons (the 3' end in all three), but were negative for the transposase-containing central region. As the central transposase-coding region is required for jumpstarting, these lines were not desired and were discarded.

G_1 progeny from insertion lines containing the transposase gene were back-crossed to Democritus wild-type flies. The G_2 progeny were screened for remobilisation events, and if an event was detected, the target individual was used to found a new 'sub-line'. The resulting progeny phenotypes from the G2 and G3 crosses are shown in **Table 7.4**.

Table 7.4. Numbers of G_2 and G_3 progeny obtained displaying various possible fluorescent phenotypes after backcrossing to Democritus wild-type. Table shows different possible marker combinations (i.e. GYR corresponds to an insertion line displaying Cop-GFP, Phi-Yellow, and J-Red, representing the transposon segments A, B, and C as defined in **Fig. 7.4**, respectively). To obtain G_2 progeny, G_1's displaying the phenotypes as described in **Table 7.3** were backcrossed to wild-type insects. If a remobilisation event was detected, a sub-line with that individual was founded. Results given here show numbers of progeny obtained from various parental genotypes. The exact genotype of a parental insertion line is indicated in brackets next to the insertion name [i.e. OX3133B(GYR) indicates the presence of the A, B and C transposon segments as defined in **Fig. 7.4**.]

Fluorescence phenotypes of G_2's							
Line	**GYR**	**GY**	**YR**	**GR**	**G**	**Y**	**R**
OX3133A(GY)	0	16	0	0	0	0	0
OX3133C(GYR)	32	0	3	0	0	0	0
OX3133D(GYR)	47	0	0	0	0	0	0
OX3133F(GYR)	21	0	0	0	0	0	0
Fluorescence phenotypes of G_3's							
Line	**GYR**	**GY**	**YR**	**GR**	**G**	**Y**	**R**
OX3133A(GY)	0	44	0	0	0	0	0
OX3133C(YR)	0	0	72	0	0	3	0
OX3133D(GYR)	63	0	0	0	0	0	0
OX3133F(GYR)	50	15	2	0	0	0	0

After three generations of backcrossing individuals from insertion line OX3133C-Bol were obtained that had lost both flanking transposons. Individuals were obtained from the OX3133A-Bol and OX3133F-Bol insertion lines that had both lost one of their original flanking transposons, but retained the other. No individuals were obtained from OX3133D-Bol that showed signs of terminal *piggyBac* transposon remobilisation. We can conclude from these crosses that in insertion lines OX3133C-Bol, OX3133A-Bol and OX3133F-Bol, functional *piggyBac* transposase was being endogenously expressed in the germline, causing remobilisation of the OX3133 flanking transposons in some gametes, and subsequently the production of offspring lacking some of the OX3133 *piggyBac* sequence present in their parent.

Remobilisation and subsequent loss of both flanking *piggyBac* regions in OX3133C-Bol was confirmed by PCR (**Fig. 7.6**). The transgene insertion in OX3133C-Bol was therefore resolved of its *piggyBac* ends and insensitive to further transposition.

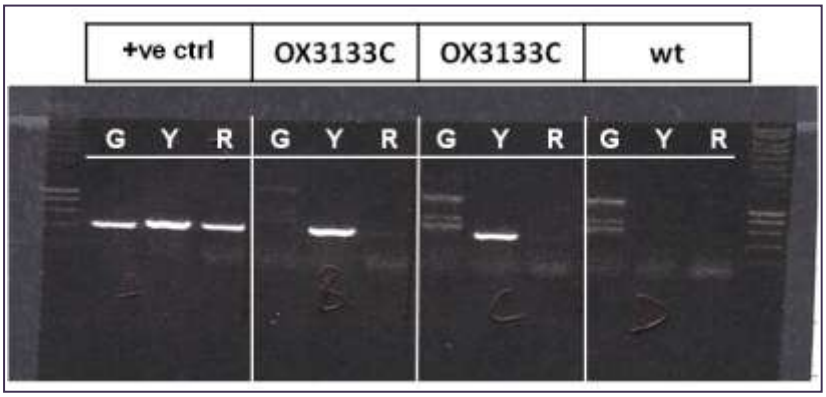


Figure 7.6. PCR results from OX3133C-Bol individuals displaying phenotypic signs of flanking transposon remobilisation. (+ve control) was an OX3133D-Bol displaying fluorescence pattern from all three transposons. (OX3133C) are two samples of OX3133C-Bol G_3 adults displaying only the PhiYellow fluorescent marker in fluorescent microscopy. (wt) is a Democritus wild-type individual and represents a negative control. PCR primers were as described in Fig. 7.5.

Attempted remobilisation of OX3713A-Bol flanking transposons

We attempted to utilise the 'jumpstarter' properties of OX3133C-Bol to achieve remobilisation of the flanking transposon sequences from the female lethal OX3713A-Bol strain. OX3713A-Bol males were out-crossed to OX3133C-Bol females, and individuals containing both insertions were backcrossed to wild-type insects. The progeny were then screened for remobilisation events. The procedure of obtaining double insertion individuals and backcrossing to wild-type was repeated for six generations. The numbers of screened pupae and remobilisation events in each generation are given in **Table 7.5.**

Table 7.5. OX3713A-Bol males and females were out-crossed in pools with fully resolved OX3133C-Bol males and females for six generations. Larvae containing the OX3713A-Bol were screened for loss of either of the flanking transposons.

Out-crossed generation	Number screened	Number with remobilisation
Generation 1	127	0
Generation 2	193	0
Generation 3	72	0
Generation 4	155	0
Generation 5	169	0
Generation 6	149	0

A total of 865 pupae containing both the OX3133C-Bol and OX3713A-Bol insertions were screened by fluorescence microscopy. However, no pupae were recovered that showed the loss of a fluorescent marker indicating the remobilisation of any OX3713A-Bol flanking *piggyBac* sequence. This was somewhat surprising as three of the four OX3133 insertion lines appeared to be reasonably sensitive to the endogenous transposase source. Variation in the remobilisation rate of a particular transposon insertion is expected depending on the surrounding genomic context and chromatin state of the insertion site (Horn et al., 2003). Indeed, chromosomal positioning effects have been shown to affect transposable element mobility (Berg& Spradling, 1991), and a range of chromatin states may alter the accessibility of required transposase binding factors. Epigenetic regulation is a well known silencing mechanism for suppressing transposable element activity in eukaryotes (Slotkin& Martienssen, 2007). It is therefore possible that the insertion site was an unfortunate one in terms of ease of flanking transposon remobilisation, and that further OX3713 insertion lines may have to be developed with the hope that they will be more amenable.

Summary and conclusions

The inverted terminal repeats of the *piggyBac* transposon are critical for transpositional activity (Li et al., 2001); their removal therefore renders the transposon inert to transposase binding and constitutes stabilisation in the host genome. Although endogenous *piggyBac* sequences have not been identified in olive fly, they have been found in several closely related *Bactrocera* species, including the Oriental fruit fly (Handler& McCombs, 2000) and the Queensland fruit fly (Raphael et al., 2011). The precautionary principle suggests that steps should be taken to stabilise a transposon-integrated transgene.

We followed the 'composite *piggyBac*' methodology introduced by Dafa'alla et al. (2006) to remove the flanking *piggyBac* sequences from OX3713A-Bol, an fsRIDL olive fly strain. We developed a range of germline transposase-expressing jumpstarter lines using composite *piggyBac* construct OX3133. Three out of four of the lines showed some germ-line *piggyBac* remobilisation after only three generations of backcrossing, with one of the three resulting in a transgene insertion with no flanking *piggyBac* sequences. This confirmed the approach of Dafa'alla et al. (2006) as a viable methodology for *piggyBac* transposon stabilisation in olive fly. OX3133C-Bol was outcrossed to OX3713A-Bol, and a large number of progeny containing both insertions were screened. However, none showed evidence of remobilisation. This may be due to a property of the OX3713A-Bol insertion site, and generating further OX3713 insertion lines may give lines with more favourable remobilisation rates.

Materials and methods

OX3713 and OX3133 strain development

Micro-injections were performed according to *Section 2.4.2.* OX3713 survivors were placed on larval dietary medium containing 100µg/ml of tetracycline. OX3713A-Bol strain assessment for female-lethal penetrance and repressibility was carried out according to *Section 2.4.4.* OX3133 G_0 survivors were sexed on eclosion and crossed in pools of 20 male G_0's with 30 wild-type females, or 20 female G_0's with 10 wild-type males. Insertion lines from seven separate cages were recovered and progeny were screened for various fluorescence phenotypes. OX3133 G_1's were back-crossed to wild-type flies and wild-type to transgenic progeny ratios were examined to check copy number. Lines were backcrossed for three generations, and remobilisation events were recorded. Once an insertion line gave a remobilisation event, the individual with a remobilisation was used to found a sub-line. Remobilisation events were confirmed by PCR.

Primer sequences (as described in **Fig. 7.5**) were as follows:

P1: 5' - CAAGATGAAGAGCACCAAGGGC - 3'
P2: 5' - TGGATGGGGTCTTGAATGTC - 3'
P3: 5' - GCAAGATCCCCTACGTGGTGG - 3'
P4: 5' - GATCTCGTGGCAGATCTTGAAGG - 3'
P5: 5' - GATCCAGTACGGCGAGCCC - 3'
P6: 5' - CGCTGGTGTCCCTCATCTGC - 3'

OX3713A-Bol *piggyBac* remobilisation

OX3713A-Bol males were outcrossed to OX3133C-Bol females. Progeny carrying both insertions were identified by fluorescence microscopy. These individuals were backcrossed to wild-type flies and the progeny were screened for remobilisation events. Simultaneously, OX3713A-Bol males were outcrossed to OX3133C-Bol females, and the

procedure was repeated. This continued for six generations with a total of 865 pupae screened.

Appendix 2: Transgene-flanking genomic DNA sequences.

Flanking sequence for OX3097D-Bol as determined by adaptor-mediated PCR (see *Section 2.2.2.*):

5' Upstream sequence:

TAGATTTGTGTATGCATTACTTATTTTTCGCGGTCATATGCATATTTGTACATAC
ATACTTACATATAGAGCAGTGCGCTCACATGTATTTATTGATAATTGATAATAT
ATTATTATATTATATAATATAACATATTGATGTGCATATGTACATACATAAATT
ATTAATTCTGTAAATATTGAAATTAGAAAATTATGAAAACAGTAAAATATCAT
TTTTTGCATAATCTTTCACATTTTATCAATGTAACATTTGTGCAAAAAAAATCA
TATTTTATCAATTTTTCATCATAAAAATCGTTTATATGGCAAAAAATARTCATG
CATATGTGAAGTGGCATTTGTATTTCTTGTTTTCTCATTCATTTCATTTCATTTT
TCTCTTTATATTGGTAGATACGTATTYTGTCAGAGAACGYTGTTAAAAATTCTC
ATTTCTG**TTAA<OX3097>**

3' Downstream sequence:

<OX3097>TTAAATAACAGCAAAACTATACAGAAAGTGGTGAACTCCTTGATCT
CAAATTTTCAGCCAACCAATCGAGCATCATACAAAAWTCAATTTGCACCTTCT
CATTGTTTTAAGTTCTCTGGTACGGGATAGYAATCATCAAACTTCTAGCATCTG
AACTCGGTTTAACTTTGCAGAAGCTAATTTTACAAAGTTAARTAGAGAACTTTC
AACAATAACATGGCCTAATTATAATGATGGTATTAAATTGAATGTCTCGCATT
GTTATAATATAATAAAGAAATTATTTGAAAAGTATGTCCCAATAGTAAAAACG
AATATTGTAAGTAATCCAAAATCGTTTTATGATTTCGTCATCTCCAAACGCAGG
AAAAAATATATTTGCTTGCCTTTGATAAGGATATTTAATTTCTCTCTTAAACAC
GGTATATTTCCTTCAATATGGAAACAATCAGTTATAATCCCTTTGCATAAAAGT
GGATTTAGGTCTAACATAGATAACTATAGAGCTATTGCAAAACTATCAGTTAT
ACCTAAACTTTTTGAAACTATCATCACAAACCTTTTCGATTTCTCCGTTAATTTC
CTCATCTCAGCATTGATTTCGTAAAGGGAAATCGACTCTAATAAACTTGCTTGA
ATTTGTAAACCATATACCATTGGGTTTTAGGGAGCATAAGCAGACGGATGTTA
TATATGTACACAGACTTTTGCAAAGCTTTCGATAAAGTATCTAAAGATAGAAG
TGATTTGCCATTGAATCTCTATAAATGTAAGACGATGTGCTTTTCCCGTAGATC
WGTGCAACCCTCTCCTTATGTAATAAAACATTATCTTGAAAGCAAAAGCTGTC
CTCAGTTTTATTAAACGTTGATCTAAAGAATTTAAAGATCCGTATATTACTAAA
AGACTTTTTACAACTTTGGTTAGACCTATATTGGAATATGGCTCTGTTGTATGG
AACCCTATCTATCAAATCTATTCTGACAAGTAGGAGTCGGTTCAAAAACAATT
TTTCGCCTTAGGGCATTTCCGACGGGCTTCCTAGGGTAAGTCTACCTCCATACA

TTAGTCGTTCAAAAATCAACCTACACTTTTTAGTCGCAGGGAAATGCTTGGCAT
AATATACTTAGTGAAAATCTTGATTGGAAAAGTTTGCAGTTCTTTTCACCTGCC
TAAAATAAATTTTATATCCCGGTTCGGTTTTCGAGGCAGTTTAGATCGTTACTG
TTAAAATGCTGTAGATAAATTCCG

Flanking sequence for OX3097C-Bol as determined by adaptor-mediated PCR (see *Section 2.2.2.*):

5' Upstream sequence:

CGACAGAAAGGGCGTGGTGCGGAGGGCGGTGTCGGGCGATGATATTGTATTT
ATTTATTCTTATATTGGCATTTCGATAGAATTCGATTACTGCTGAGTTCTTTTTA
ATAGATGGGTAATTGTTTCTTGCCTTAA<**OX3097**>

3' Downstream sequence:

<**OX3097**>CAGATCTTCCGGATGGCTCGAGTTTTTCAGCAAGATGGCGACTGAG
ATGTCCTAAATGCACAGCGACGGATTCGCGCTATTTAGAAAGAGAGAGCAATA
TTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAACAGA
AATGAGAATTTTTAACAACGTTCTCTGACAAAATACGTATCTACCAATATAAA
GAGAAAAATGAAATGAAATGAATGAGAAAACAAGAAATACAAATGCCACTTC
ACATATGCATGATTATTTTTTGCCATATAAACGATTTTTATGATGAAAAATTGA
TAAAATATGATTTTTTTTGCACAAATGTTACATTGATAAAATGTGAAAGATTAT
GCAAAAAATGATATTTTACTGTTTTCATAATTTTCTAATTTCAATATTTACAGA
ATTAATAATTTATGTATGTACATATGCACATCAATATGTTATATTATATAATAT
AATAATATATTATCAATTATCAATAAATACATGTGAGCGCACTGCTCTATATGT
AAGTATGTATGTACAAATATGCATATGACCGCGAAAAATAAGTAATGCATACA
CAAATCTACATACATTTAAGCGTACAAATGTAAGTACGTCAGCCAATAAGAAA
AATACAAACATTGTTGTACAAAAACAACAATTGTCTCAAATAGCATCAGCACC
AGAAATCAATATGGCAGCCAAATTGACAAATCCTAAATTTGTAGTAAATCACA
CACCAACAATATTTTCATTCATGAACGCTGGCGCACAAGCAATAAGCTAAGTC
GTTTCATTCATAAAAGCAAACGCCTTACACATCTCCCCGAGCATGCGTTTGCCT
ACAAGCGTCTTTTCGCGACGATGGCAAAAGCTAGAAATCATAAATTGAACAAA
TTTCTGATATTCCCTCTAGAAGGAAAAGTGACACCCCTCAAATATGAGTATTA
AAATATTTAGAATAAAGTGACACATAGTAATTTGTCGATTACATTCAGAACTT
TTAAAGAAATATTCATATTCCTCTGATTATGTATACAGTAAACATCGGTAGTAT
CACTCTCATCCCTCTTATCTGTCGACACGCCTCCGCACCAGCCTATCTTTCAGA
AGATCTCTACAATTTCTCAGCTGCATGGAAATCGAGTTCTTCTTTTATTCTCTC
AGATTTCAGCTGTTATTAACCTATATAAGAACAATGCTTACCACCCTCTAGAA
ACGGTGGAGGGTGGCGCGGTTCTTGGCACATCGAACCTCATG

Bibliography

ALCOCK, J., 1994. Post insemination associations between males and females in insects: The mate-guarding hypothesis. *Ann. Rev. Entomol.,* **39**, 1-21.

ALEKSANDROV, A., SCHULDT, L., HINRICHS, W. and SIMONSON, T. 2008. Tet repressor induction by tetracycline: a molecular dynamics, continuum electrostatics, and crystallographic study. *J. Mol. Biol.,* **378**(4), 898-912.

ALLEE, W.C. 1939. *The social life of animals.* London.

ALPHEY, L., BAKER, P., BURTON, R.S., CONDON, G.C., CONDON, K.C., DAFA'ALLA, T.H., EPTON, M.J., FU, G., GONG, P., LI-JIN, L., LABBE, G., MORRISON, N.I., NIMMO, D., O'CONNELL, S., PHILLIPS, C.E., PLACKETT, A., SCAIFE, S. and WOODS, A. 2006. Genetic technologies to enhance the Sterile Insect Technique (SIT). *Proceedings of the 7th International Symposium on Fruit Flies of Economic Importance,* 319-326.

ALPHEY, L. and ANDREASEN, M. 2002. Dominant lethality and insect population control. *Mol. Biochem. Parasitol.,* **121**(2), 173-178.

ALPHEY, N., BONSALL, M.B. and ALPHEY, L. 2011. Modeling resistance to genetic control of insects. *J. Theor. Biol.,* **270**(1), 42-55.

ALPHEY, N., BONSALL, M.B. and ALPHEY, L. 2009. Combining pest control and resistance management: synergy of engineered insects with Bt crops. *J. Econom. Entomol.,* **102**(2), 717-732.

ALPHEY, N., COLEMAN, P.G., DONNELLY, C.A. and ALPHEY, L. 2007. Managing insecticide resistance by mass release of engineered insects. *J. Econom. Entomol.*, **100**(5), 1642-1649.

ANT, T., KOUKIDOU, M., REMPOULAKIS, P., GONG, H.F., ECONOMOPOULOS, A., VONTAS, J. and ALPHEY, L., 2012. Control of the olive fruit fly using genetics-enhanced sterile insect technique. *BMC Biol.,* **10**, 51-7007-10-51.

ARGOV, Y., KUSLITZKY, W. and HOELMER, K.A., 2012. Biological control of olive fruit fly, *Bactrocera oleae*, in Israel. *European Meeting in the IOBC/WPRS Working Group*, 79-85.

ARNQVIST, G. and NILSSON, T., 2000. The evolution of polyandry: multiple mating and female fitness in insects. *Anim. Behav.,* **60**(2), 145-164.

ASAMI, Y., JIA, D.W., TATEBAYASHI, K., YAMAGATA, K., TANOKURA, M. and IKEDA, H., 2002. Effect of the DNA topoisomerase II inhibitor VP-16 on illegitimate recombination in yeast chromosomes. *Gene,* **291**(1-2), 251-257.

AUGUSTINOS, A.A., STRATIKOPOULOS, E.E., DROSOPOULOU, E., KAKANI, E.G., MAVRAGANI-TSIPIDOU, P., ZACHAROPOULOU, A. and MATHIOPOULOS, K.D., 2008. Isolation and characterization of microsatellite markers from the olive fly, *Bactrocera oleae*, and their cross-species amplification in the Tephritidae family. *BMC Genom.,* **9**, 618-2164-9-618.

AVIDOV, Z. and HARPAZ, I., 1969. *Plant pests of Israel.* Jerusalem: Israel University Press.

BANNO, H., 1990. Plasticity of size and relative fecundity in the aphidophagous lycaenid butterfly: *Taraka hamada*. *Ecol. Entomol.* **15**, 111-113.

BARGIELOWSKI, I., ALPHEY, L. and KOELLA, J.C., 2011. Cost of mating and insemination capacity of a genetically modified mosquito *Aedes aegypti* OX513A compared to its wild type counterpart. *PloS one,* **6**(10), e26086.

BATEMAN, A.J., 1948. Intra-sexual selection in Drosophila. *Heredity,* **2**(Pt. 3), 349-368.

BEECH, C.J., KOUKIDOU, M., MORRISON, N.I. and ALPHEY, L., 2012. Genetically Modified Insects: Science, Use, Status and Regulation. *Biosafety Rev.,* **6**, 66-124.

BENELLI, G., CANALE, A., BONSIGNORI, G., RAGNI, G., STEFANINI, C. and RASPI, A., 2012. Male wing vibration in the mating behaviour of the olive fruit fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae). *J. Insect Behav.,* **25**, 590-603.

BEN-YOSEF, M., AHARON, Y., JURKEVITCH, E. and YUVAL, B., 2010. Give us the tools and we will do the job: symbiotic bacteria affect olive fly fitness in a diet- dependent fashion. *Proc. Biol. Sci.,* **277**, 1545-1552.

BERG, C.A. and SPRADLING, A.C., 1991. Studies on the rate and site-specificity of P element transposition. *Genetics,* **127**(3), 515-524.

BIGLER, F. and DELUCCHI, V., 1981. Main mortality factors affecting prepupal development of the olive fly, *Dacus oleae* Gmel. (Dipt., Tephritidae) on oleasters and cultivated olives in western Crete, Greece. *Z. Angew. Entomol.,* **92**, 343-363.

BOORMAN, E. and PARKER, G.A., 1976. Sperm (ejaculate) competition in *Drosophila melanogaster*, and the reproductive value of females to males in relation to female age and mating status. *Ecol. Entomol.,* **1**, 145-155.

BRICENO, R.D. and EBERHARD, W.G., 2002. Decisions during courtship by male and female medflies: correlated changes in male behaviour and female acceptance criteria in mass-reared flies. *Florida Entomol.,* **85**(1), 14-31.

BRICENO, R.D. and EBERHARD, W.G., 1998. Medfly courtship duration: A sexually selected reaction norm changed by crowding. *Ethol. Ecol. Evol.,* **10**, 369-382.

BRICENO, R.D., RAMOS, D. and EBERHARD, W.G., 1996. Courtship behavior of male *Ceratitis capitata* (Diptera: Tephritidae) in captivity. *Florida Entomol.,* **79**, 130-143.

BURK, T., 1981. Signaling and sex in acalyptrate flies. *Florida Entomol.,* **64**, 67-83.

BURRACK, H.J., FORNELL, A.M., CONNELL, J.H., O'CONNELL, N.V., PHILLIPS, P.A., VOSSEN, P.M. and ZALOM, F.G., 2009. Intraspecific larval competition in the olive fruit fly (Diptera: tephritidae). *Environ. Entomol.,* **38**(5), 1400-1410.

BURTIS, K.C. and BAKER, B.S., 1989. Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell,* **56**(6), 997-1010.

BUSH, G.L. and KITTO, G.B., 1979. Research on the genetic structure of wild and laboratory strains of olive fly. *F.A.O.Report, Development of Pest Management Systems for OliveCulture Program.* Rome: F.A.O. of the United Nations.

CACERES, C., 2002. Mass rearing of temperature sensitive genetic sexing strains in the Mediterranean fruit fly (Ceratitis capitata). *Genetica,* **116**(1), 107-116.

CAPUZZO, C., FIRRAO, G., MAZZON, L., SQUARTINI, A. and GIROLAMI, V., 2005. *'Candidatus Erwinia dacicola*', a coevolved symbiotic bacterium of the olive fly *Bactrocera oleae* (Gmelin). *Int. J. Syst. Evol. Micr.,* **55**(Pt 4), 1641-1647.

CAREY, J.R., PAPADOPOULOS, N.T., MULLER, H.G., KATSOYANNOS, B.I., KOULOUSSIS, N.A., WANG, J.L., WACHTER, K., YU, W. and LIEDO, P., 2008. Age structure changes and extraordinary lifespan in wild medfly populations. *Aging cell,* **7**(3), 426-437.

CARY, L.C., GOEBEL, M., CORSARO, B.G., WANG, H.G., ROSEN, E. and FRASER, M.J., 1989. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology,* **172**(1), 156-169.

CAVALLORO, R. and DELRIO, G., 1974. Mating behavior and competitiveness of gamma-irradiated olive fruit flies. *J. Econ. Entomol.,* **67**(2), 253-255.

CAYOL, J.P., CORONADO, P. and TAHER, M., 2002. Sexual compatibility in Medfly from different origins. *Florida Entomol.,* **85**(1), 51-57.

CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W.W. and PRASHER, D.C., 1994. Green fluorescent protein as a marker for gene expression. *Science (New York, N.Y.),* **263**(5148), 802-805.

CHAPMAN, T., 2001. Seminal fluid-mediated fitness traits in Drosophila. *Heredity,* **87**(Pt 5), 511-521.

CHAPMAN, T., MIYATAKE, T., SMITH, H.K. and PARTRIDGE, L., 1998. Interactions of mating, egg production and death rates in females of the Mediterranean fruit fly, *Ceratitis capitata*. *Proc. Bio. Sci. Roy. Soc.,* **265**(1408), 1879-1894.

CHARLESWORTH, D. and WILLIS, J.H., 2009. The genetics of inbreeding depression. *Nat. Rev.Gen.,* **10**(11), 783-796.

CHRISTOPHIDES, G.K., SAVAKIS, C., MINTZAS, A.C. and KOMITOPOULOU, K., 2001. Expression and function of the Drosophila melanogaster ADH in male *Ceratitis capitata* adults: a potential strategy for medfly genetic sexing based on gene-transfer technology. *Insect Mol. Biol.,* **10**(3), 249-254.

CLARKE, A.R., ARMSTRONG, K.F., CARMICHAEL, A.E., MILNE, J.R., RAGHU, S., RODERICK, G.K. and YEATES, D.K., 2005. Invasive phytophagous pests arising through a recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. *Ann. Rev. Entomol.,* **50**, 293-319.

CONDON, K.C., CONDON, G.C., DAFA'ALLA, T.H., FU, G., PHILLIPS, C.E., JIN, L., GONG, P. and ALPHEY, L., 2007. Genetic sexing through the use of Y-linked transgenes. *Insect Biochem. Mol. Biol.,* **37**(11), 1168-1176.

CORBETT, J.R., 1974. *The Biochemical Mode of Action of Pesticides.* London, UK: Academic Press.

CT/EFSA/GMO/2009/03, 2010. *Defining Environmental Risk Assessment Criteria for Genetically Modified Insects to be placed on the EU Market.*

DAANE, K.M., RICE, R.E. and ZALOM, F.G., 2005. Arthropod pests of olive. In: G.S. SIBBET and L. FERGUSON, eds, *Olive Production Manuel.* 2 edn. UC ANR Pub, 105-114.

DAANE, K.M., SIME, K.R. and WANG, X., 2008. *Psyttalia lounsburyi* (Hymenoptera: Braconidae), potential biological control agent for the olive fruit fly in California. *Biol. Control,* **44**, 79-89.

DAANE, K.M. and JOHNSON, M.W., 2010. Olive fruit fly: managing an ancient pest in modern times. *Ann Rev. Entomol.,* **55**, 151-169.

DAFA'ALLA, T.H., CONDON, G.C., CONDON, K.C., PHILLIPS, C.E., MORRISON, N.I., JIN, L., EPTON, M.J., FU, G. and ALPHEY, L., 2006. Transposon-free insertions for insect genetic engineering. *Nat. Biotechnol.,* **24**(7), 820-821.

DHILLON, M.K., SINGH, R., NARESH, J.S. and SHARMA, H.C., 2005. The melon fruit fly, *Bactrocera cucurbitae*: a review of its biology and management. *J. Insect Sci.,* **5**, 40.

DIAMANT, G., 1963. Screwworm eradication in southeastern United States. *Am. J. Public Health N.,* **53**, 22-26.

DOUGLAS, A.E., 2007. Symbiotic microorganisms: untapped resources for insect pest control. *Trends Biotechnol.,* **25**, 338-342.

EBERHARD, W.G., 1996. *Female control: sexual selection by cryptic female choice.* Princeton, NJ: Princeton Univ. Press.

ECONOMOPOULOS, A.P., 1972. Sexual competitiveness of gamma-ray sterilized males of *Dacus oleae*. Mating frequency of artificially reared and wild females. *Environ. Entomol.,* **1**, 490-497.

ECONOMOPOULOS, A.P., HANIOTAKIS, G., MATHIOUDIS, J., MISSIS, N. and KINIGAKIS, P., 1978. Long-distance flight of wild and artificially-reared *Dacus oleae* (Gmelin) (Diptera, Tephritidae). *Z. Angew. Entomol.,* **87**, 101-108.

ECONOMOPOULOS, A.P. and ZERVAS, G.A., 1982. The quality problem in olive flies produced for SIT experiments: *IAEA STI/PUB.*

ECONOMOPOULOS, A.P., 2002. *The olive fly, Bactorcera (Dacus) oleae (Gmelin) (Diptera:Tephritidae): its importance and control; previous SIT research and pilot testing.* Vienna, Austria: International Atomic Energy Agency.

ECONOMOPOULOS, A.P., AVTZIS, N., ZERVAIS, G., TSITSIPIS, J., HANIOTAKIS, G., TSIROPOLOUS, G. and MANOUKAS, A., 1977. Control of the olive fly, *Dacus oleae* (Gmelin), by the combined effects of insecticides and release of gamma sterilised insects. *J. Appl. Entomol.,* **83**, 201-215.

ENGELS, W.R., 1992. The origin of P elements in Drosophila melanogaster. *BioEssays : news and reviews in molecular, cellular and developmental biology,* **14**(10), 681-686.

ESTES, A.M., NESTEL, D., BELCARI, A., JESSUP, A., REMPOULAKIS, P. and ECONOMOPOULOS, A.P., 2011. A basis for the renewal of sterile insect technique for the olive fly, *Bactrocera oleae* (Rossi). *J. Appl. Entomol.,* **136**, 1-16.

EUROPEAN UNION REGULATION (1830/2003), *Concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending*. Directive2001/18/EC.

EUROPEAN UNION REGULATION (543/2011), 2011. *Commission Implementing Regulation (EU) No 543/2011 of 7 June 2011 laying down detailed rules for the application of Council Regulation (EC) No 1234/2007 in respect of the fruit and vegetables and processed fruit and vegetables sectors* .

EUROPEAN UNION REPORT (1829/2003), *Report from the Commission to the Council and the European Parliament on the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council on genetically modified food and feed.* .

FAO/IAEA, 2007. *Guidance for packing, shipping, holding and release of sterile flies in area-wide fruit fly control programmes.* Rome: Food and Agricultural Organisation of the United Nations.

FAO/IAEA/USDA, 2003. *Product quality control and shipping procedures for sterile mass-reared tephritid fruit flies.* Vienna, Austria: International Atomic Energy Agency.

FIELD, S.A. and YUVAL, B., 1999. Nutritional status affects copula duration in the Mediterranean fruit fly, *Ceratitis capitata* (Insecta Tephritidae). *Ethol. Ecol. Evol.,* **11**(1), 61-70.

FINNEY, G.L. and FISHER, T.W., 1964. Culture of entomophagous insects and their hosts. In: P. DE BACH, ed, *Biological Control of Insect Pests and Weeds.* London: Chapman and Hall, .

FOX, C.W., WADDELL, K.J. and MOUSSEAU, T.A., 1995. Host-associated Wtness variation in a seed beetle (Coleoptera: Bruchidae): evidence for local adaptation to a poor quality host. *Oecologia,* **99**, 329-336.

FRASER, M.J.,JR, 2012. Insect transgenesis: current applications and future prospects. *Ann. Rev. Entomol.,* **57**, 267-289.

FRASER, M.J., SMITH, G.E. and SUMMERS, M.D., 1983. Acquisition of Host Cell DNA Sequences by Baculoviruses: Relationship Between Host DNA Insertions and FP Mutants of *Autographa californica* and *Galleria mellonella* Nuclear Polyhedrosis Viruses. *J. Virol.,* **47**(2), 287-300.

FU, G., CONDON, K.C., EPTON, M.J., GONG, P., JIN, L., CONDON, G.C., MORRISON, N.I., DAFA'ALLA, T.H. and ALPHEY, L., 2007. Female-specific insect lethality engineered using alternative splicing. *Nat. Biotechnol.,* **25**(3), 353-357.

FU, J., WENZEL, S.C., PERLOVA, O., WANG, J., GROSS, F., TANG, Z., YIN, Y., STEWART, A.F., MULLER, R. and ZHANG, Y., 2008. Efficient transfer of two large secondary metabolite pathway gene clusters into heterologous hosts by transposition. *Nucleic Acids Res.,* **36**(17), e113.

GABRIELI, P., GOMULSKI, L.M., BONOMI, A., SICILIANO, P., SCOLARI, F., FRANZ, G., JESSUP, A., MALACRIDA, A.R. and GASPERI, G., 2011. Interchromosomal duplications on the *Bactrocera oleae* Y chromosome imply a distinct evolutionary origin of the sex chromosomes compared to Drosophila. *PloS one,* **6**(3), e17747.

GAUNT, M.W. and MILES, M.A., 2002. An insect molecular clock dates the origin of the insects and accords with palaeontological and biogeographic landmarks. *Mol. Biol. Evol.,* **19**(5), 748-761.

GILBERT, C., SCHAACK, S., PACE, J.K.,2ND, BRINDLEY, P.J. and FESCHOTTE, C., 2010. A role for host-parasite interactions in the horizontal transfer of transposons across phyla. *Nature,* **464**(7293), 1347-1350.

GINGRICH, J.R. and RODER, J., 1998. Inducible gene expression in the nervous system of transgenic mice. *Ann. Rev. Neurosci.,* **21**, 377-405.

GONG, P., EPTON, M.J., FU, G., SCAIFE, S., HISCOX, A., CONDON, K.C., CONDON, G.C., MORRISON, N.I., KELLY, D.W., DAFA'ALLA, T., COLEMAN, P.G. and ALPHEY, L., 2005. A dominant lethal genetic system for autocidal control of the Mediterranean fruit fly. *Nat. Biotechnol.,* **23**(4), 453-456.

GOULD, F. and SCHLIEKELMAN, P., 2004. Population genetics of autocidal control and strain replacement. *Ann. Rev. Entomol.,* **49**, 193-217.

GROTH, A.C., FISH, M., NUSSE, R. and CALOS, M.P., 2003. Construction of transgenic Drosophila by using the site-specific integrase from Phage φC31. *Gen. Soc. Am.,* **166**, 1775-1782.

GUTIERREZ, A.P., PONTI, L. and COSSU, Q.A., 2009. Effects of climate warming on Olive and olive fly (*Bactrocera oleae* (Gmelin)) in California and Italy. *Climate Change,* **95**, 195-217.

HAGLER, J.R. and JACKSON, C.G., 2001. Methods for marking insects: current techniques and future prospects. *Ann. Rev. Entomol.,* **46**, 511-543.

HAGLER, J.R. and MILLER, E., 2002. An alternative to conventional insect marking techniques: detection of a protein mark of pink bollworm by ELISA. *Entomol. Exp. et App.*, **103**, 1-9.

HALDANE, J.B., 1931. Mathematical Darwinism: A discussion of the genetical theory of natural selection. *Eugen. Rev.,* **23**(2), 115-117.

HALLMAN, G.J. and THOMAS, D.B., 2011. Evaluation of the efficacy of the methyl bromide fumigation schedule against Mexican fruit fly (Diptera: Tephritidae) in citrus fruit. *J. Econom. Entomol.,* **104**(1), 63-68.

HAMILTON, M.B., 2009. *Population genetics.* Wiley-Blackwell.

HANDLER, A.M., ZIMOWSKA, G.J. and HORN, C., 2004. Post-integration stabilization of a transposon vector by terminal sequence deletion in *Drosophila melanogaster*. *Nat. Biotechnol.*, **22**, 1150-1154.

HANDLER, A.M., 2002. Use of the *piggyBac* transposon for germ-line transformation of insects. *Insect Biochem. Mol. Biol.,* **32**(10), 1211-1220.

HANDLER, A.M. and HARRELL, R.A.,2ND, 2001. Transformation of the Caribbean fruit fly, *Anastrepha suspensa*, with a *piggyBac* vector marked with polyubiquitin-regulated GFP. *Insect Biochem. Mol. Biol.,* **31**(2), 199-205.

HANDLER, A.M. and JAMES, A.A., 2000. *Insect Transgenesis: Methods and Applications.* Boca Raton, FL: CRC Press.

HANDLER, A.M. and MCCOMBS, S.D., 2000. The *piggyBac* transposon mediates germ-line transformation in the Oriental fruit fly and closely related elements exist in its genome. *Insect Mol. Biol.*, **9**(6), 605-612.

HANDLER, A.M., MCCOMBS, S.D., FRASER, M.J. and SAUL, S.H., 1998. The lepidopteran transposon vector, *piggyBac*, mediates germ-line transformation in the Mediterranean fruit fly. *PNAS.,* **95**(13), 7520-7525.

HAWKES, N.J., JANES, R.W., HEMINGWAY, J. and VONTAS, J., 2005. Detection of resistance-associated point mutations of organophosphate-insensitive acetylcholinesterase in the olive fruit fly, *Bactrocera oleae* (Gmelin). *Pest. Biochem. Physiol.,* **81**, 154-163.

HEINRICH, J.C. and SCOTT, M.J., 2000. A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *PNAS,* **97**(15), 8229-8232.

HENDRICHS, J., 2000. Use of the Sterile Insect Technique Against Key Insect Pests. *Sus. Devel.,* **2**, 75-79.

HENDRICHS, J. and HENDRICHS, M.A., 1990. Mediterranean fruit flies (Diptera: Tephritidae) in nature: location and diel pattern of feeding and other activities on fruiting and nonfruiting hosts and nonhosts. *Ann. Entomol. Soc. Am.,* **83**, 632-641.

HENDRICHS, J., ORTIZ, P.L. and SCHWARZ, A., 1983. Six years of successful medfly program in Mexico and Guatemala. In: R. CAVALLORO, ed, *Fruit Flies of Economic Importance.* Rotterdam: A.A. Balkema, 353-365.

HENDRICHS, J., ROBINSON, A.S., CAYOL, J.P. and ENKERLIK, W., 2002. Medfly areawide sterile insect technique programmes for prevention, suppression or eradication: the importance of mating behavior studies. *Florida Entomol.,* **85**(1).

HOENIGSBERG, H.F., 1999. Female competition in mate choice decreases fitness in flies (*Drosophila pseudoobscura*) from Colombia. *Genet. Mol. Biol.,* **22**, 213-215.

HONĚK, A., 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. *N. Soc. Oikos.,* **66**(3), 483-492.

HOOPER, G.H., 1972. Sterilization of the Mediterranean fruit fly with gamma radiation: effect on male competitiveness and change in fertility of females alternately mated with irradiated and untreated males. *J Econ. Entomol.,* **65**(1), 1-6.

HORN, C., OFFEN, N., NYSTEDT, S., HACKER, U. and WIMMER, E.A., 2003. *piggyBac*-based insertional mutagenesis and enhancer detection as a tool for functional insect genomics. *Genetics,* **163**(2), 647-661.

HORN, C. and WIMMER, E.A., 2000. A versatile vector set for animal transgenesis. *Develop. Genes Evol.,* **210**(12), 630-637.

HOY, M.A., 2003. Transgenic insects for pest management programs: status and prospects. *Environ. Biosafety Res.,* **2**(1), 61-64.

IAEA/USDA/FAO, 2003. Required routine quality control tests. *Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies*.

INTERNATIONAL OLIVE OIL COUNCIL (IOOC)., 2011, Available: http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures.

IRVIN, N., HODDLE, M.S., O'BROCHTA, D.A., CAREY, B. and ATKINSON, P.W., 2004. Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes. *PNAS,* **101**(3), 891-896.

ITO, Y. and KAWAMOTO, H., 1979. Number of generations necessary to attain eradication of an insect pest with sterile insect release method: a model study. *Res. Popul. Ecol.,* **20**, 216-226.

JAROŠIK, V., 1989. Mass vs. length relationship for carabid beetles (Col., Carabidae). *Pedobiologia*, **33**

KAKANI, E.G., ZYGOURIDIS, N.E., TSOUMANI, K.T., SERAPHIDES, N., ZALOM, F.G. and MATHIOPOULOS, K.D., 2010. Spinosad resistance development in wild olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae) populations in California. *Pest Man. Sci.,* **66**(4), 447-453.

KASPI, R., TAYLOR, P.W. and YUVAL, B., 2000. Diet and size influence sexual advertisement and copulatory success of males in Mediterranean fruit fly leks. *Ecol. Entomol.,* **25**, 279-284.

KNIPLING, E.F., 1959. Sterile-male method of population control. *Science (New York, N.Y.),* **130**(3380), 902-904.

KOUKIDOU, M., KLINAKIS, A., REBOULAKIS, C., ZAGORAIOU, L., TAVERNARAKIS, N., LIVADARAS, I., ECONOMOPOULOS, A. and SAVAKIS, C., 2006. Germ line transformation of the olive fly *Bactrocera oleae* using a versatile transgenesis marker. *Insect Mol. Biol.,* **15**(1), 95-103.

KRAAIJEVELD, K. and CHAPMAN, T., 2004. Effects of male sterility on female remating in the Mediterranean fruit fly, *Ceratitis capitata*. *Proc. Bio. Sci. Roy. Soc.,* **271**, S209-11.

KRAFSUR, E.S., WHITTEN, C.J. and NOVY, J.E., 1987. Screwworm eradication in North and Central America. *Parasitol. Today*, **3**(5), 131-137.

KUMANO, N., KURIWADA, T., SHIROMOTO, K., HARAGUCHI, D. and KOHAMA, T., 2010. Assessment of effect of partial sterility on mating performance in sweetpotato weevil (Coleoptera: Curculionidae). *J. Econom. Entomol.,* **103**(6), 2034-2041.

KWIATKOWSKI, D., PHILLIPS, P.C.B., SCHMIDT, P. and SHIN, Y., 1992. Testing the Null Hypothesis of Stationarity against the Alternative of a Unit Root. *J. Econom.,* **54**, 159-178.

LAGOS, D., KOUKIDOU, M., SAVAKIS, C. and KOMITOPOULOU, K., 2007. The transformer gene in *Bactrocera oleae*: the genetic switch that determines its sex fate. *Insect Mol.Biol.,* **16**, 221-230.

LANCE, D.R. and MCINNIS, D.O., 2005. Biological Basis of the Sterile Insect Technique. In: V.A. DYCK, J. HENDRICHS and A.S. ROBINSON, eds, *Principles and Practice in Area-Wide Integrated Pest Management.* Dordrecht, The Netherlands: Springer, 69-94.

LANCE, D.R., MCINNIS, D.O., RENDON, P. and JACKSON, C.G., 2000. Courtship among sterile and wild *Ceratitis capitata* (Diptera: Tephritidae) in field cages in Hawaii and Guatemala. *Ann. Entomol. Soc. Am.,* **93**, 1179-1185.

LANDE, R., 1983. The response to selection on major and minor mutations affecting a metrical trait. *Heredity,* **50**(1), 47-65.

LEE, S.G., MCCOMBC, S.D. and SAUL, S.H., 2003. Sperm Precedence of Irradiated Mediterranean Fruit Fly Males (Diptera: Tephritidae) . *Proc. Hawaiian Entomol. Soc.*, **36**, 47-59.

LEVINSON, H.Z. and LEVINSON, A.R.:., 1984. Botanical and chemical aspects of the olive tree with regards to fruit acceptance by *Dacus oleae* (Gmelin) and other frugivorous animals. *Z. Angew. Entomol.*, **98**, 136-149.

LI, X., LOBO, N., BAUSER, C.A. and FRASER, M.J.,JR, 2001. The minimum internal and external sequence requirements for transposition of the eukaryotic transformation vector *piggyBac*. *Mol. Gen. Genom. : MGG*, **266**(2), 190-198.

LIIMATAINEN, J.O., HOIKKALA, A. and SHELLY, T.E., 1997. Courtship behavior in *Ceratitis capitata* (Diptera: Tephritidae): comparison of wild and mass reared males. *Ann. Entomol. Soc. Am.*, **90**(6), 836-843.

LIPHSCHITZ, N., GOPHNA, R., HARTMAN, M. and BIGER, G., 1991. The beginning of olive (olea europaea) cultivation in the old world: A reassessment. *J. Archaeol. Sci.* **18**(4), 441-453.

LORETO, E.L., CARARETO, C.M. and CAPY, P., 2008. Revisiting horizontal transfer of transposable elements in Drosophila. *Heredity*, **100**(6), 545-554.

LOWE, R.E., FOWLER, E.F., BAILEY, D.L., DAME, D.A. and SAVAGE, K.E., 1981. Separation of sexes of adult *Anopheles albimanus* by feeding of insecticide-laden blood. *Mosq. News*, **41**, 634-638.

LUX, S.A., VILARDI, J.C., LEIDO, P., CAGGL, K., CALCAGNO, G.E., MUNYIRI, F.N., VERZ, M.T. and MANSO, F., 2002. Effects of irradiation on the courtship behaviour of medfly mass-reared for the sterile insect technique. *Florida Entomol.*, **85**(1), 102-112.

MACBEAN, I.T. and PARSONS, P.A., 1967. Directional selection for duration of copulation in Drosophila melanogaster. *Genetics*, **56**(2), 233-239.

MANOUKAS, A.G., 1972. Free and peptide-bound amino acids in the insect *Dacus oleae* (Gmelin) grown under natural and artificial conditions. *Comp. Biochem. Physiol.*, **43**(4), 787-794.

MARGARITOPOULOS, J.T., SKAVDIS, G., KALOGIANNIS, N., NIKOU, D., MOROU, E., SKOURAS, P.J., TSITSIPIS, J.A. and VONTAS, J., 2008. Efficacy of the pyrethroid alpha-cypermethrin against *Bactrocera oleae* populations from Greece, and improved diagnostic for an iAChE mutation. *Pest Manag. Sci.*, **64**(9), 900-908.

MARKSTEIN, M., PITSOULI, C., VILLALTA, C., CELNIKER, S.E. and PERRIMON, N., 2008. Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. *Nat. Gen.*, **40**(4), 476-483.

MARRELLI, M.T., MOREIRA, C.K., KELLY, D., ALPHEY, L. and JACOBS-LORENA, M., 2006. Mosquito transgenesis: what is the fitness cost? *Trends Parasitol.*, **22**(5), 197-202.

MAVRAGANI-TSIPIDOU, P., 2002. Genetic and cytogenetic analysis of the olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae). *Genetica,* **116**(1), 45-57.

MAZZI, D., KESANIEMI, J., HOIKKALA, A. and KLAPPERT, K., 2009. Sexual conflict over the duration of copulation in *Drosophila montana*: why is longer better? *BMC Evol. Biol.,* **9**, 132-2148-9-132.

MCCOMBS, S.D. and SAUL, S.H., 1992. Linkage analysis of five new genetic markers of the oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae). *J. Hered.*, **83**(3), 199-203.

MCGUIRE, S.E., ROMAN, G. and DAVIS, R.L., 2004. Gene expression systems in Drosophila: a synthesis of time and space. *Trends Gen.: TIG,* **20**(8), 384-391.

MCINNIS, D.O., TAM, S.Y.T., GRACE, C. and MIYASHITA, D., 1994. Population suppression and sterility rates induced by variable sex-ratio, sterile insect releases of *Ceratitis capitata* (Diptera: Tephritidae) in Hawaii. *Ann. Entomol. Soc. Am.,* **87**, 231-240.

MCINNIS, D.O., SHELLY, T.E. and KOMATSU, J., 2002. Improving male mating competitiveness and survival in the field for medfly, *Ceratitis capitata* (Diptera: Tephritidae) SIT programs. *Genetica,* **116**(1), 117-124.

MEREDITH, J.M., BASU, S., NIMMO, D.D., LARGET-THIERY, I., WARR, E.L., UNDERHILL, A., MCARTHUR, C.C., CARTER, V., HURD, H., BOURGOUIN, C. and EGGLESTON, P., 2011. Site-specific integration and expression of an anti-malarial gene in transgenic Anopheles gambiae significantly reduces Plasmodium infections. *PloS one,* **6**(1), e14587.

MITRA, V., FAIN-THORNTON, J. and CRAIG, N.L., 2008. *piggyBac* can bypass DNA synthesis during cut and paste transposition. *EMBO J.*, **27**, 1097-1109.

MKIZEA, N., HOELMERB, K.A. and VILLETA, M.H., 2008. A survey of fruit-feeding insects and their parasitoids occurring on wild olives, *Olea europaea* ssp. cuspidata, in the Eastern Cape of South Africa. *Biocontrol Science and Technology*, **18**(9), 991-1004.

MONTIEL-BUENO, A. and JONES, O., 2002. Alternative methods for controlling the olive fly, *Bactrocera oleae*, involving semiochemicals. *IOBC wprs Bulletin,* **25**.

MORRISON, N.I., SEGURA, D.F., STAINTON, K.C., FU, G., DONNELLY, C.A. and ALPHEY, L.S., 2009. Sexual competitiveness of a transgenic sexing strain of the Mediterranean fruit fly, *Ceratitis capitata*. *Entomol. Exp. Appl.,* **133**, 146-153.

MORRISON, N.I., FRANZ, G., KOUKIDOU, M., MILLER, T.A., SACCONE, G., ALPHEY, L.S., BEECH, C.J., NAGARAJU, J., SIMMONS, G.S. and POLITO, L.C., 2010. Genetic Improvements to the Sterile Insect Technique for Agricultural Pests. *AsPac J. Mol. Biol. Biotechnol.,* **18**(2), 275-295.

NARDI, F., CARAPELLI, A., DALLAI, R., RODERICK, G.K. and FRATI, F., 2005. Population structure and colonization history of the olive fly, *Bactrocera oleae* (Diptera, Tephritidae). *Mol. Ecol.,* **14**(9), 2729-2738.

NATESAN, S., RIVERA, V.M., MOLINARI, E. and GILMAN, M., 1997. Transcriptional squelching re-examined. *Nature,* **390**(6658), 349-350.

NEILSON, W.T.A. and MCALLAN, J.W., 1965. Effects of mating on fecundity of the apple maggot, *Rhagoletis pomonella* (Walsh). *Can. Ent.,* **97**, 276-279.

NITZAN, Y., ROSSLER, Y. and ECONOMOPOULOS, A.P., 1993. Mediterranean fruit fly: effective control of the Mediterranean fruit fly by genetic sexing male only sterile insect technique releases during 1989-1990. In: J. HENDRICHS, ed, *Management of insect pests: nuclear and related molecular and genetic techniques.* Vienna, Austria: International Atomic Energy Agency, 331-334.

NIYAZI, N., LAUZON, C.R. and SHELLY, T.E., 2004. Effect of probiotic adult diets on fitness components of sterile male Mediterranean fruit flies (Diptera: Tephritidae) under laboratory and field cage conditions. *J. Econom. Entomol.,* **97**(5), 1570-1580.

O'BROCHTA, D.A. and ATKINSON, P.W., 1996. Transposable elements and gene transformation in non-drosophilid insects. *Insect Biochem. Mol. Biol.,* **26**(8-9), 739-753.

O'BROCHTA, D.A., SETHURAMAN, N., WILSON, R., HICE, R.H., PINKERTON, A.C., LEVESQUE, C.S., BIDESHI, D.K., JASINSKIENE, N., COATES, C.J., JAMES, A.A., LEHANE, M.J. and ATKINSON, P.W., 2003. Gene vector and transposable element behavior in mosquitoes. *J. Exp. Biol.,* **206**(Pt 21), 3823-3834.

OCHIENG-ODERO, J.P.R., 1990. Critical, pupal and adults weights in the size related metamorphosis of the black lyre leafroller '*Cnephasia jactatana*. *Entomol. Exp. Appl.* **54**: 21-27.

OGAUGWU, C.E., SCHETELIG, M.F. and WIMMER, E.A., 2013. Transgenic sexing system for *Ceratitis capitata* (Diptera: Tephritidae) based on female-specific embryonic lethality. *Insect Biochem. Mol. Biol.,* **43**(1), 1-8.

OGAUGWU, C.E., WILSON, D., COBBLAH, M. and ANNOH, C., 2012. Gamma radiation sterilization of *Bactrocera invadens* (Diptera: Tephritidae) from southern Ghana. *Af. J.of Biotechnol.,* **11**(51), 11315-11320.

PANE, A., SALVEMINI, M., DELLI BOVI, P., POLITO, C. and SACCONE, G., 2002. The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development (Cambridge, England),* **129**(15), 3715-3725.

PARKER, A., 2005. Mass-rearing for sterile insect release. In: A. DYCK, J. HENDRICHS and A.S. ROBINSON, eds, *Sterile Insect Technique Principles and practice in area-wide integrated pest management.* The Netherlands: Springer, 209-232.

PARKER, A. and MEHTA, K., 2007. Sterile insect technique: a model for dose optimization for improved sterile insect quality. *Florida Entomol.,* **90**, 88-95.

PARKER, G.A., 1970. Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.*, **45**, 525-567.

PARSONS, P.A. and KAUL, D., 1966. Mating speed and duration of copulation in *Drosophila pseudoobscura*. *Heredity,* **21**(2), 219-225.

PARTRIDGE, L. and FARQUHAR, M., 1983. Lifetime mating success of male fruit flies (*Drosophila melanogaster*) is related to their size. *Anim. Behav.*, **31**, 871-877.

PARTRIDGE, L., HOFFMANN, A. and JONES, J.S., 1987. Male size and mating success in *Drosophila melanogaster* and *D. pseudoobscura* under field conditions. *Anim. Behav.*, **35**, 468-476.

PEREZ-STAPLES, D., SHELLY, T.E. and YUVAL, B., 2012. Female mating failure and the failure of 'mating' in sterile insect programs. *Entomol. Exp. Appl.*, **146**, 66-78.

PHUC, H.K., ANDREASEN, M.H., BURTON, R.S., VASS, C., EPTON, M.J., PAPE, G., FU, G., CONDON, K.C., SCAIFE, S., DONNELLY, C.A., COLEMAN, P.G., WHITE-COOPER, H. and ALPHEY, L., 2007. Late-acting dominant lethal genetic systems and mosquito control. *BMC Biol.*, **5**, 11.

PITNICK, S. and MARKOW, T.A., 1994. Large-male advantages associated with costs of sperm production in *Drosophila hydei*, a species with giant sperm. *PNAS,* **91**(20), 9277-9281.

QUESNEVILLE, H. and ANXOLABEHERE, D., 1997. A simulation of P element horizontal transfer in Drosophila. *Genetica,* **100**(1-3), 295-307.

RAPHAEL, K.A., SHEARMAN, D.C., STREAMER, K., MORROW, J.L., HANDLER, A.M. and FROMMER, M., 2011. Germ-line transformation of the Queensland fruit fly, *Bactrocera tryoni*, using a *piggyBac* vector in the presence of endogenous *piggyBac* elements. *Genetica*, **139**(1), 91-97.

REMPOULAKIS, P. and NESTEL, D., 2011. Dispersal ability of marked, irradiated olive fruit flies [*Bactrocera oleae* (Rossi) (Diptera: Tephritidae)] in arid regions. *J. Appl. Entomol* .

RENDON, P., MCINNIS, D., LANCE, D. and STEWART, J., 2004. Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *J. Econom. Entomol.*, **97**(5), 1547-1553.

REYES, J., SANTIAGO, G. and HERNÁNDEZ, P., 2000. The Mexican fruit fly eradication programme. In: K.H. TAN, ed, *Area-wide control of fruit flies and other pests.* Penang: Penerbit Universiti Sians Malaysia, 377-380.

ROBERT, M.A., LEGROS, M., FACCHINELLI, L., VALERIO, L., RAMSEY, J.M., SCOTT, T.W., GOULD, F. and LLOYD, A., 2012. Mathematical Models as Aids for Design and Development of Experiments: The Case of Transgenic Mosquitoes. *Entomol. Soc. Am.*, **49**(6), 1177-1188.

ROBINSON, A.S., 2002. Genetic sexing strains in medfly, *Ceratitis capitata*, sterile insect technique programmes. *Genetica,* **116**(1), 5-13.

RULL, J., ENCARNACION, N. and BIRKE, A., 2012. Mass rearing history and irradiation affect mating performance of the male fruit fly, *Anastrepha obliqua*. *J. Insect Sci.,* **12**, 45.

SACCHERI, I.J., WILSON, I.J., NICHOLS, R.A., BRUFORD, M.W. and BRAKEFIELD, P.M., 1999. Inbreeding of Bottlenecked Butterfly Populations: Estimation Using the Likelihood of Changes in Marker Allele Frequencies. *Gen. Soc. Am.,* **151**, 1053-1063.

SAKURAI, H., MURAKAMI, Y., KOHAMA, T. and TERUYA, T., 2000. Sterilizing mechanism of gamma-radiation in the female of West Indian sweet potato weevil, *Euscepes pastfasciatus*. *Bulletin of the faculty of agriculture, Gifu University, Japan,* **65**, 13-20.

SAOUR, G. and MAKEE, H., 2004. A kaolin-based particle film for suppression of the olive fruit fly *Bactrocera oleae* Gmelin (Dip., Tephritidae) in olive groves. *J. Appl. Entomol.,* **128**(1), 28-31.

SCHAACK, S., GILBERT, C. and FESCHOTTE, C., 2010. Promiscuous DNA: horizontal transfer of transposable elements and why it matters for eukaryotic evolution. *Trends Ecol. Evol.,* **25**(9), 537-546.

SCHETELIG, M.F. and HANDLER, A.M., 2012. A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). *Insect Biochem. Mol. Biol.,* **42**(10), 790-795.

SCHETELIG, M.F., SCOLARI, F., HANDLER, A.M., KITTELMANN, S., GASPERI, G. and WIMMER, E.A., 2009. Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly *Ceratitis capitata*. *PNAS,* **106**(43), 18171-18176.

SCHROEDER, W.J. and MITCHELL, W.C., 1981. Marking Tephritidae fruit fly adults in Hawaii for release-recovery studies. *Proc. Hawaiian Entomol. Soc.,* **23**(3), 437-440.

SHELLY, T., RENDON, P., MOSCOSO, F. and MENENDEZ, R., 2010. Testing the Efficacy of Aromatherapy at the World's Largest Eclosion Facility for Sterile Males of the Mediterranean Fruit Fly (Diptera: Tephritidae). *Proc. Hawaiian. Entomol. Soc.,* **42**, 33-40.

SHELLY, T.E., 2006. Aromatherapy and Medfly SIT. *Proceedings of the 7th International Symposium on Fruit Flies of Economic Importance,*, 59-69.

SHELLY, T.E., WHITTIER, T.S. and KANESHIRO, K.Y., 1994. Sterile insect release and the natural mating system of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.,* **87**, 470-481.

SIME, K.R., DAANE, K.M., MESSING, H.R. and JOHNSON, M.W., 2006. Comparison of two laboratory cultures of *Psyttalia concolor* (Hymenoptera: Braconidae), as a parasitoid of the olive fruit fly. *Biol. Control,* **39**, 248-255.

SIMMONS, L.W. and SIVA-JOTHY, M.J., 1998. Sperm competition in insects: mechanisms and the potential for selection. In: T.R. BIRKHEAD and A.P. MOLLER, eds, *Sperm competition and sexual selection.* London: Academic press, 341-434.

SINGH, S.R., SINGH, B.N. and HOENIGSBERG, H.F., 2002. Female remating, sperm competition and sexual selection in Drosophila. *Genetics and molecular research : GMR,* **1**(3), 178-215.

SIVINSKI, J. and BURK, T., 1989. Reproductive and mating behaviour. In: A.S. ROBINSON and G. HOOPER, eds, *Fruit flies, their biology, natural enemies, and control.* Amsterdam, The Netherlands: Elsevier Science Publishers, 343-351.

SKOURAS, P.J., MARGARITOPOULOS, J.T., SERAPHIDES, N.A., IOANNIDES, I.M., KAKANI, E.G., MATHIOPOULOS, K.D. and TSITSIPIS, J.A., 2007. Organophosphate resistance in olive fruit fly, *Bactrocera oleae*, populations in Greece and Cyprus. *Pest Manag. Sci.,* **63**(1), 42-48.

SLOTKIN, R.K. and MARTIENSSEN, R., 2007. Transposable elements and the epigenetic regulation of the genome. *Nat. Rev.Gen.,* **8**(4), 272-285.

SORENSEN, J.G., ADDISON, M.F. and TERBLANCHE, J.S., 2012. Mass-rearing of insects for pest management: Challenges, synergies and advances from evolutionary physiology. *Crop Protection,* **38**, 87-94.

SPRADLING, A.C. and RUBIN, G.M., 1982. Transposition of cloned P elements into Drosophila germ line chromosomes. *Science (New York, N.Y.),* **218**(4570), 341-347.

STEARNS, S., 1989. Tradeoffs in life-history evolution. *Func. Ecol.,* **3**, pp. 259-268.

TAYLOR, P.W., KASPI, R., MOSSINSON, S. and YUVAL, B., 2001. Age-dependent insemination success of sterile Mediterranean fruit flies. *Entomol. Exp. et Appl.,* **98**, 27-33.

THOMAS, D.D., DONNELLY, C.A., WOOD, R.J. and ALPHEY, L.S., 2000. Insect population control using a dominant, repressible, lethal genetic system. *Science (New York, N.Y.),* **287**(5462), 2474-2476.

THORNHILL, R. and ALCOCK, J., 1983. *The Evolution of Insect Mating Systems.* Boston, MA: Harvard University Press.

THORPE, H.M. and SMITH, M.C., 1998. In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. *PNAS,* **95**(10), 5505-5510.

TOLEDO, J., RULL, J., OROPEZA, A., HERNANDEZ, E. and LIEDO, P., 2004. Irradiation of *Anastrepha obliqua* (Diptera: Tephritidae) revisited: optimizing sterility induction. *J. Econ. Entomol.,* **97**(2), 383-389.

TSAKAS, S. and KRIMBAS, C.B., 1975. How many genes are selected in populations of *Dacus oleae*. *Genetics,* **79**(4), 675-679.

TSAKAS, S. and ZOUROS, E., 1980. Genetic differences among natural and laboratory-reared populations of the olive fruit fly *Dacus oleae* (Diptera: Tephritidae). *Entomol. Exp. Appl.,* **28**, 268-276.

TSITSIPIS, J.A., 1977. Improved method for mass rearing of olive fruit-fly, *Dacus oleae* (Gmel) (Diptera, Tephritidae). *J. Appl. Entomol.,* **83**, 419-426.

TSITSIPIS, J.A. and ABATZIS, C., 1980. Relative humidity effects, at 20 degrees Celsius, on eggs of the olive fruit fly, *Dacus oleae*, reared on artificial diet. *Entomol. Exp. et Appl.,* **28**, 92-99.

TZANAKAKIS, M., 2003. Seasonal Development and Dormancy of Insects and Mites Feeding on Olive: A Review. *Neth. J. Zoo.,* **52**(2), 87-224.

TZANAKAKIS, M., TSITSIPIS, J., PAPAGEORGIOU, M. and FYTIZAS, E., 1966. Gamma radiation-induced dominant lethality to sperm of olive fruit fly. *J, Econ. Entomol.,* **59**, 214-216.

TZANAKAKIS, M.E., 1989. Small-scale Rearing. *Dacus oleae*. In: A.S. ROBINSON and G. HOOPER, eds, *Fruit Flies: Their Biology, Natural Enemies and Control* Elsevier edn. Amsterdam, The Netherlands, 105-116.

TZANAKAKIS, K.E., TSITSIPIS, J.A. and ECONOMOPOULOS, A.P., 1968. Frequency of mating in females of olive fruit fly under laboratory conditions. *J. Econ. Entomol.,* **61**, 1309-1312.

VAN DER VLOEDT and KLASSEN, [Homepage of FAO], [Online]. Available: http://www.fao.org/ag/AGa/AGAP/FRG/FEEDback/War/u4220b/u4220b0j.htm [08/08, 2012].

VAN LEEUWEN, W., RUTTINK, T., BORST-VRENSSEN, A.W., VAN DER PLAS, L.H. and VAN DER KROL, A.R., 2001. Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. *J. Exp. Botany,* **52**(358), 949-959.

VONTAS, J.G., HEJAZI, M.J., HAWKES, N.J., COSMIDIS, N., LOUKAS, M., JANES, R.W. and HEMINGWAY, J., 2002. Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*. *Insect Mol. Biol.,* **11**(4), 329-336.

VREYSEN, M.J., 2001. Principles of area-wide integrated tsetse fly control using the sterile insect technique. *Medecine tropicale : revue du Corps de sante colonial,* **61**(4-5), 397-411.

WANG, Z., GERSTEIN, M. and SNYDER, M., 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev.Gen.,* **10**(1), 57-63.

WHITTEN, M.J., 1969. Automated sexing of pupae and its usefulness in control by sterile insects. *J. Econ. Entomol.,* **62**, 272-273.

WICKER, T., SABOT, F., HUA-VAN, A., BENNETZEN, J.L., CAPY, P., CHALHOUB, B., FLAVELL, A., LEROY, P., MORGANTE, M., PANAUD, O., PAUX, E., SANMIGUEL, P. and SCHULMAN, A.H., 2007. A unified classification system for eukaryotic transposable elements. *Nat. Rev.Gen.,* **8**(12), 973-982.

WICKER-THOMAS, C., 2007. Pheromonal communication involved in courtship behavior in Diptera. *J. Insect Physiol.,* **53**(11), 1089-1100.

WILLHOEFT, U. and FRANZ, G., 1996. Identification of the sex-determining region of the *Ceratitis capitata* Y chromosome by deletion mapping. *Genetics,* **144**(2), 737-745.

WILSON, C., BELLEN, H.J. and GEHRING, W.J., 1990. Position effects on eukaryotic gene expression. *Ann. Rev. Cell Biol.,* **6**, 679-714.

WISE DE VALDEZ, M.R., NIMMO, D., BETZ, J., GONG, H.F., JAMES, A.A., ALPHEY, L. and BLACK, W.C.,4TH, 2011. Genetic elimination of dengue vector mosquitoes. *PNAS,* **108**(12), 4772-4775.

WOLFNER, M.F., 1997. Tokens of love: functions and regulation of Drosophila male accessory gland products. *Insect Biochem. Mol. Biol.,* **27**(3), 179-192.

WYSS, J.H., 2000. Screwworm eradication in the Americas. *Ann. New York Ac. Sci.,* **916**, 186-193.

ZERVAS, G.A., 1983. Sexual and reproductive maturation in wild and lab cultured olive fruit flies *Dacus oleae* (Gmelin) (Diptera: Tephritidae). *Fruit Flies of Economic Importance.* Rotterdam: A.A. Balkema, 429-438.

ZERVAS, G.A., 1981. Sexual behaviour of the olive fruit fly, *Dacus oleae*. *Proceedings of a FAO/IAEA symposium (Neuherberg),* 427-429.

ZERVAS, G.A. and ECONOMOPOULOS, A.P., 1982. Mating frequency in caged populations of wild and artificially reared (normal or gamma-sterilized) olive fruit flies, *Dacus oleae* (Gmelin) (Diptera:Tepritidae). *Environ. Entomol.,* **11**, 17-20.

Peer reviewed paper.

The following paper is a published peer-reviewed article resulting from the work described in this thesis.

Ant et al. BMC Biology 2012, 10:51
http://www.biomedcentral.com/1741-7007/10/51

BMC Biology
incorporating Journal of Biology

RESEARCH ARTICLE Open Access

Control of the olive fruit fly using genetics-enhanced sterile insect technique

Thomas Ant[1,2], Martha Koukidou[1], Polychronis Rempoulakis[1,3], Hong-Fei Gong[1], Aris Economopoulos[3], John Vontas[3] and Luke Alphey[1,2*]

Abstract

Background: The olive fruit fly, *Bactrocera oleae*, is the major arthropod pest of commercial olive production, causing extensive damage to olive crops worldwide. Current control techniques rely on spraying of chemical insecticides. The sterile insect technique (SIT) presents an alternative, environmentally friendly and species-specific method of population control. Although SIT has been very successful against other tephritid pests, previous SIT trials on olive fly have produced disappointing results. Key problems included altered diurnal mating rhythms of the laboratory-reared insects, resulting in asynchronous mating activity between the wild and released sterile populations, and low competitiveness of the radiation-sterilised mass-reared flies. Consequently, the production of competitive, male-only release cohorts is considered an essential prerequisite for successful olive fly SIT.

Results: We developed a set of conditional female-lethal strains of olive fly (named Release of Insects carrying a Dominant Lethal; RIDL®), providing highly penetrant female-specific lethality, dominant fluorescent marking, and genetic sterility. We found that males of the lead strain, OX3097D-Bol, 1) are strongly sexually competitive with wild olive flies, 2) display synchronous mating activity with wild females, and 3) induce appropriate refractoriness to wild female re-mating. Furthermore, we showed, through a large proof-of-principle experiment, that weekly releases of OX3097D-Bol males into stable populations of caged wild-type olive fly could cause rapid population collapse and eventual eradication.

Conclusions: The observed mating characteristics strongly suggest that an approach based on the release of OX3097D-Bol males will overcome the key difficulties encountered in previous olive fly SIT attempts. Although field confirmation is required, the proof-of-principle suppression and elimination of caged wild-type olive fly populations through OX3097D-Bol male releases provides evidence for the female-specific RIDL approach as a viable method of olive fly control. We conclude that the promising characteristics of OX3097D-Bol may finally enable effective SIT-based control of the olive fly.

Keywords: olive fly, Bactrocera oleae, sterile insect technique, SIT, release of insects carrying a dominant lethal, RIDL, autocidal control, insect transgenics

Background

The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the major insect pest of olives. Females typically lay a single egg per olive [1], injected into the fruit through the female's ovipositor. The developing larva tunnels through the olive, feeding on the fleshy mesocarp. Heavy olive fly infestation can reduce the quality and therefore the value of the olive oil by up to 80%, and cause the rejection of entire harvests of table olives [2]. Control currently relies overwhelmingly on the use of chemical insecticides, and because of the high economic and environmental costs of chemical control, together with the appearance of insecticide-resistant populations [3], there is an urgent need for improved control methods.

The sterile insect technique (SIT) is an environmentally friendly and species-specific method of pest control based on the release of large numbers of sterilised insects [4]. Competition for mating between wild and sterile males results in a decrease in the number of fertile matings, and

* Correspondence: luke.alphey@oxitec.com
[1]Oxitec Limited, 71 Milton Park, Oxford OX14 4RX, UK
Full list of author information is available at the end of the article

a decline in the overall population size. SIT has been successfully implemented against various pest insect species including several Tephritidae. However, despite decades of research aimed at developing an olive fly SIT programme using radiation-sterilised flies [5], the consistently poor results led to eventual abandonment of the trials. Key issues included low quality of the radiation-sterilised mass-reared flies, economical production of sufficient numbers of sterilised flies, and assortative mating of released and wild populations because of different preferred mating times [5-7]. Laboratory-reared wild-type flies were found to mate several hours earlier than wild flies [7] (presumably due to differential selective pressures in the artificial laboratory-rearing environment). The proposed solution was male-only release [6]. Male-only release using a genetic sexing strain has also been shown to give a threefold to fivefold improvement in the performance of released radiation-sterilised *Ceratitis capitata* (Medfly) males used for SIT [8].

The RIDL® (Release of Insects carrying a Dominant Lethal) system is a transgene-based derivative of SIT [9-14], one version of which involves the mass release of insects carrying a female-specific lethal transgene (fsRIDL) [15-17]. Encouraged by work advancing olive fly mass rearing by the International Atomic Energy Agency (IAEA) and others, we set out to develop fsRIDL strains of olive fly to overcome the remaining historic limitations of olive fly SIT. We therefore transformed the olive fly with construct OX3097, an fsRIDL system that had previously provided suitable fsRIDL strains for Medfly, incorporating genetic sexing, genetic sterilisation, and a heritable fluorescent marker [15].

We report the development of the first transgenic control strains for the olive fly. The lead strain, OX3097D-Bol, provides highly penetrant, dominant, female-specific lethality when reared in the absence of a transgene repressor. Using a series of behavioral studies, we found that OX3097D-Bol males show fully synchronous and strongly competitive mating behavior with wild olive flies, and display no reduction in their ability to induce refractoriness to wild female re-mating. This is a more stringent test than using laboratory wild-type flies, which may have co-adapted to laboratory rearing conditions. Furthermore, we describe results from experiments showing the ability of periodic releases of OX3097D-Bol males to suppress large caged wild-type olive fly populations.

Results

Olive fly transformation with OX3097 and phenotypic analysis

Six transgenic lines were generated carrying the OX3097 transgene (Figure 1A). Of the five lines with single insertions, two showed female-specific lethality that was fully penetrant and efficiently repressed by dietary tetracycline (Figure 1C, D). Of these, line OX3097D-Bol (Figure 1E), which also showed the brightest expression of the DsRed2 fluorescent marker, was selected for further analysis. Consistent with previous observations of the OX3097 construct in Medfly [15] and Mexfly (Stainton *et al.,* unpublished data), female lethality occurred at the early pupal stage. Analysis of transgene-derived transcripts indicated that the alternative splicing pattern of the *Cctra* intron in OX3097D-Bol olive fly is equivalent to that in its native context [15,18,19] (Figure 1B). The OX3097D-Bol strain was selected for further development, and made homozygous for the transgene.

Mating tests with OX3097D-Bol and wild olive flies

Caged mating competitiveness tests challenging homozygous OX3097D-Bol males to compete with wild males for copulations with wild females were performed based on established guidelines [20], using wild olive flies from infested olives collected in Crete. In total, 15 experiments were performed, each with 50 OX3097D-Bol males, 50 wild males, and 50 wild females, thus more than 400 total couples were assessed. Wild males outperformed OX3097D-Bol males, gaining an average of 56% of total mates ($P = 0.01$, degrees of freedom (d.f.) = 1, likelihood ratio test for goodness of fit, n = 406). Nonetheless, this near-equal outcome far exceeds established thresholds for SIT [20]. Copulation initiation times were also recorded (Figure 2A). No significant difference was seen in mating initiation times for each type of male ($P = 0.44$, d.f. = 1, circular statistics *F*-test), suggesting synchronicity in mating activity between OX3097D-Bol males and wild flies.

Olive fly females typically only mate once [21]; males transfer more sperm than the female needs to fertilise all her eggs, and mating induces long-lasting refractoriness to re-mating, although if the females are held in close proximity to males some re-mating occurs. For Medfly, radiation sterilisation not only reduces the ability of male flies to compete for mates but also increases the likelihood that mated females will subsequently re-mate; that is, radiation reduces the ability of the males to induce refractoriness to re-mating in females [22]. Furthermore, the second mate taken is also preferentially wild rather than sterile. These effects of reduced male mating success, increased female re-mating, and preferential mating to wild (fertile) males on re-mating each reduce the efficiency of SIT. We therefore investigated the effect of first-male genotype on wild female re-mating propensity and second-mate genotype. Wild females who had initially mated either an OX3097D-Bol male (n = 188) or a wild male (n = 296) were caged with and allowed to mate with equal proportions of OX3097D-Bol and wild males over 15 days. No significant differences in re-mating rate ($P = 0.7$, d.f. = 1, χ^2 test), or second-mate genotype were seen ($P = 0.38$, d.f. = 1, χ^2 test) (Figure 2B).

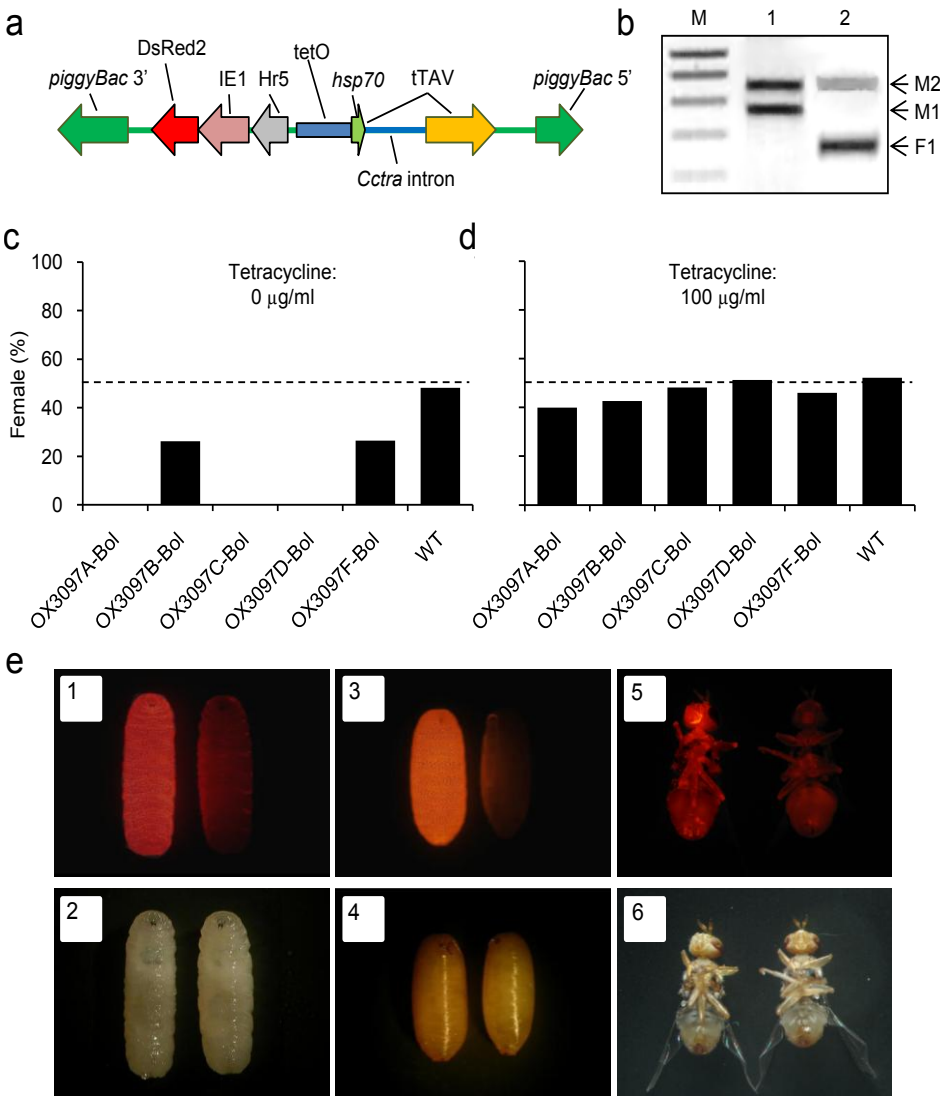


Figure 1 The OX3097 transposon and induced phenotypes in olive fly. (**A**) Diagrammatic representation of the OX3097 transposon. OX3097 comprises a fluorescent marker (hr5-IE1-DsRed2), and the female-specific tTAV expression system (tetO-*Dmhsp70* minimal promoter - Cctra:tTAV) [15]. Sex-specific alternative splicing of the *Cctra* intron leads to production of tTAV and the initiation of a lethal tTAV positive-feedback loop in females only [14,15,34]. (**b**) Products of alternative splicing of *Cctra*:tTAV in (lane 1) male and (lane 2) female OX3097D-Bol olive flies. Three splice variants were detected, corresponding to Cctra transcripts M1, M2 and F1 [18] (identity confirmed by sequencing). Only females produce the F1 splice variant, corresponding to the reconstitution of the tTAV open reading frame and leading to production of functional tTAV. Lane M shows DNA size standards: 200-1,000 bp in 200-bp increments (Eurogentec Smartladder). (**C**) Penetrance and (**D**) tetracycline repressibility of female lethality in five OX3097 olive fly lines. Strains OX3097A-D-Bol &F-Bol are five insertion lines of OX3097 in olive fly. Penetrance and repressibility of female-specific lethality was assessed by crossing heterozygous males of each strain to virgin wild-type (WT) females, and collecting eggs on filter paper saturated with water containing either 0 µg/ml tetracycline or 100 µg/ml tetracycline. The sex ratio of adult progeny expressing the DsRed2 fluorescent marker is shown for each strain compared with wild-type (WT) progeny. Lines A, C and D showed fully penetrant female-specific lethality when reared in the absence of tetracycline (off-tet); that is, they produced no female progeny off-tet in this assay. In lines C and D, female-specific lethality was also efficiently repressed on-tet. (**E**) Fluorescence microscopy allows discrimination of OX3097D-Bol from wild type at larval, pupal, and adult stages. Photomicrographs of OX3097D-Bol and wild-type olive flies under (upper panels) fluorescence and (lower panels) bright-field illumination. Each panel shows OX3097D-Bol to the left and wild-type to the right: OX3097D-Bol and wild-type (1,2) larvae, (3,4) pupae, and (5,6) adults are shown. Expression of DsRed2 is clearly visible all over the OX3097D-Bol larvae and pupae, and in areas of less opaque cuticle (for example, the labellum, upper thorax, leg joints, and anus) of OX3097D-Bol adults.

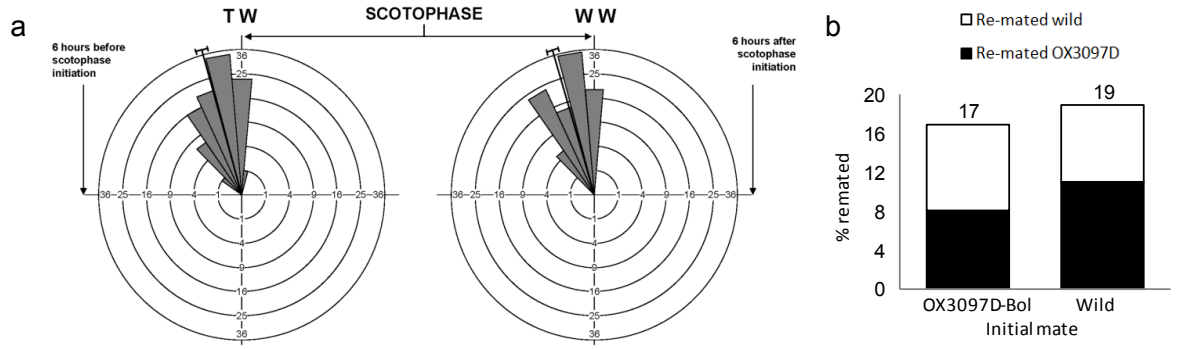


Figure 2 Mating initiation times and re-mating propensity of OX3097D-Bol with wild olive flies. (**A**) Copulation initiation times were similar for OX3097D-Bol males and wild males. Copulation initiation times were recorded for all mating pairs; each pair contained a wild female and either an OX3097D-Bol male (left circle, n = 216) or a wild male (right circle, n = 161). Scotophase is the dark phase of a light/dark cycle. Each 'wedge' on the circular graphic represents a time-interval of 45 minutes; the radial length of the wedge indicates the proportion of total matings of that type that occurred in each time segment. Mean copulation initiation time for wild females and either OX3097D-Bol or wild males was 63 and 66 minutes before scotophase respectively. Peak mating activity times were not significantly different between the two types of male ($P = 0.45$, degrees of freedom (d.f.) = 1, circular statistics *F*-test). (**B**) Genotype of first mate (OX3097D-Bol or wild) did not affect female re-mating frequency or genotype of second mate. Of 188 females initially mated to OX3097D-Bol males 32 (17%) re-mated, of which 17 (9%) re-mated to wild males (open portion of left bar), and 13 (8%) to OX3097D-Bol males (solid portion of left bar). Of 296 females initially mated to wild males 55 (19%) re-mated, of which 23 (8%) re-mated to wild males (open portion of right bar) and 32 (11%) to OX3097D-Bol males (solid portion of left bar). Re-mating propensity of wild females initially mated with either an OX3097D-Bol male or a wild male were not significantly different ($P = 0.7$, d.f. = 1, χ^2 test). Furthermore, the re-mating preference of the wild females was not found to differ significantly depending on first-mate choice ($P = 0.38$, d.f. = 1, χ^2 test).

Elimination of caged olive fly populations

We tested the ability of periodic release of OX3097D-Bol males to suppress target populations of wild-type olive fly, as published previously [17]. Wild-type populations totaling approximately 400 male and female olive flies were established in each of four large (8 m^3) cages in a greenhouse at the University of Crete. At week 13 from first establishment, approximately 1,600 OX3097D-Bol male pupae were introduced per week. Population density was monitored by measuring egg production (Figure 3A) and female mortality (Figure 3B). To track mating outcomes, pupae taken from the cage were scored for fluorescence (Figure 3C). Fluorescent pupae were detected at three weeks post-first release (PR) and the proportion increased rapidly thereafter to 100% by week 10 PR. Death of fluorescent (transgenic) female pupae as a result of expression of the lethal phenotype would reduce the number of egg-laying adult females; based on the fluorescence data, no wild-type females emerged (eclosed) in the treatment cages from week 10 PR. Egg production was lower in the treatment than in the control cages in week 6 PR, and declined rapidly thereafter. The numbers of dead females recovered from treatment cages started to decrease from around week 6 to 7 PR, presumably due to a decline in overall female numbers. Defining extinction as 2 weeks of zero egg production [17], both treatment cage populations were extinct by week 12 PR.

Discussion

An effective non-chemical approach to olive fly management is highly desirable, and SIT could provide this if specific technical issues can be overcome. Considerable progress has been made by others in respect of olive fly mass-rearing and release, and we set out to overcome the key remaining issues through genetics.

The use of genetic technology in food production has been controversial in some countries, notably within the European Union (EU). It is possible that the use of genetically modified arthropods to protect the crop in a sustainable way, which leaves little or no residue in the food product (here olives or olive oil) might be more acceptable in the EU than food products that are produced directly from genetically modified organisms. For comparison, the Standing Committee on the Food Chain and Animal Health stated that foods and feeds produced by fermentation with genetically modified microorganisms that are not present in the final product are "excluded from the scope of regulations" [23]. Additionally, it is recognized in EU legislation that the presence of technically unavoidable traces of genetically modified organisms in food and feed products should not trigger labeling requirements (Regulation 1830/2003/EC), as long as appropriate steps to avoid the presence of the materials have been taken. This is also analogous to the situation regarding the presence of wild-type insect parts as contaminants in food products [24].

Using modern genetic engineering techniques, we have developed OX3097D-Bol, an fsRIDL olive fly strain that incorporates engineered phenotypes, directly addressing problems encountered in previous unsuccessful olive fly SIT trials [5]. OX3097D-Bol is able to provide 100%

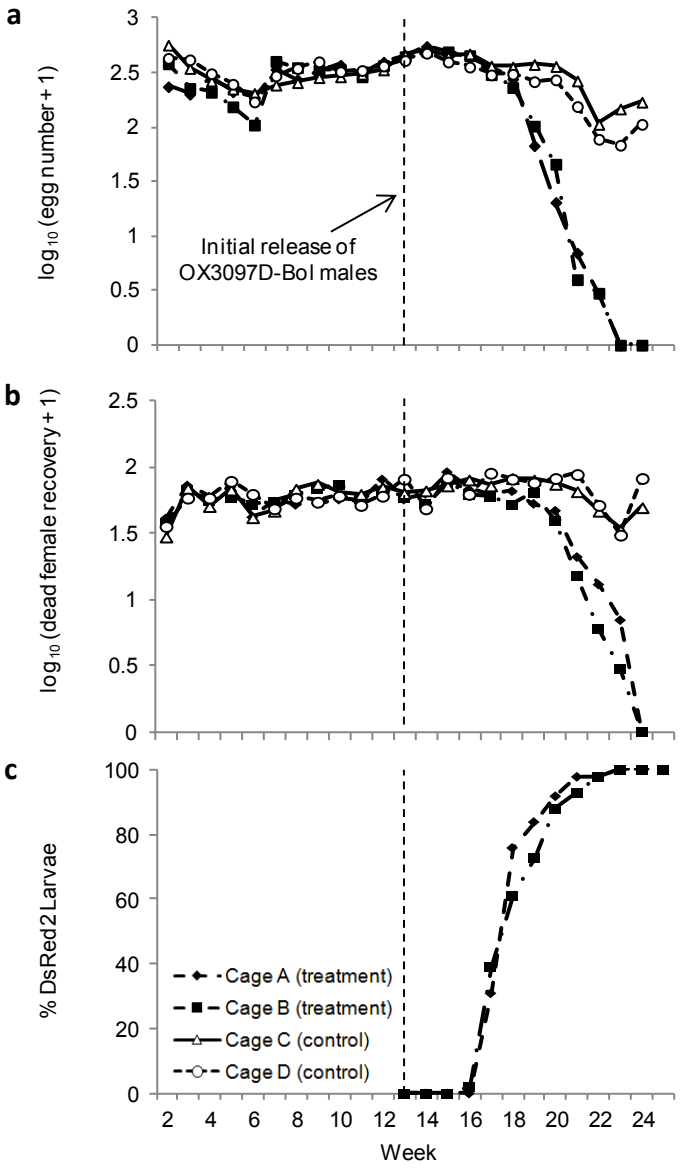


Figure 3 Population elimination by periodic release of OX3097D-Bol males. (**A**) The average daily egg production for each cage. Weeks 1 to 12 was the population stabilization period with 250 pupae added in the first week, and 200 pupae added to each cage per week thereafter. From week 13, 1,600 OX3097D-Bol pupae were added weekly into cages A and B. After week 13, weekly pupal return to the treatment cages was made proportional to the weekly egg production in the cage relative to the control cages. From 5 weeks after initiation of RIDL introductions, egg production in each treatment cage was consistently lower than in either control cage; the difference increased until eventual extinction of the wild-type population in both treatment cages by week 24 (12 weeks after the first RIDL release). Extinction was defined as 2 weeks of zero egg production. Egg numbers in control cages remained relatively stable. (**B**) Dead flies were removed from the cages weekly, and the numbers of dead females are shown. From 7 weeks after the initiation of RIDL release, increasingly fewer such females were recovered from the treatment cages than from the control cages. (**C**) Frequency of DsRed2 in treatment cages. Larvae selected for return were screened for presence of DsRed2 marker by fluorescence microscopy before being returned to the treatment cage (see Methods). The proportion of returning pupae carrying the OX3097D-Bol transgene reached 100% in both treatment cages by week 23 (10 weeks post-RIDL release). Olive fly females typically mate only once [21] (Figure 2B). Females start to lay eggs approximately 2 days after mating, and lay most of their eggs within the next 10 days. Egg to pupa development time was approximately 12 days. These pupae therefore indicate female mating choice of approximately 3 weeks before each measurement.

male-only release generations when reared in the absence of a dietary transgene repressor. Genetic sexing encourages random mating between the wild and the released sterile populations, providing established performance benefits [8]. Female lethality renders redundant the requirement of performance-reducing and costly

sterilisation using ionising radiation. Furthermore, the dominant fluorescent marker has the potential to improve field monitoring, and the requirement of tetracycline for female development mitigates risks of accidentally released transgenic populations establishing in the wild. Introgression of susceptibility alleles through the female line provides a resistance management option for other control methods used with fsRIDL in an integrated pest-management programme [25-27].

Conclusions

The good mating characteristics of OX3097D-Bol males seen in this study, including high sexual competitiveness, photo-period compatibility, and efficient induction of re-mating refractoriness in wild females, suggest a strong potential for application in a SIT control programme. Although the results presented here need field confirmation, the proof-of-principle suppression and elimination of caged wild-type olive fly populations through OX3097D-Bol male releases provides evidence for the fsRIDL approach as a viable method for olive fly control. Independently, significant advances have been achieved in olive fly mass-rearing [28,29] and release [30] techniques. We therefore propose that the development of OX3097D-Bol will help form the basis of more effective and more sustainable control of this ancient and destructive olive pest.

Methods

Olive fly strains, rearing, and transformation

Olive fly rearing was carried out using standard methods. Approximately 4,500 pre-blastoderm stage olive fly embryos of the Democritus laboratory (Greece) *B. oleae* strain were micro-injected with the OX3097 plasmid and *piggyBac* mRNA. as described by Koukidou *et al.* [31]. This resulted in 138 surviving G_0 adults. After back-crossing pools of five male survivors with ten wild-type females, and five female survivors with five wild-type males, six OX3097 lines were isolated (transformation efficiency ~4%). Transgenics were outcrossed for five generations to the Argov wild-type strain (Israel), and these outcrossed Argov derivatives were used for all experiments. The Argov strain was derived from field collections of wild male olive fly in Israel, outcrossed to Democritus females. To develop a homozygous strain, a pool of homozygous and heterozygous OX3097D-Bol adults was generated by crossing OX3097D-Bol heterozygotes. DNA from these parents was analysed by PCR using primers (5'-CCTGCGTTTGGAGATGACGAAATC-3' and 5'-CTTACATATAGAGCAGTGCGCTCACATG-3') that anneal to genomic sites flanking the insertion site producing a WT (no insertion) amplicon were discarded. Homozygotes were thereby identified, and a homozygous line was developed from 15 female and 13 male founder flies. The key phenotypic properties identified in OX3097D-Bol heterozygotes (Figure 1, 2) were also found in the homozygous line.

Reverse transcriptase PCR

Male and female tTAV [14,34] transcripts were amplified by reverse transcriptase (RT)-PCR using cDNA synthesized using a commercial kit (Superscript II; Life Technologies, Grand Island, NY, USA) on RNA extracts prepared using TRI reagent (Life Technologies). The major RT-PCR products were sequenced (GATC Biotech, Konstanz, Germany) and analysed using VectorNTi (Life Technologies).

Mate competition and re-mating tests

Mating competitiveness tests were carried out in accordance with the United Nations Food and Agriculture Organization (FAO)/IAEA/United States Department of Agriculture (USDA) guidelines [20] in semi-natural caged conditions (cages were 1.25 m high with a base of 0.25 m^2 and contained a large olive branch) in greenhouse facilities at the University of Crete under natural light. Adult male OX3097D-Bol flies were obtained from larvae reared in the absence of tetracycline ("off-tet") at low density (1 larva/0.8 g larval medium). Wild pupae were recovered from infested olives gathered from olive orchards near to the University of Crete. Each mating test used 50 OX3097D-Bol males, 50 wild males, and 50 wild females. Mated males were scored for the presence of the DsRed2 fluorescent marker by epifluorescence microscopy [32,33] (Clontech Laboratories, Inc, Mountain View, CA, USA). OX3097D-Bol mating competition tests were performed in 15 replicates with more than 400 couples assessed in total. Each experiment yielded a mating propensity of greater than 0.2 [20]. Mating cages for the first step of re-mating tests contained either wild females and OX3097D-Bol males or wild females and wild males. Mating couples were removed from cages. Mated females were grouped in accordance with the genotype of the first mate (OX3097D-Bol, n = 188 or wild-type, n = 296) and were transferred the following day to new cages with sufficient fresh wild and OX3097D-Bol males to give a 1:1:1 ratio of mated females, wild males, and OX3097D-Bol males. Cages were checked daily for re-mating events over the following 15 days. Re-mating couples were removed, and the male genotype assessed by epifluorescence microscopy.

Caged suppression of stable wild-type populations

The protocol for the caged suppression experiment was based on that of Wise de Valdez *et al.* [17]. Stable populations of wild-type (Argov) olive flies were established in four field cages (each 8 m^3 and containing an olive tree 1.5 m tall; all the cages were contained within a single

large glasshouse) over a 12-week period by introducing a fixed number of pupae to each cage weekly (250 in week 1, and 200 from weeks 2 to 12). To assess egg production rates and to sustain caged olive fly populations, four ceresin-wax cones (combined surface area approximately 550 cm^2) were added to each cage daily for female oviposition. Pupal additions for the first 4 weeks during population establishment originated from a wild-type stock colony; thereafter, experimental cages were self-sufficient. Eggs were collected from the cages and counted daily. On week 12, the experimental cages were randomly divided into two RIDL-treatment and two no-treatment (control) cages. From week 13 onwards, RIDL-treatment cages received weekly additions of 1,600 OX3097D-Bol male pupae reared off-tet (an initial recruitment rate ratio of approximately 16 OX3097D-Bol males to 1 wild-type male). Once the OX3097D-Bol introductions began, pupal introductions to the RIDL-treatment cages were proportional to the cage's respective rate of egg production, with the control cages providing a coefficient of weekly egg production to pupal return. Numbers of females in a cage were monitored by collecting and counting dead females. The ratio of RIDL heterozygous to wild-type pupae returning to RIDL-treatment cages was monitored by fluorescence microscopy (OX3097D-Bol heterozygous females reared off-tet pupate but fail to eclose). Larvae were screened only after the returning population was separated, to remove the possibility of bias in selecting individual flies for reintroduction to the cage populations.

Statistical analysis

Comparison of mating competitiveness between OX3097D-Bol and wild males was performed using a likelihood ratio test for goodness of fit with numbers of successfully mated males from both genotypes pooled across experiments, and compared against a theoretical 1:1 genotype ratio expected in random mating. Differences in copulation initiation time between the two possible mating combinations (OX3097D-Bol/wild female and wild male/wild female) were analysed using a circular statistics *F*-test with the statistical software Oriana for Windows (Kovach Computing Services, Anglesey, UK). The Pearson's χ^2 test was used to compare numbers of re-mated to non-re-mated females for both initial mating combinations. The same test was used to compare initial and second-mate choice. Unless otherwise stated, all statistical tests were performed using SPSS for Windows (version 10.0; SPSS Inc., Chicago IL USA), with significance set at $P < 0.05$.

Abbreviations

CcTra intron: *Ceratitis capitata* transformer intron; fsRIDL: female-specific Release of Insects carrying a Dominant Lethal; PR: post-first-release; RIDL: Release of Insects carrying a Dominant Lethal; SIT: Sterile Insect Technique.

Acknowledgements

We thank the Agricultural Research Organisation, Israel (ARO) and Bio-Fly Ltd for the Argov wild-type strain. We thank Zoe Lokhon, Chrysargyris Antonios, Sian Morgan, Camilla Beech, and the Oxitec molecular biology team for technical assistance. TA was supported by an Industrial Case DPhil studentship from the UK Biotechnology and Biological Sciences Research Council.

Author details

[1]Oxitec Limited, 71 Milton Park, Oxford OX14 4RX, UK. [2]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK. [3]Faculty of Biotechnology and Applied Biology, Department of Biology, University of Crete, Heraklion, Crete, Greece.

Authors' contributions

TA and MK created the transgenic olive fly lines. TA conducted the molecular analysis TA, MK, PR, carried out mating tests and suppression trial. HG, JV and AE provided advice, LA conceived the project, and MK supervised the project. TA and LA wrote the paper. All authors discussed results and commented on the manuscript

Competing interests

TA, MK, PK, HFG and LA are employees or students of Oxitec Ltd and have employment, stipend and/or equity interest in Oxitec. Oxitec and the University of Oxford have intellectual property related to the subject matter of this paper. All other authors declare no interest.

Received: 9 March 2012 Accepted: 19 June 2012
Published: 19 June 2012

References

1. Burrack HJ, Fornell AM, Connell JH, O'Connell NV, Phillips PA, Vossen PM, Zalom FG: **Intraspecific larval competition in the olive fruit fly (Diptera: tephritidae).** *Environ Entomol* 2009, **38**:1400-1410.
2. Daane KM, Johnson MW: **Olive fruit fly: managing an ancient pest in modern times.** *Annu Rev Entomol* 2010, **55**:151-169.
3. Vontas J, Hernandez-Crespo P, Margaritopoulos JT, Ortego F, Feng H, Mathiopoulos KD, Hsu J: **Insecticide resistance in Tephritid flies.** *Pest Biochem and Physiol* 2011, **100**:199.
4. Dyck VA, Hendrichs J, Robinson AS: *Sterile Insect Technique: Principles and practice in area-wide integrated pest management* Dordrecht, Netherlands: Springer; 2005.
5. Economopoulos AP, Avtzis N, Zervas G, Tsitsipis J, Haniotakis G, Tsiropoulos G, Manoukas A: **Control of the olive fly, Dacus oleae (Gmelin), by the combined effects of insecticides and release of gamma sterilised insects.** *J Appl Entomol* 1977, **83**:201-215.
6. Economopoulos AP: **The olive fly, Bactrocera (Dacus) oleae (Gmelin) (Diptera:Tephritidae): its importance and control; previous SIT research and pilot testing.** Austria: International Atomic Energy Agency; 2001, 44.
7. Zervas GA, Economopoulos AP: **Mating frequency in caged populations of wild and artificially reared (normal or gamma-sterilized) olive fruit flies, Dacus oleae (Gmelin) (Diptera:Tepritidae).** *Environ Entomol* 1982, **11**:17-20.
8. Rendon P, McInnis D, Lance D, Stewart J: **Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala.** *J Econ Entomol* 2004, **97**:1547-1553.
9. Alphey L, Nimmo D, O'Connell S, Alphey N: **Transgenesis and the management of vector-borne disease.**Edited by: Aksoy S. Berlin: Springer-Verlag; 2008:**627**:93-103.
10. Alphey L, Andreasen M: **Dominant lethality and insect population control.** *Mol Biochem Parasitol* 2002, **121(2)**:173-178.
11. Alphey L: **Re-engineering the sterile insect technique.** *Insect Biochem Mol Biol* 2002, **32(10)**:1243-1247.
12. Morrison NI, Franz G, Koukidou M, Miller TA, Saccone G, Alphey LS, Beech CJ, Nagaraju J, Simmons GS, Polito LC: **Genetic improvements to the sterile insect technique for agricultural pests.** *Asia Pac J Mol Bio* 2010, **18**:275-295.
13. Thomas DD, Donnelly CA, Wood RJ, Alphey LS: **Insect population control using a dominant, repressible, lethal genetic system.** *Science* 2000, **287(5462)**:2474-2476.

14. Gong P, Epton MJ, Fu G, Scaife S, Hiscox A, Condon KC, Condon GC, Morrison NI, Kelly DW, Dafa'alla T, Coleman PG, Alphey L: **A dominant lethal genetic system for autocidal control of the Mediterranean fruit fly.** *Nat Biotechnol* 2005, **23(4)**:453-456.
15. Fu G, Condon KC, Epton MJ, Gong P, Jin L, Condon GC, Morrison NI, Dafa'alla TH, Alphey L: **Female-specific insect lethality engineered using alternative splicing.** *Nat Biotechnol* 2007, **25(3)**:353-357.
16. Fu G, Lees RS, Nimmo D, Aw D, Jin L, Gray P, Berendonk TU, White-Cooper H, Scaife S, Kim Phuc H, Marinotti O, Jasinskiene N, James AA, Alphey L: **Female-specific flightless phenotype for mosquito control.** *Proc Natl Acad Sci USA* 2010, **107(12)**:4550-4554.
17. Wise de Valdez MR, Nimmo D, Betz J, Gong HF, James AA, Alphey L, Black WC4th: **Genetic elimination of dengue vector mosquitoes.** *Proc Natl Acad Sci USA* 2011, **108(12)**:4772-4775.
18. Pane A, Salvemini M, Delli Bovi P, Polito C, Saccone G: **The transformer gene in Ceratitis capitata provides a genetic basis for selecting and remembering the sexual fate.** *Development* 2002, **129(15)**:3715-3725.
19. Dafa'alla T, Fu G, Alphey L: **Use of a regulatory mechanism of sex determination in pest insect control.** *J Genet* 2010, **89(3)**:301-305.
20. FAO/IAEA/USDA: *Product quality control and shipping procedures for sterile mass-reared tephritid fruit flies* Vienna, Austria: International Atomic Energy Agency; 2003.
21. Tzanakakis KE, Tsitsipis JA, Economopoulos AP: **Frequency of mating in females of olive fruit fly under laboratory conditions.** *J Econ Entomol* 1968, **61**:1309-1312.
22. Kraaijeveld K, Chapman T: **Effects of male sterility on female remating in the Mediterranean fruit fly, Ceratitis capitata.** *Proc Biol Sci* 2004, **271**:209-11.
23. European Union: **Report from the Commission to the Council and the European Parliament on the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council on genetically modified food and feed.**[http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:52006DC0626:EN:HTML].
24. European Union: **Commission Implementing Regulation (EU) No 543/2011 of 7 June 2011 laying down detailed rules for the application of Council Regulation (EC) No 1234/2007 in respect of the fruit and vegetables and processed fruit and vegetables sectors.** *Official Journal of the European Union* 2011, 543/2011.
25. Alphey N, Bonsall MB, Alphey L: **Modelling resistance to genetic control of insects.** *J Theor Biol* 2011, **270(1)**:42-55.
26. Alphey N, Bonsall MB, Alphey L: **Combining pest control and resistance management: synergy of engineered insects with Bt crops.** *J Econ Entomol* 2009, **102(2)**:717-732.
27. Alphey N, Coleman PG, Donnelly CA, Alphey L: **Managing insecticide resistance by mass release of engineered insects.** *J Econ Entomol* 2007, **100(5)**:1642-1649.
28. Dimou I, Rempoulakis P, Economopoulos AP: **Olive fruit fly [Bactrocera (Dacus) oleae (Rossi) (Diptera:Tephritidae)] adult rearing diet without antibiotic.** *J Appl Entomol* 2009, **134**:72-79.
29. Estes AM, Nestel D, Belcari A, Jessup A, Rempoulakis P, Economopoulos AP: **A basis for the renewal of sterile insect technique for the olive fly, Bactrocera oleae (Rossi).** *J Appl Entomol* 2011, **136**:1-16.
30. Rempoulakis P, Nestel D: **Dispersal ability of marked, irradiated olive fruit flies [Bactrocera oleae (Rossi) (Diptera: Tephritidae)] in arid regions.** *J Appl Entomol* 2012, **136**:171-180.
31. Koukidou M, Klinakis A, Reboulakis C, Zagoraiou L, Tavernarakis N, Livadaras I, Economopoulos A, Savakis C: **Germ line transformation of the olive fly Bactrocera oleae using a versatile transgenesis marker.** *Insect Mol Biol* 2006, **15(1)**:95-103.
32. Lukyanov KA, Fradkov AF, Gurskaya NG, Matz MV, Labas YA, Savitsky AP, Markelov ML, Zaraisky AG, Zhao X, Fang Y, Tan W, Lukyanov SA: **Natural animal coloration can Be determined by a nonfluorescent green fluorescent protein homolog.** *J Biol Chem* 2000, **275(34)**:25879-25882.
33. Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, Lukyanov SA: **Fluorescent proteins from nonbioluminescent Anthozoa species.** *Nat Biotechnol* 1999, **17(10)**:969-973.
34. Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ, Pape G, Fu G, Condon KC, Scaife S, Donnelly CA, Coleman PG, White-Cooper H, Alphey L: **Late-acting dominant lethal genetic systems and mosquito control.** *BMC Biol* 2007, **5**:11.

doi:10.1186/1741-7007-10-51
Cite this article as: Ant *et al.*: Control of the olive fruit fly using genetics-enhanced sterile insect technique. *BMC Biology* 2012 **10**:51.