Development and Preparation of *Aedes aegypti* Strains For The Purpose of Genetic Population Control

Zoë Curtis

Department of Life Sciences,

Imperial College London

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Authors Declaration

I declare that all the work presented here is my own original research subject to the following acknowledgements: technical assistance was provided by Pam Gray, Dr Kelly Matzen and Heather Haines for egg collection for the individual penetrance of phenotype (Chapter 2.2.6). Dr Sarah Scaife identified the plasmid backbone in line OX4358E4 and characterised the SINE insertion isolated in the OX4358B line (Chapter 3.3.2 and Chapter 9). Caroline Phillips provided PCR services to aid the homozygosing process of OX4358B (Chapter 3.2.3). These services are acknowledged in the text.

Copyright Declaration

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Abstract

Aedes aegypti is the primary vector of dengue fever, a disease with an estimated incidence of 390 million cases per year. Vector control methods, which are used to control the spread of this disease, are proving to be unsatisfactory at preventing the spread and increasing incidence of the disease. A novel species-specific vector control tool is currently being trialled as an alternative method to limit transmission. This method, termed self-limiting uses tetracycline repressible dominant gene constructs to elicit population suppression when transgenic mosquitoes are released into the field.

This thesis examines self-limiting strain development and characterisation as well as developing a better understanding of the fitness costs and effects on released males. The lead *Ae. aegypti* strain, OX513A is characterised in preparation for global use while a novel male-selecting strain is taken through its initial selection criteria to determine its suitability to become a product.

These two self-limiting strains are assessed to determine their fitness, as well as developing our understating of what the influential components of rearing are on released males fitness. The tetracycline analogue used to rear the transgenic strains is shown to have a significant effect on transgene suppression and population selection. Male body size is shown to significantly affect the mating competiveness of released males depending on the body size of the female being competed for. Finally novel field relevant fitness assays are developed to assess insect quality more rapidly, with a view to being used at the point of male release.

In conclusion this thesis develops the understanding of self-limiting strains both in terms of phenotype and insect fitness. It highlights changes that should be made to the strain maintenance and mass production process to prevent laboratory population selection, as well as increasing the fitness and quality of the insects as they are released into the field.

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Acronyms

AeAct-4	*Aedes aegypti* Actin 4
*AeAct-4*SINE	*Aedes aegypti* Actin 4 with SINE insertion
AealbAct-4	*Aedes albopictus* Actin 4
AWT	*Aedes aegypti* Asian wild type
DALYS	Disability adjusted life years
DMEB	Dengue midget exit barrier wild type
GSS	Genetic sexing strain
IMP	Integrated pest management
IRS	Indoor residual spraying
L1	First instar larvae
LWT	*Aedes aegypti* Latin wild type
NGO	Non-governmental organisation
SD`	Standard deviation
SEM	Standard error of the mean
SIT	Sterile Insect Technique
ST	Serotype
tTAV	Tetracycline transcriptional transactivator
tetO	Tetracycline operator
tetR	Tetracycline repressor
WT	Wild type

Chapter 1 Introduction

There are approximately 3,200 species of mosquito (Diptera: Culicidae) globally (Becker, 2010), of which a small proportion are vectors of human disease, responsible for causing in excess of 1.5 million deaths annually (WHO, 2009, 2013). Most of the deaths are caused by malaria, a parasite transmitted by mosquitoes in the *Anopheles* genus however other diseases impose significant global disease burden, notably dengue fever of which there is an estimated 390 million cases annually (Bhatt *et al.*, 2013).

1.1. Dengue

Dengue is the most rapidly spreading mosquito borne disease in the world. In the past five decades, the incidence of dengue fever has increased 30 fold, with over 2.5 billion people, more than 40% of the world's population, now living in areas where dengue can be transmitted (Figure 1-1 and Figure 1-2) (WHO, 2012, 2013). This is in part due to rapid human population growth in tropical countries along with unprecedented urbanization in those same countries (Gubler, 2011a). The full global burden is uncertain, but it is estimated that 264 disability adjusted life years (DALYS) per million population are lost at an estimated cost of US$514 to US$1394 for ambulatory and hospitalized cases (WHO, 2012).

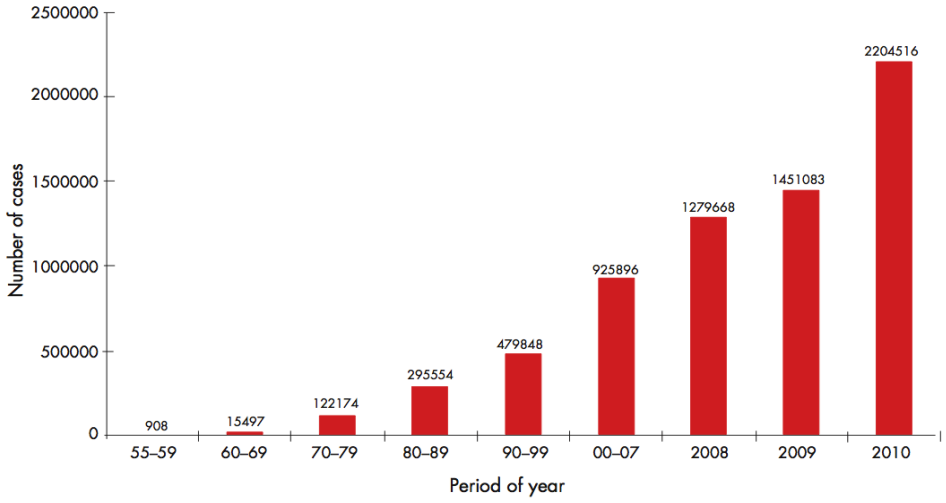


Figure 1-1 Average number of dengue and severe dengue cases reported to WHO for each time interval stated.

Annual reports from 1955-2007 and number of cases reported in recent years, 2008-2010. Note the changing scale on the x-axis. Figure source (WHO, 2012).

Dengue fever is caused by a virus in the genus *Flavivirus*, of which there are four closely related but distinct serotypes, and potentially a fifth serotype having been discovered in Malaysia (CIDRAP, 2014; Pasteur, 2014; Mustafa *et al.*, 2015). Infection and recovery from one serotype results in lifelong immunity against that specific serotype (Yacoub *et al.*, 2013), however, subsequent infection with a different serotype can result in severe dengue; dengue hemorrhagic fever (WHO, 2013).

To date, there is no specific medication available for dengue other than early diagnosis and intravenous rehydration (CDC, 2012). This practice can reduce mortality to almost zero, however, it relies upon access to health centres and the availability of rapid diagnostic tests that many people living in dengue risk areas do not have (WHO, 2013).

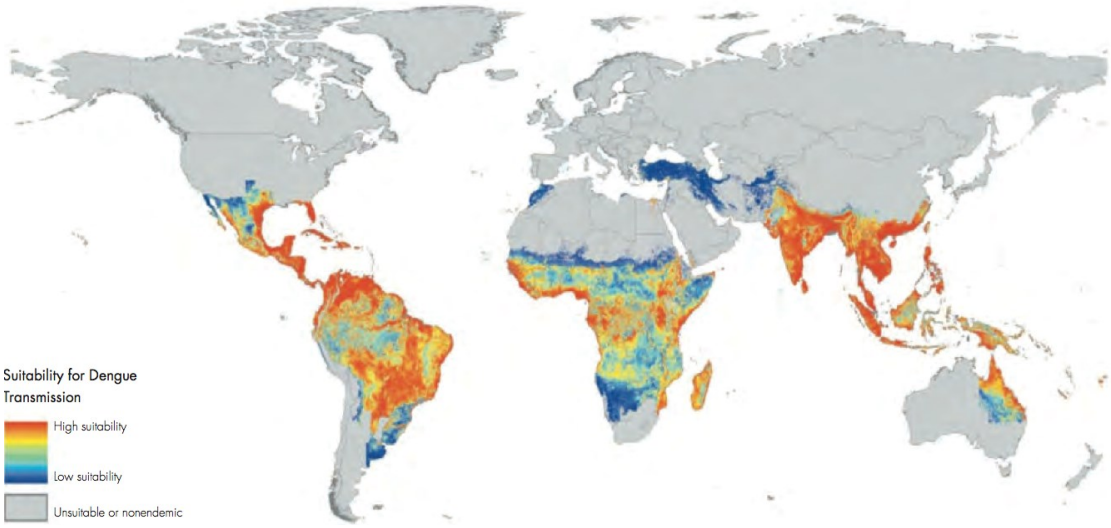


Figure 1-2 Distribution of global dengue risk.
Determination of risk status based on combined reports from WHO, United States Centres for Disease Control and Preventions, Gideon online, ProMed, DengueMap, Eurosurveillance and published literature). Figure source (WHO, 2012).

Several vaccines are being developed with the most advanced being a live-attenuated, chimeric, yellow fever-dengue virus vaccine (ChimeriVax-Dengue) having recently completed phase III clinical trials (Guy *et al.*, 2011). This vaccine has shown to have a varied efficacy against each of the four serotypes (ST); ST1 50.3%, ST2 42.3%, ST3 74.0%, ST4 77.7% (Sabchareon *et al.*, 2012; Mullard, 2014) with the results raising a particular concern at the low level of immunity provided to serotype 1 and 2 (Mullard, 2014). For the vaccine to reach this level of immunity three separate injections are required (Guy *et al.*, 2011), making

the timely delivery of such a vaccine to communities complicated for public health programmes, and with a greater associated cost (Ghosh & Dar, 2015). Further to this, the long-term safety of the vaccine has recently been called to question as vaccinated children under 9 years old appear to be developing an increased incidence of severe dengue requiring hospitalization (Hadinegoro *et al.*, 2015).

1.2. Biology of the mosquito vector

Dengue fever is transmitted by two species of *Aedes* mosquito; *Aedes aegypti* and *Aedes albopictus*. Both of these species are highly anthropophilic and closely associated with urban environments (Jansen & Beebe, 2010). Unlike most other mosquito species, the eggs of these two species are able to withstand desiccation and remain viable for up to 12 months (Christophers, 1960). This has enabled these species to be transported around the world by various means for example in used car tyres, and has resulted in them establishing in many countries; they are now considered endemic in most tropical and some sub-tropical counties (Jansen & Beebe, 2010; WHO, 2013).

Figure 1-3 Adult *Aedes aegypti*

Male (left) and female (right). Figure original.

Ae. aegypti (Figure 1-3) is considered the dominant vector of the dengue virus. The first part of this species lifecycle (Figure 1-4) is aquatic, passing through four larval instars (L1, L2, L3 & L4) which under optimum conditions take around seven days. Factors that contribute to the optimum conditions include nutrition (Van Handel, 1988; Couret *et al.*, 2014), resource availability (Kesavaraju *et al.*, 2014), and temperature (Eisen *et al.*, 2014; Lopes *et al.*, 2014; Reiskind & Janairo, 2015). Individuals then enter a pupal stage for approximately 48 hours before eclosing as flying adults. Males generally pupate and eclose as adults before females

and take approximately 18 to 24 hours to reach sexual maturity (Roth, 1948), whereas females are receptive to mating soon after they have emerged (Christophers, 1960).

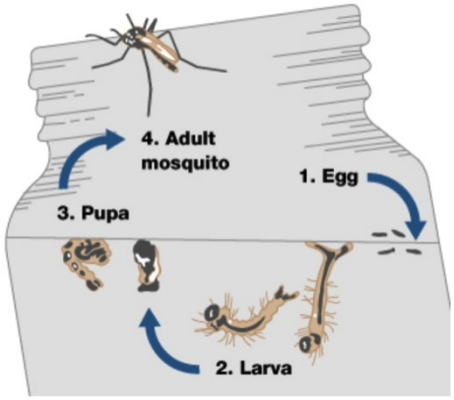


Figure 1-4 *Aedes aegypti* lifecycle.
Eggs are laid at the water surface, larvae hatch and then pupate. After this they eclose as flying adults. Figure source (Nature Education, 2011).

Many mosquito species form swarms to attract and compete for mates, however *Ae. aegypti* are not strictly a swarming species, rather the congregation of males results in a swarm but swarming is not part of the mating process (Oliva *et al.*, 2014). Males locate females by being attracted to the same locations as females for example, blood meal hosts and potential breeding sites (Oliva *et al.*, 2014). It is also thought that both males and females produce an aggregation pheromone that attracts other competing males as well as females to the location (Cabrera & Jaffe, 2007; Fawaz *et al.*, 2014), however this chemical has yet to be isolated and identified. *Ae. aegypti* males and females recognise each other and distinguish between other species by the frequency emitted by their wing beat whist in flight (Brogdon, 1994; Cator *et al.*, 2011; Arthur *et al.*, 2014; Oliva *et al.*, 2014). It is thought the potential mating couple will then harmonise their wing beats and proceed to mate whist still in flight (Cator *et al.*, 2009; Cator & Harrington, 2011). Mating takes approximately 10 seconds (Roth, 1948; Jones, 1974). Males are polygamous and able to mate at least five times in their lifetime (Ponlawat & Harrington, 2007; Bargielowski *et al.*, 2011a; Helinski & Harrington, 2011) whereas females will generally only mate once (Richardson *et al.*, 2015), with the single mating event providing enough sperm and accessory gland secretions to fertilise all of her eggs (Helinski & Harrington, 2011; Helinski *et al.*, 2012b; Boes *et al.*, 2014).

Adult males and females feed on nectar for energy but females also require blood meals to provide protein for egg production (Christophers, 1960). Being a highly anthropophilic species, the females will preferentially take blood meals from humans; this is the route of transmission of the dengue virus between human individuals (Jansen & Beebe, 2010; Guha *et al.*, 2012). As *Ae. aegypti* is closely associated with urban environments it has adapted to breed in its surroundings, consequently females will lay eggs in water collected in refuse containers, flower vases, water storage tanks, discarded tyres etc (Christophers, 1960; Jansen & Beebe, 2010; Rubio *et al.*, 2011). Females can lay eggs two days after taking a blood meal (and having mated) and will continue to lay egg clutches throughout her lifetime (Christophers, 1960). An average egg clutch will contain 85 eggs (Christophers, 1960).

Ae. aegypti acts as a vector of the dengue virus when the female mosquito takes a blood meal from an infected human host. The virus is taken up in the blood, enters the midgut and multiplies. From here the virus disseminates to the hemocoel and over the following 10 days, travels to and concentrates in her salivary glands (Salazar *et al.*, 2007). Once in the salivary glands, the virus will be transmitted to human hosts when the mosquito takes a blood meal (Chan & Johansson, 2012). Males do not require blood meals and are therefore not vectors of disease (Wahid *et al.*, 2002).

1.3. Control of the Vector

To date there are no registered vaccines available to protect humans from dengue fever and therefore control of dengue fever has been, and still is, focused on eliminating the mosquito vector (Guzman & Harris, 2014).

In the 20th Century a few major *Ae. aegypti* control efforts were undertaken and achieved success, notably in the Americas by the Pan American Sanitary Board, in Cuba and in Singapore. These programmes were large-scale, rigorous, military-like vector control programmes that used broad-spectrum insecticides and ultimately managed to eliminate disease transmission (Alphey *et al.*, 2010; Gubler, 2011b). However, the control operations stopped in the Americas due to their own success and ensuing complacency (Soper, 1963; Schliessmann, 1964), and in Cuba they were terminated due to the fall of the Soviet Union and consequent lack of funds (Gessa & Gonzalez, 1986; Gubler, 2011b). Since this time, *Ae. aegypti* populations have soared with Brazil reporting 1.2 million dengue cases in 2010 (Gubler, 2005; WHO, 2012). Singapore has continued its vector control operations with a

combination of larval source reduction, public education and law enforcement but still, the authoritative driven approach does not eliminate the endemic dengue incidence with the current vector control tools (Ooi *et al.*, 2006; Shepard *et al.*, 2013; Singapore Government, 2014; National Enviroment Agency, 2015; Struchiner *et al.*, 2015).

1.3.1. Adult control

The arsenal available to target dengue-transmitting mosquitoes is limited; DDT (dichlorodiphenyltrichloroethane), which was effectively used in control efforts in the 1950s to 1970s (Gahan *et al.*, 1945; Wright *et al.*, 1972; Klassen, 2009), is now prohibited in many countries due to its lethal bioaccumulation in species higher in the food chain (Stokstad, 2007). Other insecticides developed for vector control include pyrethrins, pyrethroids and malathion which have desirable chemical characteristics, for example a short contact time (the time the insect must be in contact with the chemical for it to have a lethal effect), a high residual efficacy (the duration the chemical maintains its effectiveness) and a low human toxicity profile (N'Guessan *et al*., 2007). This made these chemicals effective for control, however over use in agriculture and in vector control have resulted in most populations having a high degree of resistance which makes their use ineffective (Harris *et al.*, 2010; Oxborough *et al.*, 2010). Behavioural traits of adult *Ae. aegypti* also make their control using insecticides more difficult as the adults tend to rest inside houses making space spraying (fogging) with these insecticides ineffectual (Marcombe *et al.*, 2011; Bonds, 2012). Instead, vector control operators must enter people's houses and spray the walls within the dwelling, a technique known as indoor residual spraying (IRS). This process is intrusive, time consuming and does not always have public compliance (Montgomery *et al.*, 2010). Insecticide treated bed nets, which have lowered malaria incidence in many communities (Binka *et al.*, 1998; Clarke *et al.*, 2001; Minakawa *et al.*, 2015), are ineffective against *Ae. aegypti* as this species takes blood meals during the day when hosts are at school or work and not protected by the net (Anders & Hay, 2012; Wilson *et al.*, 2014).

1.3.2. Larval control

To date, the most effective methods of *Ae. aegypti* vector control are based around controlling or destroying breeding sites. These methods include control teams scouring urban areas and private proprieties for containers that might act as egg laying sites for *Ae. aegypti* and either removing them or treating them with a larvicide to kill developing larvae.

A number of different larvicide approaches are available: chemicals, for example temephos, malathion and pyriproxyfen (Wang *et al.*, 2013; Marcombe *et al.*, 2014) and non-chemical approaches for example Bti (*Bacillus thuringiensis israelensis*). Bti is a naturally occurring bacterium that during reproduction produces an endotoxin that is eaten by the developing larvae (Boyce *et al.*, 2013). This lyses the midgut epithelia cells and results in larval death. This bacterium can be applied to water storage tanks that act as breeding sites to eliminate mosquito larvae, whilst still being safe for human consumption (Tan *et al.*, 2012; Jacups *et al.*, 2013). Bti has been shown to still have a high efficacy at killing *Ae. aegypti* where chemical larvicides fail due to evolved resistance (Wang *et al.*, 2013; Koou *et al.*, 2014), and can be effectively used to control populations (Jacups *et al.*, 2013) however, the application of the larvicide must encompass all larval breeding sites and therefore Bti's overall effectiveness to population reduction can be reduced.

A second larvicide option is the use of copepods, a minute fresh water crustacean, which can be added to large water storage containers to establish populations and predate the mosquito larvae (Phong *et al.*, 2008; Mahesh Kumar *et al.*, 2012; Sinh Nam *et al.*, 2012). Elimination of *Ae. aegypti* has been achieved in some communities and a corresponding decrease in dengue incidence observed (Kay & Vu, 2005; Phong *et al.*, 2008; Soumare & Cilek, 2011; Mahesh Kumar *et al.*, 2012; Sinh Nam *et al.*, 2012), however this method is only effective when there are large identifiable *Ae. aegypti* breeding sites that represent suitable habitats for copepods (Lazaro *et al.*, 2015). The method also involves rigorous community engagement to maintain the copepod populations in the water containers (Jansen & Beebe, 2010).

1.3.3. Population replacement

A novel approach to vector control which is currently being tested is using *Wolbachia pipientis,* an intracellular bacterium that naturally infects many arthropod species and is known to affect their lifespan (Iturbe-Ormaetxe *et al.*, 2011). *Wolbachia* spreads through insect populations by a process known as cytoplasmic incompatibility (CI) that occurs when an uninfected female mates with an infected male and produces non-viable embryos (unidirectional CI). Conversely an infected female mated to an infected or uninfected male produces viable embryos, all of which carry the bacteria, consequently selecting for *Wolbachia* infection and allowing it to spread through populations. This attribute allows for a process known as population replacement (Alphey, 2009; Alphey *et al.*, 2013; Robert *et al.*,

2013) whereby a natural wild population are replaced with a more favourable strain, in this case a strain with a lower dengue vectorial capacity along with a reduced lifespan which should further prevent dengue transmission.

Ae. aegypti has been artificially infected with two strains of *Wolbachia* known as *w*Mel and *w*MelPop (McMeniman *et al.*, 2008) each of which have been shown to produce differing phenotypes. *w*MelPop has been shown to halve the average laboratory lifespan of individuals (McMeniman *et al.*, 2009; Yeap *et al.*, 2011) and strongly blocks dengue transmission by exerting a strong inhibition on the dissemination of the dengue virus from the mosquito's midgut, therefore lowering the vectorial capacity (Moreira *et al.*, 2009a; Frentiu *et al.*, 2010; Iturbe-Ormaetxe *et al.*, 2011) however, this strain also appears to have a high fitness cost including reduced fecundity (Yeap *et al.*, 2011; Turley *et al.*, 2013), reduced ability to blood feed (Moreira *et al.*, 2009b; Turley *et al.*, 2009), reduced egg viability (Ritchie *et al.*, 2015) and altered development (Yeap *et al.*, 2011; Yeap *et al.*, 2013). The reduced lifespan may further decrease the ability of these populations to spread dengue by rendering individuals unable to survive the extrinsic incubation period of dengue virus (Tjaden *et al.*, 2013). The second strain, *w*Mel, is less effective at blocking dengue transmission (Moreira *et al.*, 2009a) but exerts a lower fitness cost and therefore can be considered a fitter strain (Walker *et al.*, 2011).

Open field trials of *w*Mel and *w*MelPop *Ae. aegypt*i have been carried out in Queensland, Australia and have further highlighted the differing fitness costs. *w*Mel has successful invaded two natural populations of *Ae. aegypti* (Hoffmann *et al.*, 2011) and has now been established for three years (Hoffmann *et al.*, 2014), however dengue virus replication was only significantly lower than wild type controls for serotype 2 seven days after infection but was significantly lower for all four serotypes 14 days after infection (Frentiu *et al.*, 2014). This raises a concern to the degree of disease prevention that this strain would be able to provide. Releases of the second strain, *w*MelPop, failed to establish in the wild populations due to the high fitness cost of the infection, as described above (Segoli *et al.*, 2014; Yeap *et al.*, 2014). Consequently the potential of *Wolbachia* to eliminate dengue transmission is currently unlikely to be feasible unless the fitness of the *w*MelPop strain can be increased. A drawback to this technology is that for the infection to establish within the wild population, a large number of *Wolbachia* infected females have to be released into the trial area; this may have a negative impact on the human population due to the increased nuisance biting

and depending on the strain being released, has differing degrees of dengue vectoral transmission capacity.

1.3.4. Female immigration

An issue that plagues many *Ae. aegypti* vector control efforts is the immigration of adults from untreated areas into the control areas (Dame *et al.*, 2009; Klassen, 2009). This can occur by females flying from neighbouring urban areas, being transported in vehicles e.g. cars and planes or being transported as eggs, which particularly affects shipping and transport hubs (Gubler, 2011a). This movement of individuals can result in the rapid re-colonization of an area that has been treated. Only a low-density population is required to maintain dengue transmission in an urban area (Koh *et al.*, 2008) and therefore the re-colonisation of a city can quickly resume disease transmission. Consequently if a city or suburb can reduce the vector population to below the disease transmission threshold level (Focks *et al.*, 2000), control effects must be maintained to prevent re-colonisation. This practice is necessary but costly and currently has to be performed with tools that have a decreasing effectiveness with each passing season.

1.4. Sterile Insect Technique

1.4.1. Principle and past applications

The sterile insect technique (SIT) is a concept pioneered by Edward F Knipling who hypothesised that if sterile males were released at a rate high enough to cause decline in the wild population, then the sustained released of this constant number of sterile males into the wild population each generation will achieve a progressively greater degree of suppression in the wild population in each successive generation (Knipling, 1955; Klassen, 2009). Furthermore Knipling hypothesised that this method would be even more effective if used as part of an integrated pest management system (IPM) where cultural, biological and chemical measures would be first used to decrease the wild population by 90% to 98% and then SIT would be used to drive the population to elimination (Knipling, 1979; Klassen, 2009).

This species-specific pest control method has been successfully used over the last 60 years to control a variety of agricultural and public health pests. In the 1950s the method was used to eradicate New World Screw Worm from the USA, Mexico, and from Central America

down to Panama (Wyss, 2000). In the 1970s Mediterranean fruit fly was eradicated from Central America to southern Mexico (Hendrichs *et al.*, 1983). In the 1980s Japan used SIT to control melon fly (Ito *et al.*, 2003) and in 1997 tsetse fly was eradicated from Zanzibar (Vreysen *et al.*, 2000). SIT has also been successfully used to control mosquito populations in El Salvador in the 1960s and in India in the 1970s. India showed that *Culex quinquefasciatus* could be mass reared, sexed to 99.8% accuracy (based on pupal size), and either sterilised with a chemosterilant or sterility was induced by male-linked chromosome translocations combined with cytoplasmic incompatibility or sex-ratio distortion due to meiotic drive (Klassen & Curtis, 2005; Klassen, 2009). Released sterilised males were competitive with wild males, but the trial was less successful than hoped due to immigration of pre-mated females into the trial site (Klassen & Curtis, 2005; Klassen, 2009). In El Salvador, *Anopheles albomanius* was suppressed using males with a chromosome translocation linked resistance to the insecticide propoxur. Treatment with propoxpur at the egg stage resulted in elimination of 99.8% of the female population for release and the released males were almost fully competitive (Lofgren *et al.*, 1974; Weidhaas *et al.*, 1974). Unfortunately, due to political unrest in both countries, the trials were terminated prematurely (Klassen & Curtis, 2005).

Although some of the mosquito SIT trials were successful they highlighted issues that need to be addressed before SIT can be used as a stable part of a mosquito control programme, these are;

1 Sex separation – some mosquito species are readily separated at the pupal stage due to morphological size differences, while with others this is not an option (Gilles *et al.*, 2014). Male only mosquito releases are important for two reasons; firstly released females could increase the transmission of disease and secondly co-releases of males and females result in males courting released females rather than dispersing in search of wild females (McInnis *et al.*, 1994; Alphey & Andreasen, 2002; Rendon *et al.*, 2004).
2 Mating competitiveness – the greater the mating competitiveness of released males for mating opportunities with wild females, the fewer males that need to be released, which reduces the programme cost (Koenraadt *et al.*, 2010). Reductions in mating competitiveness have been highlighted in previous trials (Dame & Woodward, 1964; Baker *et al.*, 1980; Reisen *et al.*, 1981) and point towards irradiation (Patterson *et al.*,

1975; Patterson *et al.*, 1977), handling (Dame *et al.*, 2009), transport, and distribution (Klassen & Curtis, 2005; Klassen, 2009).

3 Sufficient production – the consistent and reliable production of a genetically diverse population is crucial to allow regular, high-quality releases of fit individuals to achieve the desired over flooding ratio (sterile:wild male ratio) (Benedict *et al.*, 2009; Dame *et al.*, 2009; Carvalho *et al.*, 2014).

4 Immigration of pre-mated females into the suppression site – these females are generally not receptive to mating by the sterilised males but lay fertilised viable eggs, which maintain the mosquito population (Klassen & Curtis, 2005; Klassen, 2009).

1.4.2. Self-limiting Insects

Sterilisation of mosquitoes using irradiation or chemosterilisation results in random dominant lethal mutations which can have negative fitness effects on the surviving adults (Helinski *et al.*, 2009). A novel approach is to insert a repressible, dominant lethal gene into the mosquitoes genome which can be induced or repressed when required (Figure 1-5). In this system, termed self-limiting, males are released to mate with wild females and their progeny inherit a copy of the dominant lethal transgene. Without the addition of the lethal genes antidote, these progeny die. As with other SIT programmes, this principle requires the sustained release of cohorts of the self-limiting insects as the released cohort dies, along with their progeny, resulting in the transgene being eliminated from the environment.

In this self-limiting system, individuals reared in the absence of the antidote produce a toxic protein, tTA (tetracycline transcriptional transactivator), that binds to tetO (tetracycline operator) and drives more expression of tTA and results in the individual's death (Figure 1-5) (Phuc *et al.*, 2007). When individuals are reared in the presence of the antidote, tetracycline, tTA undergoes a conformational change that prevents it from binding to tetO and consequently prevents the positive feedback loop of tTA production (Kisker *et al.*, 1995; Baron *et al.*, 1997). This allows the individual to survive to normal adulthood. This repressible control system allows large numbers of insects to be reared to adulthood in a factory by adding tetracycline to the larval diet, but progeny of the released males will not have access to the antidote in the larval breeding pools, and consequently die before becoming disease vectors. This will result in population suppression (Phuc *et al.*, 2007).

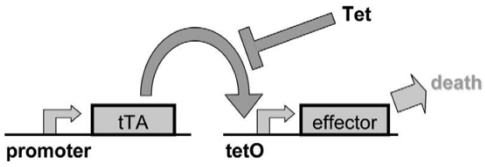


Figure 1-5 The tetracycline repressible system.

Composed of two parts: tetracycline transcriptional activator (tTA) protein is under the control of a promoter. When expressed, tTA binds to tetO driving expression of a minimal promoter and then expression of an effector gene. In the presence of tetracycline (Tet), the tTA protein is unable to bind to tetO, preventing the expression of the effector gene (Alphey, 2002). Figure adapted from (Gong *et al.*, 2005)

The design of this system allows a substantial amount of flexibility and control; for example, the initial production of tTA is under the control of a promoter that can be chosen to be tissue specific or developmental stage specific (Alphey & Andreasen, 2002). This will determine the time of the lethal effect, the location and impact of the toxic effects or even the sex specificity of the lethal effect. Secondly the effector gene can be moderated so that it is lethal at low levels, or has an accumulator lethal effect (Marrelli *et al.*, 2006; Catteruccia *et al.*, 2009). In addition to this, a fluorescent marker can be incorporated into the transgene construct. This is an important tool in the self-limiting system which although it does not play a part in the lethal effect of the transgene, it allows the identification of individuals which are carriers of the transgene, both in the laboratory and in the field. This aids the identification, processing and handling of strains in the laboratory whereas in the field, it is a vital tool to allow the monitoring of the success of the suppression trial by the percentage of individuals in the whole population that are fluorescent (Harris *et al.*, 2012; Facchinelli *et al.*, 2013). This tool is absent from traditional SIT methods and therefore makes monitoring of the trial harder to estimate (Simmons *et al.*, 2011; Juan-Blasco *et al.*, 2013).

1.4.3. Oxitec Product self-limiting strains

A product self-limiting *Ae. aegypti* strain has been developed at Oxitec Ltd that results in both male and female death at a late larval/pupal stage. This strain, OX513A, was first transformed in 2002 and the initial characterisation resulted in a phenotype of >95% death of both males and females when larvae were reared in the absence of tetracycline (Phuc *et*

al., 2007). When reared in the presence of tetracycline, survival was comparable to wild-type individuals (Phuc *et al.*, 2007).

The OX513 construct consists of tTAV (a variant of tTA) under the control of its own binding site tetO, a minimal promoter hsp70 and a 3' UTR sequence (Figure 1-6). tTAV is a fusion of two proteins: tetR, the tetracycline repressor protein and VP16, a transcription activating domain (Baron *et al.*, 1997; Berens & Hillen, 2003). In the absence of tetracycline, tTAV tetracycline binds to tetO and drives expression of more tTAV in a positive feedback loop. High levels of tTAV expression are toxic, likely due to transcriptional squelching resulting from an overabundance of the transactivating domain VP16 (Kuhnel *et al.*, 2004), this results in the individuals' death. In the presence of the antidote, tTAV binds to the tetracycline inducing a conformation change in the tetR component (Berens & Hillen, 2003), which prevents its binding to tetO. This inhibits further expression of tTAV and allows normal development (Figure 1-5).

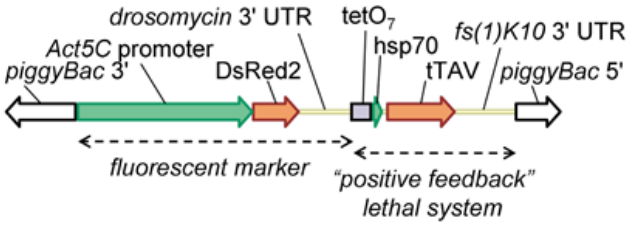


Figure 1-6 Plasmid construct OX513.

Figure source (Phuc *et al.*, 2007).

1.4.4. Field trials with self-limiting Insects

The product strain OX513A was first released into the field in 2009 in Grand Cayman as part of a proof of principle field trial (Harris *et al.*, 2011). In 2010 the strain entered a suppression trial which, over the course of six months of male only releases, 85% suppression of the wild *Ae. aegypti* population was achieved in the 16 Ha treatment area (Harris *et al.*, 2012). Subsequent field trials using OX513A have resulted in suppression of the wild population by 85% in Jacobina, Brazil (personal communication with Dr A McKemey), 94% in Itaberaba Brazil (Carvalho *et al.*, 2015) and 93% suppression in Panama (Gorman *et al.*, 2015). Together, these results demonstrate that self-limiting strains are effective at supressing wild populations of *Ae. aegypti* and can be used as effective vector control tools.

1.4.5. Mass production

Self-limiting insects have a limited lifespan when released into the environment and consequently large numbers of males need to be reliably and consistently produced for their sustained release. This process must be to a high standard to produce quality insects and be as cost efficient as possible. Figure 1-7 shows the current mass production process (Carvalho *et al.*, 2014). Eggs are hatched under vacuum to induce a synchronous hatch rate (Christophers, 1960). The first instar larvae are aliquoted into trays to a final density of 3 larvae mL^{-1} and fed a standard regimen of fish food. Upon the onset of pupation, the pupae are separated from the larvae using a Larval-Pupal Separator (LPS), which draws the larvae through the gaps while trapping the pupae. After this the collected pupae are sexed based on size using a wire sorter: the males are smaller than the females and therefore controlling and adjusting the gap between the wires allows sex sorting to an accuracy of >99.8% (Harris *et al.*, 2012). For field release, the male pupae are collected and aliquoted into release devices, allowed to eclose and released into the field. For egg production, the male and female pupae are aliquoted into cages, allowed to eclose, blood fed and eggs collected.

1.4.1. Fitness

Insect fitness is an integral part of the success of an SIT programme (Alphey, 2002), indeed, previous SIT programmes which have failed to supress wild populations often cite a reduced insect fitness as being a causative agent (Benedict & Robinson, 2003; Dame *et al.*, 2009). Fitness is best understood as the ability to produce offspring (Scott *et al.*, 2006). In the context of an SIT programme, the released males must be able to fly and locate females, be recognised as a potential mate, successfully out-compete other male suitors, mate with the female and ideally repeat the process. Our understanding of the components involved in *Ae. aegypti* fitness are only just coming to light, and there is still much to learn (Lees *et al.*, 2014; Oliva *et al.*, 2014).

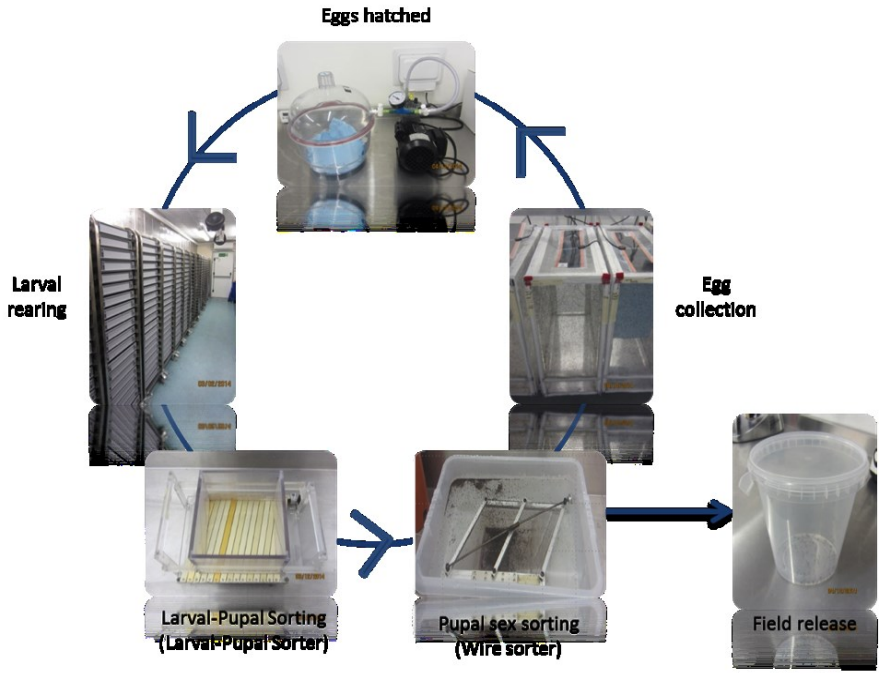


Figure 1-7 Mass production process of self-limiting mosquitoes.
Eggs are hatched under vacuum and the hatched larvae aliquoted into trays. Pupae are sorted from the remaining larvae using a Larval-Pupal Sorter (LPS). The isolated pupae are sexed based on size using a Wire Sorter. For field release, male pupae are aliquoted into the release device and allowed to eclose, and released into the field. For egg production, the male and female pupae are aliquoted into cages, allowed to eclose, blood fed and eggs collected. (Figure original).

The fitness of a self-limiting strain can be impacted both within a generation and between generations with three main sources: i) the transgene, ii) colonisation of the genetic background and inbreeding of the transgene and iii) the mass production process.

i) The insertion of a transgene into the genome can result in a fitness penalty and the severity of the penalty depends on the location into which it has inserted (Meza *et al.*, 2014). It is likely that insertions will be fatal if they have inserted into genes that are important in the maintenance and regulation of physiological processes (Marrelli *et al.*, 2006). It has been shown however that many insertions do not result in fatal mutagenesis as it is presumed many transgenes insert into non-coding regions of the genome or into areas which do not significantly disrupt native gene function (Lyman *et al.*, 1996; Marrelli *et al.*, 2006).

The expression of the transgene itself can result in a fitness penalty due to the accumulation of foreign proteins in cells. In terms of self-limiting insects, the accumulation of toxic proteins to result in cell death is desirable, however the leaky expression of these proteins or off target expression would result in an undesirable fitness cost (Alphey, 2002). This also applies to the addition of the antidote, tetracycline: in the absence of a sufficient quantity of the antidote, it is feasible to presume that there could be sub-lethal fitness penalties associated with a low level accumulation of toxic proteins which may result in significant fitness costs within the laboratory rearing system. In addition the accumulation of accessory proteins, for example the fluorescent marker could have a fitness cost (Marrelli *et al.*, 2006), although evidence suggests that under laboratory conditions this may not be significant (Liu *et al.*, 1999; Irvin *et al.*, 2004). This emphasises the control required when designing the construct to ensure that regulation of the timing and level expression of the effector gene is maximised.

ii) At the beginning of any SIT programme, a strain of the desired species must be colonised from the field into the laboratory. This process often results in a high degree of selection and Koenraadt *et al.* (2010) has shown that a wild type colony maintained in the laboratory for over 10 years was significantly less fit (in terms of survival, development rate and size) than the second generation wild collection. The process of making transgenics requires further selection: a single transgenic individual will be selected and outcrossed to the desired background strain and then made homozygous, after which the strain will be inbred within the mass production process (Harris *et al.*, 2012). The loss of fitness is thought to be as a result of inbreeding depression: most organisms carry numerous recessive mutations (Simmons & Crow, 1977; Halligan & Keightley, 2003) that have the capacity to reduce fitness and fixation of these alleles will result in inbreeding depression. In the processes of making a transgenic strain homozygous it is possible that the strain will also become homozygous for the recessive mutational alleles; this is known as the hitchhiking effect (Marrelli *et al.*, 2006). The process of maintaining a strain as homozygotes rather than heterozygotes (whereby the transgenic individuals are effectively outcrossed to wild type each generation) has been shown by Moreira *et al.* (2004) to induce a greater fitness cost. Therefore the transgenic strain selection and homozygosing process results in the loss of genetic diversity and the consequent loss of fitness of the transgenic population.

iii) Due to cost limitations, the mass rearing process for egg production and male release results in a process which may be considered stressful for the developing insects. Until we have a better understanding of what rearing aspects contribute to an insect's fitness, it is difficult to combine a cost efficient rearing process with the requirements to make a fit insect. It is likely that the mass rearing process itself exerts a selection pressure on the insect and consequently selects traits that are desirable in the mass production process for example high fecundity, a fast larval development time and a shorter duration to reach sexual maturity, which may not be desirable in insects destined for field release. The importance of colony management was highlighted in an SIT trial with *Culex tarsalis*, which found the colony to be highly competitive in 1980 (Reisen *et al.*, 1982) but further releases with the same colony in 1981 found the males unable to seek and mate with wild females. This loss of male fitness was attributed to selection during colonisation a few months prior to the 1981 releases (Dame *et al.*, 2009). This highlights the importance of colony management to minimise selection within the colony that is likely to have a negative impact on the effectiveness of the released males.

1.5. Aims of this thesis

To date, self-limiting insects have been shown to be effective at supressing populations in small geographic areas. This technology is now moving towards being used as part of an IPM system and larger stand-alone field trials, which involves a detailed understanding of how the self-limiting strain will perform throughout the world, along with improving the cost effectiveness of the strain by improving our understanding of its fitness. The work in this thesis is split into two sections which represents this process: Chapter 2 and Chapter 3 focus on strain development and characterisation, while the work in Chapter 4, Chapter 5 and Chapter 6 is directed towards understanding and measuring the fitness of the selected self-limiting strains. The aims of this thesis are to further our understanding of the phenotype of self-limiting strains under different conditions to prepare for the scale up in operations around the globe. Further to this, for the strains to become a cost effective and an accessible vector control tool to a range of control budgets, the fitness of the strains are assessed, measured and improved so that fewer insects can be released into the field to achieve wild population suppression.

1.6. Overview of chapters

Chapter 2 is aimed at understanding how the bi-sex lethal strain OX513A will behave under field conditions. It is hypothesised that the rearing conditions of OX513A heterozygotes (temperature, larval food availability, mate background) will not impact the phenotype, and in particular the number of viable adults emerging when reared in the absence of tetracycline. However the results show that the phenotype is impacted by genetic background, and to a lesser extent the rearing conditions.

Chapter 3 is focused around the identification of a male-selecting strain as a cost saving alternative to using the bi-sex lethal strain OX513A. The putative strains are tested for a desirable phenotype, suitable strains are selected and primary strain characterisation is carried out.

Chapter 4 is aimed at understanding the interaction of tetracycline analogues and dose used on the impacts they have on fitness of the two selected self-limiting strains. This work identifies the optimum tetracycline analogue to use in rearing which is likely to minimise fitness costs. This hypothesis is subsequently tested by maintaining populations over multiple generations to determine how the fitness of a strain can be impacted by the tetracycline analogue used. The results determine that doxycycline is the superior tetracycline analogue to use to rear self-limiting strains, rescuing the strains from the self-limiting phenotype at lower concentrations and preventing the accumulation of fitness costs.

Chapter 5 is focusing on quantifying the fitness implications of rearing conditions on strain mating competiveness. The work is orientated around the hypothesis that firstly the two transgenic strains in question do not have a significantly different mating competiveness compared to their non-transgenic counterparts, and secondly, the mating competiveness of the strains cannot be negatively influenced by altered rearing conditions. The results show that mating competitiveness can be reduced by compromised rearing conditions, and in particular the mating success of males is affected by their body size in comparison to the female's body size.

Chapter 6 identifies that the current arsenal of fitness assays are unsuitable to provide quick feedback on changes in insect fitness and therefore aims to develop assays to measure differences in a shorter duration of time. Two assays are developed, both of which are

aimed to measure field relevant fitness components of released males. Along the process of developing these assays, the composition of the male's diet is assessed and optimised with the aim of increasing male longevity and therefore the released male's fitness.

Chapter 2 OX513A strain characterisation

2.1. Introduction

OX513A is a self-limiting bi-sex lethal strain of *Ae. aegypti*. As reported in Phuc *et al.* (2007) when this strain is reared in the absence of tetracycline >95% of the population die before becoming viable disease vectors, however, when sufficient tetracycline is added to the larval rearing medium, the population develops to adulthood. This strain was first used in open field release trials in 2010, when it was successfully used to supress the wild population by >80% (Harris *et al.*, 2012). Since then, additional proof of principle field trials have been performed in Brazil and Panama with similar levels of suppression achieved (Personal communication Dr A McKemmy and Dr K Gorman) (Carvalho *et al.*, 2015).

The open field release of OX513A is the world's first deployment of a genetically modified human disease vector and therefore there is considerable public scrutiny in the way it performs. Since the strain has been shown to be effective at supressing small populations of wild *Ae. aegypti*, focus will now move towards larger control operations and the commercialisation of the product in different countries around the world. For this strain to be operationally successful, and for the product to be commercialised, it is important to further our understanding and parameterise how the strain will perform under different conditions it may encounter in the field, particularly in terms of penetrance of phenotype.

Analysis of the OX513A phenotype is generally performed under permissive laboratory conditions: a constant nominal rearing temperature of 24^{o}C, with an adequate amount of food per mouth (Phuc *et al.*, 2007; Massonnet-Bruneel *et al.*, 2013). Larval development sites in the field will vary from these conditions (Eisen & Moore, 2013; Chaves *et al.*, 2014) and therefore it can be hypothesised that the phenotype of OX513A may deviate from that found in the wild when reared under the permissive conditions of the laboratory. Further to this, it is important to determine if OX513A are able to survive at temperatures outside of the normal habitable range of *Ae. aegypti* to ensure that when released, OX513A are not able to extend the range of this disease vector. By simulating these conditions in the laboratory, it is possible to determine how these two factors, temperature and larval diet, impact the penetrance of the OX513A phenotype to better understand how the strain will perform in field sites located around the world.

Aside from the larval rearing conditions impacting the phenotype of the OX513A progeny in the field, the genetic contribution from the wild female mate (maternal genetic contribution) could hypothetically impact the penetrance of the phenotype. This could occur as a result of other genes altering the expression of the OX513A transgene, impacting the timing of expression or even the level of expression (Yan *et al.*, 2014; Bonasio, 2015). Studies into the genetic diversity of *Ae. aegypti* populations from around the world have shown that in the Pantropical New World and Asia, *Ae. aegypti* is a highly domesticated subspecies of *Ae. aegypti aegypti* originating from Africa (Brown *et al.*, 2014; Kraemer *et al.*, 2015). It is thought that the species left Africa in a single sub-speciation event and has since been intermixed worldwide by globalisation (Mousson *et al.*, 2005; Brown *et al.*, 2014). This has resulted in a relatively homogenous *Ae. aegypti* population in the Pantropical and Asian regions, but much more variation still exists in the African continent (Brown *et al.*, 2014): this may pose an issue with speciation barriers or impacts on phenotype if OX513A or any other self-limiting strain was released in Africa. To date, suppression trials are only focusing on the Pantropical and Asian regions and therefore understanding the impacts of genetic differences in regional *Ae. aegypti* populations from these areas on the phenotype of OX513A is a priority.

As the use of OX513A expands from proof of principle trials to operational control programmes treating greater areas in multiple countries, it is important to understand and be able to predict how the strain will perform and behave. Further to this, as the strain moves to become a commercial product it becomes increasingly important to maintain strain integrity through a rigorous Quality Control system. The data collected here will contribute to the basis of this system, allowing monitoring of the strain integrity to ensure that the released individuals are of a known quality and that they will perform to a consistent and expected standard.

The work in this chapter provides a detailed analysis of the penetrance of phenotype of OX513A reared under permissive and restrictive conditions. Further to this, parameters are investigated which are hypothesised to affect the OX513A penetrance of phenotype of heterozygous progeny developing in the field, these include larval rearing temperature, larval food abundance and maternal genetic contribution.

2.2. Methods

2.2.1. Mosquito Strains

OX513A Rockefeller: The original strain of OX513A, which was transformed in 2002 (Phuc *et al.*, 2007). The OX513A construct was injected into the Rockefeller (ROCK) wild type genetic background which was collected from Cuba ca. 1930 (Kuno, 2010).

OX513A Latin: Introgressed from a Rockefeller background into a Mexico-derived genetic background by five generations of back-crossing, and then made homozygous**.** Unless otherwise stated, this OX513A strain was used for experiments.

OX513A Asian: Introgressed from a Rockefeller background into a Malaysian-derived genetic background by five generations of back-crossing, and then made homozygous**.**

Latin wild type (LWT): A non-transgenic version of the same Mexican genetic background of OX513A Latin. Field collected in 2006.

Asian wild type (AWT): A non-transgenic version of the same Malaysian derived genetic background of OX513A Asian. Field collected in 1975.

Singapore lab wild type: A non-transgenic strain collected from Singapore and maintained for >20 generations in the laboratory.

Singapore field wild type: A non-transgenic strain recently collected from Singapore and maintained for approximately 10 generations in the laboratory.

DMEB (Dengue Midgut Exit Barrier) wild type: a non-transgenic wild type strain isolated from a Houston Laboratory strain, originally collected from Houston, Texas in 1998 (Bennett *et al.*, 2005). This strain has been reported to have a naturally occurring reduced dengue vector competence (Mercado-Curiel *et al.*, 2008).

2.2.2. Insect Rearing

Unless stated otherwise insects were reared at 26^{o}C [+/- 2^{o}C], 70% [+/- 10%] relative humidity, 12h: 12h light: dark cycle. Five cohorts of 200 larvae for each treatment were reared at 1 larvae mL^{-1} in 16 oz. pots (Roberston Packaging, UK SICC65) and fed by a standard regimen of finely ground Tetramin© fish flakes (Tetra GmbH, Germany) (Table 9-1). Live pupae were counted and placed into cages (15 cm x 15 cm x 15 cm, Bugdorm-

Megaview, Taiwan). Adults were provided with 10% sucrose solution *ad libitum*. To obtain eggs, females were fed defibrinated horse blood (TCS Biosciences Ltd., UK) and provided with a wet filter paper (Whatman, No. 3) as an oviposition substrate.

2.2.3. Assessment

Dead larvae and dead pupae from larval rearing pots were counted and discarded. Live pupae were placed into cages and allowed to eclose. Adult cages were assessed three days after the last pupa was added. Cage assessment included counting the total number of dead pupae, non-viable adults (dead adults on the water, dead adults on the floor of the cage and non-flying adults) and functional adults (flying adults).

2.2.4. Homozygous outcrosses

OX513A homozygous larvae were reared in the presence of 30 µg mL^{-1} chlortetracycline hydrochloride (Sigma Aldrich, UK), the standard tetracycline supplement used in rearing. Wild type (WT) larvae were reared at a similar density in the absence of tetracycline. Larvae were fed *ad libitum*. Unless otherwise stated, OX513A males were outcrossed to WT females. Heterozygous eggs were collected and used for the following experiments.

2.2.5. Penetrance of phenotype

Homozygous OX513A Latin larvae and heterozygous progeny from OX513A Latin ♂ x LWT ♀ and OX513A Latin ♀ x LWT ♂ were reared in the presence of 30 µg mL^{-1} chlortetracycline and in the absence of tetracycline. LWT were also reared in the presence and absence of chlortetracycline as a control.

2.2.6. Individual penetrance of phenotype

Single homozygous OX513A Latin males were crossed with approximately five LWT females, 50 crosses were set. Each cross represented a 'family'. The same was performed with OX513A Asian males with AWT females. Eggs were collected from each family. Approximately 20 families of each strain were selected based on the abundance of eggs, and the progeny were assessed. The eggs were hatched and reared in the absence of tetracycline. A cohort of 200 larvae was also reared in the presence of chlortetracycline from a population cross of OX513A Latin ♂ x LWT ♀ and OX513A Asian ♂ x AWT ♀. All the larvae were used from each family so the number of larvae in each cohort varied.

2.2.7. Effect of strain background on the penetrance of phenotype

Homozygous OX513A Latin and OX513A Asian were outcrossed to three naive WT strains; DMEB WT, Singapore lab WT and Singapore field WT. OX513A males were crossed to WT females and OX513A females were crossed to WT males. Progeny were reared in the absence of tetracycline.

2.2.8. Effect of temperature on the penetrance of phenotype

OX513A Latin heterozygous progeny and LWT were reared in the absence of tetracycline at the following temperatures: 9°C, 18°C, 24°C, 30°C and 37°C. Temperatures were maintained using incubators for the 9°C, 18°C and 37°C experiments and heat mats (Habistat, UK) for the 24°C and 30°C experiments. Evaporation from the pots was compensated for by adding deionised water as required to maintain pots at starting levels (200 mL). Water temperatures were monitored using Thermochron iButtons (Maxim, UK).

2.2.9. Effect of feeding and aeration on the phenotype

OX513A Rockefeller homozygous males were crossed to AWT. The resulting heterozygotes were reared in the absence of tetracycline and in the presence of ½ rations of food, ¾ rations of food or normal food rations based on the standard feeding regimen (Table 9-1). In addition to this, cohorts were reared in the presence of aeration (air-stone: Angel Aquarium, UK, and pump: Sera air 110 plus) with normal food rations (Table 9-1). Four cohorts of heterozygotes were run for each treatment along with three AWT cohorts as rearing controls.

2.2.10. Statistical Analyses

Data were analysed using the RStudio software package version 0.97.318 (RStudio, USA). Normality of data was tested using the Shapiro-Wilk method. Homogeneity of variance was tested for using the Bartlett test.

For data that was normally distributed (temperature, feeding and strain background studies), parametric significance tests were carried out using ANOVA and post-hoc testing using the Tukey HSD method, using the multcomp package. For data that was not normally distributed (penetrance and aeration data), non-parametric data was tested for significance using the Wilcoxon rank sum test. For all comparisons significance was set to $p < 0.05$.

2.3. Results and Discussion

2.3.1. Penetrance of phenotype

The phenotype of OX513A was first assessed in 2002. Since then, the strain has been through nearly 100 generations and has been introgressed into a younger genetic background. Here, a thorough analysis of the phenotype of homozygotes and heterozygotes was performed with larvae reared in the presence and absence of tetracycline to produce a complete characterisation of the strain. This dataset will be used as a baseline for the subsequent experiments. Larvae were reared in cohorts of 200 in the presence of 30 µg mL^{-1} chlortetracycline, or in the absence of tetracycline. The results show that of the OX513A homozygotes reared in the absence of tetracycline, no individual's eclosed as adults. However of the heterozygotes (OX513A Latin ♂ x LWT ♀), 10% of the individuals eclosed but only 2.1 % of those were classed as flying adults (Figure 2-1 A). These fractions were also observed when analysing the reciprocal cross (OX513A Latin ♀ x LWT ♂) (Figure 2-1 B). No significant difference was found between any of the classes of the heterozygotes as a result of the cross direction (dead pupae W=22, p=0.06; non-viable adults W=15, p=0.67; functional adults W=16, p=0.50).

When comparing these heterozygous data to the data presented in Phuc *et al*. (2007) (also presented in Figure 2-1 A) it would appear that the fraction of individuals eclosing as adults has increased. However, upon further assessment of these data, a clear separation can be made in the 'eclosed adult' fraction: those that are fit and could mate and reproduce, and those that are not. For this reason, adults in these experiments will be classified into two groups, those with zero potential fitness: dead adults on the water, dead adults on the cage floor and non-flying adults which will be defined as 'non-viable adults', and 'functional adults' which are adults that are able to fly at the time of assessment. It is unclear from the data presented in Phuc *et al*. (2007) whether any individuals would have fallen in the non-viable adult category or whether all individuals were dead larvae, dead pupae or eclosed viable adults. From these data it appears there has been a shift in the time of death of heterozygotes from the time of initial examination. However, the proportion of adults which eclose and have any substantial degree of fitness has remained constant, that is

below the regulatory threshold of 5%[1], and therefore these data do not negate the permitted use of this strain in the field.

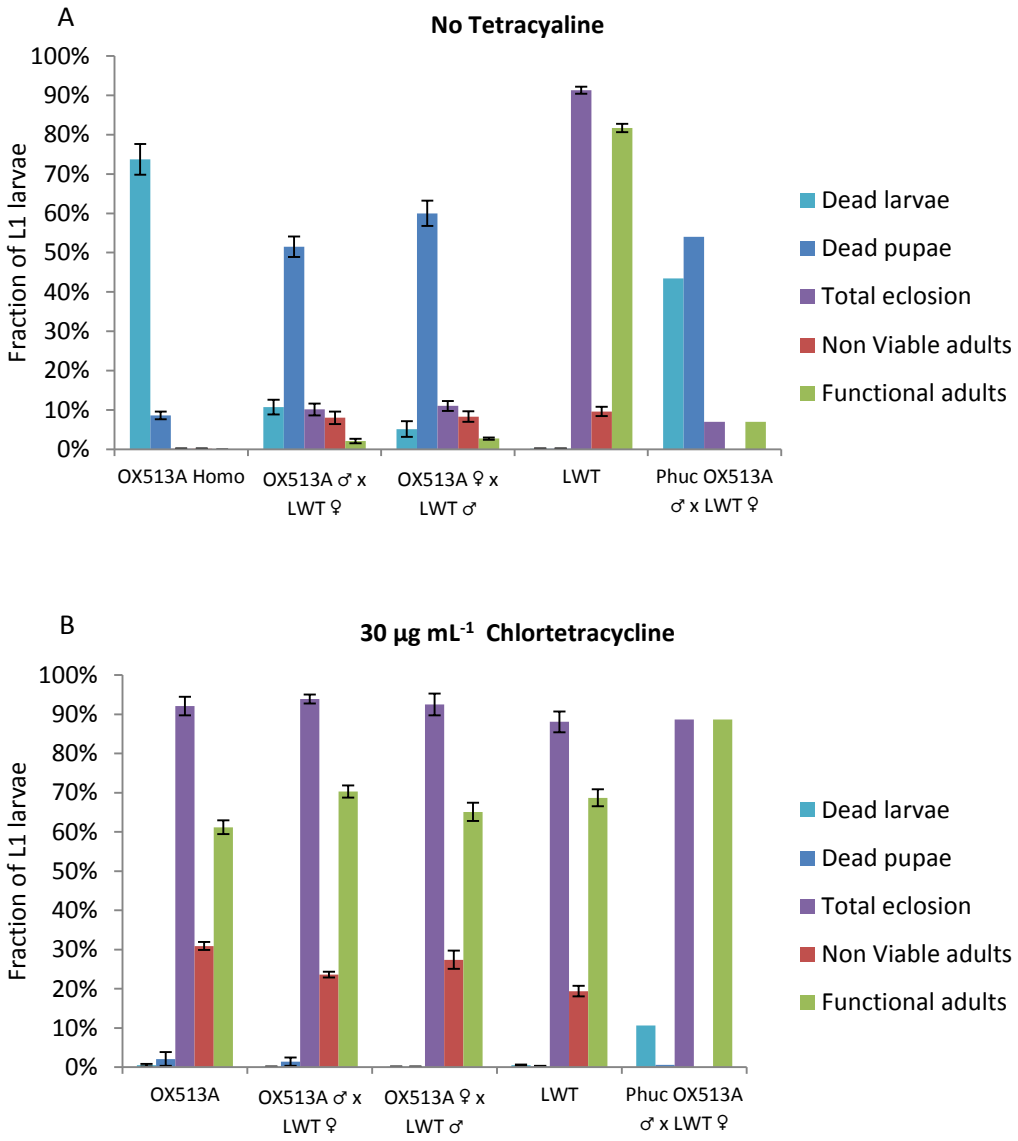


Figure 2-1 Penetrance of the phenotype of OX513A Latin.

A) Represents larvae reared in the absence of tetracycline. B) Represents larvae reared in the presence of 30 µg mL^{-1} chlortetracycline. For each treatment larvae were reared in five cohorts of 200 first instar larvae, N= 5. Data are the means for each treatment. Error bars represent SEM.

[1] This 5% value has been set by Oxitec as the maximum fraction of functional adults which will eclose when OX513A are reared in the absence of tetracycline (personal communication C. Beech)

Comparing the data of OX513A heterozygous and homozygous larvae reared in the presence of 30 µg mL^{-1} chlortetracycline shows there is no significant difference in the fraction of functional adults compared to WT also reared in the presence of chlortetracycline (Figure 2-1 B) (functional adults; OX513A ♂ x OX513A ♀ *W*=2.5, *p*=0.05; OX513A ♂ x WT ♀ *W*=10, *p*=0.67; OX513A ♀ x WT ♂ *W* =16.5, *p*=0.46). However, comparing these same fractions to LWT reared in the absence of chlortetracycline shows a significant decrease in the fraction of functional adults eclosing (functional adults; OX513A ♂ x OX513A ♀ *W*=25, *p*=0.01; OX513A ♂ x WT ♀ *W*=25, *p*=0.10; OX513A ♀ x WT ♂ *W*=25, *p*=0.10). This result therefore indicates that the addition of chlortetracycline to the rearing environment can result in a decreased survival of the cohort, with more individuals being classed as non-viable adults than functional adults. In an egg mass production facility this could result in a lower yield of egg to functional adult, which could decrease the rearing efficiencies and increase the overall cost. However the strain requires the addition of tetracycline to rescue the individuals from the lethal phenotype and therefore these losses will need to be taken into account when costing and planning an egg production programme. Further investigations into the interaction of tetracycline and OX513A are carried out in Chapter 4.

2.3.2. Individual penetrance of phenotype

The experiment performed above examined the phenotype of OX513A at a population level and potentially identified a shift in the time of death from approximately 97% pre-eclosion death (as reported in Phuc *et al.* (2007)) to approximately 8% of the population being classified as non-viable adults and an additional 3% as functional adults. This drift in phenotype could be due to either a population level change in the phenotype or a few individuals within the population that have a different phenotype and consequently shift the population means. These isolated individuals could hypothetically be resistant to the lethal effects of tTAV or could carry a mutated form of the transgene that does not produce functional tTAV. If a variation in the phenotype of progeny from single individuals is found, then the population could be screened to remove those with undesirable characteristics to leave a 'pure' strain.

To examine this hypothesis, individual OX513A homozygous males in two genetic backgrounds, Latin and Asian were crossed to five WT females from their respective genetic

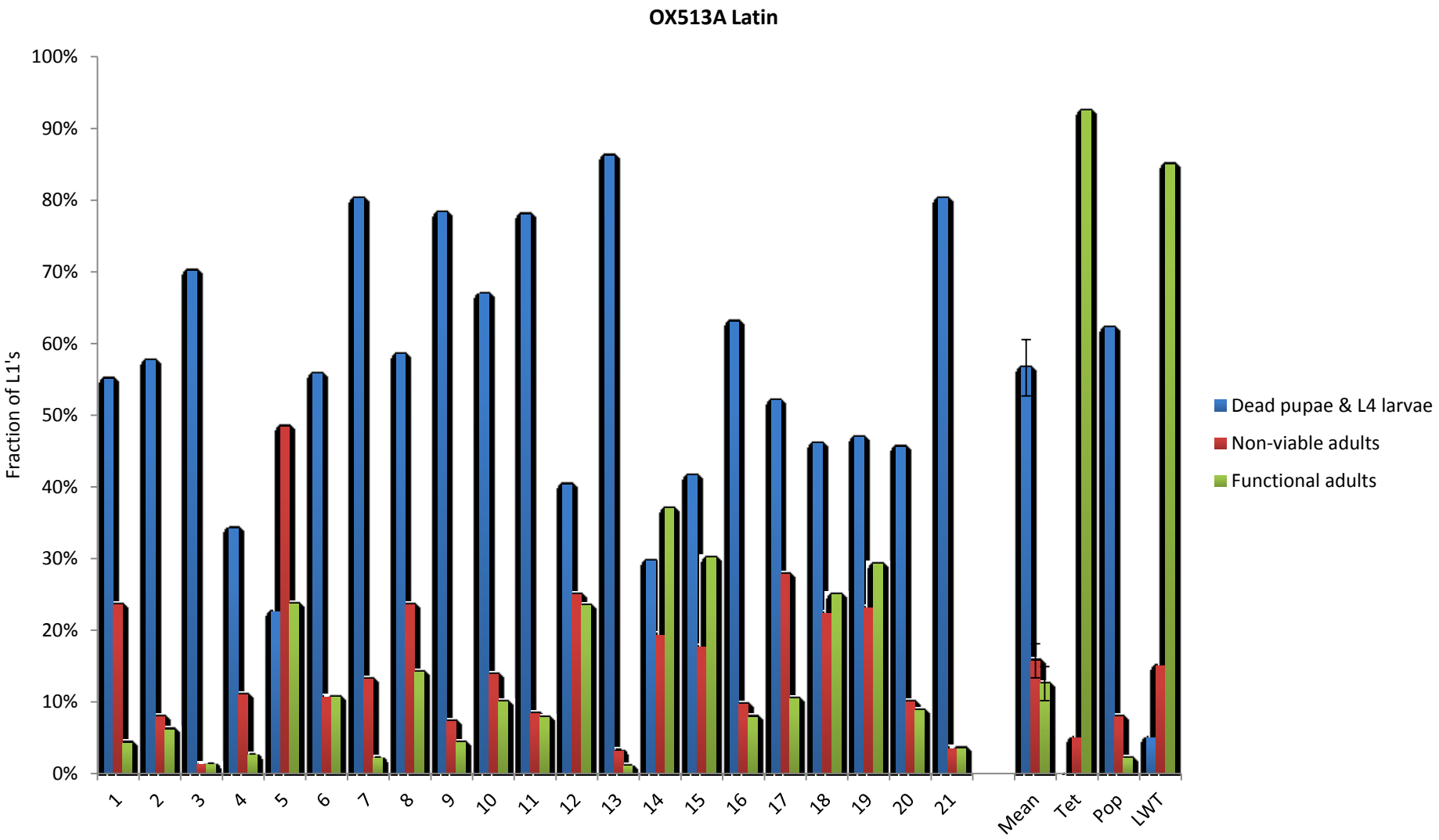
backgrounds[2], and their progeny reared in the absence of tetracycline. Wild type were also reared as a control to determine the expected fraction of functional adults if there was a complete failure of the transgene. The results (Figure 2-2) from both genetic backgrounds show that the fraction of functional adults arising from each individual cross is lower than the fraction of functional adult's eclosing from the control (wild type) crosses (Asian background $U = 0$, $p = 0.008$, Latin background $U = 0$, $p = 0.006$) and therefore this implies that there are not individuals in the population that have, for example, a dominant resistance to the effects of the transgene, rather the population phenotype is the combined effect of an array of many phenotype intermediates. There was no significant difference between the fractions of functional adults in the Latin background compared to the Asian background ($U = 258.5$, $p = 0.10$). However, the results show that the mean functional adult fractions across all the individual phenotypes within a background was higher than the population means[3] collected in subsequent experiments (Figure 2-2). This is likely to be as a result of low-level tetracycline contamination as the experiment was performed using pre-used deli pots; this practice has since been stopped. This low-level tetracycline contamination would provide rescue from the lethal effects of the transgene to a limited fraction of the reared cohort. The sensitivity of OX513A to tetracycline is discussed in Chapter 4. In the experiments that contributed to forming the population mean, brand new deli pots were used and consequently the presumed low level of rescue provided by the tetracycline contamination was eliminated. This population mean is likely to represent the true fraction of functional adults to eclose when OX513A heterozygous larvae are reared in the absence of tetracycline.

Although there is likely to have been low level tetracycline contamination in this experiment, the aim of the experiment was to identify individual crosses that displayed a significantly different phenotype to the rest of the population, and was similar to that of the wild type control, representing a likely failure in the action of the transgene. This has not been identified and therefore it can be assumed that the shift in OX513A phenotype is as a result

[2] The crosses and egg collection were a collaborative effort with assistance from Kelly Matzen, Pam Gray and Heather Haines.

[3] Mean generated from experiments performed by Z. Curtis with OX513A Latin, some of which are reported in this thesis (Chapter 4). These experiments were performed under 'super off tetracycline' methods including brand new deli pots for each experiment, isolated larval food, avoidance of equipment contamination with lab benches etc.

of an overall population level drift, rather than individual contributions increasing the population's mean.



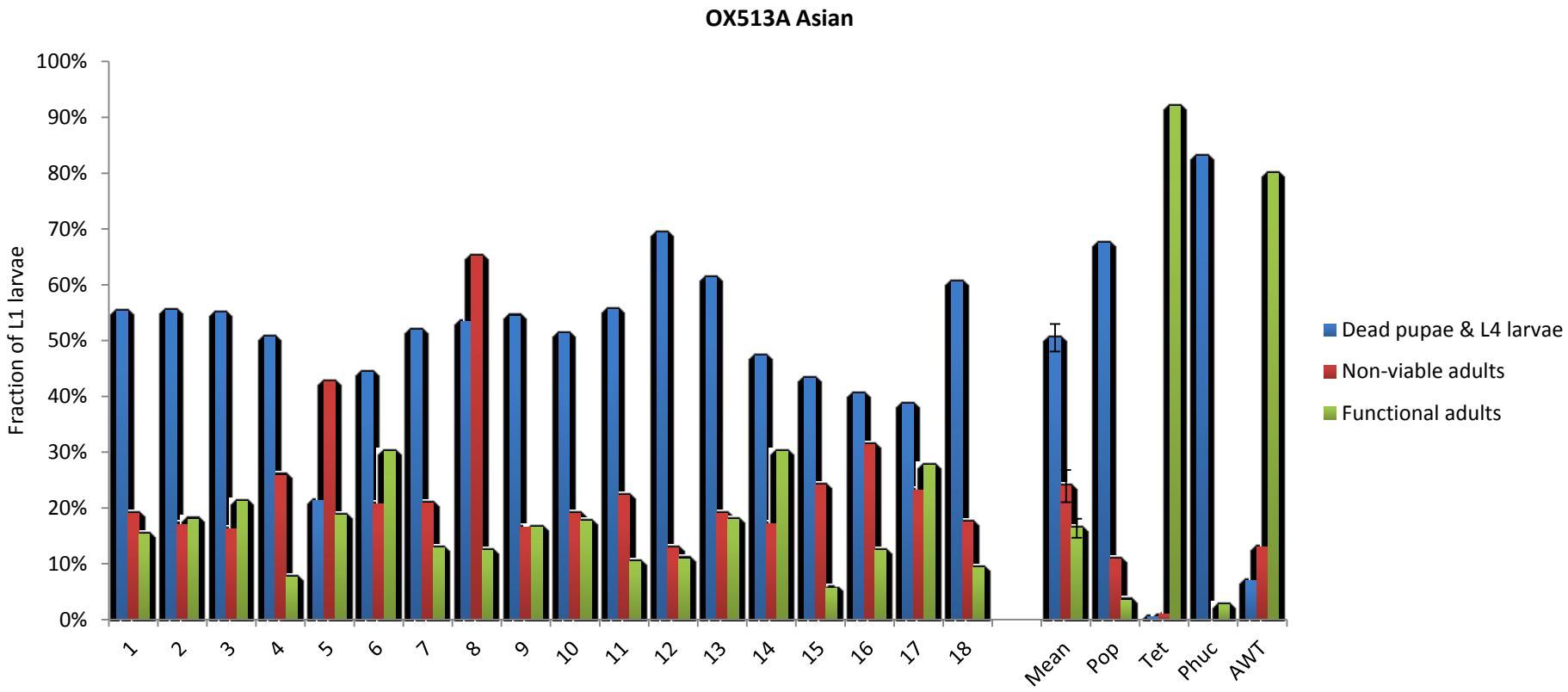


Figure 2-2 OX513A Latin and OX513A Asian heterozygotes resulting from single male crosses.

Larvae were reared in the absence of tetracycline. A cohort (+ Tet) was reared in the presence of 30 µg mL^{-1} chlortetracycline as a control as well as Asian wild type (AWT) and Latin wild type (LWT), in the absence of tetracycline. The mean of all the families are shown including the SEM. Data from Phuc *et al.* 2007 and population (pop) data are included for reference.

2.3.3. Effect of strain background on the penetrance of phenotype

The results collected in the previous experiment using OX513A individuals in two different genetic backgrounds produced results which inferred that there was a difference in the phenotype depending on the background of the OX513A strain and the wild type contribution. To examine this effect further, OX513A Latin (the product strain background) was outcrossed to three naive WT strains originating from different places or laboratory colonisation duration: DMEB WT, a strain collected in approximately 1998 from Texas USA, Singapore Lab WT, collected in approximately 2005 and Singapore Field WT, which was colonised in approximately 2010. Heterozygous progeny from both cross directions (OX513A ♂ x WT ♀ and OX513A ♀ x WT ♂) were reared in the absence of tetracycline and their phenotype analysed. New deli pots were used to rear larvae to prevent potential tetracycline contamination.

The results, displayed in Figure 2-3 show that there is a considerable variation in the fraction of individuals falling into each category, depending on their parental genotype. Examining the fraction of functional adults, the most important category in terms of regulation, only two crosses produced fractions above the 5% threshold: Singapore field ♂ x OX513A Latin♀ and DMEB ♂ x OX513A Asian ♀, both of which resulted in 6% functional adults. These results arise from an OX513A female outcross and therefore given that >99.8% of the released OX513A individuals are males, their impact on a suppression trial should be minimal.

In terms of the pre-functional adult death, there are considerable differences in the timing of death. Figure 2-3 shows that there is a highly variable fraction of death at L4, which is dependent on the WT background ($F(1,63)=104$, $p=<0.001$), rather than the OX513A strain background ($F(1,63)=0.44$, $p=0.51$) or sex ($F(1,63)=0.05$, $p=0.82$). This trend is also carried through to the fractions of dead pupae and the total eclosion. An interesting comparison is that of OX513A Latin ♂ x Latin WT ♀ (the control) compared to the other crosses. Here it can be seen that this cross combination results in the highest value of non-viable adults and consequently the highest total eclosion compared to the other cross combinations. However, OX513A Latin ♂ x Latin WT ♀ has been shown in numerous experiments to consistently produce a functional adult fraction of ≤ 5%, therefore although the fraction of non-viable adults is the greatest, the strain produces a repeatable and desirable fraction of functional adults, which can be used in open releases.

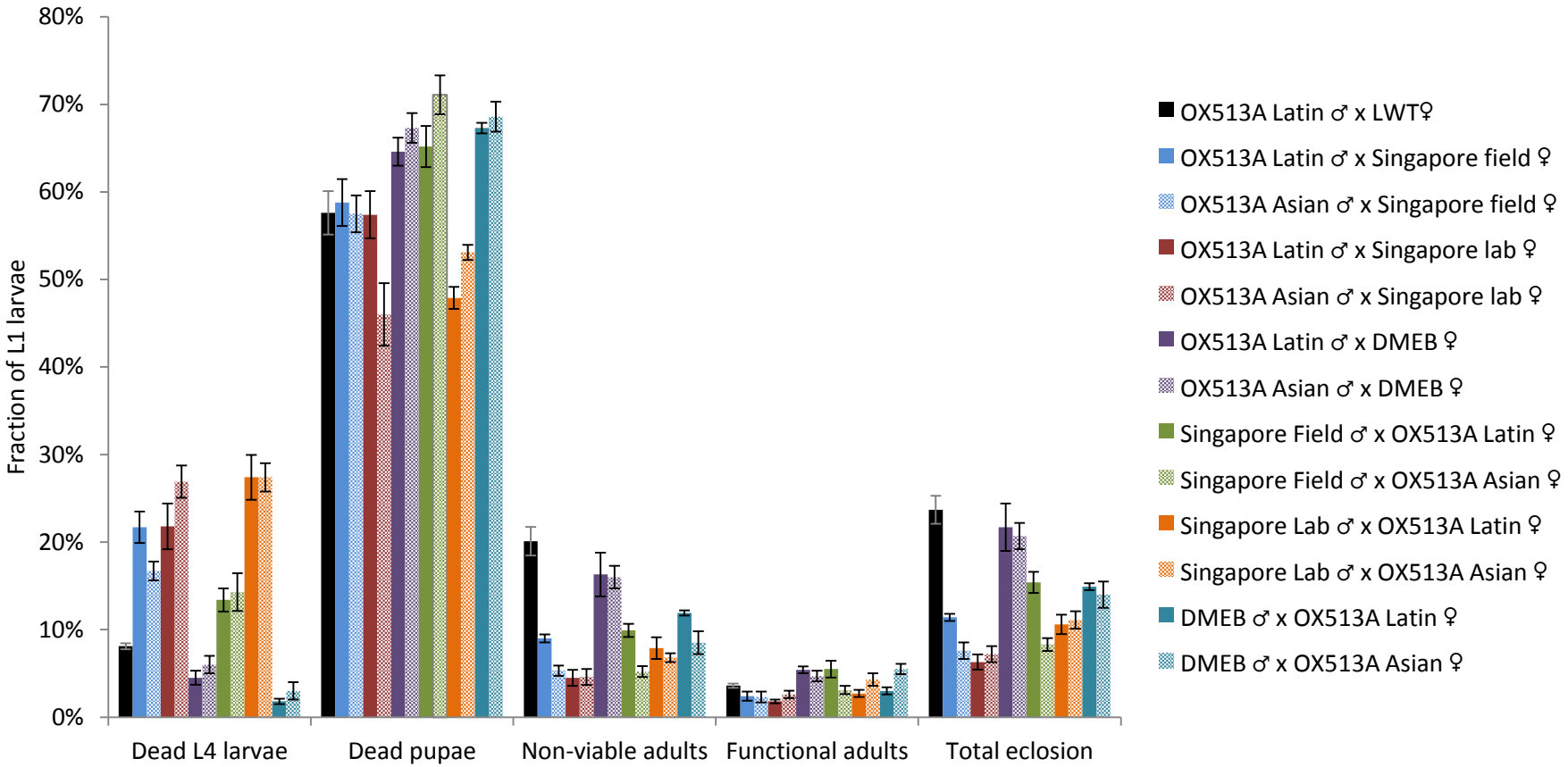


Figure 2-3 OX513A Latin and OX513A Asian outcrossed to naïve WT backgrounds.

The resulting progeny were reared in the absence of tetracycline. Error bars represent the SEM. For each treatment larvae were reared in five cohorts of 200 first instar larvae, N=5.

2.3.4. Effect of temperature on the penetrance of phenotype

The nominal rearing temperature of OX513A in the laboratory is 24˚C, however in the field the developing heterozygous progeny are likely to encounter a range of temperatures. The purpose of the experiment performed here was twofold, firstly to establish the phenotype of OX513A heterozygotes when they are reared in the absence of tetracycline at temperatures they are likely to encounter in the environment but outside of their nominal rearing range, and secondly at reported temperatures at the limit of the *Ae. aegypti* geographical range (Hemme *et al.*, 2009; Richardson *et al.*, 2011; Carrington *et al.*, 2013) to establish that OX513A will not be able to survive and extend the habitable range of *Ae. aegypti*. Hemme *et al*., (2009) measured the water temperature of *Ae aegypti* positive larval containers in Trinidad and found the maximum temperature to be 35˚C, while Brady *et al.,* (2013) used modelling and field data to estimate that the minimum habitable temperature to be approximately 10˚C. Consequently two cohorts of temperatures were tested, 9°C and 37°C[4] which represent temperature points somewhat beyond the lower and upper bounds of the reported habitable temperature range of *Ae. aegypti* (Hemme *et al.*, 2009; Richardson *et al.*, 2011; Brady *et al.*, 2013), and three temperatures within the reported habitable range which larvae are likely to encounter in the field; 18°C, 24°C (control) and 30°C. OX513A heterozygous larvae and LWT larvae were reared at the specified temperatures. Each temperature had five repeats of each strain with 200 larvae per repeat, reared in the absence of tetracycline.

The results demonstrated that all OX513A and LWT larvae reared at 9°C and 37°C die before reaching pupation. These data establish that OX513A will not be able to extend the geographical range of *Ae. aegypti* further than its current range limits as a result of tolerance to temperature extremes.

Of the OX513A heterozygotes reared at the permissive temperatures of 18°C, 24°C and 30°C the results displayed in Table 2-1 show that at all temperatures, the proportion of functional adults is below the 5% regulation threshold limit, in fact at 18°C, no functional adults

[4] Single temperature points were used in this study to limit the size of the experiment, as well as representing the temperature stability that can be achieved during rearing. To determine the true limits of the habitable range of OX513A, temperature intervals, for example 2˚C intervals could have been used but this was not feasible here given resources available.

eclosed. A very high proportion (95.5%) of OX513A larvae reared at 30°C died prior to pupation; this was consistent between repeats but not seen in the LWT (Table 2-1).

OX513A also showed a variation in the total eclosion between these intermediate temperatures: 18°C and 30°C had a significantly lower total eclosion compared to the standard laboratory rearing temperature of 24°C ($F(2,12)=104.82$, $p=<0.001$) (Table 2-1). No significant difference was seen in the total eclosion of LWT reared at 18°C, 24°C and 30°C ($\chi^2(2)=0.19$, $p=0.91$). The increased pre-eclosion death of OX513A heterozygotes away from the nominal rearing temperature, which is not seen in LWT could indicate that the OX513A strain is less fit than the LWT strain and therefore the increased stress from deviations in temperature result in a higher fraction of death.

Table 2-1 Impact of larval rearing temperature on OX513A and LWT larvae.
Heterozygous OX513A and LWT larvae were reared at temperatures within the normal habitable range for *Ae. aegypti*. For each treatment larvae were reared in five cohorts of 200 first instar larvae in the absence of tetracycline, N = 5. Bootstrapped 95% confidence intervals in parentheses.

Strain	Temperature	Dead larvae %	Total eclosion %	Functional adults %
OX513A	18°C	27.6	0.8	0.0
		(24.8-30.3)	(0.3-1.4)	(0.0-0.0)
	24°C	39.0	16.2	1.0
		(36.3-42.3)	(13.9-18.5)	(0.4-1.7)
	30°C	95.5	2.0	2.0
		(94.2-96.7)	(1.2-2.9)	(1.2-2.9)
Latin WT	18°C	18.7	71.2	59.6
		(16.3-21.1)	(68.4-74.0)	(56.5-62.6)
	24°C	30.0	69.4	68.3
		(27.2-32.9)	(66.6-72.2)	(65.4-71.2)
	30°C	30.1	67.0	65.8
		(27.4-32.9)	(64.2-69.8)	(62.9-68.6)

2.3.5. Effect of feeding and aeration on phenotype

Strains of *Ae. aegypti* reared in the laboratory are provided with conditions which promote a desirable rate of pupation and high cohort survival; this is managed and manipulated by

controlling temperature and the larval food per mouth. In field breeding sites, larval food availability is known to vary and often there can be considerable inter-species and intra-species competition for this resource (Murrell *et al.*, 2011; Bagny Beilhe *et al.*, 2013; Couret *et al.*, 2014). As a consequence, it is important to consider how the phenotype of OX513A progeny are affected by developing in food rich or food poor environments. It is hypothesised that larvae developing in food poor environments are likely to be under more stress and have longer development times. This is predicted to reduce the fraction of functional adult's eclosing. Conversely, larvae developing in food rich environments, like those of permissive lab rearing, are expected to exhibit the same fraction of functional adults as seen in previous standard laboratory experiments.

Further to this experiment, anecdotal evidence suggested that the rate of development of larvae reared in aerated water reached pupation faster than those reared in non-aerated water. It is unclear why this should occur but maybe the result of faster bacterial growth in aerated water, which is a food source for young larvae (Christophers, 1960). This could promote their development over and above those of their non-aerated cohorts. It was hypothesised that if larvae were able to develop faster, they could almost 'outrun' the lethal effects of the OX513A transgene and a greater fraction of functional adults could eclose than nominally seen. Although water aeration is not an applied rearing variable as *Ae. aegypti* larvae tend to develop in still pools (e.g. (Saleeza *et al.*, 2013; Vijayakumar *et al.*, 2014), the potential effect of aeration on the development time of OX513A was considered as a useful tool to further test the stability of the phenotype under different rearing conditions.

For these experiments OX513A Rockefeller was used, the original background strain of OX513A, outcrossed to AWT females. This was chosen so that the results could be compared to the data presented in Phuc *et al.* (2007). OX513A heterozygotes were reared in the absence of tetracycline in cohorts of 200 with four repeats for each treatment. Three cohorts of AWT were reared as a control for each treatment. Larvae were fed according to the standard feeding regimen for permissive rearing (Table 9-1), along with two treatments of reduced food that received 75% or 50% of the permissive amount. A separate treatment of water with aeration and normal food rations was also included.

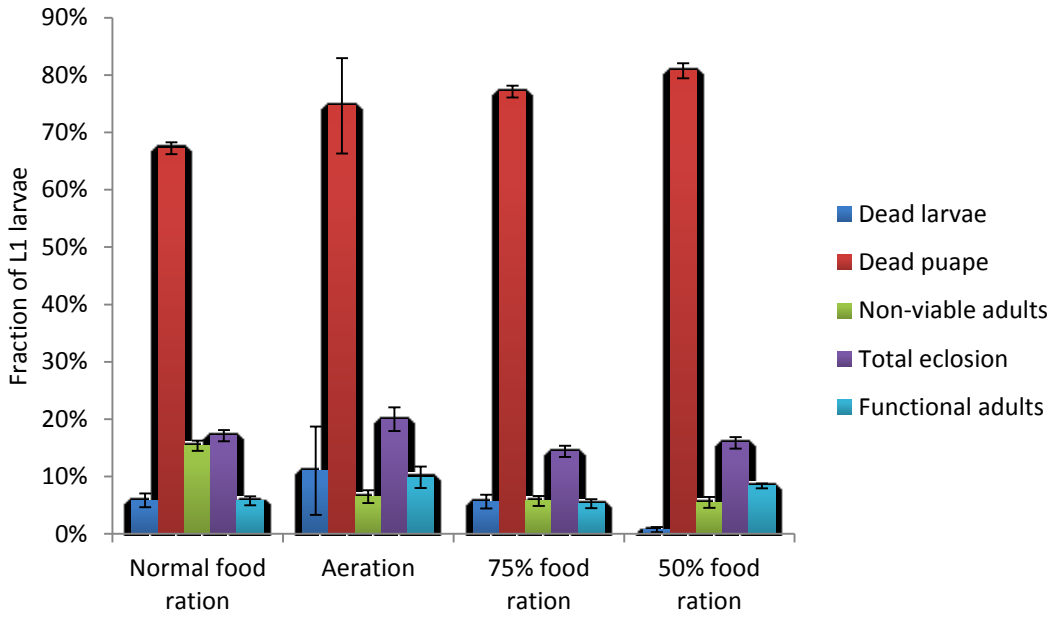


Figure 2-4 OX513A heterozygotes reared with different rations of larval diet and with water aeration.

All cohorts were reared in the absence of tetracycline. Food rations were based on the standard permissive rearing regimen. Larvae were fed reduced rations of 75% or 50%. Additionally cohorts were reared in the presence of aeration with normal food rations. For each treatment larvae were reared in four cohorts of 200 first instar larvae, N=4. Error bars represent SEM.

The results show that there was no significant difference in the fraction of functional adults arising from the 75% food rations compared to the normal food rations (control), however, provision of only 50% rations resulted in significantly more individuals reaching functional adulthood ($F(2,9)=7.41$, $p=0.01$. TukeyHSD: 50% rations $p=0.04$. 75% food rations $p=0.84$) (Figure 2-4). Further analysis of the data showed that there was no significant difference between the total eclosion from each treatment ($F(2,9)=1.11$, $p=0.37$), and therefore the higher fraction of functional adults scored in the half food rations indicates that more adults were flying at the time of assessment, rather than a greater fraction of individuals having eclosed to adulthood. In addition to this 50% food rations also exhibited a significantly lower larval death ($F(2,9)=1.73$, $p=0.003$. TukeyHSD: 50% rations $p=0.005$. 75% food rations $p=0.98$) but a significantly higher pupal death than the normal food ration cohort ($F(2,9)=12.76$, $p=0.002$. TukeyHSD: 50% rations $p=0.002$. 75% food rations $p=0.02$). This was also seen in the 75% food ration cohorts.

These data infer that that larval diet availability can have an impact on the phenotype of OX513A and can influence the fraction of functional adults. It is likely that the time of death of the OX513A individuals is somewhat impacted by the environmental circumstances of the larvae and their rate of development, with the faster development rate (normal and 75% food rations, Figure 2-5) exhibiting a lower fraction of functional adults.

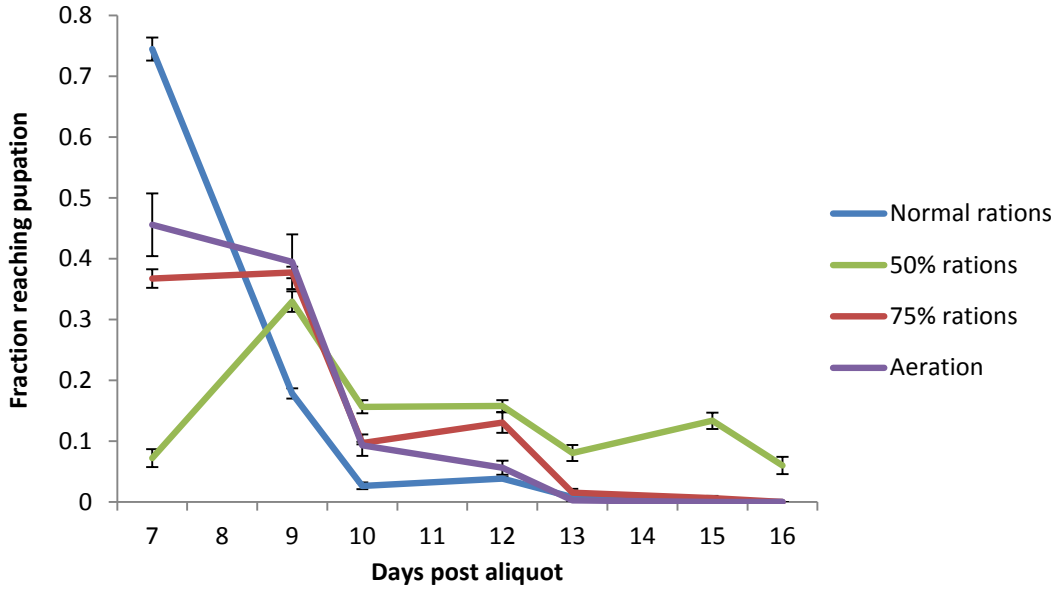


Figure 2-5 Rate of pupation of OX513A heterozygotes reared with different food amounts OX513A Rockefeller heterozygotes were reared with normal food rations (Table 9-1), 75% food rations or 50% food rations. In addition, individuals were reared in the presence of normal food rations and water aeration. The rate of pupation is displayed here. Larvae were aliquoted into five cohorts of 200 L1's for each treatment, N=5. Errors bars represent SEM.

Examination of the effect of aeration on the phenotype of OX513A heterozygotes shows that the fraction of functional adults is not significantly different from non-aerated cohorts ($F(1,6)=4.40$, $p=0.08$). No significant difference was found between the fractions of dead larvae ($W=5$, $p=0.47$), although there was more variation in the individual results of the cohorts reared with aeration (refer to Figure 2-4). This is likely to be due to the increased water disturbance as *Ae. aegypti* usually prefer still water (Christophers, 1960). There was also no significant difference in the total eclosion ($F(1,6)=1.11$, $p=0.33$). The rate of pupation was expected to be faster in the aerated cohorts compared to the non-aerated cohort fed

the same feeding regimen. This was not seen; in fact the rate of pupation was marginally slower than that of the control (Figure 2-5).

Comparing both the feeding and aeration data to the data collected in Phuc *et al.* (2007) presented in Figure 2-1 indicates the fraction of adults eclosing in the Rockefeller background is still greater than was originally reported (3% compared to 6% respectively) and is also greater than the fraction eclosing from the product strain, OX513A Latin (2% functional adults). This implies that there has been a phenotype shift in the Rockefeller genetic background over the generations since the original phenotype examination in 2002. Further to this, limiting the food intake of OX513A Rockefeller appears to result in an increased fraction of functional adults. This experiment has not determined if the same effect is true of product strain OX513A Latin. This should be investigated in future work. Owing to an undesirable phenotype, with a consistent fraction of functional adults in excess of 5%, OX513A Rockefeller should not be released into the field.

2.4. Conclusions

As the self-limiting bi-sex lethal strain OX513A moves from small scale suppression trials into large area-wide control programmes, it is important to understand the penetrance of its phenotype under the different circumstances heterozygous progeny might encounter. Here a detailed analysis of the OX513A heterozygous phenotype has been performed to characterise the phenotype, and these data will help to support risk assessments and commercial applications.

The release of OX513A homozygous males into the field to supress the wild population is dependent on the heterozygous offspring having a very low fraction of functional adults eclosing. Modelling presented in Phuc *et al.* (2007) shows that the fraction of functional adults can be up to 10% to still achieve suppression. However under regulation, Oxitec has stated that no more than 5% functional adults should eclose. Some of the data collected here indicates that under certain circumstances, the fraction of OX513A functional adults arising may exceed 5%. The first cause identified to impact this is the difference in the genetic backgrounds of the wild type and the OX513A strain. A recent global genetic study by Brown *et al.* (2014) showed that *Ae. aegypti* around the Pantropical New World and Asia is relatively homogenous which infers that there should not be a compatibility issue with OX513A, however the data collected here has shown that the genetic background

(determined by the location of collection) does appear to influence the penetrance of the lethal phenotype. This appeared to be impacted by both the genetic background of OX513A and the wild type background of the other parent. In terms of the results specific to the product strain OX513A Latin, when OX513A males were outcrossed to WT females, the functional adult fraction was consistently below the 5% regulation threshold however, the time of death varied with the female's genetic background. The reason for this is unclear; it could be due to epigenetic factors influencing the timing and expression of the OX513A transgene or due to any number of loci that influence the sensitivity to tTAV overexpression (Handler, 2004; Faulk & Dolinoy, 2011; Yan *et al.*, 2014). These factors could influence the expression level, timing and toxicity of the OX513A transgene, resulting in marginal shifts impacting the time of the insects death. For this reason, it is important that the OX513A product strain be tested against each wild population into which it is to be released, to ensure that the fraction of functional adults remains below the designated 5%, which is crucial to the regulations of the use of the strain in the field.

The second set of conditions examined were abiotic components of the larval rearing environment, these included temperature, food supply and water aeration. With respect to the larval rearing temperature two questions were posed: firstly, does OX513A show differential penetrance at temperatures other than those typically used in laboratory culture, and secondly, does the presence of the OX513A gene preferentially allow the insects to colonise areas that were previously uninhabitable to wild *Ae. aegypti*. The data presented here show that at the larval rearing temperatures of 9°C and 37°C, somewhat outside the permissive range for wild *Ae. aegypti* development, neither OX513A nor Latin WT were able to survive to pupation. This demonstrates that released OX513A would not represent an establishment hazard to *Ae. aegypti*-free areas outside the current temperature-limited range of the species. In addition to this, rearing OX513A heterozygotes at temperatures away from the nominal rearing temperature resulted in a greater penetrance and fewer individuals eclosing as functional adults. This demonstrates that the penetrance of the OX513A lethal trait will not be adversely affected by the temperature of the larval habitats in the receiving environments.

Ae. aegypti larvae are generally found in clean, still water, and their main food source is provided by bacteria and rotting detritus (Christophers, 1960). Under laboratory conditions, OX513A is fed a generous diet based on a regimen of diet per mouth (Carvalho *et al.*, 2014).

In field breeding sites, food availability is likely to be limited so that the developing larvae are in competition with one another for the available food (Murrell *et al.*, 2011; Bagny Beilhe *et al.*, 2013; Couret *et al.*, 2014). The results collected here show that competition for food can alter the penetrance of OX513A with more functional adults eclosing when less food is available. It is unclear at present as to the reason for this result, however it is known that adults resulting from larvae with limited diets tend to have lower teneral reserves and a shorter life expectancy (Maciel-De-Freitas *et al.*, 2007). It is possible therefore, that although there is an increased fraction of functional adults in lower food environments, which are likely to closer represent food environments in the field (Barrera *et al.*, 2006), the adults eclosing from these are likely to have shorter life expectancies than those eclosing from food rich environments. In addition, this experiment was performed with a strain background, the Rockefeller strain, which has been shown here to produce an unfavourable phenotype under many different rearing circumstances. It is possible therefore that the interaction of food availability and penetrance of the OX513A transgene is a function of this genetic background rather than a function of the transgene. This result should be challenged using the product strain OX513A Latin to confirm the effect, as well as further exploring the interaction of food limitation and penetrance, both on the fraction of functional adults eclosing, and the fitness of those adults.

The work in this chapter has characterised the phenotype of the product strain of OX513A under permissive and non-permissive conditions. The experimental data have shown that the penetrance of the phenotype and time of death can be influenced by the strain genetic background, mate genetic background and by abiotic factors. Collectively these data show that OX513A should be effective at supressing wild populations in the field around the world, however, to maintain regulatory standards of having <5% functional adults, it is recommended that OX513A should be tested against local strain backgrounds before being deployed. Furthermore, these data indicate that it may be possible for the penetrance of the OX513A phenotype to shift over time. As part of the scale-up of operations with OX513A, a quality control system has been implemented to monitor and detect phenotype changes ahead of time with the data collected here being used as the baseline for future comparisons.

Chapter 3 Selection of a novel female flightless insertion

3.1. Introduction

OX513A is a tetracycline repressible bi-sex lethal strain of *Ae. aegypti*. It has been successfully used in field suppression trials in Cayman, Panama and Brazil to suppress wild populations of *Ae. aegypti* in limited geographical areas (Harris *et al.*, 2012; Carvalho *et al.*, 2015). The use of this strain for field suppression requires the sustained release of millions of male mosquitoes for multiple weeks, with regulatory standards requiring that fewer than <1% of the release cohort be female (personal communication C. Beech), as only this sex bites and spreads disease.

The rearing of the OX513A strain requires the addition of tetracycline to the larval rearing medium to produce adult males, however, this also allows females to develop to adulthood. Upon pupation, pupae are sorted from the remaining larvae (which are assumed to be mostly female due to the development timings of males and females, (refer to section 1.2)), and then the pupae are sorted by size to separate the males from the unwanted females. These processes are labour-intensive, and consequently costly. Taking the mass rearing process for field release as a whole (up to the point of transporting the males into the field), the proportion of the cost that is spent on sorting and processing the pupae equates to approximately 15%. The total cost of this system can be prohibitive to very large countries with vast *Ae. aegypti*-infested areas to treat, or countries with small vector control budgets. Therefore an improved self-limiting strain is sought that will reduce the labour required for production, and consequently decrease the cost of a programme.

An alternative to a bi-sex lethal strain where both sexes die when reared in the absence of tetracycline, is a genetic sexing strain (GSS) that results in female death and male only survival and therefore removes the cost of sex sorting the pupae. Variations of these GSS or 'male-selecting strains' have been produced in different insects including *Bactrocera carambolae* (Isasawin *et al.*, 2014), *Anopehles arabiensis* (Yamada *et al.*, 2015), *Anastrepha ludens* (Orozco *et al.*, 2013) and *Ceratitis capitata* (Barry *et al.*, 2002; Robinson, 2002) and have successfully been used in field operations to reduce wild populations (Robinson, 2002).

There are two approaches to this male-selecting system: early-acting female death or late-acting female death, of which both systems have pros and cons. An early-acting female lethal system would have a significant impact on reducing the cost of the mass production of males for release. If females are killed at the egg or first instar larval stage, for example, the rearing capacity of a production facility could be doubled compared to that of rearing OX513A. The space occupied to rear female larvae would be replaced by developing male larvae, hence the male pupae output from a single tray would be twice that of OX513A. The pupae collected would be 100% male and any remaining larvae would also be male so that the labour intensive sorting process could be eliminated, and the female contamination of the released adults would reliably be 0%. This system would therefore have a great impact on reducing the cost of male mass production for field release. A down side to this early-acting female lethal system is that some benefits of transgenic versus wild larval competition in the field would be lost. Phuc *et al.* (2007) showed that by using a late acting lethal system, the critical input ratio is lowered, i.e., fewer males have to be released for each estimated wild male in a population, and that the wild population is suppressed more readily. If an early-acting female lethal system is used, half of the progeny (the females) will die and not compete with the wild larvae, however the transgenic males will still be present. Therefore, there will still be some advantageous effect, but not as great as that of a late-acting system.

In the late-acting system, females are killed or incapacitated late in development, i.e. at pupation or adulthood. If this system is to be successfully introduced into the field, it is imperative that the females do not represent any threat of disease transmission. The reduction in mass production costs are less than in the early acting system; the females survive to pupation and therefore occupy larval development space. However, upon pupation, the female pupae and remaining larvae will represent no threat of disease transmission and consequently the larvae and pupae will not require sex sorting. This will have a positive impact on reducing production costs. In terms of the benefits of larval competition in the field, a late-acting female system is expected to have the same impact as a late acting bi-sex lethal strain.

Further advantages of a female lethal strain, either late-acting or early-acting, are the additional benefits gained by the surviving male progeny. These F_1 males will be heterozygous for the transgene and will be able to mate with the wild females, resulting in

50% of their F_2 progeny carrying a copy of the transgene, further reducing the wild population. This additional suppression will help to drive the population towards elimination, while also being self-limiting ensuring that after a few generations the transgene will become extinct (Alphey *et al.*, 2007; Atkinson *et al.*, 2007; Black *et al.*, 2011). The heterozygous male progeny can also be exploited to introduce desirable characteristics into the wild population as the surviving males will mate with wild females, of which half the offspring will be wild type, but will inherit half of the male's genes (Alphey *et al.*, 2007). Consequently, if the released males also carry other desirable characteristics, for example dengue refractory genes or insecticide susceptibility, the wild population can be suppressed not only by the dissemination of the lethal transgene, but the remaining mosquitoes are less capable of spreading disease and can also be targeted using appropriate insecticides as part of an integrated pest management programme (IPM) (Alphey, 2009; Okamoto *et al.*, 2014).

Another advantage of a male-selecting system is the potential for community based egg-release programmes. This concept would allow mosquito egg packages to be delivered for example to school children or isolated communities, where they can submerge the eggs in a container of water and one to two weeks later the self-limiting males would eclose to begin their mating mission. This concept could be vital to remote communities where it would be impossible to regularly visit with factory reared mosquitoes, either bi-sex lethal or male-selecting. Alternatively, in cities it could form a component of the community engagement in schools, enhancing the profile and acceptability of a suppression programme.

Based on the evidence presented above, a self-limiting, early-acting female lethal strain would be desirable. It would significantly reduce the costs of mass production, and any loss of larval competiveness in the field could be replaced by releasing additional cheaply produced males. A late acting female flightless *Ae. aegypti* strain, OX3604C, has previously been generated using the *Aedes Actin-4* (*AeAct-4*) promoter (Munoz *et al.*, 2004) driving expression of the tetracycline repressible transactivator, tTAV (Fu *et al.*, 2010). As discussed in section 1.2 the ability of mosquitoes to fly is fundamental to their ability to mate, evade predators, find food sources (both blood meals and nectar) and oviposition sites. If the ability to fly is eliminated then the individual will not be able to spread disease or be able to contribute progeny to the next generation. OX3604C produced 100% flightlessness in females when reared in the absence of tetracycline, and 77.6% rescue of the flightless females when tetracycline was added to the larval rearing medium (Fu *et al.*, 2010) (and my

data). The males were able to fly when reared in the presence or absence of tetracycline. This strain was used in large laboratory cage trials to demonstrate population suppression: after successive releases of male and female OX3604C pupae into initially WT cage populations, the treatment populations were eliminated after 10 to 20 weeks (Wise de Valdez *et al.*, 2011). This demonstrates that the female flightless approach is an effective method to control *Ae. aegypti* populations. However the future use of this strain has some negatives; the degree of female rescue exhibited by the addition of tetracycline is less than 80%. This results in >20% of the adult female population effectively being lost in each mass rearing batch, not contributing to the egg production and consequently decreasing the efficiency of the mass production process. OX3604C also contains four *piggyBac* ends (the vector system by which the DNA construct is transposed into the host genome (Handler, 2002)) as it was designed with a view to removing these elements, leaving only the functioning parts of the transgene in place. The theoretical possibility that the transgene could remobilize and insert into a different location in the genome, potentially changing the functionality of the transgene, motivated this design (Beech *et al.*, 2009). This remobilization process has been observed at a high rate in a number of species including *Drosophila melanogaster* (Horn *et al.*, 2003), *Tribolium castaneum* (Lorenzen *et al.*, 2003) *Bombyx mori* (Uchino *et al.*, 2008) and *Ceratitis capitata* (Dafa'alla *et al.*, 2006). However the removal of the *piggyBac* ends or remobilization of any *piggyBac* insertion has not been possible in *Ae. aegypti* (Sethuraman *et al.*, 2007; St John, 2012; Palavesam *et al.*, 2013). Although this implies that the remobilization of a *piggyBac* mediated insertion is highly unlikely, the presence of four *piggyBac* ends is less desirable in a transgenic insect destined for field release than the minimum of two ends.

To improve on the design of OX3604C, a new construct was designed by Dr Scaife at Oxitec Ltd, based on the principle components of OX3604 (Fu *et al.*, 2010). The construct, OX4358, (Figure 3-1) was designed using the Actin-4 promoter isolated from *Ae. albopictus* (Labbe *et al.*, 2012) instead of *Ae. aegypti* so that it could also be used to transform *Ae. albopictus* in addition to *Ae. aegypti*. As shown in Figure 3-1, the *Ae. albopictus* Actin-4 (*AealbAct-4*) promoter drives expression of tTAV2 (a variant of tTA (Fu *et al.*, 2010) composed of the tetracycline repressor TetR fused to the VP16 activation domain (Baron *et al.*, 1997), with a sex specific intron allowing the production of functional tTAV2 in females but preventing expression of functional tTAV2 protein in males (Labbe *et al.*, 2012). In females, the tTAV2 protein binds to the bi-directional enhancer tetO, and drives expression of VP16, which

causes cellular toxicity (Gill & Ptashne, 1988; Berger *et al.*, 1990; Lin *et al.*, 2007) and ultimately a flightless phenotype. When reared in the presence of the antidote, tetracycline, TetR of the tTAV2 complex binds to the tetracycline resulting in a conformational change in shape and therefore cannot bind to tetO (Kisker *et al.*, 1995). Consequently, toxic VP16 is not produced and females can develop to flying adults. The construct also contains a second promoter, Hr5IE1 that controls the expression of a fluorescent marker AmCyan (Clontech) to give punctate but widely distributed fluorescence throughout the body for easy identification of individuals positive for the transgene at all life stages.

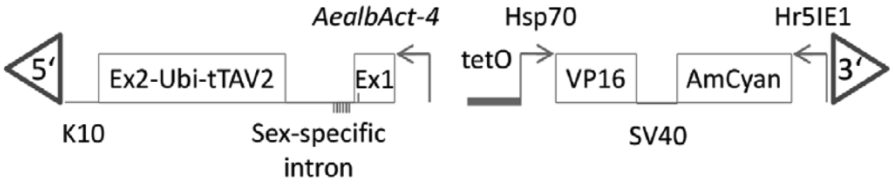


Figure 3-1 OX4358 construct.

Aedes albopictus Actin-4 (*AealbAct*-4) promoter drives sex specific production of tTAV2. This in turn binds to tetO driving expression of the toxic VP16 protein. Figure source (Labbe *et al.*, 2012).

This chapter presents the initial phenotype testing of OX4358 *Ae. aegypti* putative insertion events in the presence and absence of tetracycline. Lines with desirable phenotypes are selected and taken forwards to determine strain integrity in terms of the strain being a single insertion, homozygous lethality, sex linkage and the preservation of the genetic background. Together these data will determine whether one or more of the OX4358 insertion strains will be taken forward for extended testing to confirm its suitability to become a product strain.

3.2. Methods

OX4358 was microinjected into the eggs of *Ae. aegypti* Asian WT (AWT) and into *Ae. albopictus* by Dr Labbe in 2009 (Labbe *et al.*, 2012). Dr Labbe retained and analysed the resulting *Ae. albopictus* lines, while I received G_1 eggs of the putative *Ae. aegypti* lines. The following characterizations of the *Ae. aegypti* strains, along with the characterizations of the *Ae. albopictus* strains have been published in Labbe et *al*. (2012), of which I am a co-author.

All insects were reared according to Chapter 2.2.2 Insect Rearing. The strains used in the following experiments are described in Chapter 2.2.1 Mosquito Strains.

3.2.1. Phenotype testing of OX4358 lines

Each independent line was tested for phenotype in the presence and absence of tetracycline. Larvae were reared at a density of <1 larvae mL^{-1} and fed as required with Tetramin fish flakes (Tetra, Germany). For cohorts reared in the presence of tetracycline, chlortetracycline (Sigma-Aldrich, UK) was added to a final concentration of 30 µg mL^{-1}. AWT were also reared under the same conditions as a control. The total number of larvae in each cohort varied depending on the number of eggs but was approximately 300 to 400 L1s (first instar larvae). Pupae were picked, sexed and screened for the presence of the fluorescent marker, AmCyan using an epifluorescent microscope (Leica, Germany) with the filters: exciter D436/20x, emitter D480/40m (Chroma Technology, USA). OX4358 positive pupae were separated by sex and housed in cages to eclose. Phenotype was assessed three days after the last pupa was placed into each cage. The phenotype classes were either flying (individuals which could be seen to hold a flight pattern in the cage) or flightless (those which could not hold a flight pattern and often seen to be hopping). Dead pupae were also counted.

3.2.2. Homozygous lethality test

Heterozygous males were crossed to heterozygous females and eggs collected. The resulting progeny were reared in the presence of 30 µg mL^{-1} chlortetracycline and fed as required with Tetramin fish flakes. The pupae were picked and screened for the presence of the AmCyan fluorescent marker. A Chi-square test was used to detect a significant deviation from the expected fluorescence ratio of 75% fluorescent and 25% non-fluorescent individuals.

3.2.3. Introgressing OX4358B3 into a Latin genetic background and making homozygous

Outcrosses of OX4358B3 to LWT were performed four times, alternating between males and females of OX4358B3 being outcrossed to LWT of the opposite sex and maintaining crosses above 200 individuals. The line was enriched for the insertion by crossing male heterozygotes to female heterozygotes to produce 25% homozygous offspring. Putative

homozygous single males were mated with two to three putative homozygous virgin females. The males were removed and PCR analysed by C. Philips to determine their genotype. Females mated to a heterozygous male were discarded. G_1 eggs were collected from each female mated to a homozygous male separately; this constituted a 'family'. After eggs were collected, the females were PCR analysed and any families with a heterozygous mother were discarded. Each putative homozygous family was taken through another generation to collect G_2 eggs. The G_2 eggs were hatched and the larvae screened; any families with non-fluorescent (WT) larvae present were discarded, along with any families that did not produce >100 G_2 larvae. Families with 100% fluorescent larvae were considered homozygous and reared to pupae. Upon pupation, 30 male and 30 female pupae were collected from each homozygous family and placed into a homozygous male cage or homozygous female cage to eclose. 48 hours after the last adult eclosed, the mixed homozygous males were transferred into the mixed homozygous female cage. This process allowed individuals from each family to reach sexual maturity before any individual had the chance to mate, allowing even mixing of the genetic background. Homozygous OX4358B3 eggs were collected, hereafter referred to as OX4358B.

3.2.4. Homozygous and Heterozygous phenotype of OX4358B Latin

OX4358B Latin homozygous males were crossed to LWT females and heterozygous eggs collected. OX4358B Latin homozygotes, OX4358B Latin heterozygotes and OX4358B Asian homozygotes were hatched and L1s were counted into cohorts of 200 individuals and assigned to be reared in the presence or absence of 30 µg mL^{-1} chlortetracycline. Five repeats were set up for each treatment.

3.2.5. Sex linkage

OX4358B Latin homozygotes were crossed to LWT females and heterozygous F_1 eggs collected. F_1 male heterozygotes were then crossed to LWT females and F_1 female heterozygotes crossed with LWT males, and F_2 eggs from the two crosses collected. The F_2 generation were hatched and counted into five cohorts of 200 individuals and reared in the absence of tetracycline. Pupae were picked, sexed and screened and the numbers recorded.

3.2.6. Single insertion

OX4358B Latin homozygous males were crossed to LWT females and F_1 eggs collected. These F_1 eggs were hatched and counted into a single cohort of 200 individuals. Upon pupation, the pupae were picked and screened. Males were crossed to LWT females and F_2 eggs were collected. This was repeated for four generations.

3.2.7. Flightless female fecundity

OX4358B Latin heterozygous larvae were reared in 15 cohorts of 200 larvae in the absence of tetracycline. Larvae were fed a standard feeding regimen (Table 9-1). Pupae were picked and sexed and allowed to eclose in separate male and female cages. Adult males were added to the flightless female cages and monitored for mating. The females were offered a blood meal and two days later an oviposition substrate was provided for the females to lay eggs. After allowing the eggs to mature, the egg papers were submerged in water and hatched under vacuum (Desiccator: Nalgene. Vacuum pump: Compton D351VM). Egg hatching pots were inspected for larvae 24 hours and 72 hours later.

3.3. Results and Discussion

3.3.1. Phenotype testing of OX4358 lines

Injected G_0 eggs were reared to adulthood and outcrossed to AWT by Dr Labbe. Received G_1 eggs were hatched and immature stages were screened for expected AmCyan expression; 15 transformation events were identified and selected for testing. Heterozygous larvae of each of the 15 putative OX4358 lines were reared in the presence and absence of 30 µg mL^{-1} chlortetracycline to determine which lines displayed a desirable phenotype. Male and female pupae were allowed to eclose separately and their ability to fly was assessed. The total number of pupae collected for each treatment for each line was between 290 and 400. The results in Figure 3-2 show the phenotypes achieved with the OX4358 construct. The variation in observed phenotypes is due to the transgene inserting into the genome at different positions: if it has located into an actively transcribed area of the genome it is likely to have a higher protein production than if it inserts into an area that remains tightly folded and is therefore transcribed less, this is known as the position effect (Handler, 2004; Labbe *et al.*, 2010). Of the lines analysed here, F4 does not display a female flightless phenotype when reared in the presence or absence of tetracycline. Lines C2, E8, F8 and H5 show a

marginal female flightless phenotype, with varying degrees of flightlessness when reared in the absence of tetracycline, but a very high percentage of flying females when reared in the presence of tetracycline indicating that there is an insufficient level of expression of the transgene to cause the desired phenotype. The males of all lines appear to be unaffected by the insertion with >95% males flying (calculated from total pupae) when reared in the presence or absence of tetracycline. This is comparable to the WT control. Lines B3, C3, D7 and E4 all show a desirable tetracycline repressible phenotype: >96% of the males can fly when reared in the presence or absence of tetracycline, and the females are 100% flightless when reared in the absence of tetracycline but in the presence of tetracycline >96% of the females can fly.

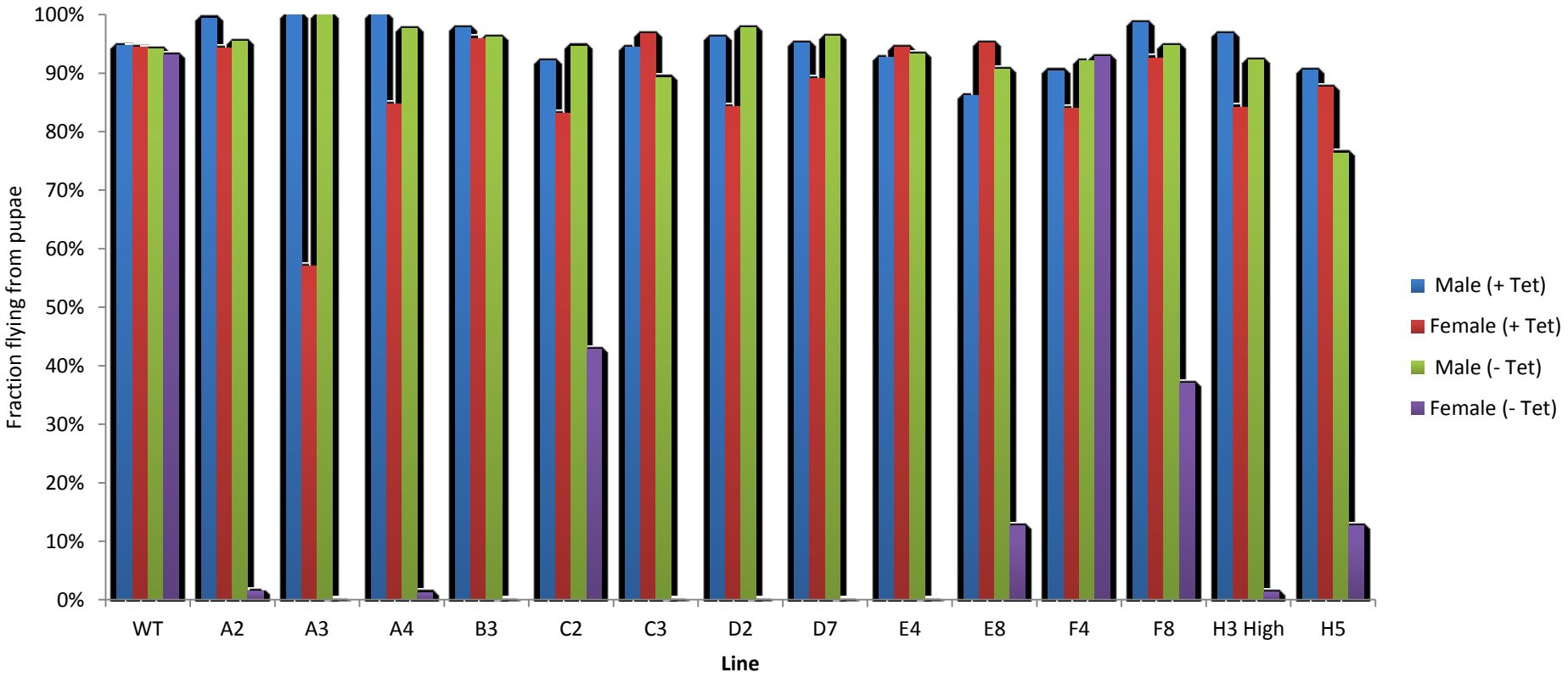


Figure 3-2 Phenotype testing of OX4358 heterozygous insertion lines.

Larvae are reared in the presence (+Tet) and absence (-Tet) of 30 µg mL^{-1} chlortetracycline. Percentages of flying adults were calculated from the total pupae into the cage. Wild type (WT) included as a reference for the phenotype of the genetic background. N for each line varied between 300 to 400 L1 larvae depending on the number of eggs available.

3.3.2. Homozygous lethality test

The location of the transgene's insertion can result in a deleterious phenotype if it inserts into, for example, a housekeeping gene and prevents its proper functioning. Many such effects only become apparent when the transgenic strain is made homozygous as the individuals will only have interrupted copies of the gene rather than a single copy of the functioning gene and a single copy of the mutated gene. From the phenotype analysis above, lines B3, C3, D7 and E4 were taken forwards and tested to determine if these insertions result in a lethal phenotype when an individual carries two copies of the allele at the same locus. If the homozygous insertion does not have a lethal effect, the progeny from the cross of heterozygotes to heterozygotes should show the following ratio: 25% WT, 25% homozygotes and 50% heterozygotes, therefore a ratio of 25% non-fluorescent to 75% fluorescent should be seen. Table 3-1 shows that lines B3 and E4 did not show a significant difference from the expected ratio of 75% fluorescent with 25% WT. Lines C3 and D7 did show a significant difference in the expected ratio, implying that the insertion could be causing lethality when individuals are homozygous for the transgene.

Table 3-1 Survival to pupation of four separate insertion lines of OX4358.
Heterozygotes were crossed together to determine if homozygotes resulted in a lethal phenotype. A Chi-square test was used to determine if there was a significant deviation from the expected 75% fluorescence ratio. If no homozygotes survived the fluorescent fraction would be 50%.

Line	WT Total	OX4358 Total	OX4358 (%)	*P* value
B3	635	1991	75.8	0.9
C3	570	1484	72.3	<0.005
D7	560	1365	70.9	<0.005
E4	881	2594	74.7	0.9

Based on these results it was decided that insertion line B3 and E4 were the preferred candidates to take forward for further testing. However, in the interim, molecular analysis (performed by Dr Scaife) of line E4 showed the presence of plasmid backbone (parts of the

plasmid vector involved in the replication and delivery of the construct into the genome), indicating that it had been an irregular integration. This line would not be suitable to pass through regulatory approval for field release and consequently only line B3 was selected for further testing.

3.3.3. Introgressing OX4358B3 into a Latin genetic background and making homozygous

OX4358 was injected into an *Ae. aegypti* laboratory colony originating from Asia (Asian WT) which was colonized in 1975. However, due to the success of the field trial in Grand Cayman with the bi-sex lethal line in the Latin genetic background (Harris *et al.*, 2012) and with the prospect of performing a field trial with OX4358B3 in Grand Cayman it was decided that OX4358B3 should be introgressed into the Latin WT background and be made homozygous. This was on the basis that it is likely that the younger colony of Latin WT colonised in 2006 compared to the colony of Asian WT, would be fitter and more closely related to wild populations. The bi-sex lethal strain has continued to be used in field suppression trials, successfully competing with wild males for mates and releases have resulted in wild population suppression suggesting the genetic background is suitable for effective field application.

To introgress the transgene into an alternative genetic background, sufficient outcrosses must be made to transfer the desirable genetic background into the new strain. Here four outcrosses were performed which will result in an exchange of 97% to 99% of the genetic background. After this, OX4358B3 positive males were crossed to OX4358B3 positive females to enrich the strain for OX4358B3 alleles and produce OX4358B3 homozygotes. At the point of screening this population for the fluorescent marker, it became evident that individuals with two OX4358B3 alleles (homozygotes) fluoresced more brightly than those with only one allele (heterozygotes) (Figure 3-3). Consequently putative homozygous males and females could be isolated from the population, resulting in a greater number of successful homozygous families being produced. Single males were crossed to two to three virgin females and eggs collected. These adults were PCR analysed by C. Philips to determine if they were homozygous but due to the accuracy of the PCR at detecting homozygotes being only approximately 80% (personal communication C. Philips), fluorescent screening of the G_2 offspring was also performed. 31 homozygous families were produced with 51 founding individuals. After combining these families together in equal

numbers to allow the balanced addition of genetic material, this OX4358B3 Latin homozygous line (hereafter OX4358B Latin) was used for the subsequent testing described below.

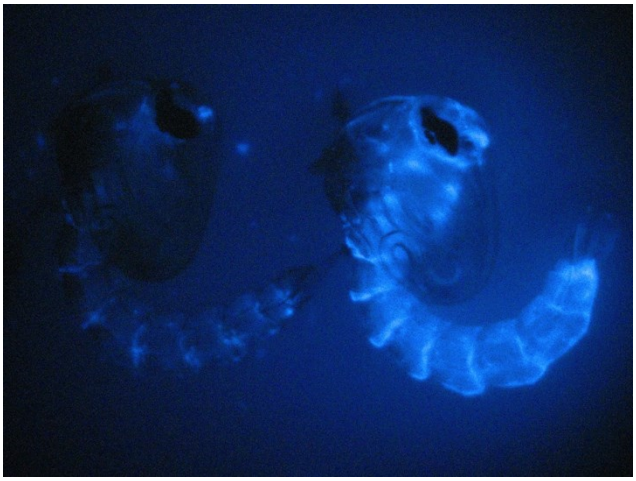


Figure 3-3 OX4358B fluorescent pupae
Homozygous (right) and heterozygous (left) pupae showing the different intensities of AmCyan fluorescence. Figure original.

Five filter colonies of OX4358B Latin were also produced from the homozygous families. There were a total of 31 homozygous families that were allocated to one of five filter colonies. This resulted in each filter colony being composed on average of six families and eight founding parents.

The principal behind mosquito filter colonies is currently only theoretical, but aims to maintain the genetic diversity of a strain by keeping separate populations with the expectation that different alleles will go to fixation (Fisher & Caceres, 1998; Dyck *et al.*, 2006). These isolated populations can be mixed together to replace lost genetic diversity (Dyck *et al.*, 2006). The theory behind this is termed heterosis, and has been shown to have significant improvements in the fitness of progeny compared to that of the parents (Baeshen *et al.*, 2014). On a large scale, the isolation of OX4358B Latin homozygous filter colony populations may negate the need to outcross the mainline colony to a recently colonised wild population to increase the genetic diversity, which in turn would require the whole colony to be processed through the homozygosing bottleneck process. By producing filter colonies early on, the maximum genetic diversity can be maintained, and homozygous

individuals from each colony can be periodically feed into the main colony without the need to interrupt the mass production process or contaminate the colony with WT alleles.

3.3.4. Determining OX4358B is a single insertion

Ensuring that the OX4358B strain has only a single transgene insertion is important for the integrity and reproducibility of the strain phenotype over multiple generations. If, for example, there were multiple insertion events, the desirable phenotype of OX4358B may manifest as a result of a combination of the insertions and therefore, if an insertion event was 'lost' by outcrossing, the phenotype could change. Here, homozygous OX4358B males were crossed to WT females and F_1 eggs collected. The eggs were hatched and screened for the presence of the fluorescent marker. Fluorescent individuals were outcrossed to WT and their offspring screened. This was repeated for four generations to determine if there was any deviation from the expected 50% fluorescence ratio in each generation, based on Mendelian inheritance. Chi-square results, displayed in Table 3-2, show that there was no deviation from the expected fluorescence ratio in each of the four generations, therefore OX4358B should be a single insertion.

Table 3-2 Mendelian inheritance ratios of OX4358B over four generations.
The founding cross was homozygous OX4358B males to WT females. Thereafter, OX4358B males were crossed to WT females. A Chi-square test was used to determine if there was a significant deviation from the expected ratios.

Generation	Expected Fluorescence %	WT	Fluorescent	Observed Fluorescence %	*P* value
F1	100	0	206	100	NA
F2	50	97	84	46	0.42
F3	50	91	93	51	0.84
F4	50	97	90	48	0.69

Determining that the phenotype of OX4358B is the result of a single insertion by this method of fluorescence screening is only effective if all possible OX4358 transgene insertions are inserted into a region where they are expressed, and functional fluorescent protein is

produced. It is plausible that there could be insertions in this line that are silenced by their location, and consequently cannot be detected by fluorescence screening. To detect these insertions a FISH (fluorescence in situ hybridisation) was performed at Virginia Tech, USA, which determined that OX4358B was indeed a single insertion and that the insertion was located on Chromosome 1.

3.3.5. Sex linkage of OX4358B

This experiment was performed to determine if the OX4358B transgene was inherited equally between males and females. OX4358B heterozygous males were crossed to WT females and OX4358B heterozygous females were crossed to WT males and eggs were collected. The progeny were reared to pupation and individuals were sexed and screened for the fluorescent marker. The results represented in Figure 3-4, show that there is a distortion from the expected sex ratio of 50% of males and 50% of the females carrying the transgene (χ^2 = 249.9, N = 1019, p=< 0.001). However, when OX4358B females are crossed to WT males, the sex ratio inheritance distortion is not seen (χ^2 = 0.06, N=1005, p= 0.80). These data demonstrate that the OX4358B insertion, located on chromosome 1 is linked to the sex-determining locus, which in *Ae. aegypti* is also located on chromosome 1 at band 1q21 (Hall *et al.*, 2014).

Using data of the frequency of the recombination of progeny it is possible to determine the distance between the transgene insertion location and the M-locus: based on the data collected here, the OX4358 transgene has inserted 25 cM from the M-locus.

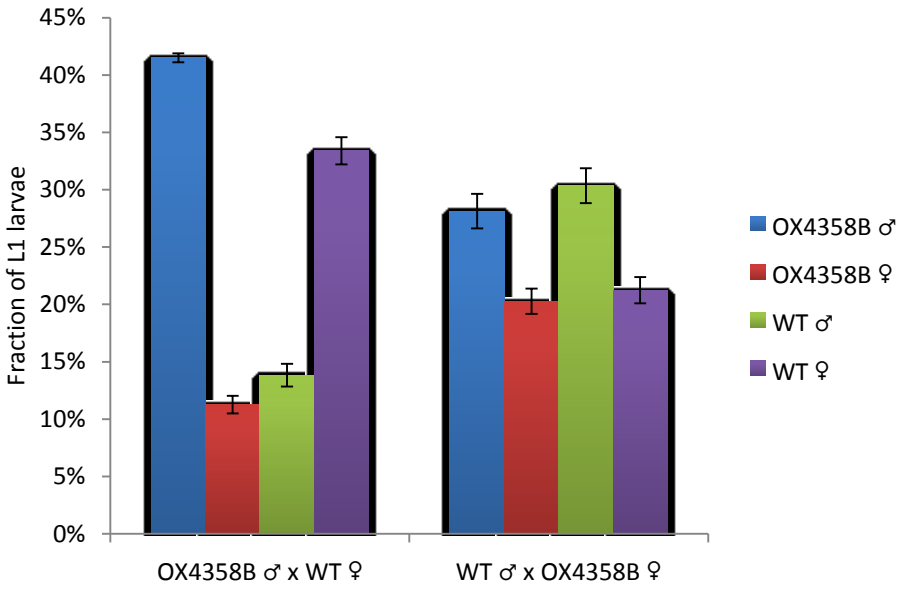


Figure 3-4 Sex linkage of OX4358B.

Fluorescent status and sex of progeny from OX4358B heterozygous males crossed to WT females and the reciprocal cross are presented. First instar larvae were reared in 5 cohorts of 200 for each cross direction, N= 5. Error bars are the standard error of the mean (SEM).

In terms of field functionality of this strain, the proximity of the transgene to the M-Locus will not affect the released insects as they will be homozygous and therefore 100% of the progeny will inherit a single copy, whether male or female. However, the progeny of the surviving heterozygous males will result in a skewed inheritance of the transgene (Figure 3-5). There are two possible impacts of this. Firstly, there will be a greater proportion of wild type females in the F_2 progeny that will be able to fly and potentially transmit disease. Secondly, with more males inheriting the OX4358B transgene than expected, the transgene could persist in the environment for more generations than if it was equally inherited between males and females. Although reducing the number of females in the field as fast as possible is desirable, the passing on of the transgene from field developed $F_{>1}$ males to female progeny will still occur, but at lower frequencies than if the transgene was not sex-linked. Additionally, when releases stop the transgene could persist in the field for more generations than if the gene was inherited equally by each sex, but the system is still self-limiting so the transgene will be eliminated from the environment. The persistence of the OX4358B transgene over successive generations is modelled as part of the work presented in Chapter 4.

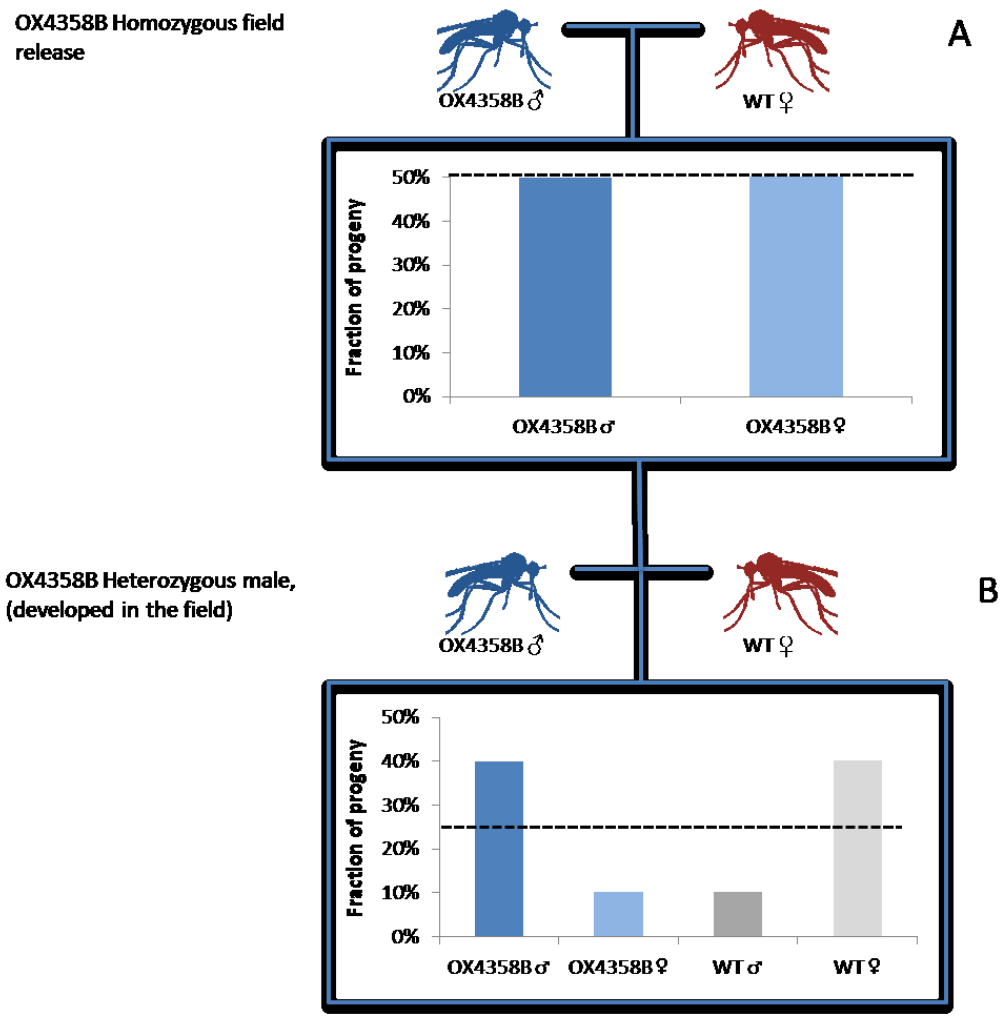


Figure 3-5 Breeding pattern of OX4358B.

Homozygous OX4358B males are released in to the field and mate with wild females (A). All of their progeny will be inherit a single copy of the OX4358B transgene and 50% will be male and 50% will be female. The female progeny will be flightless and have a fitness of zero. The male progeny will develop to flying adults and search for wild female mates (B). As the father of these progeny only has a single OX4358B allele, the offspring will be a combination of OX4358B male heterozygotes, OX4358B female heterozygotes, wild type (WT) females and WT males. If the transgene was not sex-linked, the expected proportion of each group would be 25% (indicated by the dashed line), however given the sex linkage of the transgene the actual ratios are biased towards OX4358B males and WT females. Figure original.

3.3.6. Effects of genetic background on phenotype of OX4358B

After changing the genetic background of OX4358B from an Asian origin to a Latin origin, it was important to ensure that there had not been a change of phenotype. This could arise due to differences in the expression levels of genes surrounding the integration site of OX4358B or distant alleles that render the strain less sensitive to overexpression of tTAV in the female flight muscle. The phenotype in the Latin background was assessed in both homozygotes and heterozygotes and compared to OX4358B Asian heterozygotes. In each case, five cohorts of 200 larvae were reared in the presence and absence of chlortetracycline and the phenotype assessed.

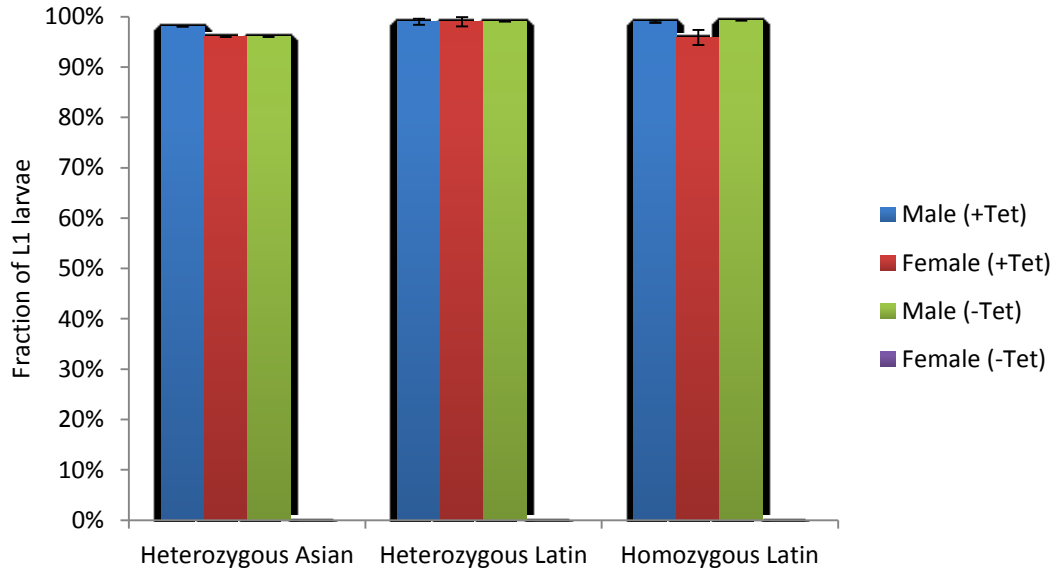


Figure 3-6 Phenotype of OX4358B Latin homozygotes and heterozygotes and OX4358B Asian heterozygotes.

Larvae were reared in the presence and absence of 30 µg mL^{-1} chlortetracycline. Data shows flying adults as a fraction of total adults eclosed. Error bars are the SEM. 200 first instar larvae reared in each of 5 cohorts for each treatment, N= 5.

The results, displayed in Figure 3-6 show that there has not been a change in the phenotype between the original testing in the Asian background and the heterozygous phenotype displayed in the Latin background. The results also show that when reared in the presence of 30 µg mL^{-1} chlortetracycline the flightless phenotype is rescued to >95% in both the heterozygous and homozygous Latin background. The examination of the homozygous phenotype has different motivations to the examination of the heterozygous phenotype. When an individual has only a single transgene allele, the purpose of the experiment is to

monitor for the potential of resistance emerging in the strain, for example by detecting the reduced production of functional tTAV protein or the decrease in sensitivity to the effects of VP16. If resistance were to develop, heterozygous phenotype testing is likely to result in the identification of flying females when reared in the absence of tetracycline. Conversely, the examination of homozygotes is to determine that when reared in the presence of tetracycline, the effects of the two transgene alleles can be fully suppressed to prevent a flightless phenotype. If a proportion of the population were either flightless or had a flight impairment, this could result in the selection of individuals in the colony and lead to a phenotype shift, as well as the cost of egg production increasing as fewer females would be contributing to the egg pool.

3.3.7. Flightless female fecundity

Ae. aegypti males and females recognise one another by the frequency emitted when they beat their wings during flight. The phenotype of OX4358B results in females that are unable to fly and are therefore expected to be unable to mate and consequently have a reproductive fitness of zero. This factor is fundamental to the motivation for the strain, that the female homozygous pupae can be released with the male pupae to reduce the cost of production, and that in the field, heterozygous female progeny will not develop into disease vectors. This experiment aims to determine whether flightless females are able to attract mates, successfully mate with males, take blood meal and lay viable eggs. Heterozygous females were used for this experiment as they only have a single transgene copy and therefore are more likely to be able to mate and fly than homozygotes, and represent progeny to be found in the field.

Individuals reared in the absence of tetracycline were sexed as pupae and the male and female pupae housed separately. When all the adults were sexually mature, males were added to the female cages and the cages were observed for mating. No mating events were witnessed. The females were offered a blood meal and eggs were collected and allowed to mature. The matured eggs were hatched under vacuum to provide the optimum conditions to encourage hatching. Of the 15 cages with approximately 80 flightless females in each cage, eggs were collected from six cages, although blood feeding was observed in all cages (Table 3-3). After vacuum hatching the eggs, no larvae were seen. On inspection of the eggs, approximately 80% appeared to have exploded along the dorsal surface, which is consistent with the laying of sterile, unfertilised eggs (Figure 3-7) (Christophers, 1960). This

was subsequently confirmed by collecting eggs from blood fed WT virgin females. These eggs also appeared to have exploded upon hatching and no larvae were observed. These data confirm that the OX4358B flightless females are unable to mate and consequently have a reproductive fitness of zero.

Table 3-3 Viability of eggs laid by flightless OX4358B heterozygous females.
15 cages of approximately 80 females reared in the absence of tetracycline were used. Cages are only listed if eggs were laid, the remaining 9 cages did not lay any eggs.

Cage	Total number eggs	Total number larvae
2	85	0
7	201	0
8	33	0
13	90	0
14	58	0
15	56	0

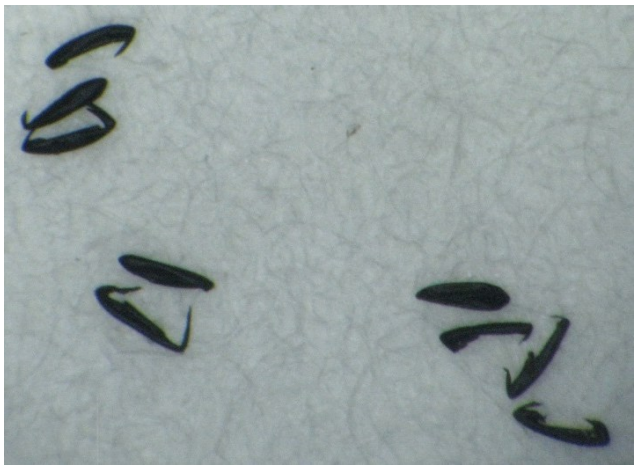


Figure 3-7 Eggs laid by flightless OX4358B females after hatching under vacuum.
Figure original.

3.4. Conclusions

The development of a fully penetrant male-selecting strain has many advantages over the existing bi-sex lethal strain in terms of reducing the cost of production and potentially the faster suppression of wild populations in the field. Fu. *et al* (2010) developed a tetracycline

repressible female flightless strain which was shown to be effective at suppressing WT populations in cage experiments (Wise de Valdez *et al.*, 2011), however this strain did not have a desirable level of rescue of the female flightless phenotype when reared in the presence of tetracycline.

Here, an array of novel putative female flightless insertions have been examined. From a collection of 15 lines, four were identified as having a desirable fully penetrant tetracycline repressible phenotype from which, after further characterization, OX4358B was selected as the strain with the most favourable characteristics.

As part of the examination of OX4358B, it has become apparent that the transgene has inserted on Chromosome 1, 25 cM from the male determining locus, which has resulted in a skewed inheritance ratio of the OX4358 transgene in favour of males in the F_2 generation. In terms of the effectiveness of this line in a suppression programme, only homozygous individuals would be released and therefore all of their progeny will inherit a single copy of the transgene. Therefore although the selected OX4358 insertion is linked to the male locus, this will not affect the overall safety or field efficacy of a suppression programme using this strain.

The work in this chapter has identified a candidate self-limiting male-selecting strain that from the initial testing is suitable to take forward for further characterisation. The next steps will be to investigate the performance of the strain in terms of laboratory and field fitness to understand how it is likely to perform when released into the field as part of a wild population suppression programme. Work towards these goals is addressed in subsequent chapters.

Chapter 4 Impacts of tetracycline and its analogues on transgenic fitness

4.1. Introduction

The mass production of self-limiting strains requires the addition of an antidote, tetracycline to supress the lethal effects of the transgene to allow individuals to develop to adulthood to breed and expand the population. As previously discussed, tetracycline interacts with the TetR component of the tTA complex to cause a conformational change in the protein that prevents it binding to tetO (Figure 4-1). This stops the production of the downstream effector gene allowing the insects to develop to functional adulthood. For this system to work effectively, tetracycline must be added to the larval rearing medium at the optimum time and to a sufficient concentration to maximally supress the effects of the transgene and to prevent sub-lethal fitness costs.

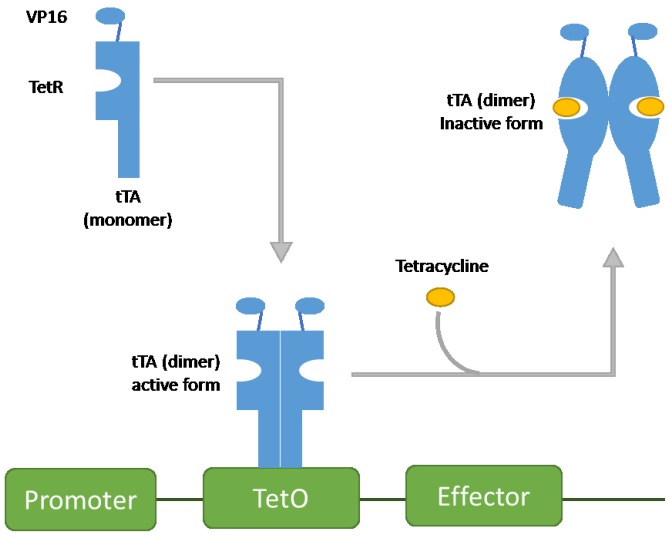


Figure 4-1 Tetracycline repressible system for effector gene regulation.

The transcriptional transactivator (tTA) is a fusion protein of TetR and VP16. It is produced as a monomer but must form a homodimer before it is able to bind to tetO. In the absence of tetracycline, tTA binds to TetO and activates expression of the downstream effector genes. In the presence of tetracycline or its analogues, tTA undergoes a conformational change which inhibits it's binding to TetO and prevents the expression of the downstream effector gene. (Figure adapted from (Rolland, 2003)).

Tetracyclines are a family of broad-spectrum antibiotics (Chopra & Roberts, 2001). They are well known in human therapy and, due to their low cost, are used in animal production as therapeutics, prophylactics and growth promoters (Chopra & Roberts, 2001; Ratasuk *et al.*, 2012; Dahshan *et al.*, 2015), although many countries have banned this use (Thormar, 2012; Figueiredo *et al.*, 2015). Agricultural use in particular can result in significant quantities being excreted, ending up in sewage systems or in manure (Ratasuk *et al.*, 2012) as well as being adsorbed to certain soils within the environment (Zhang *et al.*, 2011). Tetracycline's are highly susceptible to photolysis and hydrolysis giving some tetracycline's half-lives of only a few hours which limits their load in the environment (Loftin *et al.*, 2008), and could reduce the concentration of tetracycline available in the rearing water of self-limiting insects. There is considerable interest in antibiotic load within the environment and numerous surveys have been conducted to determine their concentrations: for tetracyclines these focus primarily on tetracycline, oxytetracycline, chlortetracycline and doxycycline, four common analogues used for therapy. A survey of the literature found maximum reported concentrations from field sites around the world as follows; tetracycline 0.096 ng mL^{-1} to 1.3 ng mL^{-1} (McQuillan *et al.*, 2002; Brown *et al.*, 2006; Gulkowska *et al.*, 2008; Le-Minh *et al.*, 2010; Locatelli *et al.*, 2011) chlortetracycline 0.04 ng mL^{-1} to 0.97 ng mL^{-1} (Yang *et al.*, 2005; Choi *et al.*, 2007), oxytetracycline 0.7 ng mL^{-1} to 1.34 ng mL^{-1} (Lindsey *et al.*, 2001; Watkinson *et al.*, 2009) and doxycycline 0.07 ng mL^{-1} to 0.4 ng mL^{-1} (Watkinson *et al.*, 2009; Le-Minh *et al.*, 2010; Xiong *et al.*, 2015). These data have largely been generated from examination of tetracycline levels from wastewater treatment plants and their downstream flow as they are expected to have particularly high levels, along with the efficiency of removal of tetracyclines during treatment. However these environments are not typical *Ae. aegypti* larval habitats which include artificial containers such as used car tyres, flower vases, water storage vessels and discarded materials in the domestic/peri-domestic environments (Christophers, 1960; Rohani *et al.*, 2014; Romero-Vivas *et al.*, 2015). Reports have also been made of *Ae. aegypti* breeding in septic tanks (Barrera *et al.*, 2008) and brackish waters (Ramasamy *et al.*, 2014) but these are regarded as unusual.

The examination of the effects of tetracycline on a self-limiting strain comes from two different angles: firstly the use of tetracycline's in human and veterinary therapy results in tetracycline being present in the environment. This could mean that the progeny of released males are exposed to sufficient tetracycline to allow their development to adulthood, which could affect the safety and efficacy of a suppression programme. To

understand this risk, determining the lowest concentration of tetracycline to allow rescue of the released male's progeny will allow an understanding of how the concentrations found in the field are likely to impact a control programme. Secondly, tetracycline is added to the larval rearing medium to allow development of individuals to adulthood for the purpose of egg collection. If the provision of tetracycline is sub-optimal, either not a high enough concentration or not delivered at the optimum time, then lethal or sub-lethal effects of the transgene may affect the insects fitness and performance. This could occur within a generation resulting in a shorter life-span, a lower mating competiveness, a lower egg yield or poor performance in the field, or over the course of multiple generations, resulting in population drift selecting for individuals which are resistant to the effects of the transgene: this could ultimately result in a strain which loses the desired self-limiting phenotype.

To understand these opposing aspects of the strain interaction with tetracycline and its concentration, a series of experiments have been designed to determine the optimum tetracycline analogue to use for mass production, along with an assessment of the fitness impacts related to the suppression of tTAV. The current tetracycline of choice for mass production is chlortetracycline, however investigating the effectiveness of three other widely available tetracycline analogues, tetracycline, oxytetracycline and doxycycline may identify a more effective antidote for future use.

Work in this chapter identifies which tetracycline has the most desirable profile by examining the changes in phenotype of OX513A and OX4358B reared at different concentrations of the four compounds: oxytetracycline, chlortetracycline, doxycycline and tetracycline (OX513A data published in (Curtis *et al.*, 2015)). Upon selecting a desirable tetracycline, longevity assays are performed to determine how different tetracyclines affect lifespan, an important aspect of insect fitness that if reduced is likely to impact egg production and male field effectiveness. Finally, the effectiveness of the selected tetracyclines at supressing the effects of the transgene over multiple generations is monitored in closed populations to ascertain if sub-lethal fitness costs are resulting in selection within the population. This assay will help to identify which tetracycline is optimal at maintaining the fitness of a population to reduce the selection of individuals, which could ultimately result in selection away from the desirable phenotype.

4.2. Methods

4.2.1. Insect rearing and strains

Insects were reared according to Chapter 2.2.2 Insect Rearing. The strains used in the following experiments are described in Chapter 2.2.1 Mosquito Strains and in Chapter 3.

4.2.2. Tetracycline and its analogues dose response

OX513A Latin homozygous males and OX4358B Latin homozygous males were outcrossed to LWT females and eggs collected. The resulting progeny were hatched and the larvae were counted into cohorts of 200. Larvae were reared in the presence of different concentrations of chlortetracycline, tetracycline, oxytetracycline and doxycycline (Sigma Aldrich, UK) along with cohorts reared as a control in the absence of tetracycline. Each concentration had three repeats. OX513A individuals were assessed according to Chapter 2.2.3 Assessment. OX4358B individuals were assessed according to Chapter 3.2.1 Phenotype testing of OX4358 lines.

4.2.3. OX4358B Tetracycline Longevity

OX4358B Latin homozygotes and OX4358B Latin heterozygotes and LWT were reared in five cohorts of 200 larvae, in the presence or absence of 30 μg mL^{-1} chlortetracycline. Pupae were picked and sexed and placed into cages (15 cm x 15 cm x 15 cm) in cohorts of 25 pupae and allowed to eclose. Four cages (repeats) were prepared for each treatment (Table 4-1). Adult cages were supplied with 10% sugar water and water *ad libitum*. Males and females were kept virgin. Daily mortality was recorded. The water feeders and sugar feeders were changed weekly.

Table 4-1 OX4358B Tetracycline longevity treatments.

Four cages of 25 pupae were prepared for each treatment with each sex. Different treatments were run in different experiments, Latin wild type (LWT) controls were included in each experimental set up.

Experimental set up	Strain	Chlortetracycline
1	OX4358B Homozygotes	+
	OX4358B Heterozygotes	-
	LWT	+
	LWT	-
2	OX4358B Homozygotes	-
	LWT	-

4.2.4. OX513A Tetracycline Longevity

OX513A Latin homozygotes were reared in four cohorts of 200 larvae, in the presence of 30 µg mL^{-1} chlortetracycline or 1 µg mL^{-1} doxycycline. Pupae were picked, sexed and placed into cages (15 cm x 15 cm x 15 cm) in cohorts of 25 pupae and allowed to eclose. Four cages (repeats) of each sex were prepared for each of the treatments (Table 4-2). Adult provision of the designated tetracycline in the sugar water and water feeder was to the same concentration as the larval rearing medium.

Table 4-2 OX513A Tetracycline longevity treatments.

Four cages of 25 pupae were prepared for each treatment and for each sex.

Treatment	Larval rearing	Adult diet	
		10% sucrose water	Water Feeder
A	Chlortetracycline	No chlortetracycline	No chlortetracycline
B	Chlortetracycline	With chlortetracycline	With chlortetracycline
C	Doxycycline	No doxycycline	No doxycycline
D	Doxycycline	With doxycycline	With doxycycline

Males were kept virgin. Upon eclosion, additional males were introduced into the female cages to allow mating. Two days later the males were removed and discarded. The females

were offered two blood meals on day 19 and day 27 post-eclosion. Eggs were collected after each blood meal. Daily mortality was recorded. The water feeders and sugar feeders were changed weekly.

4.2.5. Changes in transgene allele frequency over successive generations as a measure of fitness

OX4358B Latin homozygous males were crossed to OX4358B Latin heterozygous females to make a F_1 generation with an OX4358 allele frequency of 0.75. The F_1 eggs were reared in three cohorts of 200 larvae, in the presence of 30 µg mL^{-1} chlortetracycline or 1 µg mL^{-1} doxycycline and fed a standard feeding regimen (Table 9-1). To determine the fitness of the strain in the absence of tetracycline, OX4358B Latin heterozygous males were crossed to LWT females to produce an F_1 generation with an OX4358 allele frequency of 0.25. The F_1 eggs were reared in three cohorts of 200 larvae in the absence of tetracycline.

Each of the three cohorts in each treatment represents a population. Upon pupation, pupae were picked, sexed, screened and the numbers recorded. The ratio of homozygous to heterozygous individuals in each population was scored by the difference in the intensity of the AmCyan fluorescence seen using an epi-fluorescence microscope (Figure 3-3) at the pupal stage. Male and female pupae from each cohort were housed separately to eclose. Three days after the last pupa was added to the cage, the males were transferred into the female cage. The females were blood fed and F_2 eggs collected.

The F_2 eggs (and subsequent generations) were hatched and reared under the same conditions as the parental cohort; one cohort of 200 larvae was set and reared in the presence of 30 µg mL^{-1} chlortetracycline or 1 µg mL^{-1} doxycycline or in the absence of tetracycline.

The populations reared in the presence of chlortetracycline and doxycycline were reared for 10 generations. The populations reared in the absence of tetracycline were reared until a generation was negative for the OX4358B allele or generation 10 was reached.

4.2.6. Statistical analyses

Data were analysed using the RStudio software package version 0.97.318 (RStudio, USA). Normality of data was tested using the Shapiro-Wilk method. Homogeneity of variance was tested for using the Bartlett test. For all comparisons significance was set at $p < 0.05$.

Dose response data was analysed for significant differences between concentrations using either a Student's *t*-test if normally distributed or Wilcoxon Rank Sum if not normally distributed. The data was modelled using a Weibull model for chlortetracycline and a Log-linear model for the remaining antibiotics, using the DRC package (2.5-12). These models were used to calculate the EC_{50} (Effective Concentration$_{50}$) with 95% confidence intervals.

Longevity data were analysed using the Survival Analysis package (2.38-2). Kaplan-Meier curves were plotted and Log-rank tests used to test for significant differences between treatments.

Change in transgene allele frequency, permissive conditions: The fitness of individual genotypes were analysed by using a Chi square test comparing the observed and expected frequencies of the OX4358B allele assuming the Hardy Weinberg assumptions. The fitness of the OX4358B allele was estimated by using the formula Log(p/(1-p)) where p is the OX4358B allele frequency. The slope of the trend line provides the selection coefficient against the transgene.

Change in transgene allele frequency, restrictive conditions: Stochastic modelling was developed from a model built by Dr Gong built in Microsoft Excel (Microsoft, California, USA) using a Monte Carlo design. In this model, the progeny resulting from parental crosses (200 in each generation) were assigned two random numbers: one determining their sex, the other their genotype (OX4358B or WT). The probability of being males was set at a constant 0.5 for each generation. The probability of being assigned a self-limiting genotype was dependant on the proportion of transgenic males in the parental generation. As part of the analysis process performed here, this model was updated twice: in the first modification, the sex linkage of the OX4358B transgene was introduced so that 80% of the transgenics were male and the remaining 20% were female. In the second modification, which included the sex linkage, an adjustable fitness cost was placed onto the transgenic males to reduce their efficacy in that generation. The model assumes a panmictic closed population, of 200 individuals with a fully penetrant phenotype. The modelled transgene allele frequencies were compared to empirical data using a Nash Sutcliffe model efficiency coefficient, which compares the mean modelled values to the mean observed values, over a time series. This results in a coefficient which can range from 1 to ∞ where 1 = a perfect match.

4.3. Results and Discussion

4.3.1. Tetracycline and its analogues dose response

OX513A and OX4358B both require the addition of tetracycline to allow the development of individuals to functional adulthood. The purpose of investigating the dose response of tetracycline on the resulting phenotype of the two strains is twofold: firstly to determine the lowest concentration which allows individuals to be rescued from the lethal or flightless phenotype which has relevance for field safety and efficacy, and secondly to determine the concentration at which the lethal effects or flightless phenotype are fully supressed to allow breeding of the strains in factories without associated fitness affects. In addition to this, these experiments aimed to assess the effectiveness of different tetracycline analogues efficacy at supressing the strain phenotype. The tetracycline analogues used were chlortetracycline (the standard tetracycline used in mass production), oxytetracycline, tetracycline and doxycycline. These tetracyclines were chosen due to their common use in human and veterinary medicine and their cost effective supply. Three cohorts of 200 larvae were reared at different concentrations of each of analogue and their phenotype assessed.

Starting with OX513A, the results show that larvae reared at chlortetracycline concentrations at or below 1 ng mL^{-1} did not give rise to a significantly greater percentage of functional adults than larvae reared in the absence of tetracycline (0 µg mL^{-1}) ($t(18) = -1.31$, $p=0.21$). Chlortetracycline concentrations in excess of 1 ng mL^{-1} allowed a greater percentage of functional adults ($t(18) = -4.39$ $p=<0.001$). Figure 4-2A shows the percentage of functional adults increasing with greater chlortetracycline concentrations, with the model predicting the maximum rescue plateau beginning to appear at 1 µg mL^{-1}. This indicates that concentrations at or slightly above 1 µg mL^{-1} will give rise to the maximum percentage of functional adults.

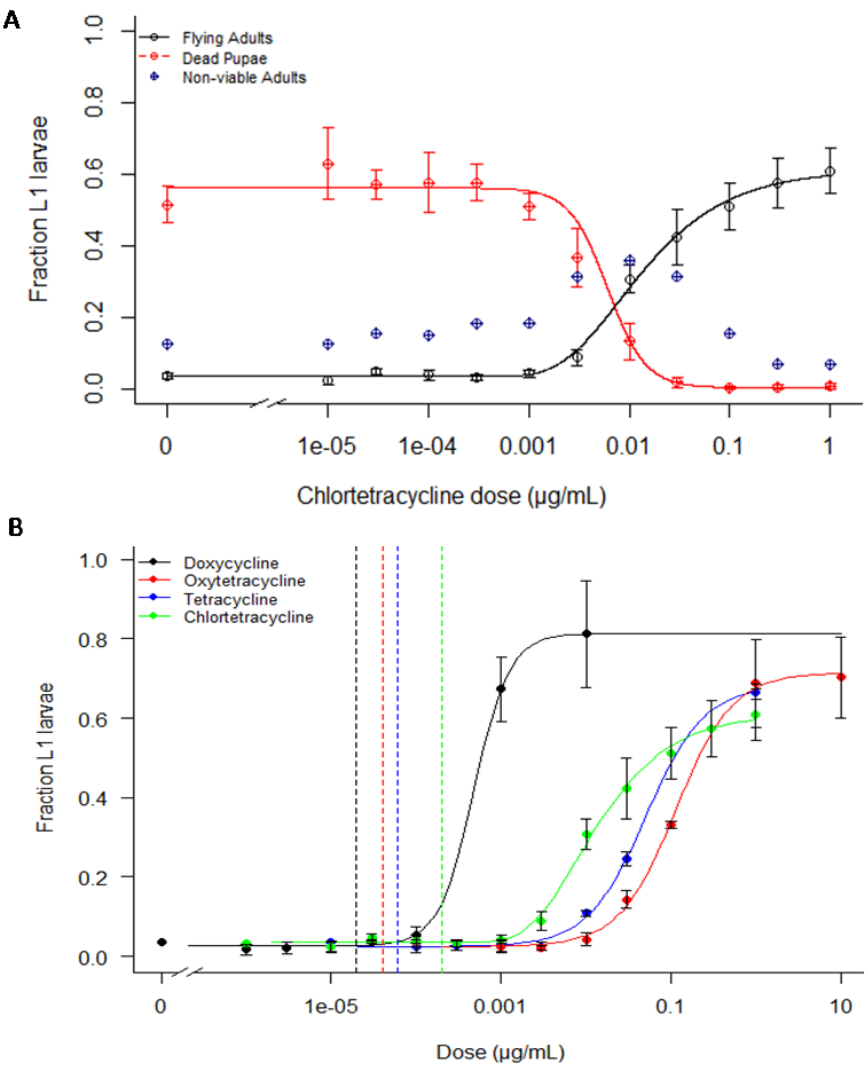


Figure 4-2 Dose response of OX513A to tetracycline and its analogues.

A) Chlortetracycline dose response. Data shown for each of the three developmental outcomes for first instar larvae (L1) is shown: mortality as pupae, eclosion as non-viable adult, eclosion as functional (flying adult) adult. B) Dose response for four tetracyclines. Proportion of L1 surviving to functional adult data are shown after rearing on various concentrations of each tetracycline. Solid lines indicate the best-fit model; see also Table 4-3 for EC_{50} calculated from this model. Dashed vertical lines represent mean concentrations of each analogue (in the same colour as the lines themselves) found in environmental bodies of water (Kolpin *et al.*, 2004; Gulkowska *et al.*, 2007; Kemper, 2007; Watkinson *et al.*, 2009; Locatelli *et al.*, 2011; Wei *et al.*, 2011; Tong *et al.*, 2014). If reported values were below the limit of quantification (LOQ), the LOQ value was used to populate the mean. Error bars represent the 95% confidence intervals. Note different scales on X-axis.

For the other tetracycline compounds tested, concentrations at or below 3 ng mL^{-1} tetracycline, 10 ng mL^{-1} oxytetracycline and 100 pg mL^{-1} doxycycline, did not give rise to a significantly greater proportion of functional adults than if reared in the absence of tetracycline (Z=6.5, p= 0.48; Z =3, p=0.64; Z =1.5, p=0.27 respectively) (Figure 4-2B).

For all four tetracyclines tested, increasing concentrations resulted in incremental shifts towards higher fitness resulting in the increased survivorship of OX513A individuals (Figure 4-2). With reference to Figure 4-2A, as the chlortetracycline concentration increases to result in more functional adults than 0 µg mL^{-1} tetracycline, the sample population shifts towards a higher fitness with an increasing fraction of non-viable adults and decreasing fraction of dead pupae. With further increases in the chlortetracycline concentration, the non-viable adult fraction drops and the population shifts to eclosing as functional adults. This demonstrates that increasing tetracycline concentrations have incremental rescue effects on OX513A resulting in an average increase in survivorship represented by an increased proportion of flying functional adults.

The EC_{50} values (half maximal effective concentration; the concentration which induces a response halfway between the baseline and maximum) shown in Table 4-3, demonstrate that doxycycline is by far the most effective analogue at rescuing the OX513A phenotype, being able to rescue individuals at a lower concentration than the other tetracyclines.

Table 4-3 EC_{50} of tetracycline and analogues at rescuing OX513A.

Tetracycline and analogues were added to the larval rearing water at various concentrations and the EC_{50} (half maximal effective concentration) modelled. 95% confidence intervals shown in parenthesis. Results are for functional (flying) adults.

Compound	EC_{50} ng mL^{-1} (95% C.I.)
Oxytetracycline	113 (89 to 138)
Tetracycline	50 (34 to 66)
Chlortetracycline	13 (9 to 16)
Doxycycline	0.48 (0.40 to 0.60)

The results of the dose response analysis with OX4358B show a similar pattern of rescue to that of OX513A. Doxycycline is again by far the most effective tetracycline at rescuing the phenotype with the concentrations of 0.03 µg mL^{-1} allowing the first females within the

population to fly (mean flying fraction at 0.03 µg mL^{-1}; 4.2% ± 1.2% SEM compared to 0.0% flying at 0.0 µg mL^{-1}) (Figure 4-2) compared to concentrations of 3 µg mL^{-1} oxytetracycline, 1 µg mL^{-1} tetracycline and 1 µg mL^{-1} for chlortetracycline (Figure 4-3). Using the model to predict the concentrations for full rescue of the females from being flightless, concentrations of chlortetracycline, oxytetracycline and tetracycline are required of at least 30 µg mL^{-1}, however, doxycycline exhibits full rescue from the flightless phenotype when larvae are reared at concentrations of 1 µg mL^{-1}. Further to this, these data indicate that a concentration of 1 µg mL^{-1} doxycycline allows an overall greater fraction of females to fly than the other tetracyclines tested, although this is not significant.

The effectiveness of doxycycline at rescuing the strain is confirmed with the EC_{50} values showing that doxycycline rescues 50% of the population at 14 to 26 times lower concentrations than the other tetracyclines tested (Table 4-4).

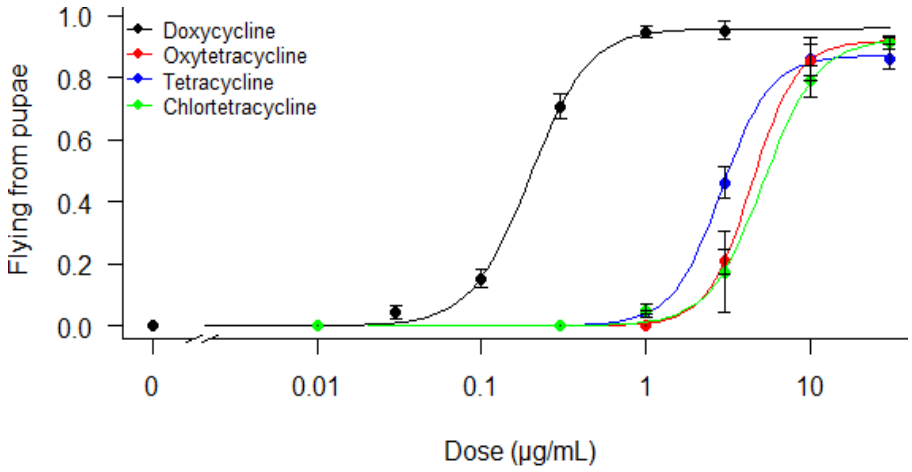


Figure 4-3 Dose response of OX4358B to tetracycline and its analogues.

Dose response of four tetracyclines. Results are of the proportion of females flying from total female pupae collected. Lines indicate the best-fit model; see also (Table 4-4) for EC_{50} calculated from this model. Error bars represent the 95% confidence intervals.

Table 4-4 EC_{50} of tetracycline and analogues at recovering OX4358B females flight ability. Tetracycline and analogues were added to the larval rearing water at various concentrations and the EC_{50} (half maximal effective concentration) modelled. 95% confidence intervals shown in parenthesis. Results are based on the fraction of flying females from female pupae.

Compound	EC_{50} µg mL^{-1} (95% C.I.)
Oxytetracycline	4.4 (4.1 to 4.7)
Tetracycline	2.9 (2.7 to 3.0)
Chlortetracycline	5.2 (4.4 to 5.9)
Doxycycline	0.20 (0.19 to 0.21)

The first motivation of this experiment was to determine the lowest concentration of tetracycline and its analogues that would rescue the lethal or female flightless phenotype to ensure that this concentration was above the concentration found in the field. Figure 4-2B demonstrates that the mean concentration of each analogue in environmental water bodies is below the concentration required to rescue the OX513A lethal phenotype, and far below the concentration required to rescue the flightless OX4358B phenotype. However a wide survey of the literature identified a few instances of reported environmental concentrations of doxycycline above the concentration which would allow a greater than the nominal fraction of OX513A larvae to develop to functional adults. Such studies are often interested in the impact of treatment and removal of antibiotics in waste-water treatment plants (e.g.(Watkinson *et al.*, 2009; Le-Minh *et al.*, 2010) and their associated rivers, and find the maximal tetracycline concentrations to be in the sewage input to treatment plants. These environments are not the typical larval habitats of *Ae. aegypti.* This species is a 'container breeding mosquito'; its preferred breeding sites are refuse, flower vases, water storage containers and other peri-domestic sites. Immature stages are found in clean, still water, not flowing river systems and are rarely found in collections of water in the ground such as earth drains (Christophers, 1960; Morrison *et al.*, 2006; Dieng *et al.*, 2012). Some reports have suggested that *Ae. aegypti* can breed in septic tanks, usually where they are cracked or broken (Barrera *et al.*, 2008; Mackay *et al.*, 2009), but this tends to be in the clear water at the top of the tank whereas tetracyclines tend to bind to the sediments located at the bottom of the tanks (Brown *et al.*, 2006; O'Connor & Aga, 2007; Mackay *et al.*, 2009; Barrera *et al.*, 2011) and are expected to represent a minority of breeding sites. Full rescue of the OX513A individuals (the maximum number surviving to functional adults) was shown in this data to require tetracycline compound concentrations that were 746 to 2500 times greater

than the highest concentrations found reported in the literature for environmental tetracyclines. Therefore although there are instances where the concentration of doxycycline is sufficient to allow for a small increase in the OX513A functional adults, these locations are unlikely to be a part of a suppression programme area. Alternatively, OX4358B could be used in preference to OX513A as the required dose to allow the rescue of the flightless phenotype to deviate from 0 µg mL^{-1} is 75 to >2000 fold higher than the maximum concentration reported in the literature.

The second motivation of these experiments was to determine which tetracycline was optimal at supressing the effects of the transgene, and at what concentration full rescue could be achieved. These data clearly show that doxycycline is superior at rescuing the phenotype from the negative effects of the transgene at lower concentrations. Krueger et *al*. (2004) identified doxycycline as a superior tetracycline to use in conjunction with the tetracycline repressible system when working with cell lines, repressing the system at lower concentrations than other tetracyclines tested (Krueger *et al.*, 2004), but the data collected here appears to be the first report of different tetracycline compounds efficacy in *in vivo* studies.

To determine why doxycycline is more effective at supressing the transgene, it is important to look at the chemical behaviour of the molecule: it has been shown that doxycycline is more stable than the other tetracyclines, having a half-life of approximately 17 hours compared to 6-9 for the other tetracyclines tested (Chopra & Roberts, 2001; Soeborg *et al.*, 2004; Kemper, 2007) and will therefore persist at high concentrations for a longer duration than the other tetracyclines, but it is most likely due to doxycycline's increased lipid solubility compared to the other tetracyclines which makes it more effective (Valentin *et al.*, 2009). Being more lipid soluble will allow an easier passage of the molecule through the phospholipid bilayer of the cells, most likely allowing a greater concentration into the cells. It is also possible that the rate of passage will be faster so that the critical concentration within the cells is reached more rapidly to bind to tTAV to prevent any toxic effects.

When comparing the data of OX513A and OX4358B it is apparent that OX513A requires a considerably lower concentration of tetracycline or its analogue than OX4358B to allow for partial or full rescue from the strain phenotype. The reason for this is not clear but it could be as a result of one or a combination of the following: Muñoz et *al.* (2004) showed that AeAct4, the promoter of OX4358B is active predominantly at the pupal stage and mostly in

the indirect flight muscles, whereas Actin5C, the promoter of OX513A is predicted to be active at all life-stages and is associated with the gut, fat body tissue surrounding the gut and in the gastric caeca (Burn *et al.*, 1989; Pinkerton *et al.*, 2000). In addition to this, the process of metamorphosing from a larva to a pupa prevents the individual feeding further (Christophers, 1960) and therefore presumably accumulating doxycycline. Consequently, OX4358B individuals need to have accumulated sufficient doxycycline by the point of pupation to supress the effects of the transgene, once it is actively expressed, and it must be in the location where the indirect flight muscles will develop. For these reasons, it can be seen how OX4358B is likely to require higher concentrations of doxycycline than OX513A to supress the transgene.

4.3.2. OX4358B Tetracycline longevity

Measuring the longevity of a population can be used to identify sub-lethal fitness effects that are not measurable in other laboratory assays. Many factors can reduce the lifespan of a strain including leaky expression, transgene insertion position, selection during the homozygosing process and adaptation to the laboratory environment. Here OX4358B homozygotes and LWT were reared in the presence of tetracycline to determine if the expression of the transgene had been repressed by the addition of the standard rearing tetracycline, chlortetracycline to a concentration of 30 µg mL^{-1}. This scenario represents the population of an egg mass production facility to ensure that both males and females have an uncompromised longevity as a result of maximal suppression of the transgene. Conversely, OX4358B heterozygotes and LWT were reared in the absence of tetracycline to determine firstly that the females had a significantly reduced life-span due to the inability to fly and to access food sources, and secondly that the males life-span was unaffected by the absence of tetracycline from the rearing medium. Heterozygotes were used for this treatment to simulate the progeny expected to develop in the field in the absence of tetracycline. Finally in a separate experiment, OX4358B homozygous males and LWT were reared in the absence of tetracycline to ensure that the males reared for release in the absence of tetracycline do not suffer from a reduced life-span compared to LWT.

The results show that homozygous OX4358B and heterozygous OX4358B females reared in the absence of tetracycline both have a significantly shorter life-span than LWT females reared in the absence of tetracycline (Table 4-5) (χ^2 (1, *N*= 195) =200, *p* =<0.001, χ^2 (1, *N*= 187) =180, *p* =<0.001 respectively) (Figure 4-4 B & D), which is expected because these

females are unable to fly and consequently struggle to access nutritional resource. However OX4358B homozygous females reared in the presence of chlortetracycline do not have a significantly shorter lifespan than LWT females reared in the presence of chlortetracycline (χ^2 (1, *N*= 178) =1.6, *p* =0.20) (Figure 4-4 B).

With respect to males, OX4358B heterozygous males reared in the absence of tetracycline do not have a significantly shorter lifespan than LWT males reared in the absence of tetracycline (χ^2 (1, *N* = 191) =1.1, *p* =0.30) (Figure 4-4 A), however OX4358B homozygous males reared in the absence of tetracycline are longer lived than LWT males with a median lifespan of 55 days compared to 51 days (χ^2 (1, *N* = 187) =7.2, *p* =<0.001) (Table 4-5) (Figure 4-4). In addition when OX4358B homozygous males are reared in the presence of chlortetracycline, the lifespan of individuals was significantly longer than LWT males reared in the presence of chlortetracycline (χ^2 (1, *N* = 181) =10.4, *p* =0.001) (Figure 4-4). It is unlikely that OX4358B has gained any fitness improvement by the addition of the transgene, rather the longer lifespan seen in OX4358B males is likely to either be a stochastic effect, or a result of strain selection and maintenance in previous generations.

These data show that the phenotype of OX4358B heterozygous and homozygous females reared in the absence of tetracycline results in the expected fitness penalty of a significantly reduced lifespan. Their lifespan in the field is expected to be further reduced by the absence of close provision of sugar water and by the addition of predators, which they will be unable to evade. In terms of the survival of OX4358B males, they do not appear to be compromised by the transgene, in fact the homozygous males live longer than WT males when reared in the presence or absence of tetracycline. These data imply that there is little detrimental expression of tTAV in males, and that males with two copies of the transgene have a comparable fitness to heterozygous males carrying only one copy.

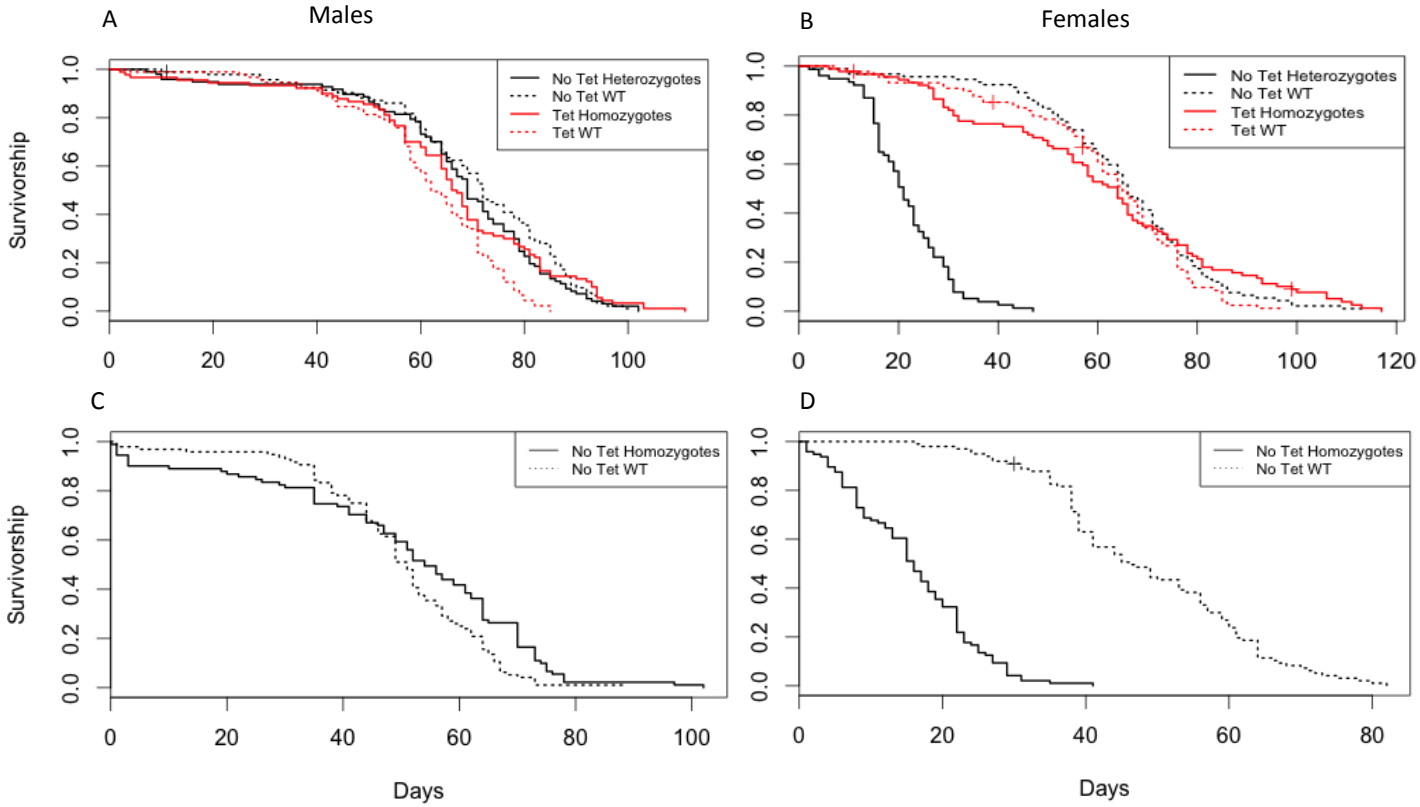


Figure 4-4 Longevity of OX4358B Latin heterozygotes, OX4358B homozygotes and LWT.

Larvae reared in the absence of tetracycline (No Tet) and OX4358B Latin homozygotes and LWT in the presence of 30 µg mL^{-1} chlortetracycline (Tet). Males are represented in panels A and C and females in panels B and D. Due to the two experimental runs, the results presented in panel C and D cannot be directly compared to panel A and B. Each treatment had 100 starting pupae. Note the differing scales on the x-axes.

Table 4-5 Longevity of OX4358B homozygotes, OX4358B heterozygotes and LWT reared in the presence or absence of 30 µg mL^{-1} chlortetracycline.

Median survival is presented with the 95% confidence intervals. The experiment was run in two separate cohorts (indicated by the dividing line). Due to environmental conditions in the insectary the results from the two runs cannot be directly compared. Each treatment had 100 starting pupae.

Tetracycline	Strain	Sex	Median (days)	95% Confidence interval
No	OX4358B/LWT	F	21	(18 to 23)
	LWT/LWT	F	66	(64 to 71)
	OX4358B/LWT	M	69	(66 to 73)
	LWT/LWT	M	72	(69 to 78)
Yes	OX4358B/OX4358B	F	63	(57 to 63)
	LWT/LWT	F	64	(60 to 68)
	OX4358B/OX4358B	M	66.5	(64 to 69)
	LWT/LWT	M	62	(60 to 67)
No	OX4358B/OX4358B	M	55	(51 to 62)
	LWT/LWT	M	51	(49 to 53)
	OX4358B/OX4358B	F	16	(15 to 18)
	LWT/LWT	F	45	(41 to 53)

4.3.3. OX513A Tetracycline longevity

Previous work carried out by Dr K. Matzen has found that OX513A homozygous males are significantly shorter lived than LWT males (personal communication with Dr K. Matzen). This result has the potential to manifest itself both in the field and in an egg production facility: in the field if OX513A males are shorter lived than their wild competitors, their ability to effectively and successfully mate with wild males may be compromised. In the laboratory, the reduced longevity of males could result in lower female fertilisation or potentially population selection if longer lived males achieve more mates. The current mass production protocol for both egg production and for field release is to rear cohorts in the presence of 30 µg mL^{-1} chlortetracycline, however results collected in Chapter 4.3.1 indicate that doxycycline maybe more efficient at supressing the lethal effects of the transgene and therefore may restore the OX513A male longevity to that of LWT. In this experiment two variables are introduced: firstly cohorts are reared in the presence of chlortetracycline and the more effective tetracycline analogue doxycycline, and secondly the adult diet is supplemented with tetracycline (the same tetracycline as added to the rearing medium,

chlortetracycline or doxycycline) to assess if addition to the adult diet extends longevity. For each treatment, larvae were reared in the presence of 30 µg mL^{-1} chlortetracycline or 1 µg mL^{-1} doxycycline. Upon pupation, 25 pupae were placed into each of four cages for each treatment and allowed to eclose. Cohorts were either provisioned with 10% sugar water and water or 10% sugar water and water supplemented with their tetracycline analogue (Table 4-1). Daily mortality was recorded.

The results show that there was no significant difference in the longevity of males reared in the presence of chlortetracycline, doxycycline and with or without adult tetracycline (χ^2 = (1, *N* = 66, 88, 96, 90)=4.7, *p* = 0.20) (Figure 4-5) (Table 4-6). This result demonstrates that rearing with the more effective analogue, doxycycline, does not prevent the fitness cost seen in previous experiment performed by Dr Matzen, nor does the addition of tetracycline to the adult diet. Consequently rearing in the presence of doxycycline or supplementing the adult diet with tetracycline does not significantly increase the longevity of OX513A compared to chlortetracycline reared males, and therefore will not return the longevity of OX513A males to that of LWT.

In the experiment performed by Dr K. Matzen the longevity of OX513A females was not compromised compared to LWT. In this experiment however, doxycycline reared females are significantly shorter lived than chlortetracycline reared females (χ^2 (1, *N* = 95, 91) =4.4, *p* =0.04 (Table 4-6) (Figure 4-5) and therefore it can be hypothesised that rearing females with doxycycline produces females which are significantly shorter lived than LWT[5]. The provision of adult tetracycline resulted in no significant difference in the longevity compared to their no adult tetracycline reared counter-parts (chlortetracycline χ^2 (1, *N* = 95, 90) =0.6, *p* =0.45, doxycycline χ^2 (1, *N* = 91)= 0, *p* =0.95).

[5] It is not possible to directly compare the longevity of treatments in this experiment to the results collected by Dr.K Matzen as longevity assays are very sensitive to environmental conditions resulting in different median longevities between experimental runs of the same treatment. As a result, the treatments within an experimental set up must be compared only against each other, not against external data.

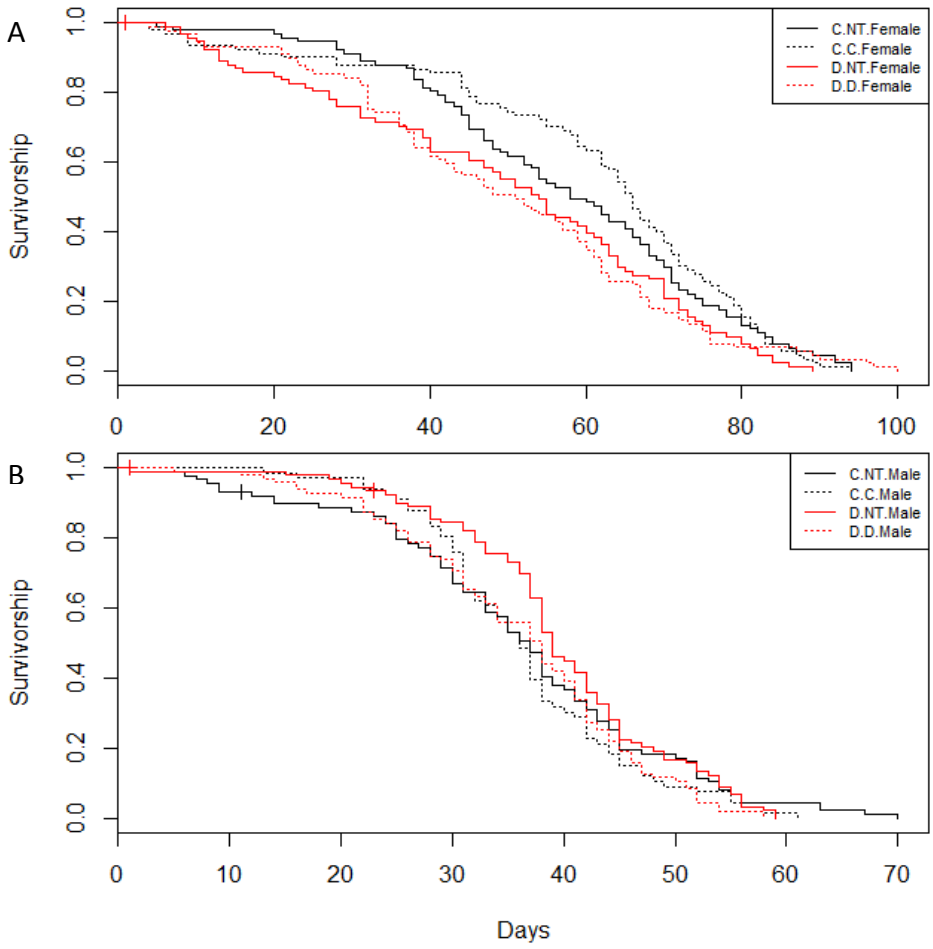


Figure 4-5 Longevity of OX513A females (A) and males (B) reared in the presence of 30 µg mL^{-1} chlortetracycline and 1 µg mL^{-1} doxycycline.

25 males or females were placed into each of four cages per treatment and their daily mortality was recorded. Adults were provided with 10% sugar water and water, with or without the larval rearing tetracycline included. C.NT. Chlortetracycline reared larvae, no adult tetracycline. C.C. Chlortetracycline reared larvae with adult chlortetracycline. D.NT. Doxycycline reared larvae, no adult tetracycline, D.D. doxycycline reared larvae with adult doxycycline. Note different scales on x-axis

The reduced longevity of the doxycycline-reared females is an unexpected outcome as results from previous experiments inferred that doxycycline was the more effective tetracycline at supressing the transgene and would therefore prevent any transgene related fitness costs. It is possible that the reduced longevity seen here is a consequence of doxycycline at 1µg mL^{-1} changing the rearing environment to be less permissive or even toxic at this concentration. Adult longevity is closely related to the individuals teneral reserves

and therefore if doxycycline is affecting the accumulation of these reserves during larval development, it is possible that the females would have a shorter lifespan (Koenraadt *et al.*, 2010; Bargielowski *et al.*, 2012). However, as the reduced lifespan was not seen in the male doxycycline reared cohort, and given the result is only just significant in the female cohort (p=0.04) it is possible that this was a stochastic result, and it should be repeated and investigated further before any decisions are made about the effectiveness of doxycycline.

Table 4-6 Longevity of OX513A homozygotes, reared in the presence of 30 µg mL^{-1} chlortetracycline or 1 µg mL^{-1} doxycycline.

Adults were provisioned with 10% sugar water and water with or without the associated larval rearing tetracycline analogue. Median survival is presented with the 95% confidence intervals. Each treatment had 100 starting pupae.

Larval rearing	Adult tet	Sex	Median Survival	95% Confidence Interval
Chlortetracycline	No	M	37	(33 to 39)
		F	58	(53 to 66)
	Yes	M	36	(33 to 38)
		F	66	(62 to 70)
Doxycycline	No	M	39	(38 to 42)
		F	54	(47 to 61)
	Yes	M	38	(34 to 41)
		F	51	(43 to 59)

4.3.4. Changes in transgene allele frequency over successive generations as a measure of fitness

Many experiments designed to test the fitness of a strain only examine isolated components of an insect's fitness, for example mating competitiveness or longevity, which can result in an unrealistic or biased assessment of the strains likely performance in the field. The experiment performed here aims to quantify the cumulative fitness effects impacting a strain both within a generation e.g. competition for larval food and mating competitiveness, as well as affects which can play out over successive generations, for example assortative mating and hybrid vigour.

As previously discussed, the fitness of a transgenic strain can be negatively affected by several factors including insertional mutagenic effects of the transgenes insertion position,

inbreeding depression or genetic drift as a result of selection or laboratory adaptation. These affects are likely to manifest themselves to a greater extent when the insect carries two copies of the transgene (homozygous) as opposed to one copy (heterozygous). By monitoring the changes in the transgene allele frequency over successive generations, it is possible to estimate the fitness cost of the transgene as a homozygote and as a heterozygote with all life-stages contributing to the insect's fitness. The experiment performed here was designed to quantify the fitness of transgenic insects under two different scenarios: firstly reared in the presence of tetracycline (permissive conditions) and secondly in the absence of tetracycline (restrictive conditions).

In the first instance, the change in frequency of the transgene allele monitored over successive generations of populations reared in the presence of tetracycline (permissive conditions) can be used to estimate the fitness cost of individuals carrying the transgene when it is expected to be supressed. By spiking the population with wild type alleles, the changes in three allele states, transgene homozygous AA, transgene heterozygous Aa, wild type homozygous aa can be monitored to quantify how quickly a wild type allele could spread through a mass production colony, as a consequence of the decreased fitness of their transgenic siblings. Where transgenic individuals do not suffer a fitness penalty the change in allele frequency over successive generations is expected to obey Hardy-Weinberg's principle, and if a deviation is seen between the observed and expected frequencies it is likely that a genotype is being selected against. The design of this experiment allows for further evaluation of the effectiveness of doxycycline at supressing the transgene compared to the standard tetracycline used, chlortetracycline. Given the data from the previous experiments, it is hypothesised that doxycycline is a more effective suppressor of the transgene and could reduce transgene related fitness costs (if found to be present) better than chlortetracycline, preventing the selection of the wild type allele over that of the transgenic allele. By comparing the allele frequencies of the populations maintained on these two tetracyclines, it will be possible to determine which tetracycline is most effective at preventing fitness penalties.

In the second instance by rearing the strain in the absence of tetracycline (restrictive conditions) it is expected that those carrying the transgene will suffer a significant fitness cost and not contribute to the following generation. This scenario simulates that of field

releases and can be used to estimate the number of generations required before the transgene is lost from the environment from the point releases stop.

For this experiment, the strain OX4358B was used as the results presented in Figure 4-3 show that they require a higher tetracycline concentration than OX513A (Figure 4-2) to repress the effects of the transgene. It is expected that the use of this strain will expose fitness effects relating to tetracycline faster and more effectively than OX513A.

For the populations reared in the presence of tetracycline, OX4358B homozygotes and OX4358B heterozygotes were reared in the presence of 1 µg mL^{-1} doxycycline and 30 µg mL^{-1} chlortetracycline. Male OX4358B homozygotes were crossed to OX4358B heterozygotes to produce an F_1 population with an OX4358 allele frequency of 0.75. For the cohorts to be reared in the absence of tetracycline, OX4358B heterozygous males reared in the absence of tetracycline were crossed to LWT females to produce an F_1 population with an allele frequency of 0.25.

The F_1 generation eggs were hatched and counted into three cohorts of 200 larvae and reared in the presence of their parental tetracycline or absence. Pupae were picked, sexed and screened to determine their allele frequency (homozygous, heterozygous or WT). Male and female pupae were housed separately, and allowed to eclose. Three days after the last adult eclosed, males were added to the female cage and F_2 eggs collected. This process was repeated for a further nine generations or until the OX4358 allele was extinct from the population.

The results of the data collected under permissive conditions clearly demonstrate a difference in the trend frequency of the genotypes of larvae reared in the presence of doxycycline compared to being reared in chlortetracycline (Figure 4-6). The data from populations maintained on chlortetracycline (Figure 4-6A) shows that over the 10 generations, the frequency of the OX4358B homozygotes decreases in favour of heterozygotes and an increasing frequency of wild type individuals. Conversely, populations reared in the presence of doxycycline result in a greater fraction of homozygotes, with only a very low proportion of wild type individuals.

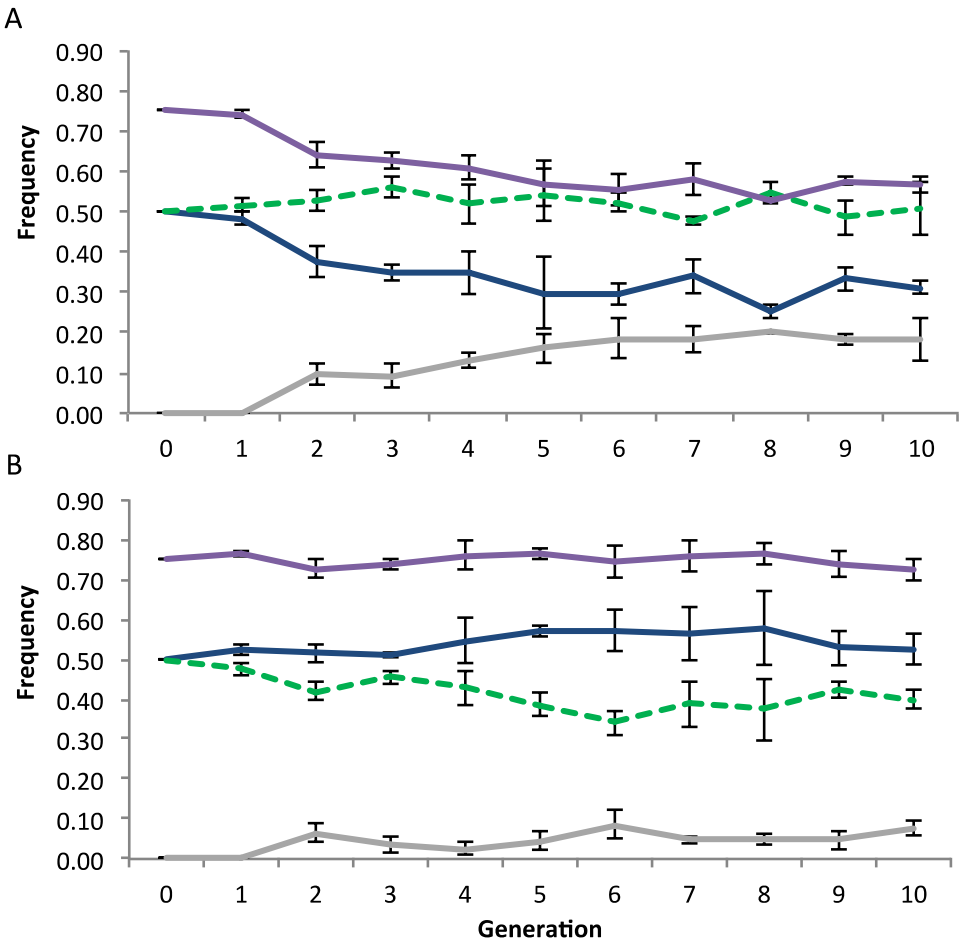


Figure 4-6 Change in OX4358B genotype frequency over ten discrete closed generations. OX4358B were reared in the presence of A) 30 µg mL^{-1} chlortetracycline and B) 1 µg mL^{-1} doxycycline for 10 generations. For each generation 200 first instar larvae were randomly selected and reared to pupation. Pupae were screened to determine genotype: OX4358B homozygous (blue line), OX4358B heterozygous (green dashed line), and homozygous wild type (grey line). The mean OX4358B allele frequency for each generation is also shown (purple line). Three populations were run for each treatment. Data presented at the means of three populations. Error bars are the SEM.

By comparing the ratio of genotypes in generations to those expected based on Hardy Weinberg's equilibrium, an estimate of the selection against the transgene can be gained. These calculations demonstrate that populations maintained on chlortetracycline result in a significant difference in the observed and expected genotype ratios after 10 generations (χ^2 (1, *N*=200) =29.8, *p* =<0.001) with the selection against the transgene. However, comparing the genotype ratios of doxycycline reared populations reveals that there is no significant

difference between the observed and expected ratios (χ^2 (1, *N*=200) =7.4, *p* =0.60) and therefore there is no significant selection against the transgene. This is further confirmed by Figure 4-7 which determines the selection against the transgene whereby if the slope is a straight horizontal line there is no selection, and if there is selection, it's coefficient is that of the slope. This determines that the selection coefficient against doxycycline reared OX4358B to be 0.005(ln) over the whole life cycle, whereas the selection coefficient against chlortetracycline reared OX4358B is 0.18(ln). What can be concluded from this data is that OX4358B homozygotes and heterozygotes reared in the presence of doxycycline do not appear to suffer a significant fitness cost as a result of the transgene, and this explains the maintenance of the OX4358B allele frequency over successive generations. Together, these data clearly demonstrate that doxycycline is the superior tetracycline analogue to rear self-limiting strains. It is able to supress the transgene of both homozygotes and heterozygotes sufficiently to maintain the fitness of populations to minimise selection in favour of wild type alleles as a result of transgene related fitness penalties.

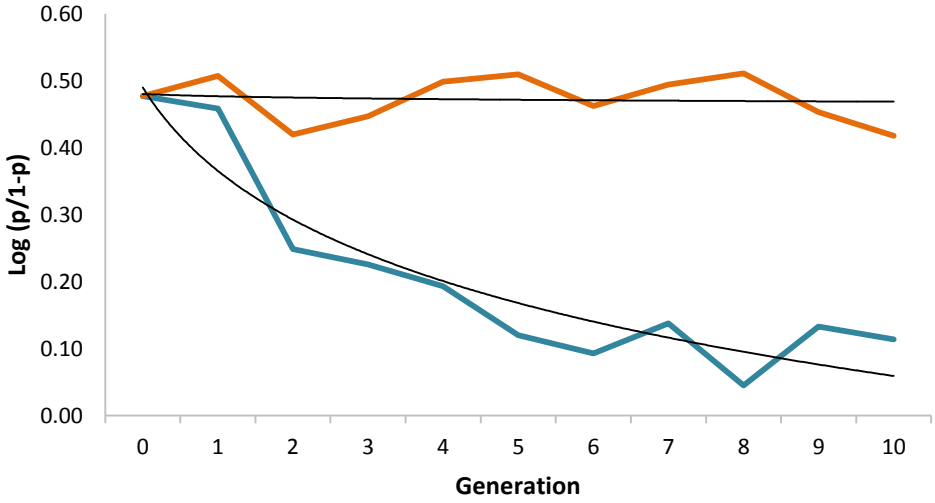


Figure 4-7 Selection of the OX4358B allele over successive generations.
OX4358B were reared in three separate populations in the presence 30 µg mL^{-1} chlortetracycline (blue line) and 1 µg mL^{-1} doxycycline (orange line) for 10 generations. The OX4358B allele frequency in each generation (p) was recorded, with the mean frequency presented here.

The second part of this experiment was to investigate the rate of loss of the OX4358B transgene over successive generations from the point that releases stop in the field. From

this point, remaining released homozygous males will mate and die and the only surviving OX4358B carrying individuals will be heterozygous male and female progeny. To determine the rate of decline of the transgene from the wild population, a stochastic model was developed to predict the number of generations that the transgene would persist. This model was developed with the assumptions that the population was panmictic, closed and of a limited size of 200 individuals. The model demonstrates that a male-selecting strain should be eliminated from a population (less than 1 individual) by generation 10 in 95% of iterations (Figure 4-8A) with a mean of 6.5 (± 1.8 SD). However, this model does not take into account the sex linkage of OX4358B to the male determining locus described in Chapter 3.2.5. Adding this parameter into the model (80% of transgenic progeny are male, 20% female) shown in Figure 4-8B, demonstrates that the transgene is lost at a much slower rate than if the transgene is not sex linked, in fact the transgene still persists after >20 generations since homozygous releases have stopped. Although the OX4358B is not expected to be harmful to the environment and therefore this should not pose a risk, the persistence of the transgene for so many generations would be undesirable from a regulatory perspective.

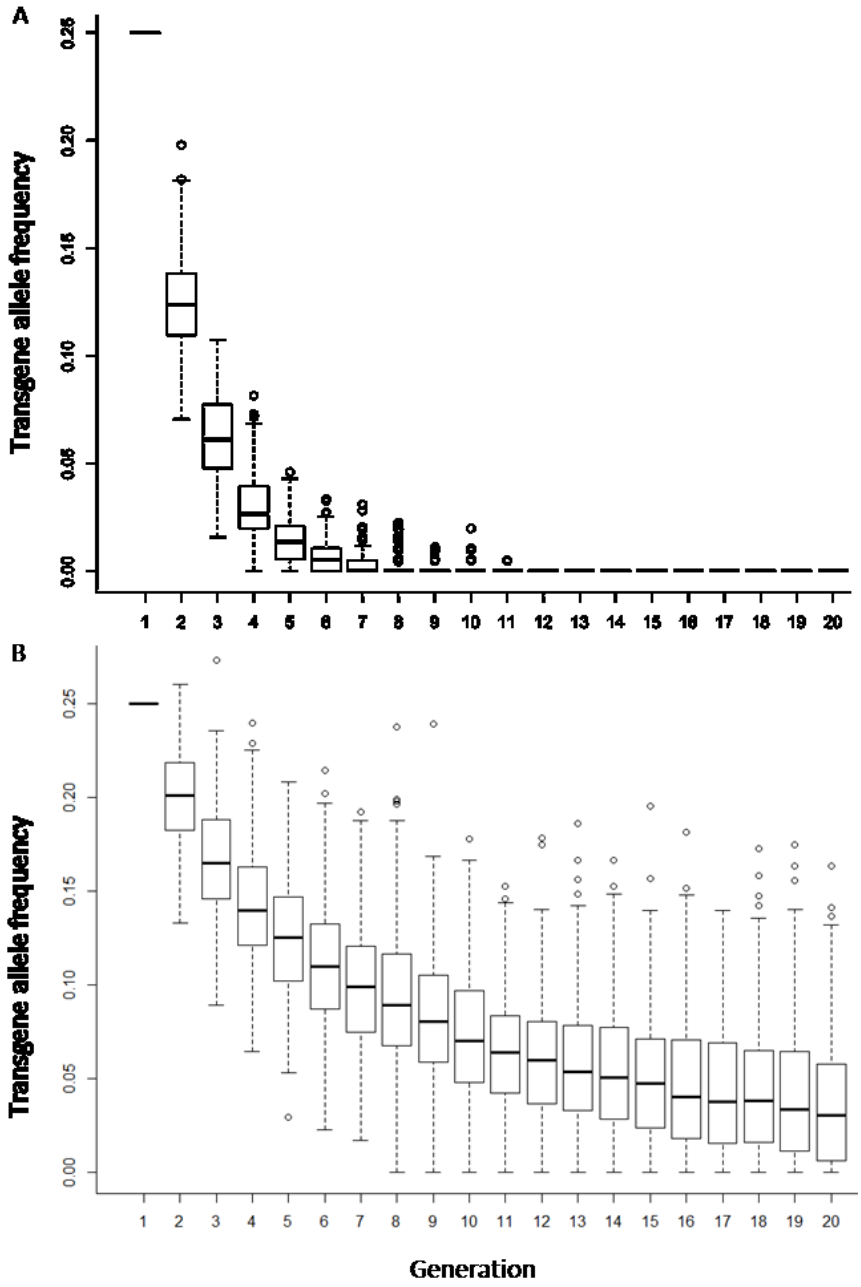


Figure 4-8 Modelling of the rate of change of a male-selecting transgene from a closed population with discrete generations.

Boxplot showing the results from 250 iterations of a stochastic model to simulate the change in frequency of the OX4358B transgene over 20 generations. The starting allele frequency was 0.25 to represent the allele frequency of viable wild populations at the point that releases end. A) This scenario is the predicted loss of a male-selecting transgene over successive generations assuming the allele is equally inherited between both sexes. B) This represents that of the male selecting strain OX4358B with the sex linkage of the transgene

to the male determining locus. Based on previously collected data, the sex ratio of the transgenic progeny in each generation is expected to be 80% male and 20% female. Horizontal bold lines represent generational medians; upper and lower box lines represent first and third quartiles, respectively; outer horizontal lines represent 1.5 × the interquartile range and dots represent data points over 1.5 × above or below the first and third quartiles, respectively.

To determine the accuracy of the modelled data, empirical data was collected of three populations maintained in the absence of tetracycline and the rate of loss of the OX4358B allele was monitored. Populations were maintained under the same conditions as the permissive populations, expect that tetracycline was withheld from the larval rearing medium. Of the three populations reared (Figure 4-9), two went to transgene elimination (at generations 4 and 7) and the third population had an allele frequency of 1% (two individuals) at the final experimental generation (generation 10). The probability of these allele frequencies occurring by generation 10 in 250 iterations of the model (Figure 4-8B), is 0/250 for elimination and 11/250 for the allele frequency to be ≤1%. These empirical data show that the transgene is being lost from the population at a considerably faster rate than the model predicts and this is most likely due to a fitness cost suffered by the transgene carrying individuals.

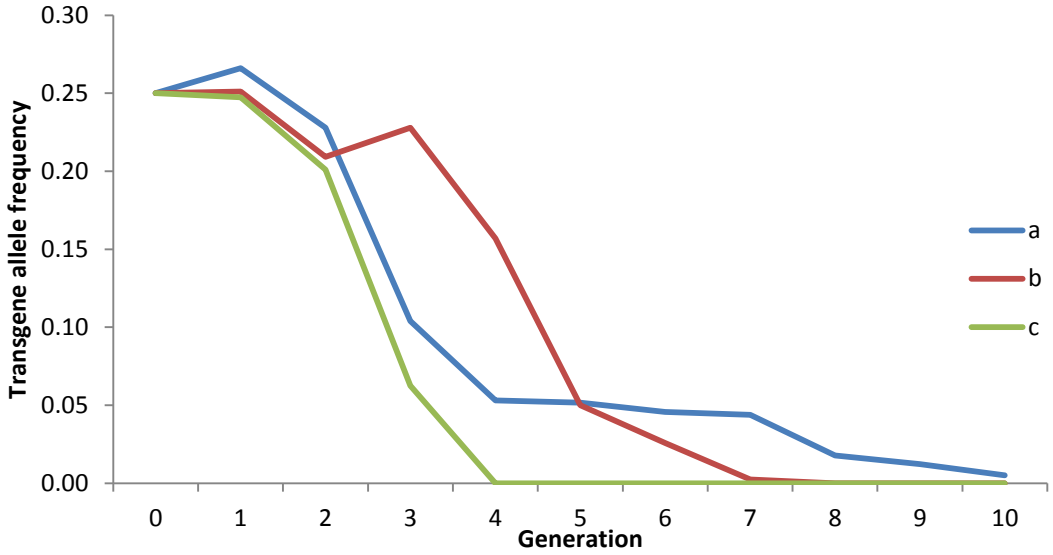


Figure 4-9 Allele frequency of three populations of OX4358B maintained in the absence of tetracycline.

Three populations were maintained in the absence of tetracycline over 10 discrete generations. For each generation 200 first instar larvae were randomly selected and reared to pupation. The starting allele frequency was 0.25 (OX4358B heterozygous crossed to wild type females).

The empirical data collected from the three populations maintained in the absence of tetracycline have shown that carrying the transgene imparts a fitness penalty to the individuals. To estimate this fitness cost, the sex-linked model of Figure 4-8B was updated to include a fitness cost parameter to reduce the effectiveness of transgenic males. Iterations of this model were run with different fitness costs ranging from 10% to 60% and the empirical data was tested against the outcomes to determine the best fit. Figure 4-10 demonstrates that when the OX4358B males suffer an increasing fitness penalty, the transgene allele is lost from the population at a faster rate, as would be expected. By comparing the model means (250 iterations) with the empirical data means (three populations), it is found that the data fits the 30% fitness penalty the best (E = 0.95 (where 1 = a perfect match. For a 20% fitness penalty E = 0.91, for a 40% fitness penalty E = 0.89) where the mean allele frequency at generation 10 is expected to be 0.68% (95% CI 0.56% to 0.80%). This implies that the OX4358B males are 30% less fit than the wild type males, and consequently the transgene is lost from the population at a faster rate than would be expected if they were equally as fit. Moreover, it is expected that in field conditions which

would typically be much more challenging for the insects, that any fitness cost seen in the laboratory will be exacerbated and it is likely that the OX4358B allele will disappear more rapidly than these experiments indicate.

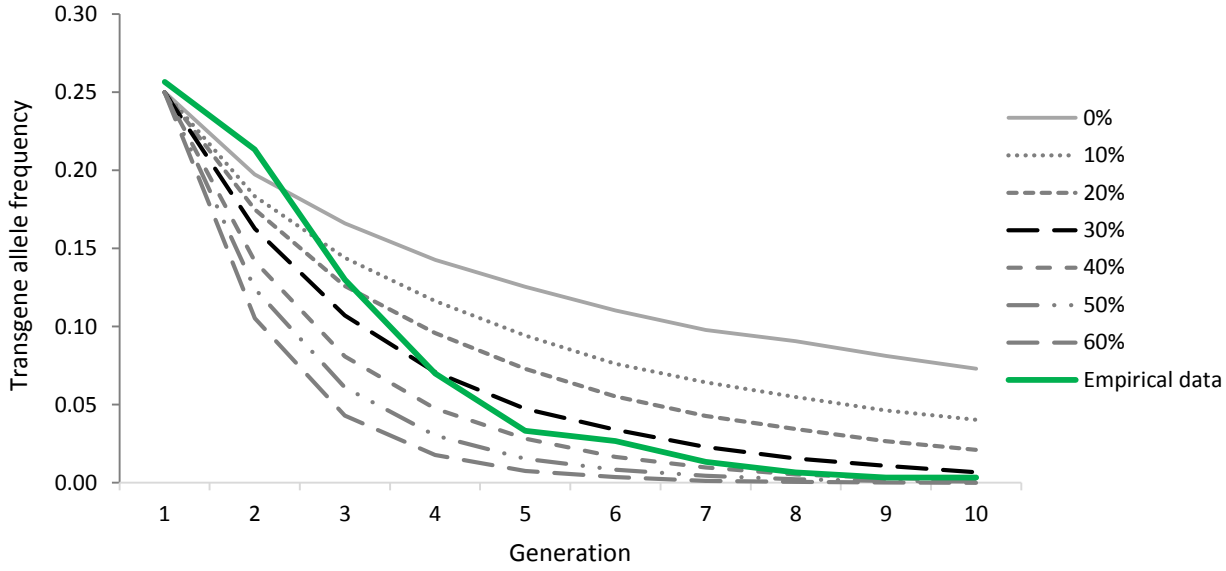


Figure 4-10 Modelling of the sex linked male selecting strain OX4358B with increasing fitness costs associated to the transgenic males.

A stochastic model was run with 250 iterations to simulate the change in frequency of the OX4358B transgene over 10 generations, with increasing fitness costs placed onto the transgenic males. The lines represent the mean allele frequencies for each fitness cost (0% to 60%). The empirical data is the mean allele frequency of three populations of OX4358B maintained in the absence of tetracycline.

4.4. Conclusions

Self-limiting strains require the addition of tetracycline to the larval rearing medium to supress the self-limiting phenotype to allow breeding and population expansion in the laboratory. Understanding the interaction of the tetracycline analogue and the required concentration on the impact on the transgene is important to understand for two reasons: firstly to determine if the strain can be rescued by concentrations of tetracycline found in the environment, and secondly the concentration required in the laboratory to supress the self-limiting phenotype to allow rescue of the population to functional adulthood with maximal fitness.

Four commonly used tetracycline analogues were tested for their efficacy at rescuing the self-limiting phenotypes of the bi-sex lethal strain OX513A and the male selecting strain OX4358B. These data determined that doxycycline was significantly more effective at rescuing individuals at lower concentrations than the other analogues tested. Doxycycline, and the other tetracycline analogues tested, are used in animal husbandry and human and animal therapy and are consequently found in the environment, although their total load is limited by hydrolysis and photolysis (Loftin *et al.*, 2008). A review of the literature has shown that the concentrations of tetracycline and its analogues present in the field is significantly lower than is required to allow rescue of the OX4358B phenotype. However, OX513A requires lower concentrations to allow an increase in the rescue of the lethal phenotype and there are occasional reports of concentrations of doxycycline in the environment that are in excess of this threshold (Watkinson *et al.*, 2009; Le-Minh *et al.*, 2010; Xiong *et al.*, 2015). These reports are usually measuring the change in tetracycline concentration between influent and effluent of wastewater treatment plants and are not representative of *Ae. aegypti* breeding sites. Studies published in Curtis *et al.* (2015) of tetracycline concentrations in actual *Ae. aegypti* breeding sites in Brazil (where samples were collected on the basis of the presence of *Ae. aegypti* larvae) found the concentration of tetracyclines in these locations to be below the limit of quantification (LOQ: 1pg mL^{-1} tetracycline, 2.5 pg mL^{-1} chlortetracycline and oxytetracycline) and therefore below the concentration required to allow rescue from the lethal phenotype. Taken together, these data infer that concentrations of tetracycline and analogues in the environment are unlikely to result in an increase in survival of progeny from self-limiting strains.

Focusing on the other end of the scale, determining the tetracycline analogue and concentration that allows the maximum rescue of the self-limiting phenotype in a population is important to prevent population selection and increasing rearing costs. The data collected here for both OX4358B and OX513A has shown that rearing larvae in the presence of doxycycline is preferable to that of chlortetracycline, requiring a lower concentration to reach full rescue. However, longevity experiments performed with OX513A indicated that doxycycline reared adults may have a marginally reduced median longevity compared to chlortetracycline reared adults, but it is not clear why this should occur. Further experiments performed with O4358B however did not find negative fitness costs of

rearing in the presence of doxycycline, in fact, rearing populations in the presence of doxycycline resulted in a higher fitness of transgenics measured by the maintenance of the transgene at higher frequencies over discrete generations better than being reared in chlortetracycline. Furthermore assessment of the OX4358B allele frequency of populations reared on doxycycline over the 10 generations determined there was very little selection against the transgene, whereas rearing in the presence of chlortetracycline resulted in population selection. The design of this experiment was to test the combined fitness of all life-stages but was not specifically aimed at exposing the fitness costs of a reduced lifespan. It is therefore possible that rearing in the presence of doxycycline could result in a high recovery of fit functional adults, but that they are marginally shorter lived than their chlortetracycline reared counterparts, and this may only be exposed in long term fitness studies. If the doxycycline reared females are shorter lived it is possible the eggs laid per female maybe fewer than expected and therefore have an impact on egg production costs. This should be investigated further before introducing doxycycline into an egg mass production facility.

Finally, the rearing of OX4358B populations under restrictive conditions has highlighted a reduced fitness of the males. Given the linkage of the transgene to the male determining locus, it was predicted that the transgene would persist in wild populations for >20 generations after homozygous male releases stopped. Although the transgene does not pose any threat in the environment, it is likely that the persistence of the transgene for this many generations will be too long to gain regulatory approval. Data collected here however, has shown that under laboratory conditions the transgene is lost from populations by between generation 4 and likely generation 12. In the harsher conditions of the field environment, it is predicted that this rate of decrease will be faster and the transgene will be lost in fewer generations than seen here. Although this is likely to be beneficial to passing through the regulatory process and gaining a positive public opinion, these data have highlighted that the OX4358B males suffer a fitness cost, and this has been quantified to approximately a 30% reduction in fitness compared to wild type males. It is not clear from this experiment at which life stage the male's fitness is compromised, but it is possible that this fitness cost could impact the efficacy of this strain at supressing wild populations. Further work will be required to investigate the different components of the OX4358B male's fitness (e.g. mating competiveness) to determine which component is being compromised.

The work in this chapter has identified that doxycycline is a superior tetracycline to use to rear self-limiting strains to supress the transgene. Data collected here has indicated that doxycycline is able to limit population selection against those carrying transgenes, however further work should be performed to ensure that the longevity of individuals are not negatively impacted and that doxycycline will not affect the cost efficiency of rearing of populations for egg production.

Chapter 5 Impact of larval rearing conditions on male mating competitiveness

5.1. Introduction

The success of a sterile insect programme is highly dependent on the released insects being able to successfully compete with wild males for wild female mates (Benedict & Robinson, 2003). If the released insects have a low mating competitiveness the programme is at risk of failing unless additional insects can be released to compensate, however, this comes at a financial cost. Understanding what makes a male insect fit and to be able to compete for wild female mates is an important question for every SIT programme and in many instances the answer is still unclear, and none more so than for *Ae. aegypti* male fitness (Lees *et al.*, 2014).

The overall cost of an *Ae. aegypti* self-limiting control programme can be simplified to two main influencing components: firstly the size of the wild population to supress and secondly the effectiveness of the released self-limiting males. Together, these factors will determine the release ratio of self-limiting males: wild males (Enkerlin, 2007). The effectiveness of the released males is determined by their ability to find wild females and successfully copulate with them, however this process has many caveats including: the males must be active at the correct time of day (Benelli, 2014), search for and be able to locate the females (Oliva *et al.*, 2014), compete with other potential suitors for the female (Massonnet-Bruneel *et al.*, 2013; Patil *et al.*, 2014) and then successfully transfer sufficient sperm to her to make her refractory to further mating attempts (Bargielowski *et al.*, 2011a; Helinski *et al.*, 2012a). Ideally the male will then be able to search for a sugar source to replenish his energy reserves (Oliva *et al.*, 2013; Chadee *et al.*, 2014), evade predators, rest and repeat approximately 12 hours later when the next mating period commences. In reality, it will be considered a success if a single released male is able to win a single female mate rather than achieve successful mating's with multiple females.

The localisation of *Ae. aegypti* males to a mating arena and their ability to successfully mate with a female is not well understood, however evidence is being collected to allow a better understanding of the process and factors to consider in planning a control programme. Male *Ae. aegypti* are not strictly a swarming species but as males are attracted to blood host

odours (Wright *et al.*, 1966; Hartberg, 1971) and breeding sites (Oliva *et al.*, 2014) where they hope to find a female mate, congregations and consequently swarms of males do appear. This male localisation is likely to be enhanced by the production of a pheromone by males that has been shown to attract both males and females (Cabrera & Jaffe, 2007; Fawaz *et al.*, 2014), however, to date this pheromone has not been isolated or characterised. Females, often singularly will enter a male swarm and mating will ensue, although the process of mate choice is unclear. Like many other mosquito species, wing beat frequency has been shown to be an important component of mating (e.g. (Gibson & Russell, 2006; Warren *et al.*, 2009; Ritchie & Immonen, 2010)), as well as being a component of species recognition (e.g. (Gibson *et al.*, 2010; Pennetier *et al.*, 2010)). Cator *et al*. (2009) showed that just before copulation, the male and female will often harmonise their wing beat frequencies, and that the successful harmonisation of wing beat frequencies before copulation frequently resulted in an improved mating success of their progeny (Cator *et al.*, 2009; Cator *et al.*, 2011). This harmonisation of wing beats infers an element of mate choice and that this trait is an inheritable component. Upon the successful grasping and orientation of the male and female (Spielman, 1964), the male transfers sperm and accessory gland secretions (Sirot *et al.*, 2008; Boes *et al.*, 2014) to the female which after a few hours results in a mating plug making her refractory to any further mating attempts (Christophers, 1960).

In Anophelines, the effect of male body size has been shown to be an important contributor to mating success with large males winning more mating opportunities than small males (Maiga *et al.*, 2012; Sawadogo *et al.*, 2013). In *Ae. aegypti* it has been shown that increased male body size results in an increased mating capacity (the number of females a male can inseminate) (Ponlawat & Harrington, 2007, 2009; Helinski & Harrington, 2011) but under the laboratory conditions this test only showed that large males were able to mate more females, not that they could compete with other males for females. Considering body size in other fitness parameters, larger males have been shown to survive for longer in the field than small-bodied males (Maciel-De-Freitas *et al.*, 2007) presumably due to their increased teneral reserves, but there was no difference in the distance dispersed by large or small-bodied males (Helinski & Harrington, 2011) in a mark release recapture study.

The body size of adult *Ae. aegypti* individuals is determined by the larval rearing conditions with three factors influencing the size of the resulting adult; diet, water temperature and density. The provision of a nutritious and plentiful larval diet has been shown to increase adult body size (e.g.(Nasci, 1987; Koella & Lyimo, 1996; Puggioli *et al.*, 2013), the increase in larval density in rearing pools with either inter-species or intra-species, has been shown to decrease the adult body size by increasing the competition for the food resources (e.g. (Ng'habi *et al.*, 2005; Bargielowski *et al.*, 2011b), and higher water temperatures have been shown to result in smaller adults (e.g. (Phasomkusolsil *et al.*, 2011; Ciota *et al.*, 2014), as a result of their faster development and presumably less time to acquire teneral reserves. As there is little understanding as to how and if females choose their male mates, it is not clear if any of these factors are selected for or against at the point of mate choice, or if the resulting body size of the adult is a fundamental determinant of a successful mating event.

In the consideration of how to tailor the fitness of OX513A males for mass release it may be important to consider the body size of the released males to increase their mating potential. Currently due to the mass production processes the released OX513A males are relatively homogeneous in size and are considerably larger than the wild population: the average OX513A male has a cephalothorax width of 1.09 mm where as wild males are approximately 0.89 mm[6]. Given that the OX513A males are approximately 20% larger than the wild males, it is possible that they are at a physical disadvantage at being able to mate with the wild females. This could be as a consequence of the size of their mating organs not being compatible, that the organs do not meet when connected in the mating clasp or that the males are not agile enough to fly to compete for and win the female within the swarm (Spielman, 1964; Peckarsky *et al.*, 2002; Scott *et al.*, 2008). Conversely, larger OX513A males may be able to overpower smaller wild males to win the female, as well as having a greater mating capacity than small males, as supported by Ponlawat et *al*. (2009). It is therefore important to ascertain whether large OX513A males have a greater mating success than small males, taking into consideration the size of the female that is being competed for. Understanding this concept would allow the production of self-limiting males to be tailored

[6] Wild specimens collected in Grand Cayman by Mosquito Research Unit survey team and measured by myself on return to the laboratory.

to be attractive mates to the receiving wild females, increasing their chances of achieving mating events and enhancing the efficacy of a control programme.

Given our current understanding of male mating biology, it is likely that much of the adult males' mating success will be determined by genetic makeup or pre-imago environmental factors. The release of a male-selecting strain will involve the F_1 progeny developing to functional adults in the field co-rearing in breeding pools with wild larvae. It is possible that the lab adaption of the strain to a food rich, stable rearing environment will make the transgenic larvae less able to compete for food with the wild larvae, resulting in males with a low mating competiveness. Understanding the mating competiveness of transgenic adults eclosing from a co-rearing larval environment will help to estimate the effectiveness of their contribution to the second generation suppression and will therefore help to inform modelling to estimate the rate and effectiveness of suppression using a male-selecting strain.

The work in this chapter aims to assess the baseline mating competitiveness of the afore mentioned self-limiting strains and to assess the impact on changing their larval rearing conditions on their mating competitiveness. Firstly, the mating competiveness of the male-selecting strain OX4358B reared under permissive laboratory conditions will be assessed to compare the results to previously published data on the mating competiveness of the bi-sex lethal strain OX513A. Secondly, the mating competitiveness of OX513A reared through the mass production will be assessed to determine the effects of high density larval rearing, and high-density adult storage. Further to this, with a view to being able to manipulate the future rearing conditions of OX513A to produce a male with any desirable body size, the effect of male mating competiveness of large and small males will be examined, in relation to the female's body size. Finally, the male mating competiveness of OX4358B males co-reared with wild type males will be assessed to determine the fitness of co-reared transgenic adults to inform on their likely contribution to the second-generation suppression.

5.2. Methods

5.2.1. Mating competition

Insects were reared and maintained at 26^{o}C [+/- 2^{o}C], 70% [+/- 10%] relative humidity, 12h: 12h light: dark cycle. Insect strains are described in Chapter 2.2.1 and Chapter 3.

Mating cages (65 cm x 65 cm x 115 cm; L, W, H) were set up with a 10 % sucrose feeder available for adults *ad libitum*. Four repeats (cages) were used for each experimental treatment described below. 50 transgenic males and 50 LWT males were hovered from their eclosion cages into the mating competition cage, and allowed to equilibrate for one hour. After this, 50 LWT females were added to the cage. The cages were left for 24 to 48 hours depending on the experiment. At the end of the mating duration, the females were collected, blood fed and placed into an entomological tube (2.5 cm x 7 cm) with wet cotton wool at the bottom to allow oviposition.

The F_1 eggs were hatched and screened for the presence or absence of the AmCyan (OX4358B experiment) or the DsRed2 (OX513A experiment) fluorescent marker. If the larvae were 100% fluorescent the father was transgenic, if they were non-fluorescent the father was LWT, if there was a mixed ratio of LWT and fluorescent larvae, the female had been double mated, and a point was awarded to both transgenic and LWT.

5.2.2. Wing length measurements

To ensure that males from one of the two strains did not have an unfair advantage deriving from a larger or smaller body size, wing length measurements or pupal cephalothorax measurements were taken from the remaining excess males as a proxy for adult body size (Yeap *et al.*, 2013).

For wing measurements, wings were removed and mounted on a microscope slide using 80% ethanol. Digital images of the wings were taken including a 10 mm graticule. For cephalothorax measurements a digital image was taken of the dorsal side of the pupae with a 10 mm graticule in the background.

The wing lengths or pupal cephalothorax were measured using ImageJ software (http://rsbweb.nih.gov/ij/). For wings, measurements were taken from the auxiliary incision to the apical margin, excluding the fringe (Figure 5-1a) and for pupal cephalothorax, the measurement was taken across the widest section of the cephalothorax (Figure 5-1b).

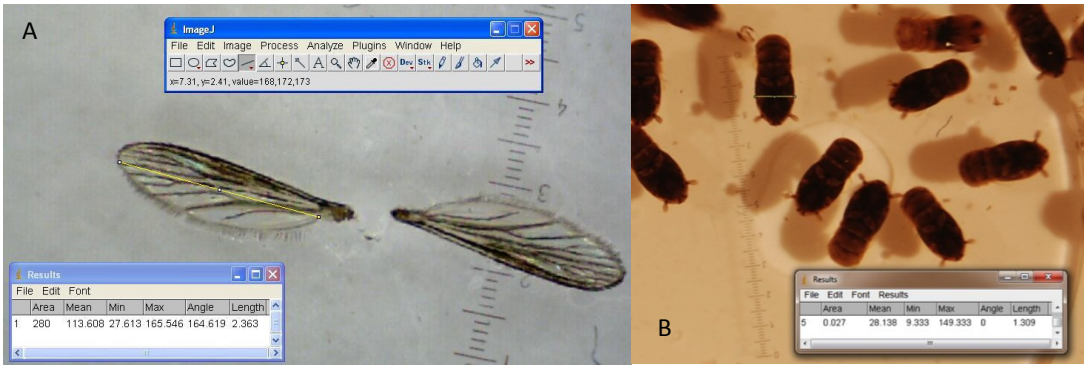


Figure 5-1 Measuring pupae and wings as a proxy for adult size.

A) Measurements of wing lengths from the auxiliary incision to the apical margin, using ImageJ software. b) Measurements of pupal cephalothorax, taken across the widest section. The graticule can be seen in the background of both images to calibrate the measurement. Figure source, Oxitec.

5.2.3. OX4358B mating competitiveness

OX4358B and LWT first instar larvae were counted into four cohorts of 200 and reared in the absence of tetracycline. Larvae were fed a standard regimen of food (Table 9-1). Upon pupation, pupae were picked and sexed and housed in 15 cm x 15 cm x 15 cm cages with no more than 150 pupae placed into each cage. Adults were transferred to the mating arena three to four days post eclosion.

5.2.4. Effect of mass rearing on mating competitiveness

OX513A were reared under mass rearing conditions: OX513A eggs were hatched under vacuum for one hour before being transferred to a tray (30 cm x 15 cm x 5 cm) overnight with additional water. The following day, the hatched larvae/egg mixture was transferred to 500 mL beaker with a magnetic flea added and placed onto a magnetic stirring plate. Five 1 mL aliquots were taken from the homogenous mixture and the number of hatched larvae recorded. From this the required volume was determined to transfer 3000 larvae into a tray. Three trays were prepared. 1 L of 30 µg mL^{-1} chlortetracycline was added to each tray, and the larvae were fed a standard feeding regimen (Table 9-1). Upon pupation (8 days after hatching), the larvae were separated from the pupae using a larval pupal sorter (Figure 1-7). The isolated pupae were sexed based on size by the pupal wire sorter (Figure 1-7): the male female mixture were placed into the device which was then submerged. The females which are larger remain in the device whereas the males, which are smaller, are able to

escape through the wire gaps and float to the surface. The male pupae were collected and 1000 were aliquoted into a release device (plastic pot with a mesh lid 15 cm x 25 cm) (Figure 1-7) with 1cm water at the bottom for eclosion. A cotton wool ball soaked in 10% sucrose solution was placed on top of the mesh lid. Two days later, the water from the release device was drained. The eclosed males were 'released' after 1 day or 3 days depending on the experiment. Release of the males involved opening the device into a large cage and allowing the males to fly out. The required number of males were then transferred to the mating cages using a micro hoover (Spider Catcher Vacuum, Lakeland, UK). LWT were reared according to Chapter 5.2.3.

5.2.5. Effect of body size on mating competitiveness

OX513A and WT were reared under two different conditions to produce large and small bodied adults. OX513A larvae were reared in the presence of 30 µg mL^{-1} chlortetracycline and LWT larvae were reared in the absence of tetracycline. To produce small adults, larvae were reared in lunchboxes (15 cm x 10 cm x 8cm) at a density of 5 larvae mL^{-1}; 1000 first instar larvae were aliquoted into each of three boxes with 200 mL of rearing medium. Larvae were fed a reduced diet described in (Table 5-1). To produce large adults, larvae were reared in 16 oz deli pots at a density of 0.5 larvae mL^{-1} and fed 150% of the standard feeding regimen (Table 5-1).

Table 5-1 Feeding regimen (grams diet) of Tetramin (Germany) to produce large and small adults.

Day	Reduced diet (g) (1000 larvae)	150% diet (g) (100 larvae)
1	0.06	0.012
2	0.00	0.000
3	0.08	0.016
4	0.16	0.032
5	0.16	0.064
6	0.16	0.064
7	0.16	0.064

Upon pupation, pupae were picked, sexed and housed in separate cages (15 cm x 15 cm x 15 cm). No more than 150 pupae were placed into each cage. Adults were transferred to the mating arena three to four days post eclosion.

5.2.6. Effect of co-reared transgenic and WT larvae on mating competitiveness

OX4358B and WT larvae were aliquoted into lunch boxes with 300 first instar larvae of each strain in each of the four containers. 1 L of water was added to each container and larvae were fed a standard feeding regimen (Table 9-1). Upon pupation, the pupae were picked, sexed and screened. Pupae were separated by strain and by sex and placed into 15 cm x 15 cm x 15 cm cages. Adults were transferred into the mating arena three to four days post eclosion. For this experiment, mating cages of 30 cm x 30 cm x 30 cm were used with ratios of 20: 20: 20 (OX4358B ♂: LWT ♂: LWT ♀) due to the lack of availability of the larger cages. Seven cages (repeats) were performed.

5.2.7. Statistical analyses

From the data of each mating experiment the Relative Sterility Index (RSI) can be calculated by the formula described in

Equation 1. A value of 0.5 represents equal competitiveness between transgenic males and WT males for WT female mates. A value <0.5 indicates that transgenic males are less competitive at successfully mating with WT females compared to WT males, and a value >0.5 infers that transgenic males are more competitive than WT males at gaining WT female mates.

$$RSI = \frac{SW}{SW + WW}$$

Equation 1 Relative Sterility Index (RSI).

SW is number of OX4358B sterile males mated to WT females and WW is the number WT males having mated a WT female.

To statistically compare the competitiveness of the transgenic and the WT males for WT female mates, a Chi square test was used.

5.3. Results and Discussion

5.3.1. OX4358B Mating competitiveness

Mating competition was performed to ascertain whether the OX4358B males were equally competitive as WT males. This is an important parameter to quantify, as a low mating competitiveness is likely to result in a decreased effectiveness when the strain is used in a field suppression programme.

Mating tests were performed with 50 OX4358B males competing with 50 LWT males for 50 LWT female mates. Four cohorts were run. The mating partner of each female was determined by screening her progeny for the presence or absence of the fluorescent AmCyan marker, which is inherited from the OX4358B males only. The results show that there was no significant difference in the number of successful mates achieved by OX4358B males compared to WT males (χ^2 (1, N = 3) =1.99, p =0.16) (Table 5-2).

Table 5-2 Mating competition results of OX4358B against LWT.

50 OX4358B males competed with 50 LWT males for matings with 50 LWT females, with four repeats. Eggs were collected from the females and paternity determined based on the presence or absence of the fluorescent marker.

Repeat	No viable eggs	Paternity			RSI	χ^2 p value
		OX4358B	WT	Both		
1	20	13	17	0	0.46 (0.41 to 0.52)	0.16
2	11	15	22	2		
3	10	15	20	5		
4	10	21	16	3		
	Pooled values	**64**	**75**	**10**		

The Relative Sterility Index (RSI) which is used to estimate the competiveness of males, estimated the OX4358B males to be 0.46 (95% CI 0.39 to 0.53) indicating that they are not less competitive than WT males at mating with WT females.

Given that the male body size has been shown to be an important factor in male mating success in other mosquito species (Maiga *et al.*, 2014), the rearing of the two strains was standardised to produce males with an equal body size. To confirm their body sizes were the same, the wings of the excess males were removed and their length measured. The

results show that there was no significant difference in the wing lengths ($t(86) = -0.67$, $p = 0.50$) and therefore neither strain should have had an advantage due to body size.

5.3.2. OX513A mass reared male mating competitiveness

The mass production of OX513A males for field release must be performed under higher density conditions than the permissive research conditions to reduce the cost of production. This has the potential to increase the stress the larvae develop under and consequently decrease their fitness. Estimates from the field of the mating competiveness of OX513A are 0.02 to 0.05 RSI (Harris *et al.*, 2011; Harris *et al.*, 2012; Carvalho *et al.*, 2015; Gorman *et al.*, 2015), whereas when mating competiveness experiments are performed with OX513A larvae reared under permissive conditions in the laboratory, the RSI rarely deviates significantly from 0.5 (Massonnet-Bruneel *et al.*, 2013; Patil *et al.*, 2014). The difference in this competiveness could be due to many factors: the mass production rearing conditions, the adult storage conditions, the travel to the release site, the location of release, the time of day of release or any behavioural differences between lab reared and wild strains e.g. peak mating time, species recognition, survival etc. As a step to understanding the differences in the mating competiveness of these released males, the competiveness of males that had passed through the mass rearing process was determined in the lab against permissive reared LWT males. This will isolate the effects of the mass production process and adult storage on the mating competiveness, without introducing behavioural variations and field release operational issues.

OX513A males were mass reared at 3 larvae mL^{-1} and fed a standard feeding regimen (Table 9-1). Upon pupation, the larvae were sorted from the pupae using a Larval Pupal Sorter and the pupae were sexed based on size using a Wire Sorter (Figure 1-7). The male pupae were aliquoted into cohorts of 1000 and placed into a release device to eclose. Adults were held in the release device for 1 or 3 days before being introduced to the mating arena. LWT males and female were reared at low density (1 larvae mL^{-1}), pupae were picked using a pastette and then separated by sex using a microscope to view their gender specific terminal segment. The adults were allowed to eclose in cages at low density and added to the mating arena at 3 days old.

Table 5-3 Mating competition of mass reared OX513A males against Latin WT.

OX513A males were held in a release device for either 1 day or 3 days. 50 OX513A males competed with 50 LWT males for mating's with 50 LWT females, with four repeats. Eggs were collected from the females and paternity determined based on the presence or absence of the fluorescent marker.

Treatment	Repeat	No viable eggs	Paternity			RSI	χ^2 *p* value
			OX513A	WT	Both		
1 day old	1	0	19	30	1	0.35 (0.31 to 0.39)	<0.001
	2	3	16	28	3		
	3	4	15	31	0		
	4	5	10	31	4		
		Pooled values	**60**	**120**	**8**		
3 days old	1	2	17	30	1	0.41 (0.36 to 0.45)	<0.001
	2	0	20	30	0		
	3	0	23	26	1		
	4	0	19	30	1		
		Pooled values	**79**	**116**	**3**		

The results show that both 1 day old and 3 day old males have a significantly lower mating competitiveness compared to LWT males (1 day old: χ^2 (1, N = 3) =58.3, p =<0.001. 3 days old: χ^2 = (1, N = 3) =17.1, p= <0.001). To control for differences in body size, pupal cephalothorax measurements were collected for each cohort and these showed there was no significant difference in the size of the LWT males and OX513A males (1 day old: t(36) = 1.59, p = 0.12. 3 day old: t(36) = -1.69, p = 0.10). The RSI indicates that 3 day old males competed more effectively than 1 day old males (Table 5-3) however, both RSI results are much lower than the usual laboratory result for OX513A of approximately 0.5 and consequently this infers that the mass rearing process and adult storage conditions maybe having a negative impact on the fitness of the OX513A males. Given that 3 day old males performed better than the 1 day old males this could indicate that the time allowed for males to reach sexual maturity had not been sufficient in the 1 day old cohort. It is known that upon eclosion the males' genitalia must rotate 180^o to be in the correct orientation for mating which is thought to take approximately 18 to 24 hours for the time of eclosion (Roth, 1948). If the 1 day old males had not completed this rotation process, the males may either not be able to copulate with the females or they may not be interested in trying to compete

for mates yet (Cabrera & Jaffe, 2007). This result should be taken into account when determining the age to release males.

5.3.3. OX513A Body size mating competition

When considering the production of OX513A males there are certain limitations in the size of the pupae (and therefore adults) that can be produced. Currently larvae are reared to produce a clear differentiation in the male and female pupal cephalothorax size so that an efficient sex sort can be achieved: this requires the larvae to be well fed and consequently the resulting adults are considerably larger than the wild males. Although the literature of other mosquito species indicates that large males are more successful at mating in the field than small males (Maiga *et al.*, 2012; Maiga *et al.*, 2014) it is possible that the OX513A males are too large and their size is beyond the compatible mating limit of wild females. Understanding whether the large OX513A males really are competitive at mating with females of different sizes will help to inform future rearing changes and to provide evidence of the size that released males should ideally be.

In this set of experiments, the rearing conditions of the OX513A and LWT larvae were manipulated to produce adults with a large body size or a small body size. OX513A males and LWT males of each size were set to compete for mates with females of large or small body size and the mating success determined by screening the progeny. Each size combination of male and female was tested so resulted in a total of eight treatments, with four repeats of each (Table 5-4).

The results show that male body size does impact the mating competitiveness when competing for females of a known body size. When assessing the controls in the experiment, large OX513A males, large LWT males competing for mates with large LWT females (treatment A) and small OX513A males, small LWT males competing for mates with small LWT females (treatment E), there was no significant difference in the mating success of LWT and OX513A males, (Table 5-4) (Figure 5-2). When comparing the mating competiveness of males for large bodied females (treatment B and C) the results show that the large bodied males were more competitive than small bodied males, however when both male strains were of small body size (treatment D), small LWT males were more competitive than small OX513A males for large female mates. When comparing the cephalothorax size of these two cohorts in treatment D, there was no significant difference

in their size ($t(53) = 1.22$, $p = 0.23$) and therefore the mating success of WT males cannot be attributed to differences in adult size.

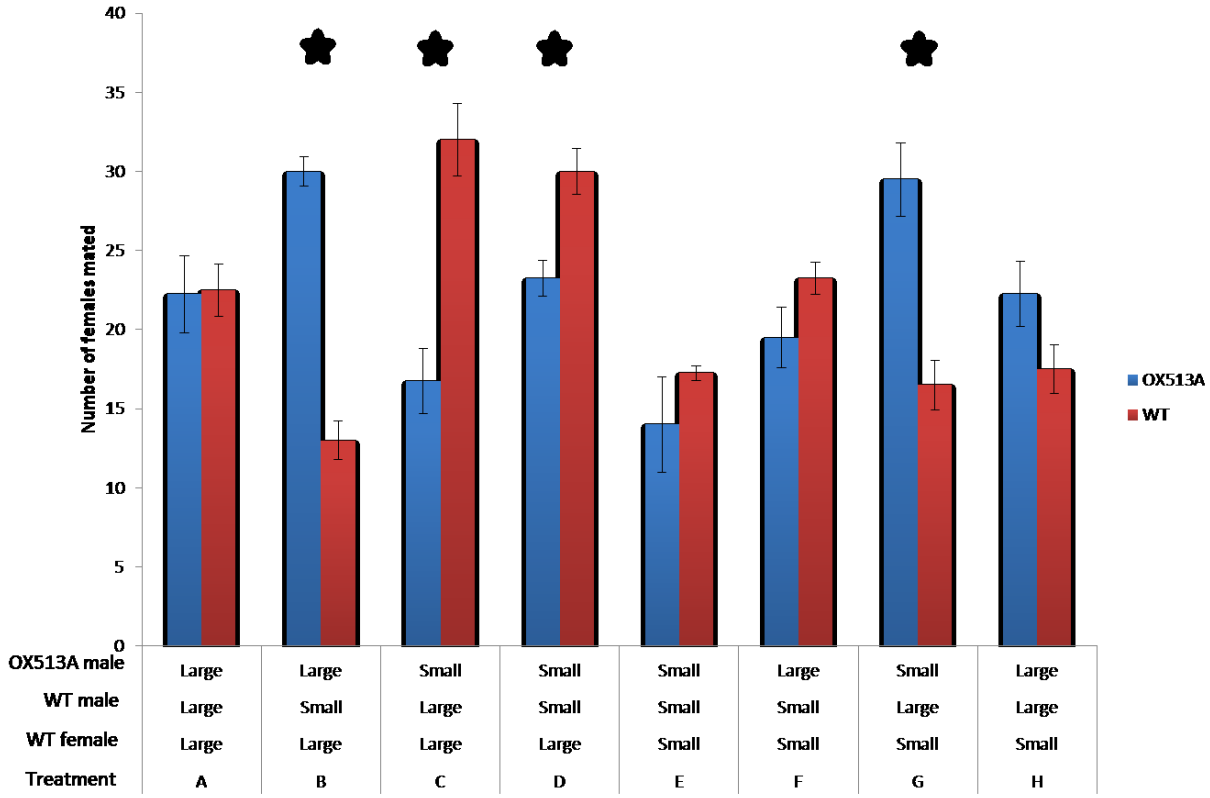


Figure 5-2 Mating results of OX513A males competing with LWT males for LWT female mates.

In each treatment the male and female body size was reared to be large or small. Treatments marked with a star showed significant differences in their mating competiveness, in these instances $p = <0.001$.

When considering the mating competitiveness of the males when the females were small bodied, the results are less clear as to which male body size is advantageous. The difference in mating competiveness is significant in treatment G when the OX513A males were small but there was no significant difference in the reciprocal treatment F when the LWT males were small (Table 5-4) (Figure 5-2), although the small LWT males achieved more mates. When comparing the control for small-bodied females (treatment H), when both OX513 and LWT males were large, there is no significant difference in their competitiveness, however, the OX513A males did achieve more mates than the LWT males. This treatment also shows that large males are able to physically mate small females, indicating that the differences in

mating competitiveness of large males competing with small males for females is not a mating incompatibility issue, rather a male being chosen, or out competing the other for the female.

From these results it is evident that when mating a large female, the male has a better chance of mating successfully with her if he is also large. When the female is small, it is not entirely clear what size male has the advantage: the OX513A males appeared to be relatively competitive whether large or small, however the results indicate smaller males maybe a preferential mate for a small female. To put this data into context, the size of males and females isolated from the field should be considered: the average cephalothorax measurement of wild males in Grand Cayman in 2010 [7] was 0.89 mm (*N*= 48, range 0.74 mm to 1.08 mm) and the wild females were 1.06 mm (*N*= 53, range 0.79 mm to 1.26 mm) (Figure 5-3). In comparison the average small female in this experiment was 1.09 mm (*N*= 120, range 0.93 mm to 1.33 mm) with the small OX513A males being 0.90 mm (*N*= 120, range 0.76 mm to 1.02 mm) and LWT males being 0.89 mm (*N*= 120, range 0.79 mm to 0.99 mm) (Figure 5-3). This makes the small rearing in this experiment comparable to body size of wild reared males and females. In contrast the large males and females in this experiment were far above the range of sizes found in the field: OX513A large males were 1.13 mm (*N*= 120, range 1.02 mm to 1.30 mm), LWT males were 1.14 mm (*N*= 120, range 0.99 mm to 1.31 mm) and LWT females were 1.43 mm (*N*= 120, range 1.26 mm to 1.64 mm) (Figure 5-3). Given that the average mass reared male for release is 1.05 mm, if small males are considered to have a mating advantage the OX513A released males are likely to be outcompeted. In the first instance this will result in the released males having a low mating competiveness index and not being as effective as they could be at obtaining female mates. This will increase the cost of a control programme, as more males will need to be produced to attempt to outcompete the wild males in opportunistic mating events. Secondly, it is possible that if the females are in any way choosing their mate based on body size they will choose not to mate with OX513A males, and if this trait is inheritable, it may result in a sub-population of *Ae. aegypti* which are not receptive to OX513A control. This selection process, termed assortative mating, has been observed in the speciation of M and S molecular forms of *Anopheles gambiae* (Dabire *et al.*, 2013) and has been raised as a concern to the success of

[7] The pupae were collected by the Grand Cayman Mosquito Research Unit survey team and measured on their return to the laboratory by myself.

Ae. aegypti SIT (Alphey *et al.*, 2010; Stone, 2013) and other insect SIT programmes (Reisen *et al.*, 1982; Hibino & Iwahashi, 1991; Boete *et al.*, 2014). Further work will be required to understand the different elements of mate choice to determine if the release of OX513A males at their current size will be negative to their chances of mating wild females.

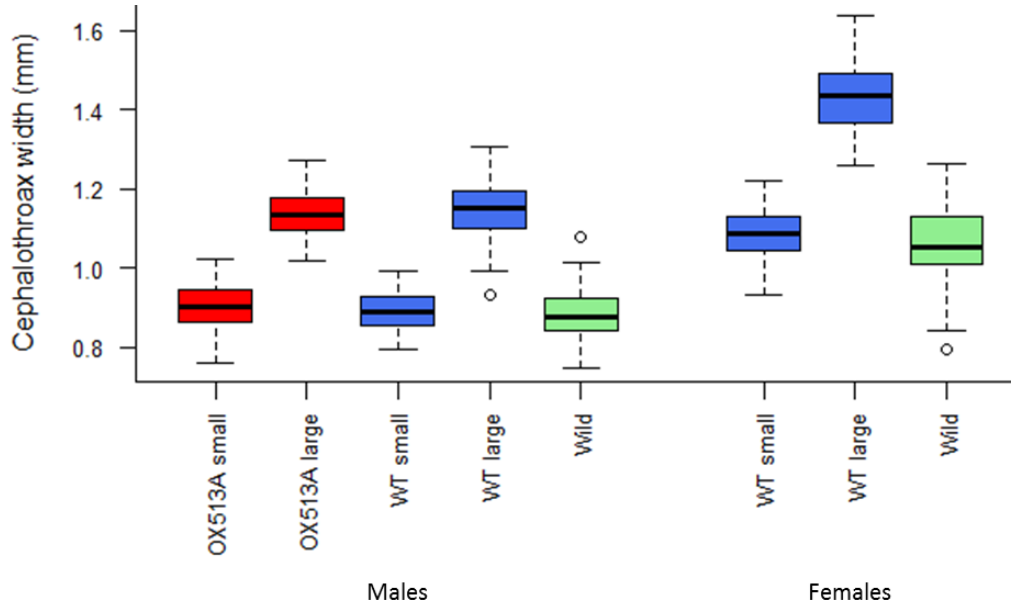


Figure 5-3 Pupae cephalothorax widths of cohorts reared to be large or small bodied. Wild collected samples are also included.

OX513A and wild type (WT) were reared to have large and small bodies by manipulating the diet per mouth and the larval rearing density. Wild pupae were collected from breeding sites in Grand Cayman in 2010.

Table 5-4 Results of the OX513A body size mating competition.

OX513A and LWT were reared to produce small and large adults. 50 OX513A males competed with 50 WT males for mating's with 50 WT females with each combination of body sizes. Each treatment had four repeats. Eggs were collected from the females and paternity determined based on the presence or absence of the fluorescent marker.

	Treatment			Repeat	No viable eggs	Paternity			RSI	χ^2 *p* value
	OX513A	WT	WT			OX513A	WT	Both		
A	L	L	L	1	1	24	25	0	0.5 (0.41 to 0.58)	0.90
				2	4	24	21	1		
				3	8	24	17	1		
				4	13	12	22	3		
					Pooled values	**84**	**85**	**5**		
B	L	S	L	1	8	32	10	0	0.7 (0.65 to 0.75)	< 0.001
				2	5	29	16	0		
				3	6	31	13	0		
				4	9	28	13	0		
					Pooled values	**120**	**52**	**0**		
C	S	L	L	1	6	11	27	6	0.34 (0.26 to 0.43)	< 0.001
				2	7	17	21	5		
				3	4	9	34	3		
				4	6	12	28	4		
					Pooled values	**49**	**110**	**18**		
D	S	S	L	1	0	18	26	6	0.44 (0.43 to 0.44)	< 0.001
				2	4	18	24	4		
				3	4	19	25	2		

				4	4	13	20	13		
					Pooled values	**68**	**95**	**25**		
E	S	S	S	1	23	10	16	1	0.43 (0.34 to 0.52)	0.15
				2	18	14	17	1		
				3	26	8	16	0		
				4	10	22	18	0		
					Pooled values	**54**	**67**	**2**		
F	L	S	S	1	6	19	25	0	0.45 (0.40 to 0.51)	0.09
				2	12	17	21	0		
				3	3	25	22	0		
				4	8	17	25	0		
					Pooled values	**78**	**93**	**0**		
G	S	L	S	1	2	31	14	3	0.64 (0.57 to 0.71)	<0.001
				2	6	26	18	0		
				3	7	24	18	1		
				4	5	33	12	0		
					Pooled values	**114**	**62**	**4**		
H	L	L	S	1	13	23	14	0	0.56 (0.48 to 0.64)	0.17
				2	13	16	20	1		
				3	8	26	15	1		
				4	9	22	19	0		
					Pooled values	**87**	**68**	**2**		

5.3.4. OX4358B Larval competition

The release of the male-selecting strain OX4358B will result in their heterozygous progeny developing in the field to produce non-functional females and functional flying males. These males are expected to further the population suppression by mating with wild females and passing the transgene on to 50% of their offspring. The effectiveness of these F_1 male progeny will be dependent, among other aspects, on their ability to compete with other co-habiting larvae for food resources. The acquisition of these resources has been shown to have an impact on the resulting adult quality (Van Handel, 1988; Gilles *et al.*, 2011; Kesavaraju *et al.*, 2014), and consequently their ability to compete for female mates.

Here OX4358B larvae were co-reared with LWT larvae at a ratio of 1:1 and fed a standard feeding regimen. Upon pupation, the pupae were picked, screened, sexed and separated into strain by sex to eclose. Adults were added to the mating arena three days after eclosion in a ratio of 10 OX4358B males with 10 LWT males competing for mates with 10 LWT females. Paternity was determined by screening the offspring for the presence or absence of the AmCyan marker.

The results show that OX4358B males co-reared with LWT were significantly less successful at mating with the WT females than the WT males (χ^2 (1, N = 6) =7.34, p =<0.001) (Table 5-5). Wing length measurements were taken for each male strain and this indicates there was no significant difference between the two cohorts (t(86)= -0.67, p = 0.50). The RSI for OX4358B was 0.30 which is significantly less competitive (U= 2, p=0.02) than the result presented above in Chapter 5.3.1 of 0.46 when OX4358B were reared separately to the WT. This decrease in mating competiveness is only likely to become more exaggerated in field rearing pools where competition for the often limited resources can be intense and larval mortality can be high.

Table 5-5 OX4358B co-reared with LWT mating competitiveness.

Larvae were reared in the absence of tetracycline. 20 OX4358B males competed with 20 LWT males for mates with 20 LWT females, in seven repeats. Eggs were collected from the females and paternity determined based on the presence or absence of the fluorescent marker.

Repeat	No viable eggs	Paternity			RSI	χ^2 *p* value
		OX4358B	WT	Both		
1	3	2	15	0		
2	7	4	6	3		
3	2	5	12	1	0.30	
4	6	3	9	2	(0.21 to 0.38)	<0.001
5	4	1	13	2		
6	6	2	8	4		
7	3	4	9	4		
	Pooled values	**26**	**67**	**16**		

Conclusions

For a self-limiting suppression programme to be successful, the released insects must be able to compete for mating opportunities with wild females. The mating biology and the concepts surrounding *Ae. aegypti* mate choice is still a relatively poorly understood subject and therefore ensuring that the self-limiting males are attractive to the wild females is difficult to implement.

Under controlled laboratory conditions, the mating competitiveness of the bi-sex lethal strain OX513A has been shown by numerous investigators to be approximately 0.5 RSI (Massonnet-Bruneel *et al.*, 2013; Patil *et al.*, 2014). Here the male-selecting strain OX4358B has also been shown to have a similar mating competiveness index of 0.46 RSI. Given that the mating process of *Ae. aegypti* is heavily dependent on flight, it is plausible to hypothesise that if there was a low level 'leaky' expression of tTAV in males, their flight ability would be compromised, limiting their competiveness for female mates, resulting in a lower RSI than was seen. This is the first quantitative measurement of the male's ability to fly and infers that they do not suffer from expression of tTAV in their flight muscles.

To produce enough males ready for a synchronous field release, a mass production system has been developed to produce insects with a high throughput and low cost. It is possible that this process places the developing insects under stress which may culminate in a lower

mating competitiveness, as well as producing a very homogenous population. Two experiments were performed to investigate the effects of the mass production process on male mating competiveness: firstly the effect of male body size in relation to the females body size, and secondly the mass production process and age at release.

The OX513A mass production of males produces a population that are relatively homogenous in size and compared to wild males and females, are considerably larger. From the data collected here, if a male is competing for a large bodied female, it is advantageous to be a large bodied male. However, if the female is small bodied, as is generally the case in the field, it is not entirely clear if small or large males have an advantage but small males certainly are competitive. The mass production of small OX513A males would represent an engineering problem: males and females are separated into their sexes based on size and when small males are produced, the differentiation in the size of males and females diminishes. This results in a high female contamination of the male population and would result in a lower male pupal collection and increased rearing costs. However, if only large males continue to be released into wild populations, it is possible that assortative mating could occur whereby the female chooses not to mate with the OX513A male in preference for a small bodied wild male. This could result in a sub-population of *Ae. aegypti* forming which would be 'resistant' to the current self-limiting control. An alternative approach would be to switch to using a male-selecting strain which could be reared under any set of conditions to produce males of heterogeneous sizes without the costly requirement to remove the females. This would allow a range of male sizes to be released which should provide the ideal male body size for every wild female to mate with. Further work should be carried out to characterise what is controlling the effectiveness of the male's body size in mating success for example, are the two bodies incompatible morphologically, is the female choosing not to mate with a male of a particular body size, are the large males poor fliers and so are not able to grab the female, or are the wing beats of the opposite body size not compatible and therefore the male is not 'recognised'. In addition to these questions, the design of the experiment manipulated body size by controlling diet. Diet composition and nutrition availability in some insects has been shown to affect pheromone production (Bogner *et al.*, 1992; Millar, 2000; Foster *et al.*, 2014), so it is possible that although pheromones have not been identified to play a role in *Ae. aegypti* mating to date (Fawaz *et al.*, 2014), the manipulation of diet to affect body size is having an as yet unidentified effect on the males mating chance, in addition to his final body size.

The second aim of the mass production experiments was to assess the effects of the mass production process as a whole on the competiveness of the males at the point of release. These data demonstrated that the mating competitiveness of OX513A was lower having been reared in the mass production process than under permissive (low density non-mechanical) rearing procedures. Further to this, the data indicated that one day old males were less competitive than three day old males, which maybe as a result of the time required to reach sexual maturity. Currently males are released at 3 days old, but this comes at a financial cost of holding the males in the insectary for two additional days, however, based on these data, the time holding time should not be reduced. To identify the particular component of the mass rearing process that is reducing the males mating success, further experiments will need to be performed, isolating each step to determine which process is detrimental. The result could indicate a single process or it could be the result of multiple components of the mass rearing process and high density holding system.

Finally this chapter estimated the competitiveness of OX4358B males co-reared with wild type males, as would be the case of progeny in the field. The results indicated that under co-reared larval conditions, the resulting transgenic adults were not as competitive at mating as the wild type males. It is unclear why the transgenic males should be less competitive at gaining resources than wild type larvae given that both strains are accustomed to laboratory rearing conditions. Koenraadt *et al.* (2010) reported that transgenic strains were less fit than their wild type counterparts and if this is the case, the transgene may be having a negative effect on the development process of OX4358B or the process of making the strain (selection and homozygosis) may have allowed recessive alleles which impact fitness to move to fixation. Given that this experiment was not testing the acquisition of limited food resources, rather, the larvae were fed by the standard feeding regimen per mouth, it is likely that under the harsher conditions of developing in the field, OX4358B $F_{>1}$ will be less competitive than seen here. These data also go some way to explaining the reduced fitness seen in the restrictive rearing conditions of Chapter 4.3.4 monitoring the loss of the OX4358B allele from populations after releases stop. These data indicated that the allele was being lost from the population at a faster rate than the modelling would predict, assuming the wild type and transgenic fitness was equal. The lower mating competiveness of OX4358B males co-reared with wild type males determined here help to explain why the transgene was lost from populations faster than models predicted.

The results collected here have demonstrated that when reared under permissive conditions, the two self-limiting strains are as competitive as wild type, however, when the conditions are less favourable these can result in reduction of the adult mating competiveness. Various larval rearing parameters have been investigated and differences in mating competitiveness have been identified, however there are still many questions about the impact these factors will have on the mate choice of wild females, as well as isolating the actual effect from confounding affects to further our understanding of mate choice and mating success.

Chapter 6 Development of novel fitness assays to measure improvements in insect fitness

6.1. Introduction

Insect fitness is the combination of events that results in an individual producing (viable) offspring. In the context of a self-limiting suppression programme, the male's fitness is the primary concern: their ability to survive and outcompete wild males for female mates will determine the success of a programme, and the more effective they are at this (i.e. the higher their fitness) the faster the wild population will be supressed. The focus of Oxitec's suppression programmes up until now has been to produce sufficient insects on a regular basis to release according to a schedule, however, the emphasis is changing with a vision to improve the insects' fitness so that the same degree of suppression can be achieved but by releasing fewer insects. This will reduce the cost of this vector control tool by increasing the efficiency of each released male mosquito. To enable this objective to be realised, the fitness of the self-limiting insects must be quantifiable so that different rearing methods and insect treatment regimens can be measured against one another to determine which processes produce the fittest insects.

A male insect's fitness is the combination of factors which allows an insect to obtain a mate and produce offspring. This includes acquisition of teneral reserves throughout larval development (Bargielowski *et al.*, 2012), locating adult food sources, locating female mates, successfully competing for females and transferring viable sperm (Boes *et al.*, 2014). For some insect species, certain components of this process have a greater impact on mating success than others, for example, the production of pheromones (Bachmann *et al.*, 2015; Bacquet *et al.*, 2015; Dweck *et al.*, 2015), having the correct morphological markings (Jaramillo *et al.*, 2015), performing the correct 'dance' (Boake & Konigsberg, 1998; Benelli *et al.*, 2015), however for *Ae. aegypti*, it is not apparent which components are pivotal to increase the males chances of success. Improvements and laboratory measurements of male fitness are therefore based around components that are perceived to increase a released male's chance at achieving a mating event in the field: these are mating competitiveness, longevity and distance flown.

As discussed in Chapter 5 mating competiveness allows the quantification of a male test population's effectiveness at competing for female mates against a control male population. The test is able to differentiate between cohorts and provides a gross estimation of the mating competitiveness of a strain. A longevity assay provides an estimate of the differential survival of populations, with the assumption that longer-lived individuals are fitter as they will survive longer in the field and have a greater chance of finding a female mate. Distance flown is an assay performed using a flight mill where an insect is flown to exhaustion and the distance and speed of flight are quantified. The underlying assumption is that the further an insect can fly, the fitter it is both in terms of available energy reserves and its ability to fly to find a female mate. Although these tests are able to differentiate between cohort batches, they are time consuming and can be resource heavy: a mating competition takes five weeks to complete, a longevity assay can take > 3 months and the distance flown, although it takes approximately two weeks to collect the data, is very resource intense. There is consequently a need to develop novel fitness tests that measure applied components of a males fitness and can provide results in a short period of time, and are ideally resource light. It is envisaged that these tests could be used as part of the routine quality control of insect batches as they leave the factory to determine batch quality and rearing issues, as well as being used to develop and assess rearing protocol changes before they become standard operating procedures.

Aside from developing insect quality assays, exploring methods and procedures to improve the fitness of the released insects is highly desirable to increase the male's efficiency at supressing the wild population. The fitness of the released insects is likely to have been negatively impacted by the mass production process as a result of the rearing system, the larval pupal processing system, the adult holding conditions and the travel conditions to the point of release. Many of the conditions which the insects encounter, for example the high larval rearing density, are driven by scale and cost and are therefore difficult to improve on a programme scale, however, adult diet is a free component which can be manipulated without interfering with the scale of an operation. Currently OX513A adult males are fed a diet of a 10% sucrose solution via a soaked cotton wool ball placed on top of the release pot (Figure 1-7). This feeding method is prone to variation between pots as a result of the technical operator administering the diet source, evaporation of the water and fungal growth on the substrate. Adult males require this food source to produce energy for metabolism (Fumio *et al.*, 1972; Nayar & Pierce, 1980), to fly (Clements, 1955), to swarm

(Maiga *et al.*, 2014) and to compete for females (Gary & Foster, 2006) and consequently the quality and access to diet pre-release is likely to directly impact the fitness of the males and their behavioural choices at the point of release. To date, research into adult diet has been focused around females (e.g. (Nayar & Sauerman, 1975; Vaidyanathan *et al.*, 2008; Vrzal *et al.*, 2010)) and less is understood about the requirements of males. Males in the wild are thought to feed on floral nectary's (Foster, 1995), which is a solution of the disaccharide sucrose and its hexose hydrolysis products fructose and glucose. It also contains other sugars, amino acids and lipids in smaller amounts (Baker & Baker, 1975; Baker & Baker, 1983; Kevan, 1983). The sugar concentration is reported to vary from between 20% to 50%, with ingested sugar concentrations isolated from the crops of wild collected mosquitoes ranging from 40% to 60% (Hocking *et al.*, 1950; Schaefer & Miura, 1972). Studies of *Anopheles gambiae* have indicated that males need to feed twice a night (Gary & Foster, 2006), however *Aedes aegypti* may display different requirements due to their differing peak activity times. Given this information, it is possible that a higher carbohydrate concentration, carbohydrate type or additives could improve the fitness of the released self-limiting mosquitoes compared to what is currently provided.

The work in this chapter describes the development of two novel fitness test assays to use to quantify differences in insect quality, and their calibration with assumed different qualities of mosquitoes. The first assay, termed release longevity is an applied longevity assay which shortens the time of an experimental longevity run from >3 months to approximately 10 days, and represents the process of insect release. The second assay is the development of flight tubes, to differentiate insect batch quality in their ability to fly vertically up a tube. The development of both of these tests are described along with the second objective of improving adult diet as a means to increase insect quality and fitness. The development of insect diet is used to aid the development of the assays and provides cohorts of differing quality to test the assays. Optimisations developed using the release longevity experiment are cross correlated with the flight tubes to determine which aspects of fitness can be distinguished using the different test options.

6.2. Methods

6.2.1. Insect strains and rearing

OX513A were reared in trays of 3000 first instar larvae in 1L of 30 µg mL^{-1} chlortetracycline or 1 µg mL^{-1} doxycycline. Alternatively insects were reared in the proprietary mass production aquaculture system using a proprietary diet. Pupae were collected, sexed and used in the following experiments.

6.2.2. Longevity experiment set up

Cages of 15 cm x 15 cm x 15cm were used for the longevity experiments. Pupae were counted into cohorts of 25 and placed into cages. Four cages were prepared for each treatment. Diet was provisioned using a 30 mL universal tube with a dental roll (ClickDental, UK) inserted through a drilled hole in the lid. Mortality was recorded daily.

6.2.3. Full term longevity

Male and female pupae were allowed to eclose for 48 hours before the pupal weighboat was removed. Female cohorts were blood fed twice a week and eggs collected. Sucrose diet was provisioned *ad libitum* and refreshed weekly. The experiment was terminated on day 100. Remaining surviving adults were counted and censored in the data analysis.

6.2.4. Release longevity

Male pupae were allowed to eclose for 48 hours before the weighboats were removed. Diet was provisioned *ad libitum* from the point of cage set up for five days, however as pupae are eclosing for two days, adults are considered to have diet for three days. After this the diet was removed and replaced with a water feeder, which was available *ad libitum*. The removal of diet for water represents release. Daily mortality was counted from this point.

6.2.5. Adult diets

Adult diets were prepared using the following ingredients, sucrose (Silver Spoon, UK), fructose (Holland and Barrett, UK) and glucose (Thornton and Ross, UK). Nipagen (Sigma Aldrich) was dissolved in ethanol to a final concentration of 10%. For diets containing Nipagen, 10% Nipagen in ethanol was added to the sugar water to a final concentration of 0.2% Nipagen.

Additives were sourced from the following places: brewers yeast (MP Biomedicals, UK), yeast extract (Fisher Scientific), egg powder (CCMoore, UK), egg albumin (CCMoore, UK), vitamin mix (Bio Serv, USA), pollen (Super Nutrients, UK), lecithin (Optima, UK) and impatiens (Wantage garden centre).

For diets of brewers yeast, yeast extract, egg albumin, egg powder and vitamin mix: for 'high' diets, 5g of additive was dissolved in 30% sucrose water with 0.2% Nipagen. For 'low' diets, 1g of additive was dissolved in 30% sucrose water with 0.2% Nipagen.

For diets containing pollen water, 1g of ground pollen was soaked for 24hrs in 300 mL of dionised water and stored in the fridge. For the low concentration 100 mL of pollen water was mixed with 45g of sucrose and 50 mL of dionisied water to make a 30% sucrose solution. Nipagen in ethanol was added to a final concentration of 0.2%. For the high concentration 45g of sucrose was dissolved in 150 mL of pollen water. Nipagen in ethanol was added to a final concentration of 0.2%.

For diets containing lecithin, 0.5 g of lecithin was dissolved in 50 mL of dionised water. For the low concentration 0.18 mL of lecithin solution was added to 150 mL of 30% sucrose solution containing 0.2% Nipagen. For the high concentration 1.5 mL of lecithin solution was added to 150 mL of 30% sucrose solution containing 0.2% Nipagen.

6.2.6. Activated longevity

Cages were prepared according to the methods described in Chapter 6.2.4. At the point of 'release' (when the diet is exchanged to a water feeder), the treatment cages were placed onto a vibration plate (Body Sculpture BM1500). The cages were vibrated for 300 seconds every hour between 08.00 and 17.00 daily.

6.2.7. Flight tubes development

Perspex tubes (Plastics Direct, UK) measuring 2 m tall by 60 mm internal diameter were coated in 10% Fluon (Blades Biological Ltd, UK). Plastic boxes (Plastic Box Shop, UK) measuring 13.5 cm x 13.5 cm x 17.5cm (W:L:H) were modified into cages by cutting a 10cm square hole in one side wall and covering in tubular gauze (Sterogauze, UK) to create a handling opening. For the cages at the bottom of the tube (the adult holding area), a 5 cm hole was drilled in the lid to serve as an exit point for the mosquitoes to enter the flight tube. For the boxes to serve as the top adult receiving cage, a 3 cm hole was cut in the

bottom of the box to allow the mosquitoes into the holding cage. Nine flight tubes, comprising of a bottom cage, tube and top cage were used to develop the assay (Figure 6-3).

As part of the development process, the hole in the bottom cage was increased to 12 cm and a 13 cm funnel was placed over the hole to effectively feed the mosquitoes into the tube more effectively. A funnel was also attached to the top of the tube to encourage the exit of the adults from the tube into the top cage.

Various attractants were used in the top cage to encourage the males to fly up the tube, these were 10% sucrose, Octanol (Mosquito Magnet) and OX513A females.

6.2.8. Flight tube differentiation test

Due to the number of pupae required for this experimental set up, OX513A were mass reared using a propriety rearing system. The rearing system uses a confidential soluble diet to allow the food to flow throughout the system. Male pupae were collected and sorted on day 8 of rearing (counted from the point of hatching), and aliquoted into cohorts of 100. For each treatment, nine cohorts were prepared, one for each of the flight tubes.

6.2.9. Statistical analyses

Data were analysed using the RStudio software package version 0.97.318 (RStudio, USA). Normality of data was tested using the Shapiro-Wilk method. Homogeneity of variance was tested for using the Bartlett test. For all comparisons significance was set at $p < 0.05$.

Longevity data (full term and release longevity) were analysed using the Survival Analysis package (2.38-2). Kaplan-Meier curves were plotted and Log-rank tests used to test for significant differences between treatments.

For normally distributed data, a Students *t*-test was used to test for significance between cohorts. For non-normally distributed data, a Mann-Whitney U test was used.

6.3. Results and Discussion

6.3.1. Full term longevity

Longevity assays are used to distinguish differences in the survival of cohorts as a measure of fitness with the assumption that longer-lived individuals are fitter as they survive for

longer to mate with females. Although this assay is effective at distinguishing differences in survival, the time taken to complete the experiment is too long and therefore a faster assay would be desirable. To enable the development of a more time efficient longevity assay, a 'full-term' longevity assay (adults provisioned with diet for the duration of their life and daily mortality counted) was run to collect a baseline data set to compare to the results of the novel longevity assay. For this experiment adult diet was used as a variable to produce cohorts with differing survival times. Three different carbohydrate sources were investigated; sucrose, fructose and glucose at the standard concentration of 10%. In addition Nipagen® was added to a 10% sucrose solution to determine if survival could be increased. Nipagen (methyl 4-hydroxybenzoate or methylparaben) is a widely used antifungal food preservative and it has been reported by Benedict et *al.* (2009) to marginally increase the survival of female *An. gambiae* and *An. arabiensis* by preserving the quality of the diet. For this experiment, male and female pupae were placed into separate cages in cohorts of 25 with four cages prepared for each treatment, and allowed to eclose. Diet was provided *ad libitum* and daily mortality was counted. In addition, the female cohorts were provided with male mates for 48 hours, after which the males were removed and discarded. The female cohorts were blood fed twice weekly (as blood is considered an important component of their nutrition) and eggs collected weekly.

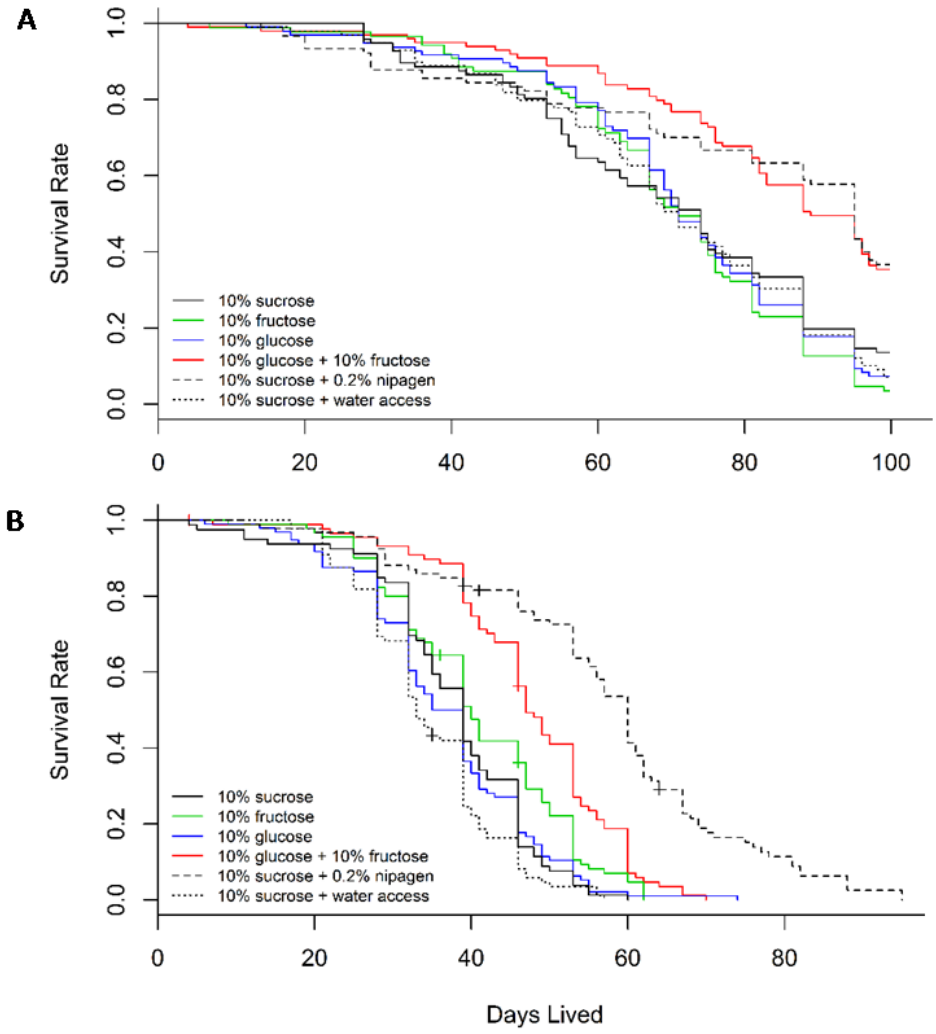


Figure 6-1 Longevity of OX513A provisioned with different adult diets.

OX513A females (A) and males (B) were provided with different adult diets *ad libitum.* Daily mortality was recorded. Each treatment consisted of four cages each with 25 pupae (N=100).

The results in Figure 6-1 show that the provision of different adult diets can significantly affect the longevity of both males and females. Comparing the longevity of the male cohorts to the control of 10% sucrose (Figure 6-1B and Table 6-1), the median longevity of cohorts maintained on the mixed diet of 10% fructose with 10% glucose was 21% longer than those of the control, while the addition of 0.2% Nipagen to 10% sucrose increased the median longevity by 58% compared to the control. The same effect was also seen with the female cohorts which showed a 25% increase in the median longevity when maintained on 10% fructose with 10% glucose and a 28% increase when maintained on 10% sugar with 0.2%

Nipagen. The other treatments tested mostly resulted in a shortened lifespan compared to provision of 10% sucrose (Table 6-1). These data infer that either a higher carbohydrate concentration is beneficial to both male and female survival, or that the combination of fructose and glucose results in an increased median longevity. Further to this, the addition of Nipagen as a preservative is beneficial to the longevity of the population, and this was seen using only 10% sucrose. It is therefore possible that the addition of Nipagen to a higher carbohydrate concentration could have an additive effect and further increase survival.

Table 6-1 Median survival of populations maintained on different adult diets.
Change in survival is compared to the control diet, 10% sucrose. Each treatment consisted of four cages each with 25 pupae (N=100).

Diet	Males			Females		
	Median survival (days)	% change	Significant difference *p*	Median survival (days)	% change	Significant difference *p*
10% Sucrose	39	-	-	74	-	-
10% Fructose	40	3	0.1	71	-5	0.13
10% Glucose	37	-6	0.77	71	-5	0.62
10% Fructose + 10% Glucose	47	21	<0.001	89	25	<0.001
10% Sucrose + water access	33	-16	0.02	71	-5	0.5
10% Sucrose + 0.2% Nipagen	60	54	<0.001	95	28	<0.001

6.3.2. Release longevity calibration

The full term longevity assay lasted 100 days, and even after this time, not all of the females had died. This results in a slow progression of understanding how different treatments affect the fitness of cohorts. This led to the design of an assay that would allow the direct comparison of treatments but would provide the results in a timely manner. Given that OX513A male cohorts destined for the field are provided with adult diet for only three days before release, an assay was designed around this concept firstly to replicate the feeding opportunities of the males as well as vastly reducing the expected longevity of the males.

Males in the field have a median longevity of approximately 2.3 days (Lacroix *et al.*, 2012) and therefore the median survival of males in the 'full term' longevity at approximately 40 days is known to be unrealistic. Cohorts of males were placed into cages to the same specification as in the full term longevity. Pupae were allowed to eclose for two days before removing the weighboat, replicating the draining of the release device. Adult diet was provided for a further three days representing the maturation time of the adults in the insectary before release. After this time, the adult diet was removed and replaced with a water feeder. This represents the males release into the field with the assumption that after this point they are unable to find any further energy source, but would be able to find water from, for example, dew.

Treatments used in this experiment were based on those used in the full term longevity so that the results could be compared and used to calibrate the assay. In addition to this, further investigations were made into optimising the energy source by investigating sugar type (glucose, fructose and sucrose) and concentration (10%, 20%, 30% and 40%). All treatments included 0.2% Nipagen as a preservative which was shown to be beneficial in the full term longevity.

The first priority of this assay was to cross correlate the results to the full term longevity to ensure that the data are comparable. Comparing the results presented in Table 6-1 and Table 6-2 shows that 10% fructose and 10% glucose (separately), which were used in both the full term longevity assay and this release assay, demonstrate similar differences in median longevity with 10% glucose eliciting a marginally shorter median longevity of 7 days compared to 10% sucrose at 8 days, and 10% fructose being the same as sucrose also at 8 days. Further to this, a combination of fructose and glucose together resulted in an increased longevity in the full term longevity (39 days compared to 47, Table 6-1), and a similar survival is seen here in the release longevity (8 to 9 days for both). These data indicate that the differences seen between test cohorts of the release longevity are comparable to the full term longevity, as well as being an applied and an appropriate design to test the optimum diet of males destined for release.

Table 6-2 Longevity of males in a release longevity assay provisioned with different adult diets.

Diet was provisioned for three days post eclosion (five days post pupal collection). After this time the diet source was removed and replaced with a water feeder. Daily mortality was recorded. Fructose & glucose was a mixture in a 1:1 ratio resulting in the total carbohydrate concentration stated. Each treatment consisted of four cages each with 25 pupae (N=100).

Treatment		Median survival		Significant difference
Sugar	Concentration (%)	Days	95% CI	*p*
Sucrose	10	8	8 to 9	-
	20	9	8 to 9	0.28
	30	9	9 to 9	< 0.001
	40	10	9 to 10	< 0.001
Fructose	10	8	8 to 8	0.97
	20	8	8 to 8	0.95
	30	9	8 to 9	0.12
	40	9	9 to 9	0.004
Glucose	10	7	7 to 8	0.08
	20	9	8 to 9	0.27
	30	9	9 to 10	< 0.001
	40	9	8 to 9	0.006
Fructose	10	8	8 to 8	1.0
& glucose	20	8	8 to 9	0.67
	30	9	9 to 9	< 0.001
	40	9	9 to 10	< 0.001

The second motivation of this experiment was to determine which sugar source and at what concentration results in the longest median male survival. The results presented in Table 6-2 indicate that for all the sugar types tested, a higher concentration elicits a significantly longer median longevity than the control of 10% sucrose. A cross comparison of the higher concentrations to determine which sugar type and which concentration (30% or 40%) is optimum demonstrates that there is no significant difference ($p > 0.05$) between these treatments, except between 30% fructose and 40% sucrose ($F(7,718)=3.46$, $p=0.001$),

TukeyHSD p= 0.03) with 40% sucrose being longer lived. The maximum concentration tested here was 40% and it is possible that higher concentrations could result in a further increase in survival, however given that the males are only provided with this sugar diet in their release pots, there is a concern that if the concentration is too high, the males could suffer from dehydration by not having access to a water source when an energy source is not required. Based on these results and the worldwide availability of sucrose, it was decided that sucrose would be the carbohydrate source to take forwards for further testing, and that given that there is no significant increase in male longevity between 30% and 40% sucrose, 30% sucrose would be used to keep the cost down.

6.3.3. Further optimisation of adult diet

Wild *Ae. aegypti* males are thought to feed on nectar which is a combination of sugars, vitamins and minerals. It is possible that components of nectar, other than the carbohydrates, could contribute to the male fitness. The composition of nectar is extremely variable between plants with examples across the insect world of symbiotic relationships with species feeding on specific plants to gain the required nutrition for their survival and mating success (e.g. (Mello & Silva-Filho, 2002; Stam *et al.*, 2014; Bachmann *et al.*, 2015)). This information is not available for *Ae. aegypti* and therefore it either does not occur in nature or these symbiotic relationships have yet to be identified.

Due to the lack of applied information about *Ae. aegypti* male diet, additives were selected to provide a range of the potential survival enhancing components; amino acids, minerals, vitamins and fatty acids. When designing an artificial insect diet it is important to consider the scale and cost effectiveness of supplementing additives to the diet, therefore additives were chosen which were globally available, cheap and soluble. Options were selected from widely used ingredients in insect diet mass production, which would provide a variety of different amino acids, vitamins and lipids (Table 6-3).

The experiment was set up as above in Chapter 6.3.2. In each case the supplements, brewers yeast, egg albumin, vitamin mix, egg powder and yeast extract were added to 30% sucrose to two final concentrations classed as ‘high’ and ‘low’. Four cages of 25 male pupae were prepared for each treatment. Adult diet was provisioned for three days post eclosion after which the diet was replaced with water. Daily mortality was recorded from the day of diet removal.

Table 6-3 Expected composition of additives to be added to adult diet (NutritionSelfData, 2015)

Ingredient	Approximate nutrition composition		
	Protein %	Carbohydrate %	Lipid %
Brewers yeast	50	45	0
Egg albumin	80	5	0
Egg powder	35	5	55
Yeast extract	50	45	0
Vitamin mix	0	0	0

The results indicate that the median longevity was not significantly increased by any of the treatments examined, in fact high brewers yeast, high egg albumin, high and low yeast extract and low egg powder all significantly reduced the median longevity compared to 30% sucrose alone ($F(10, 937)=6.46$ $p= <0.001$, TukeyHSD significant values $p = <0.001$) (Table 6-4). This is most likely due to the additive being repellent, preventing the mosquitoes imbibing the sugar water before release and consequently having reduced energy reserves after release. This could be as a result of the components of the additive being distasteful, or the concentration of the additive being too high. The other treatments tested were not significantly different from the control of 30% sucrose ($p = >0.05$) (Table 6-4).

Table 6-4 Longevity of adults fed an adult diet of 30% sucrose supplemented with additives.

Adults were provided with diet for three days post eclosion after which cages were provisioned with water only. Daily mortality was recorded. Significant differences were tested for compared to 30% sucrose. Each treatment consisted of four cages each with 25 pupae (N=100).

Treatment		Median survival		Significant difference
	Concentration	Days	95% CI	*p*
Sucrose	30%	9	9 to 9	-
Brewers yeast	High	8	8 to 8	<0.001
	Low	9	9 to 9	0.54
Egg albumin	High	8	8 to 8	<0.001
	Low	8	8 to 9	0.10
Egg powder	High	9	8 to 9	0.08
	Low	8	8 to 9	<0.001
Yeast extract	High	8	8 to 8	<0.001
	Low	8	8 to 9	<0.001
Vitamin mix	High	9	9 to 9	0.50
	Low	9	9 to 9	0.86

Based on these results brewers yeast and vitamins were taken forwards to a second experiment to further evaluate their effectiveness. Egg albumin and egg powder were not included as their ease of use was more complicated and therefore not considered fit for purpose. The data in Table 6-4 indicate that the lower concentration of brewers yeast and vitamins were more beneficial to survival than the higher concentrations, therefore in the second experiment the low concentration and a concentration of half of this was used (very low). In addition to these additives, pollen and honey were introduced as additives to represent the contents of nectar, lecithin was included as a source of fatty acids and has been shown to be beneficial to developing larvae diet (Sneller & Dadd, 1981) but its benefit to adults has not been determined, and finally impatiens plants were used as they have been reported to increase the longevity of males compared to 10% sucrose (Chen & Kearney, 2015). Impatiens are found widely throughout the tropics and could be a food source to *Ae.*

aegypti, providing nutrition through their extra floral nectaries. For each treatment except honey, the supplement was dissolved in 30% sucrose water. For the 100% honey treatment, honey was provided in a weighboat and a separate water feeder was provided. For 50% honey, honey was dissolved in water in a 1:1 ratio. For the impatiens treatment, a 10 cm tall, planted impatiens plant with three flowers was placed into each cage. The experiment was run as above, however due to marginally different environmental conditions in the laboratory, the results of the first experiment cannot be directly compared to the results of this second experiment.

Table 6-5 Longevity of adults fed an adult diet of 30% sucrose supplemented with additives.

Adults were provided with diet for three days post eclosion after which cages were provisioned with water only. Daily mortality was recorded. Significant differences were tested for compared to 30% sucrose. Each treatment consisted of four cages each with 25 pupae (N=100).

Treatment		Median survival		Significant difference
	Concentration	Days	95% CI	*p*
Sucrose	30%	10	9 to 10	-
Brewers yeast	Low	10	9 to 10	1.0
	Very low	9	9 to 10	1.0
Honey	100%	9	8 to 9	0.06
	50%	9	9 to 9	0.82
Pollen	High	10	10 to 10	0.21
	Low	10	10 to 10	0.77
Lecithin	High	10	9 to 10	1.0
	Low	10	9 to 10	1.0
Vitamin mix	Low	10	9 to 10	1.0
	Very low	10	9 to 10	1.0
Sucrose	10%	9	8 to 9	-
Impatiens	-	6	6 to 6	<0.001

The results indicate that the median longevity of all the additive treatments (first section of Table 6-5) were not significantly different from the control of 30% sucrose alone. Further to

this, in comparison to the previous experiment, the concentrations of additives do not appear to be repellent and have not had a negative impact on the longevity of the cohorts. Although not significant, the addition of pollen to 30% sucrose increased the longevity of cohorts compared to 30% sucrose alone, and therefore further work could pursue pollen as an additive to increase longevity. However caution should be taken as pollen is a variable product being dependant on the environment from which it is collected. Therefore the effectiveness of pollen at increasing the male's longevity purchased around the world could be very variable. The use of impatiens as an adult diet was shown to elicit a significantly shorter median longevity than 10% sucrose (second section Table 6-5) and therefore this will not be pursued any further. It is not clear why Chen and Kearney (2015) found that impatiens significantly increased *Ae. aegypti* male longevity but the opposite effect was seen here. It is possible that this could be as a consequence of the rearing conditions and larval diet which produces insects of different qualities at the point of eclosion, and that the impatiens were able to provide vital nutrition to their experimental cohorts but the same benefit is not seen in Oxitec reared cohorts provisioned with Tetramin diet as larvae.

6.3.4. Activated longevity

A concern with using longevity assays is considering what is actually being measured. The assumption with this assay is that long lived individuals are fitter as they would be able to survive longer to mate with females, however it is possible that the longest lived individuals arise from being lazy and not exhibiting the female search behaviours (flight) that would be expected and desirable. To determine if this is compromising the use of the release longevity assay to determine differences in field relevant fitness, the release longevity was adapted to include cage vibration to periodically force the individuals to fly. To achieve this, treatment cages were attached to a vibration plate that was set to vibrate for 300 seconds each hour between 9am and 5pm daily. Control cages were prepared which contained the same adult diet but remained stationary. The experiment was set up according to the same method as the release longevity (Chapter 6.2.4) using adult diets of 10% sucrose and 30% sucrose. However on the release day, when the adult diet is removed, treatment cages were attached to the vibration plate and vibrated periodically to encourage male flight.

The results show that the survival of the cohorts on the vibration plate was significantly shorter than the control cohorts which were resting on a shelf (10% sucrose χ^2 (1, *N*= 84, 93)= 43.6, *p* =<0.001; 30% sucrose χ^2 (1, *N*= 84, 85)= 24.6, *p* =<0.001) (Table 6-6). This result

is expected as the cohorts which are periodically vibrated are encouraged to fly and consequently use up their energy reserves more rapidly than their non-vibrated counterparts. Further to this, testing for differences between the median longevity of the control cages 10% sucrose and 30% sucrose shows an expected significant difference in the median longevity (χ^2 (1, *N*= 84, 85)= 25.6, *p* =<0.001) and the same significant difference is also seen in the median longevity between the two sucrose concentrations on the vibration plate (χ^2 (1, *N*= 84, 93)= 43, *p* =<0.001). These results indicate that the higher sucrose concentrations are enabling the mosquitoes to live longer both when forced to periodically fly and when allowed to rest for the duration of the experiment. This result infers that the results seen in the standard release assay when cages are left on the shelf provide relevant results of the fitness of the individuals, rather than measuring the potential laziness of cohorts. It is expected that the cohorts which were vibrated would have a shorter longevity as there are forced to use up their energy reserves, however, the same difference in longevity of the two sucrose treatments was seen between the control and the vibration cohorts. Given that this result confirms the validation of the release longevity assay as an applied laboratory assay, and the added complication and complexity in using a vibration plate, future release longevity assays will be run by placing cages on shelves and not vibrating them.

Table 6-6 Longevity of adults fed two sucrose concentrations and either allowed to rest or forced to periodically fly

Adults were provided with diet for three days post eclosion after which cages were provisioned with water only. Daily mortality was recorded. Cages were either allowed to rest or were agitated routinely by vibration plate. Each treatment consisted of four cages each with 25 pupae (N=100).

Treatment		Median survival	
		Days	95% CI
10 % Sucrose	Resting	7	7 to 7
	Vibration	6	5 to 6
30% Sucrose	Resting	7	7 to 7
	Vibration	7	7 to 7

6.3.5. Flight tube development

Flight is a critical function for the released males to be able to fly to find females, as well as finding safe resting places in between mating periods. Given its importance it is considered a valuable factor to measure and optimise if possible. Flight mills have been widely used with different insects as a means to measure distance flown and an individual's ability to fly, however this method is very resource intensive and can only sample individuals. It is desirable to have a fitness assay that is able to distinguish flight ability of populations (cohorts) with a considerably lower resource input.

A very simple flight assay is used in the *Ceratitis capitata* SIT programmes to ensure that the irradiated males have an acceptable level of fitness. This quality control assay consists of a black cup of 6.6 cm diameter and 10 cm tall in which 100 pupae are placed and must eclose and escape within the defined time, usually three days (FAO IAEA USDA, 2003). Dr R. Bellini (Centro Agricoltura Ambiente, Italy) has reported to be modifying this design for use with mosquitoes (personal communication) by placing individual pupae into tubes measuring 8 mm to 13 mm internal diameter by 20 cm tall, and seeing how many adults eclose and escape within a defined time. For the purpose of a self-limiting programme, this scale of assay would not be feasible to test the quality of batches of reared individuals. The time taken to load the tubes with individual pupae, and the resolution likely to be achieved by the design is unlikely to prove suitable. This design also prevents the assessment of adults leaving the factory after the adult holding; rather it only allows assessment of the rearing quality up to post-pupal sorting. It was therefore desirable to design a flight assay which could be used to test cohorts (defined as >50 individuals) that could be used at the 'factory gate' to determine the quality of mosquitoes leaving the factory taking into account the larval rearing and adult holding conditions. Building on the design of Dr R. Bellini it was envisaged that a longer tube would allow a greater differentiation of flight ability, and if the tube was wider then a greater number of individuals could be tested at the same time to determine their quality. Clear Perspex tubes were sourced which were 60 mm (internal diameter) and 2 m tall. A removable cage was attached to the bottom of the tube to hold the test cohorts, and a cage was placed onto the top of the tube to collect and hold the adults that had flown the distance. As a provisional experiment, adult mosquitoes were released at the bottom of the tube and the number reaching the top recorded (data not presented). This highlighted two points: firstly the mosquitoes rested on the way to the top of the tube and therefore could potentially walk to the top and secondly a majority

(approximately 80%) of the individuals did not make it to the top. To address the first issue, the tubes were coated in Fluon (polytetrafluoroethylene), a substance used in insect rearing to make the surface slippery so that insects are unable to grip. A literature search did not give rise to any reports of Fluon being used in conjunction with mosquitoes and therefore an experiment was performed to ensure that it was not repellent to males. If it were shown to be repellent, the mosquitoes would be inhibited from flying up the tubes, which would prevent the differentiation in insect fitness. A basic choice experiment was designed to determine if the adults found Fluon to be repellent: a 15 cm x 15 cm x 15 cm cage was modified to contain two arms, one with a coated Fluon arm and one uncoated arm (Figure 6-2). A 10% sucrose feeder was used as an attractant to make the males choose to leave the central cage, which was not provisioned with food. 20 males were hovered in the central cage of each of the three assay sets and left for 24 hours. The number of males in the cup at the end of each arm was counted. The results showed that there was no significant difference in the number of males in each cage ($t(3)= -2.5$, $p = 1$) and therefore Fluon does not appear to be repellent.

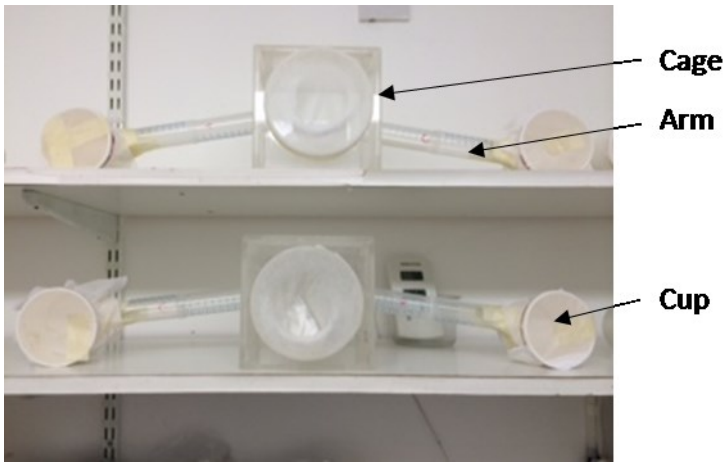


Figure 6-2 Fluon repellence assay

One arm of each test set was coated in Fluon, the other arm was not. The cups at the end of the both arms contained cotton wool soaked in 10% sucrose. 20 males were placed into the central cage and left for 24 hours.

To address the second issue highlighted in the test run (>80% of the males not making it to the top), a cohort of males were released into the tube at the bottom and the maximum height each male flew was marked. This determined that a majority of the cohort could fly

to 1.7m but few flew higher. Consequently the tubes were cut to 1.6m so that the males could fly the full tube length and escape at the top (Figure 6-3).

The next step in the development of the assay was to determine the optimum attractant to place at the top of the tube to encourage the males to leave the bottom holding cage and fly to the top. If the males do not have a reason to leave the bottom cage, the assay is less likely to be able to differentiate insect fitness. Given that this assay is to test male fitness, it is important that the attractant used is attractive to males. Published literature has focused on attractants of females for the purpose of for example baited traps (Bhami & Das, 2015; Harwood *et al.*, 2015; von Oppen *et al.*, 2015), but given that males are not the vectors of disease, their chemical attractants have not been investigated. Food sources were not provided in the bottom cage and therefore provision of a food source at the top of the tubes was considered to be an attractant. As males are reported to feed every 6 hours (Gary & Foster, 2006) it is expected that over the duration of the assay they should search for and be attracted to a food source. In the first instance 10% sucrose was provided in the top cage, the standard feeding source for mosquito research. The results indicate that after 24 hours only 50% of the mosquitoes had reached the top cage (Figure 6-4 Experiment 1). Given that these mosquitoes are considered to be fit, ideally >80% of the cohort should be able to reach the top cage so as to allow for differentiation between test cohorts in later experiments with compromised cohorts. This implies that 10% sucrose is not a sufficiently strong attractant for males to choose to try to reach the top cage.

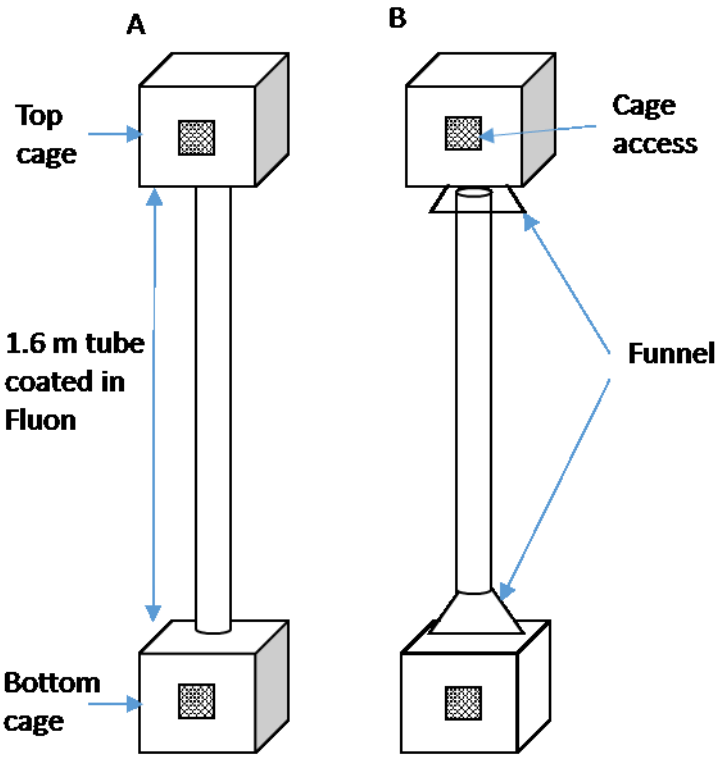


Figure 6-3 Design of the flight tube

A) The flight tube was designed so that male mosquitoes were placed into the bottom cage and allowed a defined time (usually 24 hours) to fly to the top cage. The diameter of the tube was 60 mm. An attractant was placed into the top cage to encourage the males to fly up the tube. B) The flight tube was modified so that the bottom cage had a larger hole (12 cm) covered with a funnel to feed the mosquitoes into the tube more effectively. A funnel was also placed on the top of the tube to feed the mosquitoes into the top cage.

A second experiment was set up this time using octanol as the attractant. Octanol is used to bait female mosquitoes to traps, often in conjunction with CO_2 which is reported to increase its attractiveness (Becker *et al.*, 1995; Cilek *et al.*, 2011a; Cilek *et al.*, 2011b). However, the use of CO_2 in the laboratory for this assay is not considered desirable due to the associated hazards and ability to control the concentration between repeats and experimental set ups, and therefore octanol was tested alone. The results show that octanol was not significantly more attractive than 10% sucrose ($t(5)= 0.97$, $p = 0.38$) and is therefore not suitable to pursue (Figure 6-4 Experiment 2). The difference in the proportion of males reaching the top with 10% sucrose as an attractant is also not significantly different between experiment 1 and experiment 2 ($t(4)= -2.86$, $p = 0.05$) (Figure 6-4).

In the previous two experimental runs, < 60% of the mosquito cohort were making it to the top cage and it was not clear if they were physically unable to reach the top or were choosing not to fly up the tube to the top within the 24 hour time limit. The third experiment focused the duration the mosquitoes had to get to the top to determine if leaving them for longer resulted in a greater fraction reaching the top. By allowing the experiment to run for 72 hours a significantly greater fraction of males reached the top than if left for only 24 hours (W = 63, p = <0.001) (Figure 6-4 Experiment 3). This demonstrates that the mosquitoes are able to reach the top, however within the usual 24 hour time limit, <60 % are choosing to leave the bottom cage to reach the sugar source at the top.

Sugar source alone appears to not be a strong enough attractant to encourage males to fly to the top of the tube within 24 hours, however it is desirable to run the assay for only this duration of time to obtain the results quickly and provide quick feedback as to the quality of the insects. Given the lack of known alternative male chemical cues, females were introduced to the top cage on the pretence that males should be able to detect the females either by wing beat frequency or by a pheromone cue, and should be biologically programmed to fly up the tube towards the females. Ten females were placed into the top cage along with a sugar source to prevent the female dying or the males leaving the top cage in search of food, and the experiment was run for 24 hours. Once again the results (Figure 6-4 Experiment 4) indicate that there was no significant difference in the number of males reaching the top cage with the females compared to the sucrose only control (W = 8, p = 0.9). This result was unexpected as it was hypothesised that males would be attracted to the females in the top cage and would be encouraged to fly up with the prospect of encountering an opportunity to mate. However, adult *Ae. aegypti* are known to prefer dark places to rest and it was therefore hypothesised that the males might not be attracted to the top cage as it is close to the ceiling light and they may find it undesirable to migrate directly towards the bright light, or rest in the top box due to the light intensity. Experiment 5 tested this hypothesis by covering the top box in tin foil to exclude the light and make a dark resting area. However this time the results indicated that rather than encouraging the males to migrate to the top box it was less desirable to enter the box with a significantly lower fraction making it to the top (t(3)= 4.3, p = 0.02) (Figure 6-4 Experiment 5). Given that neither females nor a dark resting place could attract the males to the top box it was considered that the males maybe struggling to find the exit from the bottom cage to enter the tube to be able to fly to the top. In the previous experiments, the exit point was a 3 cm

diameter hole in the centre of the lid and therefore the males maybe unable to locate the hole to escape, rather than simply not choosing to escape. To increase the 'feed' of males into the tubes the hole was increased to 12 cm diameter and a funnel was attached over the hole to locate the males into the tube (Figure 6-3B). In addition to this, a funnel was attached to the top of the tube to feed the flown males into the top cage. Using the attractant of 10% sucrose in the top cage, the results from this experiment (Figure 6-4, Experiment 6) shows that the addition of the two funnels at the top and the bottom significantly increased the fraction of males reaching the top cage compared to no funnels ($t(3)= -4.4$, $p = 0.03$). This indicates that the males have in the previous experiments been unable to locate the exit hole from the bottom cage and the entry hole to the top cage and this has hindered their progression to the top cage. The development process and modifications made to this assay set up have now produced a setup which can be used to test cohorts of mosquitoes with perceived differences in quality.

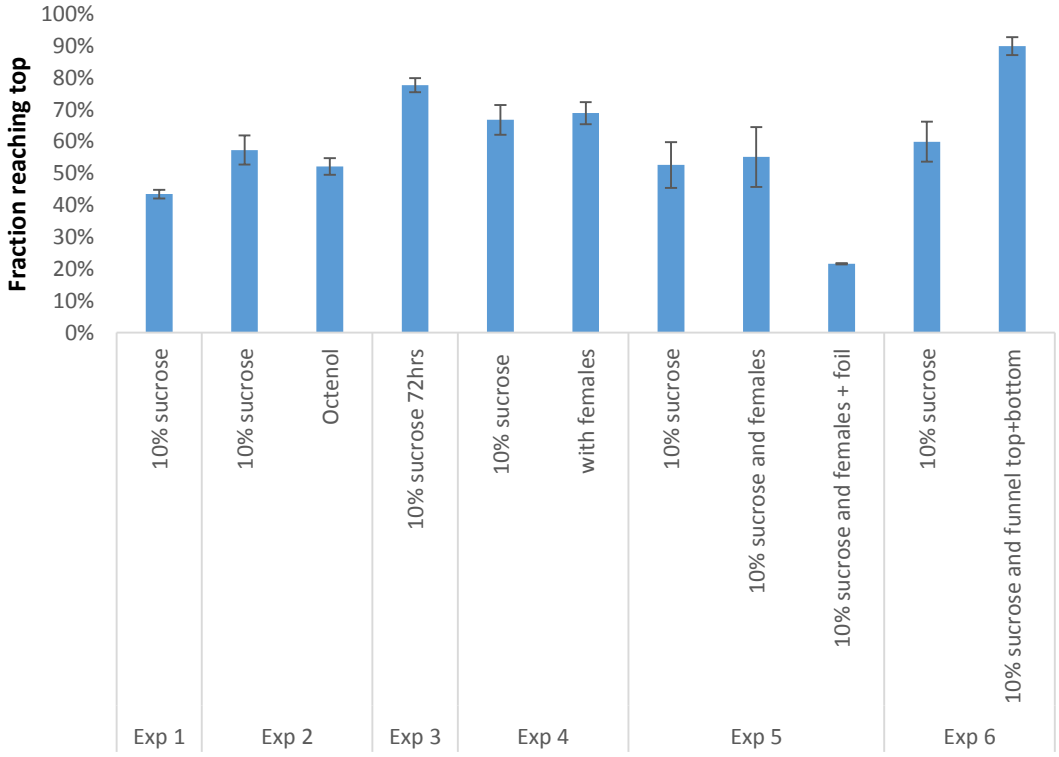


Figure 6-4 Development of the flight tube assay

Cohorts of 100 males in each of 9 tubes were released at the bottom of a vertical tube and left to fly up the tube to a holding cage at the top. Different attractants were placed into the holding cage to encourage the males to fly the 1.6 m distance.

6.3.6. Flight tube differentiation

To test the flight tube assay to determine the quantifiable differentiation achievable between test cohorts, batches of mosquitoes were prepared that were considered to have differences in fitness. These included being 'released' as young, old, having been fed a high sucrose diet, or having been stored at high temperature or high density. Due to the number of pupae required, OX513A were reared in a proprietary mass rearing system to provide sufficient males from a single rear on the same day. The male pupae were collected and counted in to nine cohorts of 100 pupae for each treatment, and placed into eclosion boxes. Cohorts were assigned to one of each of the six treatments (Table 6-7). Males were transferred into the bottom cage of the flight tube set up and allowed 24 hours to reach the top cage. 10% sucrose and 10 females were added to the top cage to provide the maximum attractant to the test males to move and collect in the top box.

Table 6-7 Treatments of test cohorts to test flight tubes

OX513A were mass reared and male pupae collected. Males were counted into nine cohorts of 100 males for each treatment.

Treatment	Sugar	Release age (days)
-	10% sucrose	3
-	30% sucrose	3
32˚C	10% sucrose	4
High density	10% sucrose	3
Young	10% sucrose	1
Old	10% sucrose	15

The results (Figure 6-5) show that there was no significant difference in the fraction of mosquitoes reaching the top cage from treatments 30% sucrose ($t(5)= -1.3$, $p = 0.24$), young ($t(7)= 2.2$, $p = 0.06$), high temperature ($t(10)= -1.5$, $p = 0.16$) and high density ($t(8)= 1.7$, $p = 0.12$), compared to the control 10% sucrose. However, the old cohorts which were released after 15 days of adult holding resulted in a significantly greater fraction of males reaching the top ($t(7)= -4.1$, $p = 0.003$) than the 10% sucrose fed males released at three days old. The overall fraction of males reaching the top cage in this set of experiments was lower than was achieved than in the previous development process (>80% compared to <50%). It is unclear why this should be the case, however it could be as a result of the rearing system

used to produce the pupae: in the development process, pupae were reared in trays at 3 larvae mL^{-1} and fed a standard diet of Tetramin. To produce the pupae in this experiment, the mosquitoes were reared in an aquaculture system and fed a proprietary diet. It is possible that the overall quality of these mosquitoes was inferior to the quality produced for the previous assays. If the quality of the larval diet is affecting the mosquitoes, for example by the accumulation of teneral reserves during larval development, the resulting adults could be less fit, and this may be being highlighted in this assay. Inferior teneral reserves may also point towards why the older cohorts performed better than the younger cohorts as they were able to accumulate more substantial energy reserves which are required for flight from adult diet acquisition in the holding time before their release (Villiard & Gaugler, 2015).

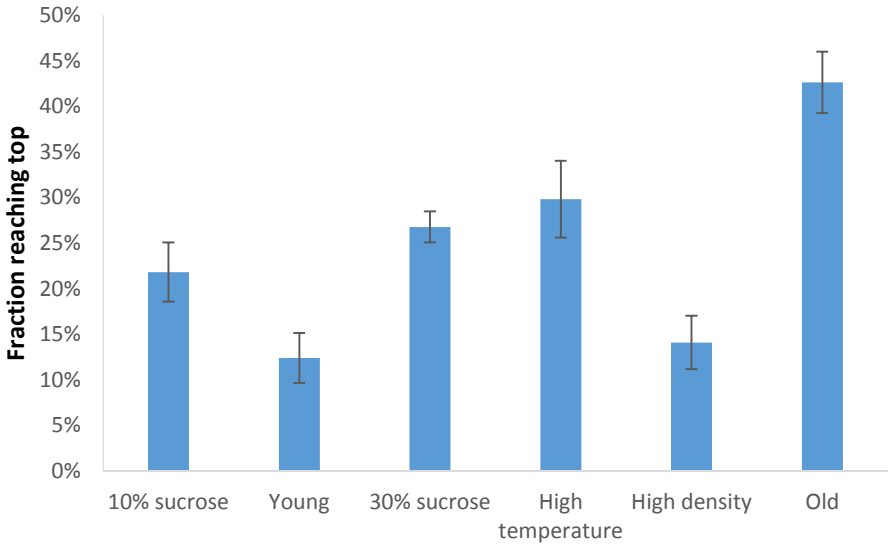


Figure 6-5 Fraction of mosquitoes with perceived differences in fitness reaching the top of a 1.6 m tube

OX513A males were counted into nine cohorts of 100 for each treatment. All cohorts were provided with 10% sucrose unless otherwise stated. Males were released into the flight tubes and the number reaching the top cage after 24 hours were counted.

As a result of the low numbers reaching the top of the tubes in this experimental run, which could be masking the potential for fitness differentiation, a follow on experiment was designed to determine if shorter tubes were more suited to the differentiation of mass reared mosquitoes. In this experiment, the lengths of tube that were cut from the original length of tube to reduce the size from 2m to 1.6m were used. These 40 cm tubes were coated in Fluon to prevent the mosquitoes from resting or walking up the sides, and were

prepared with funnels to feed the mosquitoes into the tube at the bottom, and to feed the mosquitoes into the holding container at the top. The same test cohorts were produced from the propriety rearing system, excluding cohorts held at high temperature, as these facilities were not available.

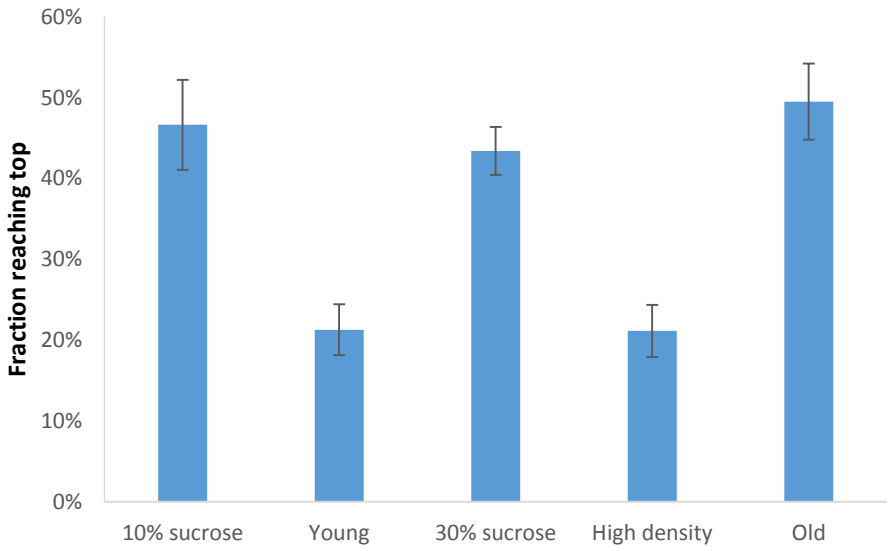


Figure 6-6 Fraction of mosquitoes with perceived differences in fitness reaching the top of a 40 cm tube.

OX513A males were counted into nine cohorts of 100 for each treatment. All cohorts were provided with 10% sucrose unless otherwise stated. Males were released into the flight tubes and the number reaching the top cage after 24 hours were counted.

The results of these experiments show that there was no significant difference in the fraction of mosquitoes making it to the top cage of the cohorts fed 30% sucrose ($t(12)= 0.5$, $p = 0.62$) or old mosquitoes ($t(15)= -0.40$, $p = 0.70$). However, significantly fewer mosquitoes reached the top in the high density cohorts ($t(13)= 4.0$, $p = 0.002$) and in the young cohorts ($t(13)= 4.0$, $p = 0.002$) compared to the 10% sucrose control cohorts. These results are different to those in the previous experiment, which indicated there was no significant difference between the cohorts except the older test subjects which flew better. In this second experiment, a greater differentiation has been observed with significant differences found between the different treatments. Further work will be required to determine why differences have been seen in this experiment but not in the previous experiment, however the shorter tubes have resulted in a greater fraction of the males reaching the top cage, and this may allow a better differentiation in quality to be detected.

The differentiation in cohorts seen here is not unexpected; it is possible that young cohorts are either not ready to fly up the tubes and still physically maturing, or they are unable to fly up the tube as they lack sufficient energy reserves as they have not had sufficient time to sugar feed. This result indicates that releasing males at 1 day old would not be beneficial to increasing field fitness and release age should be maintained at three days old. Feeding mosquito batches 10% sucrose or 30% sucrose does not appear to impact their ability to fly, although longevity experiments have shown that there is a benefit to feeding 30% sucrose by increasing their longevity. Storing adults at high density also appears to reduce the male's ability to fly which could be as a result of physical damage to the wings of individuals impacting their flight ability. Finally in this experiment, storing the cohorts for longer did not significantly increase their flight ability. This is contrary to what was seen in the first experiment, and could either indicate differences in the insect quality between experiments, or that the first result was a false positive result and that storing cohorts for 15 days does not positively impact their flight ability. Further work and experimentation with repeat batches of mosquitoes will need to be performed to determine if the differences seen in these experiments are in fact true differences or an uncontrolled effect in the experimental design.

6.4. Conclusions

Insect fitness is an important component to consider in a self-limiting strain suppression programme, and increasing the released insect's fitness should result in an increased rate of suppression. Little is understood about *Aedes aegypti* male fitness and therefore considerable research is required to understand different fitness components and develop methods to enhance these factors. To enable this work to progress, assays are required to be able to measure and differentiate field relevant components of fitness in the laboratory before applying them to the field and being able to measure the result. Here, two assays have been developed, a release longevity which is an applied field release relevant assay and flight tubes. The release longevity assay has been developed to be resource low and to provide an output in a short duration of time. The results presented here have shown it to be able to consistently and accurately differentiate between fitness effects from adult diet, and given preliminary data (not presented here) is likely to be able to differentiate adult quality from factors arising from larval rearing diet and adult diet holding conditions. This simple easy to use assay has now been transferred to Oxitec's field rearing facilities to be

used for fitness improvement experiments and for batch quality control purposes to monitor for changes as a result of rearing issues.

The second assay developed here are a series of flight tubes which have been designed to differentiate between a cohort's ability to fly up a tube to a holding container at the top. Flight is considered an important factor of male fitness as it is vital to their ability to find females and therefore distinguishing fitness by their ability to fly is a valuable tool to differentiate insect quality. Given little is known about male biology, a considerable proportion of the development process was to test attractants to encourage the males to fly up the tubes within the time limit. The assay was calibrated and successfully used to test batches of mosquitoes with perceived differences in quality, and indeed differences in quality were identified. A potential limitation of this assay to date is the variability in the total numbers reaching the top of the tubes, and this will require further work to establish if there is an issue with the setup of the experiment, or the differences seen are as a result of the differences in insect quality. Assuming the consistency and repeatability of this assay can be improved, this test will provide another tool to measure fitness differences in the laboratory.

Throughout the development of the two fitness assays, an understanding of adult diet has been developed to optimise the diet of released males in the holding period before being transported to the field. This work has indicated that by increasing the sucrose concentration from 10% to 30%, the males will live on average a day longer which equates to approximately a 10% increase in lifespan. It is unclear exactly how this increased longevity in the laboratory will transfer to field longevity of males as it is unlikely to directly translate to a day longer survival in the field, however the increased survival time will provide the males with additional time to search for and mate with females before their energy reserves fail and they either die or have to change behaviour and search for food sources. This knowledge has been transferred to the field and is being implemented in field releases. The change in longevity of the released males is yet to be measured due to resource constraints but it is hoped to ultimately have a positive impact on male fitness and to reduce the number of males required to achieve suppression. In addition to modifying the male's diet, work performed here has also identified the benefits of Nipagen in the female diet, showing that its addition to the sugar water is able to increase the median female longevity by approximately 28%. Based on these results, Nipagen has been included

in the sugar water provisioned to egg production cages, and since its introduction, the number of eggs collected per cage has increased by >20%. This effect has been seen in the UK and Brazilian egg production facilities and makes a positive impact on reducing the cost of egg production by gaining more eggs per female in the cage as a result of the increased female longevity.

The work performed here has developed at least one assay which are able to effectively differentiate insect quality in a short duration of time, and with low resource input. A second assay has been prototyped and initial testing performed however this has highlighted that additional testing is required to finalise its suitability at differentiating insect quality. Further to this, significant improvements have been made to female and male longevities by modifying the adult diet, increasing their fitness and consequently increasing their effectiveness resulting in a decrease in the cost of a suppression programme.

Chapter 7 Summary and General Discussion

7.1. Introduction

In light of the significant increase in the incidence of dengue fever over the last decade (Bhatt *et al.*, 2013), and the lack of effective tools to control the principle vector *Ae. aegypti*, novel tools are required to control the mosquito to limit the spread of the disease. Self-limiting mosquitoes, which are a genetically controlled version of SIT, have been used to successfully supress wild populations of *Ae. aegypti* by >90% in three different countries (Harris *et al.*, 2012; Carvalho *et al.*, 2015; Gorman *et al.*, 2015). The work performed here helps to further our understanding of how these self-limiting strains perform under different controlled environments, and to improve the insect fitness and consequently the cost effectiveness, to pave the way to make this technology a viable option for use in vector control programmes.

7.2. Summary of results

The research presented in this thesis is split into two sections; Section 1 looks at self-limiting strain characterisation measured in terms of phenotype, and Section 2 addresses the fitness of self-limiting strains and how this is likely to affect field performance. Beginning with Section 1, Chapter 2 focused on characterising the bi-sex lethal strain OX513A and demonstrated that the penetrance of the phenotype can be detrimentally impacted by the strain background, as well as by some larval rearing conditions. Chapter 3 reported the selection and characterisation of a novel male-selecting self-limiting strain resulting in incapacitated females, however this strain turned out to be sex-linked and therefore may not be desirable to use for open field release. From the second section of the thesis, Chapter 4 focuses on the optimum tetracycline analogue to use to rear self-limiting strains and determines that doxycycline is most effective at supressing the effects of the transgene and is likely to be most efficient at maintaining strain fitness and preventing intra-colony selection. Chapter 5 investigated the effects of larval rearing conditions on adult mating competiveness and found compromising effects from larval rearing conditions and adult holding conditions, but most notably that the male body size appears to have a significant effect on mating success depending on the size of the female the male is competing for. Finally Chapter 6 described the development of a novel applied and field relevant fitness

assay that is able to differentiate male fitness, as well as modify the male adult diet to increase their longevity by approximately 20%.

7.3. Relevance of this work for self-limiting programmes

The self-limiting strain OX513A has been used in six trial sites to supress wild *Ae. aegypti* populations, and in each location >90% suppression has been achieved (Harris *et al.*, 2012; Carvalho *et al.*, 2015; Gorman *et al.*, 2015). If or when this strain is to be used in larger field operations, a better understanding of how it will perform is critical to ensuring its success at suppressing wild populations quickly and effectively. The work performed in Chapter 2 represents the first comprehensive study of OX513A's phenotype under different larval rearing conditions and its introgression into different strain backgrounds. This in particular has highlighted the variability of strain phenotype when crossed to different genetic backgrounds, resulting in breaches of the regulatory threshold of 5% maximum functional adults when reared in the absence of tetracycline. From modelling performed in Phuc et *al*. (2007) the functional adult fraction can reach 10% before there is likely to be an impact on wild population suppression, so from an efficacy perspective this result should not pose an issue to field efficacy, however Oxitec has stated that ≤5% functional adults will eclose and therefore this could represent a problem to the strain passing through the regulatory process in new countries. Before OX513A is released in a novel country, penetrance of phenotype and mating competitiveness are performed in the laboratory to ensure they are compatible with the locally collected wild type strain and consequently the more countries which test the OX513A strain, the greater our understanding will be of the extent of the genetic background issue. Further to this, the actual field penetrance of OX513A could be determined by PCR testing females collected in BG traps from suppression sites to determine if they are heterozygous for the OX513A transgene. This fraction can be used in conjunction with the estimated population size (which can also be estimated from BG collections) to determine the penetrance of phenotype in the heterozygous progeny developing in the field, which could be considered more relevant to the efficacy of a programme than laboratory collected data. If the effect of genetic background on penetrance of phenotype does prove to be an issue, OX513A could be replaced with a fully penetrant self-limiting strain, which is less likely to suffer from variation in phenotype as a result of background.

A fully penetrant male-selecting strain OX4358B was selected and characterised in Chapter 3. This strain has the advantage that the release generation can be reared in the absence of tetracycline to result in incapacitated females, removing the costly process of sorting the male and female pupae, making the self-limiting technology cheaper. However, further characterisation showed that this strain was sex-linked and modelling performed here shows that from the point that open releases cease, the transgene could persist in the environment for >20 generations. It is unlikely that this strain will manage to pass through regulatory approval as there may be a concern over the persistence of the transgene in the environment, and consequently a new male-selecting strain should be generated. Since the time of the design of the OX4358 construct in 2010, novel promoters have been isolated in the *Ae. aegypti* genome (Salvemini *et al.*, 2013; Hall *et al.*, 2015) which could be used to produce a preferential early acting male-selecting phenotype compared to the late-acting promoter Actin4 in OX4358. If a promoter is used to produce an early male-selecting effect, the cost savings of not having to sort the pupae are still retained, while also being able to theoretically double the male outputs from a rearing tray by the absence of developing female larvae, which can be replaced with male larvae. This will result in a significant decrease in the cost of production and consequently the cost of a suppression programme.

The self-limiting system requires the addition of tetracycline to the larval rearing medium to supresses the transgene to allow the development of individuals to functional adulthood. The work performed here has shown that the use of doxycycline is more effective at supressing the transgene than that of the standard tetracycline analogue, chlortetracycline. Furthermore monitoring the changes in allele frequency within populations over successive generations (Chapter 4) has shown that doxycycline is more effective at maintaining homozygote fitness. This is important in an egg mass production colony where there is the potential for selection within the colony of individuals with differential fitness, as well as the risk of contaminating wild type alleles spreading through the colony if the selection pressure was in their favour. As a result of the data collected here, OX513A egg mass production is now using doxycycline instead of chlortetracycline. Data presented here also indicated that OX513A females reared in doxycycline had a shorter median longevity than their chlortetracycline reared counterparts, however, as a result of the switch in analogues in mass production, a data bank has been collected which indicates that there is not a negative impact on egg collection, which is ultimately the driving factor in egg mass production. An additional benefit of the increased efficacy of doxycycline, is that the concentration required

is 30 times less than that of chlortetracycline and therefore is more cost effective particularly when rearing is scaled up (personal communication N. Naish). This has the advantage that less tetracycline analogue will be entering the wastewater system at the end of the rearing process. Analysis reported in Curtis et *al*. (2015) showed that 1.4 µg mL^{-1} of chlortetracycline was left at the end of rearing when rearing at 30 µg mL^{-1} chlortetracycline. Given that this waste concentration is higher than the starting concentration of doxycycline (1 µg mL^{-1}), less tetracycline analogue will be entering the sewage system. Oxitec has recently come under criticism from NGO's for the use of tetracycline in the rearing system and therefore the less that can be used, the better the public perception.

The fitness of self-limiting males destined for release is of increasing concern when trying to scale up control operations. Numerous SIT programmes have suffered with poor insect fitness (Dame & Woodward, 1964; Baker *et al.*, 1980; Reisen *et al.*, 1981) resulting in the requirement to release more individuals to increasingly over-flood the wild population to allow the sterile males to compete for wild female mates. This is equally of a concern with self-limiting males where release ratios are currently high (>150:1) but are required to decrease to make the technology viable on a larger scale. Understanding male fitness is critical to this process so that the males can be reared and nurtured in conditions that will optimise their attractiveness and therefore fitness to the wild female mates. Work performed here has identified factors which negatively impact the mating competiveness of self-limiting males, and this has been detected within a laboratory assay. The conditions encountered in the field are known to be much harsher and it is generally assumed that an effect seen in the laboratory is likely to be exacerbated in the field, with differences becoming more evident. Released male body size is of particular concern with results here highlighting that the large male OX513A maybe at a disadvantage when competing with wild (equivalent of small bodied) females. The large male body size of OX513A is a result of requirements to rear larvae with sufficient food to produce males and females with a distinct separation in body size. To produce smaller bodied males, the larval diet per mouth would have to be decreased which in turn will result in a reduced efficiency of the sex sorting of the pupae as the differentiation of cephalothorax size of the males and females increasingly overlaps, as well as decreasing the rate of pupation. This will considerably increase the cost of rearing and is therefore not feasible to pursue. As the scale of mass production increases from laboratories to factories, the system of larval rearing is also changing from trays to a scalable proprietary rearing system. As part of this rearing system

it has been found that the current larval diet, Tetramin, is unsuitable for use due to its lack of solubility and therefore an adequate soluble larval diet has been produced. Fitness assessments of individuals arising from this soluble diet (presented in Chapter 6) have indicated that the adult quality is inferior to that of Tetramin reared individuals and therefore there is capacity to further optimise and develop this diet. This may provide an opportunity to design the diet with adult body size in mind. A study with *Anopheles arabiensis* has reported that modifying the nitrogen content of larval diets resulted in adults with differing body sizes (Hood-Nowotny *et al.*, 2012) and therefore it could be possible to modify the body size of OX513A by controlling the nutrition of the larval diet, while providing the essential nutrients for the individuals to develop at the desired rate and pupate at the optimum time. Diet modulation has also been shown in the mass production of various insects to improve insect quality and fitness (Chen *et al.*, 2014; Collins *et al.*, 2014; Augustinos *et al.*, 2015) so this route of work should be pursued to improve the quality of self-limiting insects.

Work described in Chapter 6 showed that the median male longevity can be increased by approximately 20% by increasing the sucrose concentration, and that egg production has been increased by >20% by the addition of the anti-fungal Nipagen, being added to the adult diet. Both of these show that simple optimisations can be made to existing processes to increase the fitness of the mosquitoes. The next step for the male diet improvement will be to transfer the optimisation to field release operations and to measure the field longevity of the 30% sucrose fed males compared to the 10% sucrose fed males. An outstanding concern with the male diet is the method by which the diet is provisioned to the males: cotton wool is soaked in the sucrose solution, squeezed out and placed on top of the release pot. It is highly likely that this is resulting in a mixed quality of diet access and availability, and as a result producing variability in the quality of males between pots. Further work should be performed to ensure that all the males are able to access a high quality sugar meal before their release to ensure that the gains made here with the increased longevity are realised in the field. Furthermore, additional benefits could be gained by adding an attractant to the sugar water so that the males are encouraged to take a sugar meal before being released to ensure that a high proportion of the males at the point of release are fully sugar fed. Given the lack of known male attractants, further investigations will be required to determine suitable chemicals, with work potentially building on the additives used in the sugar meals of Chapter 6. If further research is performed into the composition of preferred male *Ae.*

aegypti diet, further gains could be had by increasing longevity, as well as identifying male-specific attractants which would be used to increase the imbibing frequency of the sucrose meal in the release pots in preparation for release.

An overarching problem in *Ae. aegypti* self-limiting control is the lack of knowledge of male mating biology. Progress has been made here to begin to unwind what could be important to male mating success, however there is still a vast sea of questions yet to be answered: it is still unclear how males locate females in the field, whether pheromones are involved in mate location or selection, at what time mating occurs, how or if the female chooses her mate or if mate success if based purely on inter-male competition. By addressing these questions, advances and optimisations can be made in the operational side of a self-limiting programme; releases can be performed at the correct time of day with the correct distribution pattern, males can be treated with pheromones pre-release to gain advantages over wild males and testing can be performed to ensure OX513A males have maintained the vital biology cues since colonisation, for example female detection, diet location and predator evasion. By understanding the *Ae. aegypti* mating biology, programmes will be able to tailor the operations to effectively increase the fitness of the released males and therefore achieve suppression with fewer males. It may also be possible to overcome potential routes of assortative mating which may become apparent unless further understanding is gained. Together this knowledge will increase the programme efficacy and therefore decrease the cost of this technology, making it available to a wider range of vector control budgets.

7.4. Summary of recommendations for future work

From the work performed here there are a number of routes of further investigation to follow, some of which have been summarised here, however there are also many outstanding behavioural and biological questions to answer which have the potential to increase the efficacy of a self-limiting programme. Some suggestions are summarised in Table 7-1.

Table 7-1 Summary of suggestions for future work

Area of work	Rationale
Strain selection	
Effect of strain background on phenotype of OX513A	A better understanding is required of the effect of strain background to prepare for a switch to an alternative self-limiting strain if required.
Development of early acting male selecting strain	Generation of this strain will significantly decrease the cost of production.
Mass production	
Diet formulation	Optimising the larval diet could increase the fitness of the released males and could allow for manipulation of body size.
Insect quality and scale up	As the insect rearing process increases to a factory scale and operations become increasingly automated, the quality of the insects being produced need to be considered, rather than just production outputs.
Adult storage conditions	Adults are currently stored at high density which may impact this quality at the point of release. Modification to holding density and environment (humidity, temperature) could maintain better adult quality during the male maturation process.
Field release	
Age of release	Males are currently held for three days to sexually mature before being released. This time could be optimised to reduce the holding space required for pots, and also reducing the time males are at risk of high density damage.
Method of release	Males are released from pots in cohorts of 1000. This process needs scaling up to become viable for large scale suppression programmes, while maintaining adult fitness and delivering males effectively into the field.

Location of release	Understanding how males find females will help to dictate if point releases or blanket releases are more effective, or even hot spot releases to target high density areas.
Time of day of release	Understanding when males mate will allow males to be released ready for the female mating period, or be released at times which increases their chances of finding safe resting places.
Biology	
Male body size	Understanding the importance of male body size could help to limit the risk of assortative mating.
Mate attractants	Determining what attracts a male and what makes a male attractive to a female has the potential to be applied to self-limiting males to provide an advantage to released males.
Male field survival	Ensuring released males have maintained the field relevant cues to allow for their survival, or selecting for these cues in the laboratory, e.g. energy reserve restoration, predator evasion, etc., could increase the survival of males in the field.

7.5. Conclusions

This thesis has developed our knowledge and understanding of self-limiting strains both in terms of phenotype and in terms of insect fitness. Although some progress has been made in understanding components which impact the fitness of these mass produced strains, there is still a vast gap in knowledge which needs to be addressed before this self-limiting technology can really become an effective vector control tool. Although this seems a vast task, there is increasingly more interest in male mosquito biology and with ever more intricate methods to measure and detect insect biology both in the lab and in the field, and therefore the probability of success in this area should be considered attainable.

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Chapter 9 Appendices

Table 9-1 Feeding regime of *Ae. aegypti* larvae using Tetramin© fish flakes (Tetra GmbH, Germany)

Day	Number of Larvae						
	100	**200**	**300**	**500**	**1000**	**2000**	**3000**
1 (aliquot)	0.006	0.012	0.018	0.03	0.06	0.12	0.18
2	-	-	-	-	-	-	-
3	0.008	0.016	0.024	0.04	0.08	0.16	0.24
4	0.016	0.032	0.048	0.08	0.16	0.32	0.48
5	0.032	0.064	0.096	0.16	0.32	0.64	0.96
6	0.032	0.064	0.096	0.16	0.32	0.64	0.96
7	0.032	0.064	0.096	0.16	0.32	0.64	0.96

9.1. Mutation of the OX4358B Insertion

Studies were being performed in each of three genetic backgrounds, (Asian, Latin and DMEB) to examine the survival of individuals from L1 to adult (rearing data not presented). Five cohorts of 200 larvae of each strain were reared in the absence of tetracycline. Pupae were picked and sexed, the males were discarded and the females were caged and allowed to eclose. Upon assessment of the adults, it was discovered that two females were able to fly, one from the Latin genetic background and one from the DMEB genetic background. This represents a flying female fraction of 0.01 % in the Latin and DMEB background (Table 9-2).

Table 9-2 Phenotype of OX4358B heterozygotes introgressed into three different genetic backgrounds.

Larvae reared in the absence of tetracycline. N = 1000 L1 larvae for each background.

Strain	Female pupae	Flying females	Fraction of flying from L1 %
OX4358B Asian	470	0	0.00
OX4358B Latin	364	1	0.10
OX4358B DMEB	405	1	0.10
Total	1239	2	0.07

The two flying females from this experiment were passed to Dr Scaife for molecular analysis. Dr Scaife determined that the two individuals were positive for the OX4358 transgene and therefore eliminated the possibility of the phenotype being a result of WT contamination; instead these two individuals represented a loss of phenotype within the population.

As consequence of this identification, a larger experiment was prepared to determine the incidence of the loss of phenotype from a larger population sample. OX4358B Latin heterozygotes were used, being the primary genetic background, and out of a total larval number of 3000 individuals reared in the absence of tetracycline, four flying females were detected. This represents a flying female fraction of 0.1%.

The four flying females were outcrossed to WT males and eggs were collected. The progeny were reared in the absence of tetracycline to determine if the flying phenotype could be inherited or if it was a form of resistance to the effects of the transgene. The pupae were screened and sexed and housed separating males and females, and WT and fluorescent individuals. The results displayed in Figure 9-1 show that the inherited phenotype of the OX4358B progeny resulted in over 90% of the females flying from each of the parental crosses. This fraction is comparable to that of the WT females.

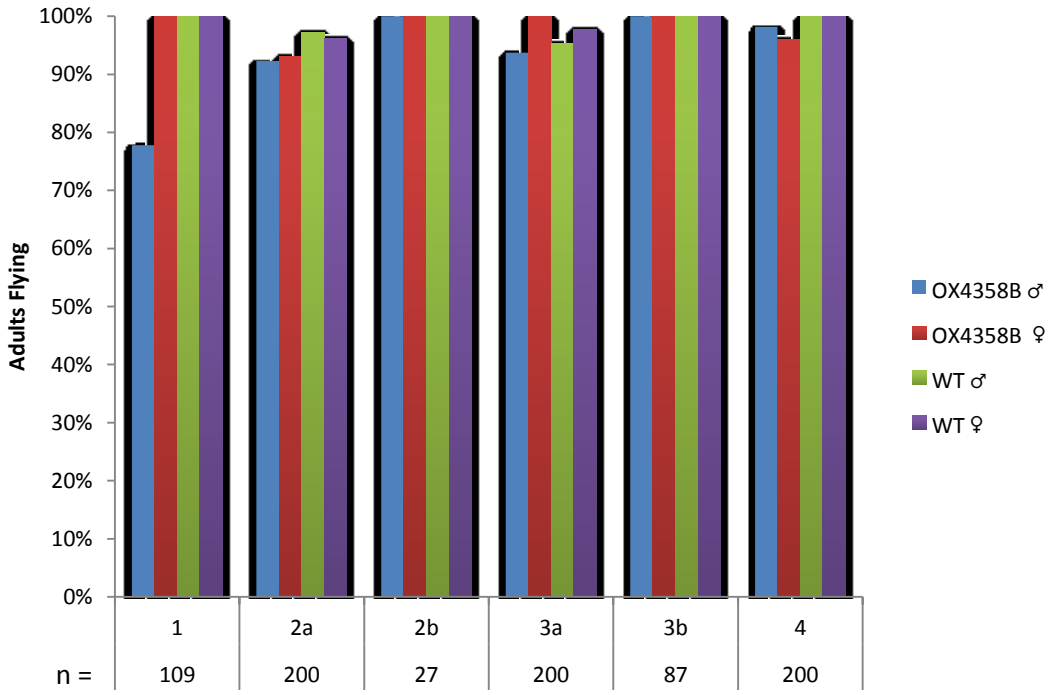

Figure 9-1 OX4358B Latin off tetracycline reared flying females mated to WT.
Here, the progeny were reared in the absence of tetracycline and assessed for phenotype. The numbers represent females 1 to 4, the letters are rearing cohorts (repeats). N (first instar larvae) in each cohort is also shown.

Given the strength of the inheritance of the loss of phenotype, it was thought that it was most likely to be caused by a change in the function of the transgene as opposed to resistance in the genetic background, which is likely to appear as a gradual increase in the fraction of flying females over multiple generations. OX4358B flying female individuals from this experiment were passed to Dr Scaife for further molecular analysis. After sequencing the transgene it was determined that there was a SINE (short interspersed elements) insertion in the VP16 component of tTAV2, preventing the production of functional tTAV2 protein. Consequently, this protein cannot bind to tetO and drive the expression of the toxic VP16 protein (Figure 3-1), to produce the female flightless phenotype. From here on this mutated OX4358B allele will be referred to as OX4358-SINE.

If this mutation had occurred in a mass production colony and males were being released as part of a suppression programme, it is important to confirm that the OX4358B-SINE allele is not dominant over the other non-mutated OX4358B allele. As the product of the SINE insertion is a disrupted protein, the hypothesis is that females carrying an OX4358-SINE allele and a canonical OX4358 allele should be flightless. OX4358B-SINE males were crossed to homozygous OX4358B females without the SINE insertion and the phenotype of their progeny was assessed. The results showed that of the 800 starting L1 larvae, zero flying females were identified. This result demonstrates that the canonical OX4358B allele is dominant over the OX4358B-SINE allele as expected as only a single allele is required to produce a flightless phenotype.

The discovery of the loss of phenotype of OX4358B was found after approximately 20 generations since the initial transformation event. The mutation was caused by SINE insertion (derived from cellular RNAs that repeatedly retropose via RNA intermediates and integrate almost randomly back into the genome (Schmitz, 2012; Ichiyanagi, 2013). Once they have placed themselves in the genome, they will be passed from generation to generation. Consequently the loss of phenotype of OX4358B is a statistical probability and is not as a result of a failing in the design or resilience of the transgene. The challenge with these very low probability events is to identify them before they become widespread in the

population. This relies on the regular screening of the population from a representative sample of the total colony. If found, the removal of the mutation from the population could be time consuming and limit field operations but the principal of filter colonies could be employed to restart the mass rearing colony.