The Genetic Control of *Aedes aegypti*

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Abstract

In the last century, we have observed the introduction, establishment and expansion of mosquito-borne diseases into diverse new geographic ranges. The utility of genetically engineered mosquitoes as tools to decrease the burden of disease by controlling disease-transmitting vectors is being evaluated. The work in this thesis contributes to this goal by exploring mechanisms to spread (or 'drive') anti-pathogenic traits (*i.e.* disease refractoriness) into target populations through the use of an engineered gene drive system in *Aedes aegypti*, and by developing additional tools for the safe, reliable, and targeted transformation of these mosquitoes for field release using a novel site-specific cassette exchange mechanism.

The proposed gene drive system is underdominance-like as it relies on the inheritance of a pair of *trans*-suppressing lethal constructs, and uses a novel design to help tackle the 'linkage problem', which is the potential dissociation of the drive system and its 'cargo' anti-pathogenic gene(s). One component of this proposed gene drive system is a lethal or fitness-reducing gene. A range of effector proteins with different biochemical modes of action was screened for their suitability in this system. Effectors that looked promising in this initial screen were evaluated further for their phenotypes when expressed under the control of selected blood-meal inducible promoters. One combination gave the interesting and novel phenotype of temporary blood-meal-induced paralysis. Partial suppression of effector expression was achieved by co-expressing a hairpin RNA for RNA interference, however it proved difficult to combine adequate fitness penalty and rescue to the degree required for a field-usable system.

The cassette exchange system combines the ΦC31-*att* integration system, and Cre or FLP-mediated excision to remove extraneous sequences introduced as part of the site-specific integration process. This provides a useful new tool for genome manipulation. Complete cassette exchange was achieved and the absence of any obvious fitness costs or positional effects in two docking strains make these lines good candidates for both research and generation of new transgenic strains for genetic control of *Ae. aegypti.*

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For Pamela Greenwell and Scott Lawton.

For enriching my beginnings as I grew ‘curiouser and curiouser’.

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Abbreviations

Ae. aegypti	*Aedes aegypti*
Ae. albopictus	*Aedes albopictus*
An. gambiae	*Anopheles gambiae*
AWT	*Ae. aegypti* wild-type (Asian strain)
BLAST	Basic local alignment search tool
bp	Base pairs
Carb	*Ae. aegypti's carboxypeptidase A* promoter
D. melanogaster	*Drosophila melanogaster*
DEN2	The target sequence for the dengue serotype 2 virus' pre-membrane transcript
DENV2	Dengue serotype 2 virus
df	Degrees of freedom
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FLP	Flippase
HEG	Homing endonuclease gene
Hex	*Aedes altropalpus hexamerin 1.2* promoter
hrs	Hours
ILP	Insulin-like protein 2
ITR	Inverted terminal repeat
IVM	Integrated Vector Management
L	Litre
M	Molar
Medea	Maternal-effect-dominant embryonic arrest
min	Minutes
mRNA	Messenger ribonucleic acid
NIPP1	Nuclear inhibitor of protein phosphatase 1
NLS-C	Nuclear localisation signal to the C-terminus
NTG	Non-transgenic
Off tet	Reared in the absence of tetracycline
On tet	Reared in the presence of tetracycline

pB	*piggyBac*-based transposon
PCR	Polymerase chain reaction
RIDL	Release of insects carrying a dominant lethal
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rotations per minute
sec	Seconds
SIT	Sterile insect technique
TAE	Buffer solution containing a mixture of Tris base, acetic acid and EDTA
Tet	Tetracycline
tetO	Response element for the tetracycline-repressible *trans*-activator protein
TG	Transgenic
tTAV	Tetracycline-repressible trans-activator
Tris	Tris(hydroxymethyl)aminomethane
vit	*Ae. aegypti's vitellogenin 1* promoter
WHO	World Health Organization
*w*Mel	*Wolbachia* Mel

Chapter 1

Introduction

1.1 Mosquitoes as vectors of disease

Collectively, the infective bite of a mosquito is responsible for more human morbidity and mortality as well as economic losses than any other animal, with millions dying annually from diseases transmitted. Mosquitoes transmit the aetiological agents of parasitic diseases such as malaria or filariasis, and viral diseases such as dengue fever, chikungunya, and yellow fever.

Adult mosquitoes feed on nectar and plant juices, and in addition females of the anoautogenous species obtain essential nutrients for the development of their eggs from a blood meal. To facilitate blood feeding, the female injects saliva rich in anti-coagulant and anti-inflammatory proteins locally (Adelman *et al.*, 2008); she also ingests blood that may be infected by viral or parasitic pathogens. These pathogens cross the mosquito's midgut, sometimes after a developmental stage, and disseminate throughout the insect's body, Figure 1.1. Eventually these reach tissues essential for the pathogen's transmission, such as the salivary glands for viral and malaria-causing *Plasmodium* species,

resulting in the secretion of infective saliva into the host during the female's next blood meal.

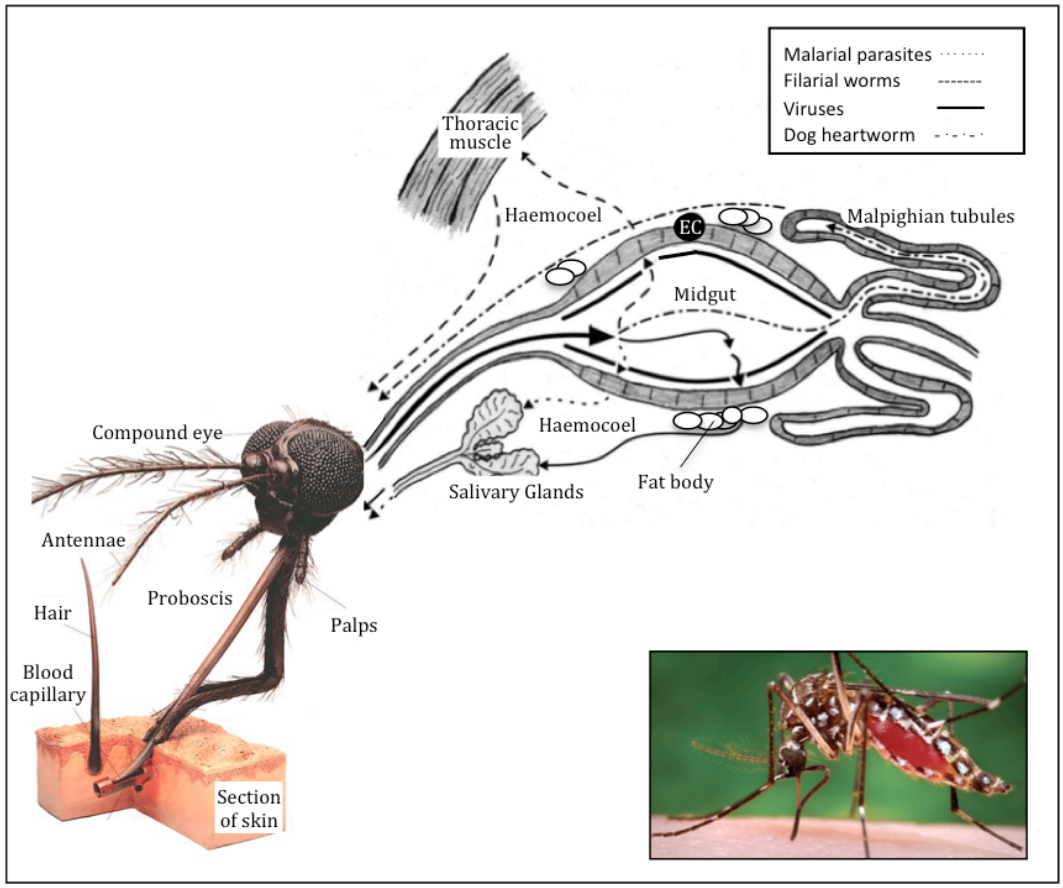

Figure 1.1 Developmental sites and dissemination routes of viral and parasitic pathogens in the mosquito. Following a blood meal, all pathogens enter the midgut. Viruses replicate in the midgut epithelial cells (EC), and malaria parasites develop in the midgut; both travel via the haemocoel to the salivary glands where they reside until injected into the host. Filarial worms migrate to and develop in the thoracic musculature and dog heartworm travels through the midgut lumen of the Malpighian tubules, and develop in the distal cells of the tubules. These worms migrate through the open circulatory system to the mosquito's head, where they emerge and are deposited on the surface of the host's skin, entering the wound made by the mosquito proboscis' piercing shafts. Figures adapted from Beerntsen *et al.* (2000) and http://www.cleanolservices.com.pk/mosquito_head_en.jpg. Inset photo source: http://www.mosquitoage .org/Portals/58/Pictures/ mOZZ.jpg.

The aim of any transmission control strategy is to disrupt this chain of infection by reducing closely interlinked factors: the pathogen, the host's susceptibility, and the vector.

Where available, drugs and vaccines have been widely used to eliminate the pathogen and reduce the host's susceptibility, or to reduce symptoms. These are often too expensive for the medical systems of resource-poor countries, whilst pathogen resistance to anti-pathogenic drugs has evolved in areas of extensive use (*e.g.* McGraw and O'Neill, 2013).

Vectors provide an attractive target to which control methods can be directed. Mosquitoes have four distinct life cycle stages, Figure 1.2, the adult, egg, larva and pupa.

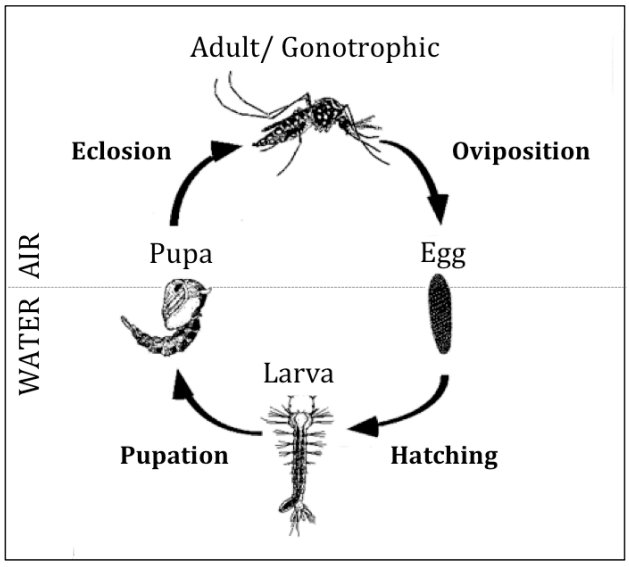

Figure 1.2 Mosquito life cycle; adapted from Hopp and Foley (2001). Submerged eggs hatch into larvae within three days, though eggs are resistant to desiccation and can survive up to a year without water. Larvae develop through four instars and pupate. Adults eclose 48 hrs later; males generally eclose 1-2 days before females. Males are sexually mature 15-24 hrs after eclosion and are sexually polygamous; females are sexually receptive 48-72 hrs after eclosion and are generally monogamous. In tropical regions adults can live up to several weeks, in temperate regions often longer, and in overwintering species female adults may survive for up to a year. Females lay eggs regularly every 2-3 days (after a blood meal). In tropical regions, the complete life cycle from egg to adult can take as little as 6-9 days.

Each mosquito species has its own niche and behaviour that also varies between its immature and adult stages, Table 1.1, and the success of each vector-based control method depends on the ecology of the target species.

Table 1.1 Important mosquito vectors of human diseases

Species	Larval habitat	Blood-feeding preference	Diseases transmitted
Anopheles gambiae sensu stricto	Clean shifting shallow freshwater: sunlight pools, hoof prints, burrow pits, rice fields	Indoor night-biter: the most anthropophagic species	Malaria, lymphatic filariasis
Anopheles stephensi	Urban: fresh, brackish or even polluted waters	Indoor or outdoor evening/night-biter: anthropophilic	Malaria
Aedes aegypti	Urban: artificial containers and tree hole	Indoor day-biter: anthropophilic	Dengue, chikungunya, yellow fever
Aedes albopictus	Sylvatic by nature: forest edges, tree holes Adaptable to urban/ rural areas: artificial containers	Outdoor day-time biter: anthropophilic and zoophilic (mammals)	Dengue, chikungunya, West Nile
Culex quinquefasciatus	Urban waters polluted with organic debris: cesspits, pit latrines, blocked drains, ditches	Indoor or outdoor night-biters: anthropophilic and zoophilic (humans, birds and cattle)	Lymphatic filariasis, West Nile, Rift Valley fever

This thesis investigates the use of transgenic methods to improve the control of the most potent arboviral (arthropod-borne viral) mosquito vector, *Aedes aegypti.*

1.2 *Aedes aegypti* and disease transmission

It is thought that *Ae. aegypti* originated in Africa, although its role as a vector for human transmission is considered to be relatively recent (Mousson *et al.*, 2005).

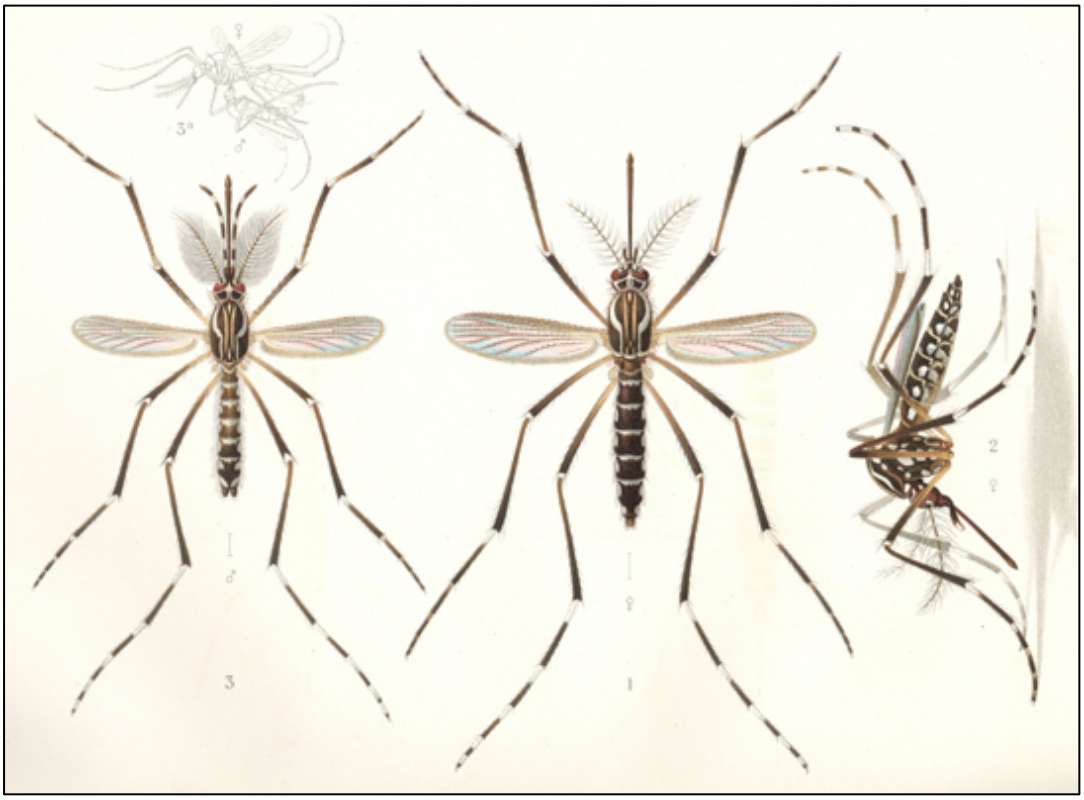

Figure 1.3 Colour print of *Ae. aegypti.* The male, left, is generally smaller than the female, middle and to the right. Above left is a flying pair in copula. By Goeldi, E.A. (1905). Source: http://www.biogents.com/html/img/pool/Goeldi _Aedes_aegypti_543 .jpg.

Ae. aegypti-transmitted dengue and yellow fever viruses were primarily involved in sylvatic, or 'jungle' transmission cycles; forest-dwelling mosquitoes transmitted them between non-human primates and sporadic epidemics occurred when susceptible human hosts were available (Holmes and

Twiddy, 2003). Eventually, widespread urbanisation and the development of modern transportation methods, coupled with rapid growth in human population sizes, led to regular accessibility of the mosquito vector to the human host, resulting in sustained disease transmission (Holmes and Twiddy, 2003).

Today, the pantropical form of *Ae. aegypti* is closely associated with human habitation, and typically lay their eggs in water-filled, manmade containers, such as discarded tyres. In addition 75-95% of the adults rest indoors with females taking a blood meal from occupants during the early morning or late afternoon (Pant and Yasuno, 1970).

The egg's ability to resist low temperatures and desiccation during diapause enables the vector to persist from year to year despite unfavourable environmental conditions (Sota and Mogi, 1992). Furthermore, the ability of arboviruses to transmit vertically in mosquitoes from adult to offspring may also facilitate the persistence of disease transmission alongside the vector (*e.g.* WHO, 2012; WHO, 2011b; Thenmozhi *et al.*, 2007; Gokhale *et al.*, 2001).

Annually, *Ae. aegypti* infect approximately 390 million people with dengue viruses (Bhatt *et al.*, 2013), 200,000 with the yellow fever virus (WHO, 2011b), and 200-250,000 with the chikungunya virus (Staples *et al.*, 2009) in the tropics and sub-tropics. Dengue is the most significant of these arboviral pathogens, with approximately 50,000 lethal cases annually and no vaccine or curative treatment, Figure 1.4 (WHO, 2012).

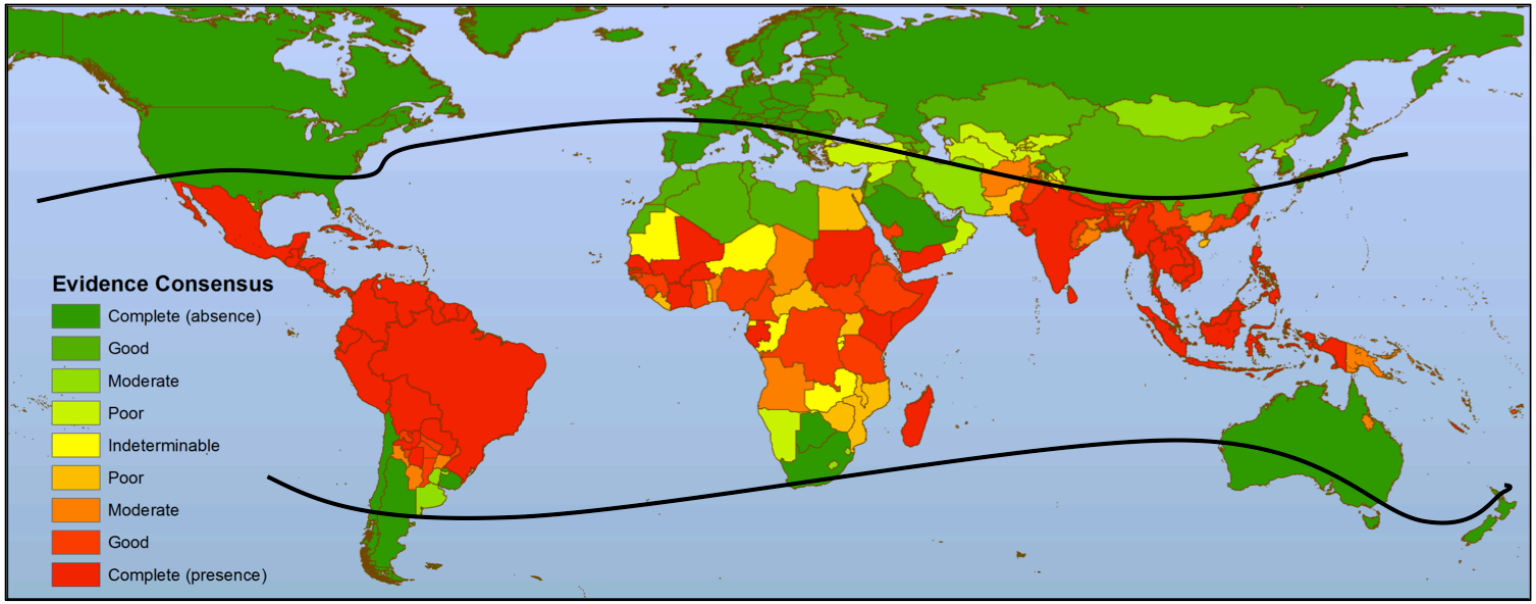

Figure 1.4 Global distribution of the dengue virus by evidence-based consensus; green represents a complete consensus on dengue absence and red represents a complete consensus on dengue presence. Solid black lines indicate proposed geographical limits for annual survival of *Ae. aegypti,* see Christophers (1960). Adult *Ae. aegypti* generally cover 50-100 metres during their lifetime and some can fly up to several hundred metres. Figure adapted from Brady *et al.* (2012).

The dengue virus is a single-stranded RNA flavivirus; there are four serotypes and the severity of disease depends on the pattern of exposure to these serotypes. Clinical manifestations include mild to severe flu-like symptoms, with ensuing complications such as dengue haemorrhagic fever or dengue shock syndrome possible (Gubler, 1998, Halstead, 2008).

Though an effective vaccine exists, yellow fever — caused by another flavivirus — is also a public health concern with 30,000 lethal cases annually, and sporadic outbreaks due to limited vaccine coverage in resource-poor settings (Gubler, 2004; Barrett and Higgs, 2007). Furthermore, outbreaks of chikungunya — which translates as 'walking bent over' in Swahili — were reported in the Indian Ocean Islands, India and Southeast Asia, and continue to threaten the Americas (Staples *et al.*, 2009). Unlike yellow fever, there is no available licensed human vaccine against this single-stranded alphavirus, and treatment usually includes anti-inflammatory drugs and analgesics to treat the severe symptoms.

Therefore, owing to the lack of adequate preventative and curative technologies for the diseases it carries, *Ae. aegypti* is highly targeted for the control of arboviral diseases.

1.3 *Ae. aegypti* as a model organism

Ae. aegypti is also considered a model species for other vector-parasite interactions. It is the natural vector for the avian parasite *Plasmodium gallinaceum* (Kilama and Craig, 1969), and strains have been selected that maintain the complete development of the filarial worms *Brugia malayi* (Macdonald, 1962a and 1962b), *Brugia pahangi* (Macdonald and Ramachan, 1965), and *Dirofilaria immitus* (McGreevy *et al.*, 1974).

As well as its public health significance, the relative ease of rearing *Ae. aegypti* has led to its significant use for basic as well as applied studies. The species can mate in pairs and their eggs can be stored for relatively long periods compared to other mosquito species, such as *Anopheles gambiae*, whose eggs hatch within a few days of oviposition and therefore cannot be stored (Beerntsen *et al.*, 2000).

As a result, the cumulative knowledge and resources gathered for this species has resulted in the progression of knowledge on a range of mosquito vectors. For example, the first molecular linkage map was assembled for *Ae. aegypti* using markers derived from random cDNA clones (Severson *et al.*, 1993), and was used subsequently to construct usable linkage maps for other mosquito vectors (*e.g.* Severson, 1995; Ferdig *et al.*, 1998).

A disadvantage of *Ae. aegypti* is the difficulty of isolating high-quality polytene chromosomes for physical mapping. Nevertheless, a large database has existed on this species for some time (Munstermann, 1990), and physiological, genetic and vector competence studies on mosquitoes were mostly derived from the study of *Ae. aegypti*. Since then, progress on mosquito genetics has been extraordinary. The genomes of the *Ae. aegypti* (Liverpool IB12 strain) was reported in 2007 (Nene *et al.*), and has led to the rapid advancement of knowledge and resources to aid the control of the diseases they transmit (*e.g.* McGraw and O'Neill, 2013).

1.4 Targeting the vector to control disease

In the last century, we have observed the introduction, establishment and expansion of mosquito borne diseases into diverse new geographic ranges (Randolph and Rogers, 2010). Socio-demographic changes resulting in more densely populated urban areas, substandard housing and other anthropogenic

modifications to the environment, as well as emerging drug resistance and climate change have all contributed to the dramatic resurgence in epidemic mosquito-borne diseases (Hemingway *et al.*, 2006). Integrated Vector Management (IVM) has been an essential part of mosquito control activities and is based on ecological, economic, and social criteria tailored to the requirements of the region. IVM incorporates surveillance, public relations, and education as well as the use of specific conventional methods such as insecticides and biological control to reduce the targeted mosquito species.

1.4.1 Traditional control of *Ae. aegypti*

Conventional mosquito control falls into four main categories:

(i) chemical control: natural or synthetic compounds or toxins of certain bacteria are utilised to attack mosquito adults and larvae;

(ii) environmental control: the environment is altered to make conditions less favourable for mosquito breeding;

(iii) biological control: natural predators or parasites of the mosquito are used to attack mosquito larvae; and

(iv) personal protection: physical barriers such as bed nets are used to reduce mosquito biting.

The diurnal behaviour of *Ae. aegypti* adults makes the species particularly difficult to control; most females rest indoors and bite during the day, rendering bed-nets ineffective. The use of insecticides to eliminate adult insects is common practice, and in some areas, space spraying of the insecticides over specific breeding sites is implemented when there is a severe risk for disease epidemic. This is achieved using hand-held fogging machines or truck-/aircraft-mounted ultra-low-volume (ULV) sprayers, and typically

the organophosphate malathion is applied. Space spraying buys valuable time to establish emergency medical facilities or introduce more effective methods of control following an outbreak of disease, and from a social perspective, is a highly visible method of control. *Ae. aegypti's* endophilic (indoor resting) behaviour, however, renders this method inefficient. Indoor residual spraying is frequently used to control *Anopheline* mosquitoes, however this is less commonly used for *Aedes* mosquitoes since they often rest on unsprayed objects such as clothes or curtains. To this end, the use of insecticide treated curtains has shown promising results (Kroeger *et al.*, 2006, Baly *et al.*, 2011). Due to the prolonged dormancy of the eggs, treatment of any given area or surface must be repeated and maintained. This repetition provides a selective advantage for resistant strains of the targeted species that subsequently thrive, whilst the insecticide continuously contaminates the environment of non-target species.

More sustainable control of *Aedes* can be achieved by targeting the immature larval stages through the elimination of aquatic breeding sites, a form of environmental control. A coordinated source reduction control program involves house-to-house checks, treatment or modification of water containers, improved storage of tyres, and education programs for local populations.

In Singapore, dengue fever was successfully controlled using environmental control against the mosquito vectors (Chan, 1985) and low incidence was maintained for decades. Nevertheless, a substantial number of cases were reported in the last few years (Reiter, 1993, Ooi *et al.*, 2006, Burattini, 2008), and this year alone has seen 16'813 reported cases of dengue fever to date (Ministry of Health Singapore, 2013). This may be due to a loss of immunity

in the human population followed by non-compliance of environmental control (Alphey *et al.*, 2010).

Biological control methods have also been deployed, such as the use of carnivorous non-blood feeding *Toxorhynchites* mosquitoes. These have been used in the United States to control inaccessible mosquito breeding sites in tyre dumps, effectively reducing the population of *Aedes* by 50% (Bailey *et al.*, 1983; Schreiber, 2007). However, the process is slow since *Toxorhynchites* larvae can consume one or two *Ae. aegypti* larvae daily, and these carnivores are not insecticide resistant and so cannot be coupled with insecticide use for effective IVM.

Another biological control agent used against mosquitoes, including *Ae. aegypti,* is *Bacillus thuringiensis israelensis* (Bti). Bti is dispensed into aquatic breeding sites and is toxic when ingested by mosquito larvae (Goldberg and Margalit, 1977). The efficacy of this control method relies on the larval feeding rates — which are sensitive to temperature, density, and the developmental stage of the larvae — and getting these carnivorous insects to a high enough proportion of the habitats for container-breeding mosquitoes. This is a difficult task. Moreover, Bti activity declines to ineffective levels after two or three days, providing only temporary control (Karch *et al.*, 1991).

The success of IVM based on these conventional control methods relies heavily on the ability of humans to seek out and access the vectors' niche and habitats. Some breeding sites are inaccessible, cover a wide area, and are subject to repeated flooding. Identifying a sufficient proportion of these sites is often an impossible task, and has been cited as a reason for the poor control of *Ae. aegypti* using conventional methods, such as aerosol

insecticides (Chadee, 1985; Hudson, 1986; Perich *et al.*, 1990; Perich *et al.*, 2000).

There is, however, one thing that is intrinsically good at seeking out female mosquitoes, and that is the male mosquito. This naturally evolved mate seeking behaviour can be exploited to introduce novel genetic traits into a target population. If the released male carries a genetic trait that renders his progeny unable to transmit disease, then the wild mosquito population can be changed to be refractory to disease, known as the population replacement strategy. If he instead passes on a gene that causes his progeny to die, the population can be reduced over time, also breaking the cycle of disease, known as the population suppression strategy. These types of efforts together are termed genetic control.

1.4.2 Genetic control of target populations

1.4.2.1 Population suppression and population replacement strategies

In 1927, Muller published substantial evidence showing that X-rays induced gene mutations and translocations. Subsequently, irradiation was shown to induce semi-sterility in *Drosophila melanogaster* (Painter, 1929), which was attributed to factors affecting the segregation of the heterozygous translocated chromosomes. Serebrovskii (1940) suggested that the mass release of detrimental engineered translocations could suppress insect populations, but his work was discontinued owing to World War II and the persecution of genetics during Lysenko's rise to power in the Soviet Union (Gould and Schliekelman, 2004; Medvedev, 1969).

The release of insect species with post-zygotic isolation from a local pest species was also proposed as a way of reducing the fitness of the targeted

species (Vanderplank, 1944). This method was successfully demonstrated through a field test with tsetse flies (Vanderplank, 1947), and was the first example of genetic control of an arthropod vector. This method is known as the sterile insect technique (SIT), and aims to reduce the ability of the target species to produce viable or fertile offspring.

The fifties saw the success of large-scale efforts in the area of genetic control. Knipling and colleagues applied irradiation-induced SIT against the screwworm *Cochliomya hominivorax*, an economically important causative agent of myiasis in cattle (Knipling, 1955 and 1979; Bushland and Hopkins, 1951 and 1953; Graham and Dudley, 1959), which was subsequently eliminated from a number of countries and regions. This paved the way for successful implementation of SIT using irradiation against a variety of agricultural pests and disease vectors, and is considered a highly species-specific and environmentally non-polluting control strategy compared to conventional insect control methods.

A major drawback for the application of classical SIT to mosquitoes is the reduced reproductive competitiveness of radiation-sterilised strains (Proverbs, 1969; Helinski *et al.*, 2009). This contributed to the failure of SIT against *Anopheles quadrimaculatus* in Florida (Dame *et al.*, 1964; Weidhaas *et al.*, 1962), and *Culex tritaeniorhynchus* in Pakistan (Reisen *et al.*, 1980). In El Salvador, a chemosterilant was used as an alternative for SIT against *Anopheles albimanus* with promising results (Lofgren *et al.*, 1974), though the toxicity of the chemical's residue was not acceptable for large-scale use, leading to the discontinuation of this line of enquiry. Improvements in mass rearing techniques and irradiation sterilisation, coupled with thorough investigations of important issues, such as gamma ray dosage and male

fitness parameters, has recently led to the successful demonstration of population suppression of *Aedes albopictus* in northern Italy (Bellini, 2013).

Arguably the most groundbreaking and compelling developments of the genetic control strategy since the discovery of translocation-induced sterility came with the ability to genetically modify (*i.e.* transform) insects. Consequently, the potential for controlling the transmission of mosquito-borne disease with genetically modified mosquitoes was openly discussed and a long-term plan was formulated (James, 1992; Meredith and James, 1990; WHO, 1991).

Since then, mosquito transformation technologies have proved to be powerful tools for genetic analysis and manipulation and have led directly to improvements in both suppression and replacement genetic control strategies. For example, the RIDL strategy, release of insects carrying a dominant lethal (Thomas *et al.*, 2000), has been genetically engineered in *Ae. aegypti* (Phuc *et al.*, 2007) for the population suppression strategy and eliminates the need for irradiation. RIDL strains carry a tetracycline-repressible lethal genetic system that can be reared on tetracycline in the laboratory; their offspring cannot survive to adulthood in its absence. The strategy was first implemented in the Cayman Islands, and showed a population reduction of approximately 80% (Alphey *et al.*, 2010; Harris *et al.*, 2011).

In addition, anti-pathogen genes targeting a range of mosquito borne pathogens have been identified and used to engineer transgenic refractory mosquitoes (Riehle *et al.*, 2003; Olson *et al.*, 2002), such as *Ae. aegypti* strains refractory to the dengue virus serotype-2 using RNA interference technology (Franz *et al.*, 2006; Mathur *et al.*, 2010).

There is usually a fitness cost associated with genetically modifying mosquitoes, and as a result there are selective pressures towards the loss of transgenes (Lambrechts *et al.*, 2008; Irvin *et al.*, 2004). Therefore, the

population replacement strategy also requires a system to spread the desirable trait through the wild vector populations to such levels that pathogen transmission no longer occurs, thus breaking the cycle of disease and reducing its burden or eliminating it altogether. Such systems are referred to as 'gene drive' mechanisms. These show non-Mendelian patterns of inheritance where more progeny inherit the selfish gene than Mendelian inheritance would predict, and are able to continue to increase in the population despite having an associated fitness cost.

Consequently, the success of a gene drive mechanism is pivotal to fix introduced novel genes into wild populations. Given the lack of practical and suitable gene drive mechanisms in mosquitoes, work to build synthetic gene drivers in the lab is actively being pursued.

The ideal driver system (Braig and Yan, 2001; James, 2000) should:

(i) maintain tight linkage with the refractory gene to spread and fix the trait into the wild population and reduce the risk of separation by processes such as meiotic recombination;

(ii) completely replace the target population with the desired genotype, thus reducing the vector-borne disease burden in any transmission setting;

(iii) spread the refractory trait controllably to avoid an unrestrained transformation of an entire species globally or elimination of the trait by selection;

(iv) be recallable lest the trait be unexpectedly hazardous and the transgenic vector must be eliminated from the population;

(v) be replaceable in the event that the modified insect no longer meets the required standards in the field;

(vi) be reusable and generic so that the same gene drive mechanism is compatible with different or improved refractory traits and can drive these through the same target vector population in the future to

make the evolution of new resistant parasitic or viral strains more difficult; and

(vii) not be transferable to non-target species with unpredictable ecological and environmental consequences.

Curtis suggested the use of translocation homozygotes to drive refractory genes into wild populations (Curtis, 1968), and this was attempted for the first time in Kenya (Lorimer *et al.*, 1976). The project failed due to severe fitness deficits of the released strain, and with the advent of novel tools for genetic analysis and manipulation, a number of potential gene drive mechanisms are currently under investigation (Figure 1.5). These include transposable elements, meiotic drive, homing endonuclease genes, intracellular symbionts (*Wolbachia*), and underdominance systems.

Transposable elements were originally identified in maize; they are fragments of DNA that move to new locations increasing their copy number within the genome, and are therefore inherited at a higher frequency than the typical 0.5 Mendelian inheritance pattern (Charlesworth and Langley, 1989). Known as 'selfish' genetic elements, Hastings suggested their use as potential gene drive mechanisms in 1994. A famous example used as a model for this system was the *P* element, shown to spread itself through the *D. melanogaster* population at a high transposition rate (Carareto *et al.*, 1997). However, high levels of internal gene sequence deletions were related to high transposition levels, especially in *P* elements carrying larger constructs (*e.g.* Carareto *et al.*, 1997). Therefore there are a number of disadvantages to using transposons as gene drive mechanisms in mosquitoes: (i) despite increasing their copy numbers at a high rate, the genes carried may not

retain anti-pathogen properties due to mutational effects; (ii) there is no mechanism for the removal of the transgene from the population; and (iii) the efficiency of the system is inversely correlated with the size of the transgene, thus limiting its usefulness. To date, transposons have been widely used to insert genes of interest into the *Ae. aegypti* genome. Unlike in *Drosophila,* identified transposons have exhibited no germline remobilisation in *Ae. aegypti*, seemingly due to some form of suppression system, rendering their use as potential gene drive mechanisms impractical (*e.g.* Sethuraman *et al.*, 2007; StJohn, 2012; Palavesam *et al.*, 2013).

Meiotic drive mechanisms are based on alterations of the normal meiotic process that result in preferential production of certain gametes (Zimmerin *et al.*, 1970). In *Ae. aegypti*, naturally occurring meiotic drive alleles have been shown to result in a distorted highly male-biased sex ratio, and meiotic drive is therefore being investigated for its potential use as a gene-driver. However, many wild strains are not sensitive to the meiotic effects of the endogenous alleles (Mori *et al.*, 2004).

Engineered systems that show real promise are based on the inheritance pattern of *Medea* (maternal-effect-dominant embryonic arrest) genetic elements originally identified in *Tribolium* beetles (Beeman, 1992). The synthetic *Medea*-like system shown in *Drosophila* is based on a genetic element, which contains both a maternally expressed toxin and an antidote expressed in the zygote. Therefore, the offspring of a *Medea*-carrying female can only survive if they also inherit at least one copy of the *Medea* element, and so are able to counter the effects of the maternal toxin during embryonic development (Chen *et al.*, 2007). This strategy leads to a rapid increase in the proportion of *Medea*-carrying individuals over time. Unfortunately, an analogous genetic system has yet to be produced in mosquitoes.

Homing endonucleases are another class of 'selfish' genetic elements. They induce double strand breaks in DNA at specific recognition sites. This activates the cell's recombination repair mechanism using the homologous chromosome carrying the homing endonuclease gene (HEG) as a template, thereby spreading the inheritable HEG throughout the population (Goddard *et al.*, 2001). Recently, Windbichler *et al.* (2011) showed the successful use of a version of this system with the transgenic target sequence in *An. gambiae* to spread transgenic *I-SceI* in mosquito cage populations efficiently, leading to the inactivation of their green fluorescent protein reporter gene. The use of HEGs in natural mosquito populations depends on the ability to re-engineer their specificity towards native mosquito sequences to disrupt genes essential for its vectorial capacity or viability, or to introduce anti-pathogenic genes at selected loci. This has not yet been achieved but would in principle allow the spread and fixation of desirable traits into the wild population.

Following observations of its natural spread through populations of *Drosophila simulans,* the use of maternally inherited intracellular reproductive parasite *Wolbachia* as a gene drive mechanism has also been suggested (Turelli and Hoffmann, 1991). *Wolbachia* manipulate their hosts to provide infected females with a reproductive advantage over their uninfected competitors since embryos from *Wolbachia*-uninfected females die if fertilised by infected males (*i.e.* cytoplasmic incompatibility), whereas embryos from infected females are not affected in this way (Turelli and Hoffmann, 1995). *Wolbachia* carrying anti-pathogenic transgenes have been proposed as a means to spread the genes into a target population of mosquitoes (Sinkins, 2004). However, the bacteria's protein expression and processing methods are poorly understood and gene transformation has yet to be achieved. Recently an Australian group reported the successful transinfection of *Ae. aegypti* with

the *Wolbachia* Mel (*w*Mel) strain, which also has a natural ability to prevent the transmission of dengue (serotype-2) by its mosquito host, indicating its potential usefulness in the control of disease (Walker *et al.*, 2011). In this study, they also showed that the *w*Mel strain increased from an original frequency of 0.65 to near fixation under semi-field conditions within approximately 80 days. This was followed up by the release of *w*Mel-infected *Ae. aegypti*, which resulted in near-fixation of the symbiont amongst a wild mosquito population in the north east of Australia after a few generations (Hoffmann *et al.*, 2011). Currently the group is seeking approval to release *Ae. aegypti* infected with *w*Mel in Vietnam, Thailand, Indonesia and Brazil, where dengue is an endemic disease. This is a major step in the fight against dengue, although it is important to note that *w*Mel has not been shown to provide its host with protection against all dengue serotypes (Walker *et al.*, 2011).

An engineered underdominance system, where inheritance of two transgenic constructs in an insect is viable, whereas the inheritance of only one construct is lethal, has been proposed as an effective gene drive mechanism in insects (Davis *et al.*, 2001). The utility of this system is its frequency-dependent drive, in which transgenic alleles have low relative fitness compared to the non-transgenic alleles at low frequency, and higher relative fitness at high frequency (Altrock *et al.*, 2010 and 2011; Marshall and Hay, 2012; Davis *et al.*, 2001). Furthermore the removal threshold frequencies for underdominance systems are considerably lower than other frequency-dependant gene drive systems such as HEGs and *Medea*. This makes engineered underdominance a more desirable gene drive mechanism as it enables the system to be recalled in the field by release of wild-type insects above the threshold frequency, and confinement of the transgenic insects to the targeted geographic location. Akbari *et al.* (2013) recently showed a

working underdominance-based gene drive system in *D. melanogaster*, however no such system currently exists in mosquitoes. Chapter 3 attempts to develop a different engineered underdominance system in *Ae. aegypti* for use in the population replacement control strategy.

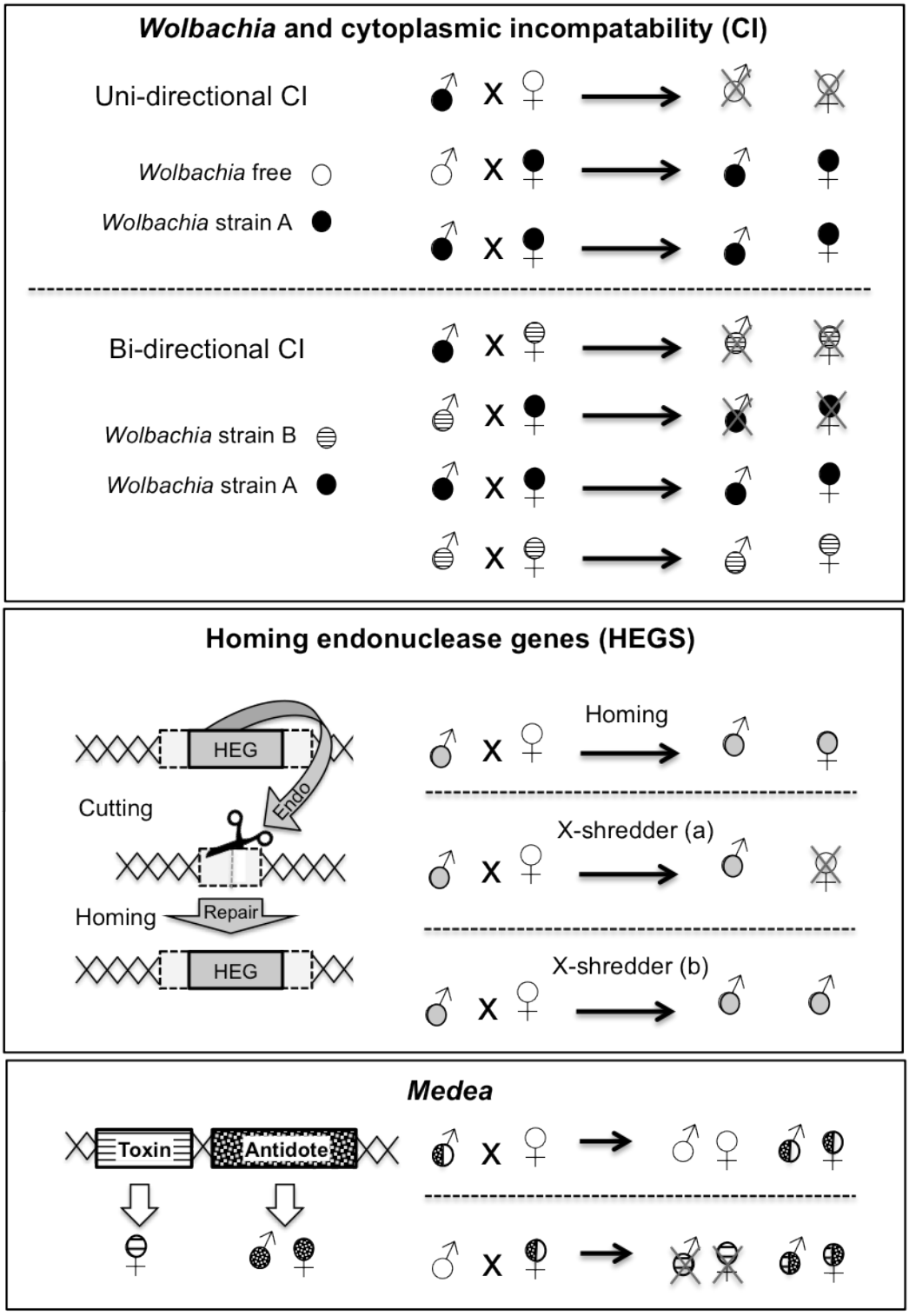
Wolbachia and cytoplasmic incompatability (CI)
Uni-directional CI
Wolbachia free
Wolbachia strain A
Bi-directional CI
Wolbachia strain B
Wolbachia strain A
Homing endonuclease genes (HEGS)
HEG
Endo
Cutting
Homing
Repair
HEG
Homing
X-shredder (a)
X-shredder (b)
Medea
Toxin
Antidote

Figure 1.5 Selected genetic control strategies. All embryos produced by infected *Wolbachia* females (♀) are infected. Embryos are non-viable if fertilised by a male carrying a *Wolbachia* strain different to the strain carried by the mother. This is the basis of SIT. Uni-directional CI results if in the presence of only one strain. If two strains are involved than CI is potentially bi-directional. Invasive strategies require release of infected females and ♂s to spread *Wolbachia* into the target species. HEGs cut at a specific nucleotide sequence. If the target sequence is X-linked ('X-shredder') and the HEG is expressed during spermatogenesis, this could result in (a) non-viable daughters, or (b) the elimination of X-bearing sperm. If the 'X-shredder' HEG is Y-linked, all sons carry it, which could lead to invasion through meiotic drive. Where the HEG is inserted into the potential cut site, repair of the HEG-induced cut can copy the HEG-bearing allele. If 'homing' occurs in the germline this causes super-Mendelian inheritance, whereby more than 50% of the offspring of a HEG heterozygote carry the HEG; this will tend to allow the HEG to invade. Synthetic *Medea* utilises a maternally expressed toxin and a zygotic antidote to achieve a somewhat similar effect. The only viable offspring of a heterozygous female are those carrying the *Medea* element. Males carrying the *Medea* element do not express the toxin. Figure adapted from Alphey, (2014).

1.4.2.2 Development of transformation technologies to improve genetic control methods in insects

Transformation of insect vectors has revolutionised the development of disease control strategies.

The first successful germline transformation system in insects was based on the *P*-element transposon in *D. melanogaster* (Rubin and Spradling, 1982). The *P*-element and all transposons later identified for insect germline transformation are referred to as 'Class II short inverted repeat' transposons. As the name suggests, these consist of inverted terminal repeats that flank an open reading frame that codes for a transposase enzyme. This transposase is able to catalyse the transposition of the whole element via a DNA-intermediate, utilising a cut-and-paste mechanism that creates a duplication of the insertion site. To achieve transformation of *in vitro* assembled genes, Rubin and Spradling (1982) used a binary system consisting of (i) a vector

construct carrying a non-autonomous *P*-element where the transposase gene was disrupted by an eye colour marker, and (ii) a helper construct carrying a functional transposase with defective terminal inverted repeats so that it cannot transpose itself. These were microinjected into *D. melanogaster* preblastoderm embryos — when the embryo is a multinucleated cell and nuclei destined for germ cells are clustered at one end of the cell — simultaneously and their offspring were screened for eye pigmentation, Figure 1.6.

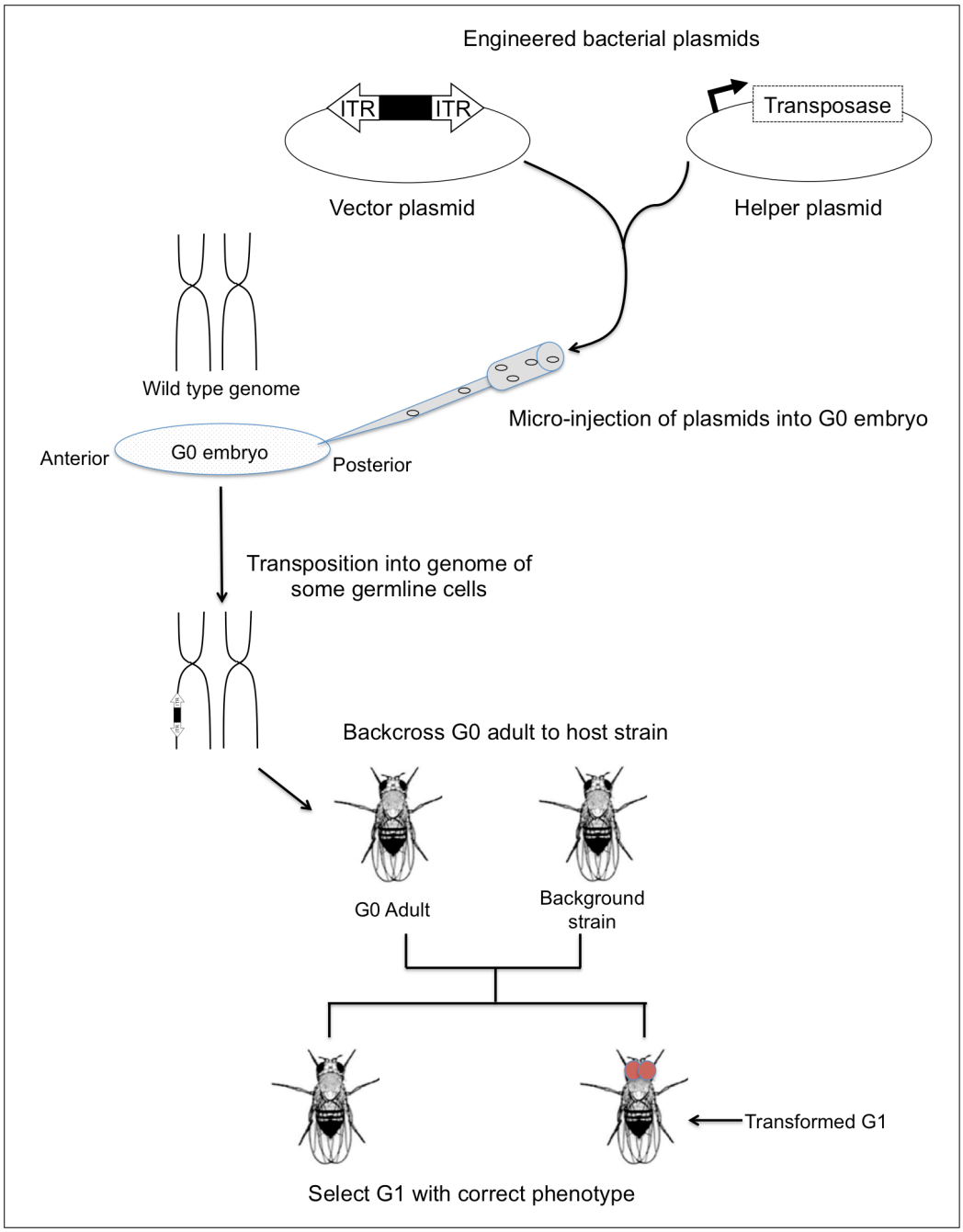

Figure 1.6 Schematic diagram of typical transformation of *Drosophila* using the binary vector-helper system; ITR= inverted terminal repeat of non-autonomous transposon; sequences containing transgenes and transformation markers in the vector plasmid are indicated in black. The transposase integrates the transposon-based transgenes into the genome using a cut-and-paste mechanism. In some cases, the co-injected helper is capped transposase mRNA, as in this thesis. Adapted from Griffiths *et al.*, (2011).

Due to limitations on the transfer of this *P*-element based system to non-Drosophilid species such as mosquitoes (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989), a detailed search for transposable element vectors suitable for germ-line transformation in a variety of insects ensued. At present, there are four transposon-based systems used to transform non-Drosophilid insect species: the *mariner* family elements *Mos1* and *Minos*, the *hAT*-related *Hermes* element, and the distinct and most widely used element *piggyBac*. *Ae. aegypti* has been successfully transformed using *piggyBac* (Kokoza *et al.*, 2001), *Hermes,* (Jasinskiene *et al.*, 1998), and *Mariner* elements (Coates, 1998) based on the binary vector system originally designed by Rubin and Spradling (1982), Figure 1.6.

Transposon-based transformation technologies have greatly improved our knowledge of vector biology and broadened our toolset for genetic manipulation and subsequently the control of these species. Moreover, the advancement of new marker systems has permitted identification of stably inherited fluorescent markers and tissue specific promoters that facilitate simple and reliable detection of transgenic individuals with minimal handling. Nevertheless, in some circumstances transposons remain limited in their utility due to remobilisation risks and positional effects that threaten the stability of the transgenes and their function.

Genomic remobilisation and loss of the transgenes may occur in the presence of a suitable source of transposase. This may affect the frequency of the transgene, and the insect's fitness depending on the new integrated position. The risk of horizontal gene transfer is also a regulatory and public perception concern if there is a perception that the transgene might be transferred to non-target organisms during any field release. Post-integrative

stability of transgenes has been studied and two dissimilar types of methods to stabilise transposon-based integrations have been devised.

The first consists of the removal of one (Handler, 2004) or both (Dafa'alla *et al.*, 2006) inverted terminal repeats (ITRs), by preferential remobilisation of smaller vectors within the larger vector. One limitation is that high transposase activity may cause repeated remobilisation, whereas low transposase activity may render the method unusable. Transposase activity varies from species to species. For example, *An. stephensi* germline remobilisation is achieved in approximately 10% of progeny (O'Brochta *et al.*, 2011), whereas germline remobilisation in *Ae. aegypti*, using the *piggyBac*-based system was not observed in the germline (StJohn, 2012; Sethuraman *et al.*, 2007) or in transgenic cell lines (Palavesam *et al.*, 2013).

The second method, demonstrated in *D. melanogaster*, consists of endonuclease and double strand break-repair with heat inducible endonucleases to remove ITRs (Tkachuk *et al.*, 2011). There is a risk of causing double-strand breaks elsewhere in the genome, which may have severe consequences to the insect if these are in important regions, although specificity of alternative endonucleases, such as engineered zinc-finger nucleases (Miller *et al.*, 2007), may increase specificity for this method. Given the high stability of *piggyBac* based transposons in *Ae. aegypti* (*e.g.* Sethuraman *et al.*, 2007; StJohn, 2012; Palavesam *et al.*, 2013), methods to stabilise transgenes in this insect have not been a research priority in this insect.

Another limitation of transposon-mediated transformation in all insect species is the inability of transposons to integrate at one pre-determined and characterised target site within the genome. *piggyBac*-based transposons are unique in that they integrate at specific nucleotide sequences within the

genome, however these sites are only four nucleotides long (TTAA) and so exist at numerous locations within the genome, and integration into any of these sites is random. This effectively random integrative nature of transposons often results in variable expression levels between insects carrying the same transgenes due to their different local genomic environments, known as positional effects. Importantly, this may mean that a transgenic phenotype from one insertion may not be replicable if the same construct is inserted at a different genomic location, which makes this an unreliable transformation method.

The benefits of using site-specific recombinases have been well publicised. They facilitate reproducible insertion into genetically active loci (*e.g.* Groth *et al.*, 2004), whilst the ability to characterise the effects of transformation into these sites and the capacity to compare a panel of transgenes inserted at the same site makes it a highly pursued technology. Unfortunately, naturally occurring integrase target sites are extremely rare, and pseudo-integration sites cannot be targeted at adequate frequencies for consistent use. Consequently, the specific target site is pre-inserted into the insect's genome using transposon-mediated transformation, and if required, post-integrative stabilisation methods can be applied.

The most studied and well-characterised site-specific transformation systems are the tyrosine-catalysed Cre-*loxP* and FLP-*FRT*, and the serine-catalysed ΦC31-*att* system. The fundamental difference between these two families is that only the serine-catalysed integrations are unidirectional, precluding recombinase-mediated excision that occurs with Cre and FLP.

Thus the ΦC31-*att* system shows great promise in improving more targeted, consistent and stable transformation technologies in insects.

Chapter 4 investigates the improvement and refinement of the system in *Ae. aegypti* for use in developing transgenic insects adequate for field release.

1.5 Personal contributions to the field

This thesis presents efforts to engineer an underdominance-based gene drive system based on the use of RNA interference (Chapter 3) and the development of a new site-specific genome engineering technique (Chapter 4) in *Ae. aegypti*. Following their construction, site-specific docking strains were evaluated further for their utility in research and the development of strains for field release in Chapter 5.

Chapter 2

General Methods

2.1. *Aedes aegypti* husbandry

An *Ae. aegypti* wild-type strain, colonised in 1975 from Jinjang, Selangor, Malaysia, was used in this study to develop all new strains, and to outcross all transgenic lines.

Mosquitoes were reared at 26°C (±1°C) and 80% (±10%) humidity with a 12:12 hour light:dark cycle and constant air circulation.

To hatch the insects, egg papers were placed in approximately 100 ml water in a 0.5 L polystyrene container. To induce hatching, pots were placed into a vacuum desiccator and a vacuum applied for between one and 15 hours. The larvae and egg papers were poured into standard rearing trays measuring 14" x 10" x 2" with 1 L of water, at a density of approximately 1 larvae ml^{-1}. On the first day after hatching, larvae were fed with two drops of Liquifry (Interpet Ltd, UK) per tray, unless a specific feeding regimen was in place. On subsequent days, larvae were fed *ad libitum*, or the specified feeding regimen, with powdered TetraMin® Ornamental Fish Flakes (Tetra GmbH, Germany).

Pupation begins approximately seven days after hatching. Pupae were removed using a 2.5 ml plastic pastette and sexed by the morphology of the terminal segments, Figure 2.1. These were separated into 100 ml weighing boats, refilled with water and placed in labelled adult cages; adults eclosed around 48 hours later.

Adult mosquitoes were provided *ad libitum* with a 10% sucrose solution with 14 U ml^{-1} penicillin and 14 μg ml^{-1} streptomycin. Females were fed on defibrinated horse blood screened for pathogens (TCS Biosciences Buckingham, UK) and provided they had mated, eggs were subsequently obtained three days after a blood meal.

Eggs were collected using a labelled Whatman filter paper laid on wet cotton wool in a 10 cm petri dish placed inside the adult cage overnight. The freshly laid eggs were kept damp for at least two days to mature, after which the wet cotton wool was removed to allow the eggs to dry before hatching. Eggs were at least four days old before hatching.

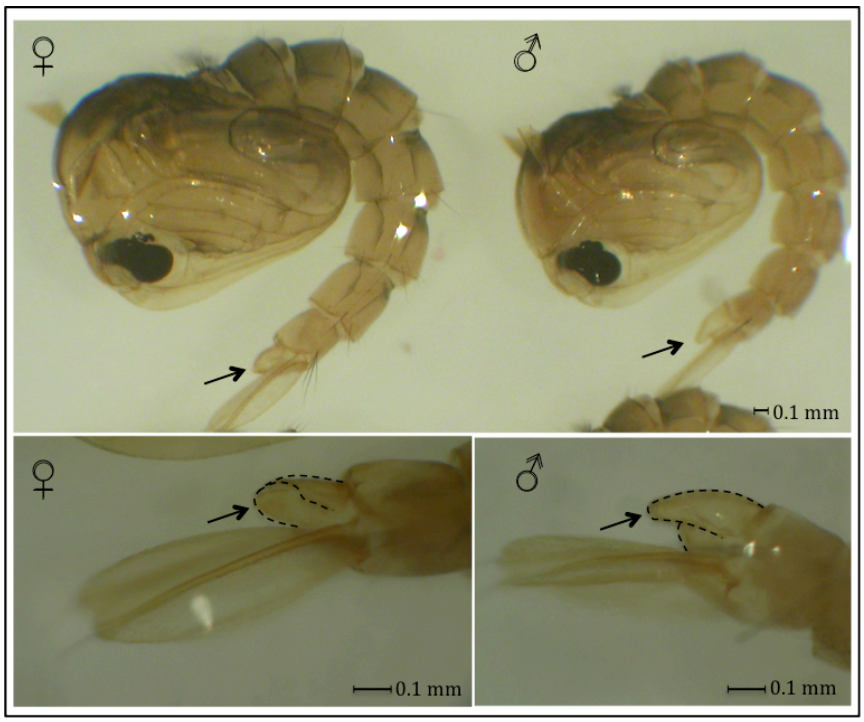

Figure 2.1. Sexual dimorphism in *Ae. aegypti* pupae. Female pupae (♀) are typically larger than male (♂) pupae; there are prominent differences in the structure of the terminal segments, shown with black arrows; structures are outlined at higher magnification with dashed black lines.

2.2 Germline transformation

2.2.1 Preparation of injection solutions

Injection solutions for transformation using *piggyBac* and ΦC31 integration systems, or recombination using FLP-*FRT* and Cre-*loxP* systems, contained the transgenic DNA plasmid and the capped mRNA helper suspended in a buffer. The buffer had a final concentration of 5 mM KCl and 0.1 mM NaH_2P0_4, pH 6.8. DNA plasmids were purified using EndoFree Plasmid Maxi Kit (Qiagen, Germany). The mRNA of *piggyBac* transposase, site-specific ΦC31 integrase, and the flippase (FLP) and Cre

recombinases were transcribed from OX3081 (Fu, 2010), OX3869 (Nimmo *et al,* 2006), OX3608 and OX4611 (Scaife, S., unpublished data) plasmids respectively, and sequences included the *Drosophila melanogaster vasa* gene's 3'UTR. *Vasa* is upregulated in the pole cells of developing embryos during germline formation and the 3'UTR was expected to assist delivery of the mRNA to these cells (Sano *et al,* 2002). The plasmids were linearised with the restriction endonucleases summarised in Table 2.1. Capped mRNA was transcribed with the mMESSAGE mMachine T7 Kit (Ambion, USA) and purified using the MEGAclear Kit (Ambion, USA).

The plasmid DNA and capped helper mRNA were diluted to specific final concentrations depending on optimum conditions required for the systems and the toxicity of the plasmid DNA. Once prepared, to confirm the integrity of the mRNA, 1 µl of the injection solution was diluted 1:10 in endonuclease free water, and nucleic acid components were separated by electrophoresis, Section 2.7; in the absence of distinct bands, indicating degradation of the mRNA or DNA, injection solutions were remade. The injected solution was divided into 10 µl aliquots and stored at -80°C.

Table 2.1 Restriction enzymes used to linearise plasmids

DNA Construct ID	Type	Restriction enzyme
OX3869	ΦC31 integrase	*BamHI*
OX3081	*piggyBac* transposase	*XbaI*
OX4611	Cre recombinase	*XbaI*
OX3608	FLP recombinase	*XbaI*

2.2.2 Preparation of injection apparatus and needles

Aluminosilicate glass filaments (10 cm) were pulled with a P-2000 Intracel LTD needle-puller (Sutter Instrument Co., England) using the programs listed in Table 2.2. Program choice depended on immediate conditions and whether a fine needle or a wider needle was required for microinjection.

Table 2.2 Needle-puller programs and the needles' characteristics. Heat (laser output power) affects the length and tip size, higher settings give longer finer tips; the filament (Fil), velocity (Vel= the point at which heat is turned off), and delay (DEL=timing of hard pull start relative to the end of laser heating) were set to standard values for aluminosilicate glass filaments. The pull strength (PUL) affects tip size, with high values resulting in smaller tips.

Program	Needle Type	Program details
3	Wide tip	Heat= 420; Fil=120; Vel=50; DEL=200; PUL=140
7	Thin tip	Heat= 360; Fil=120; Vel=50; DEL=200; PUL=180

Injection solutions (Section 2.2.1) were centrifuged at 13,000 x *g* for five minutes and stored on ice prior to injections. Using a 20 µl microloader pipette tip (Eppendorf, Germany), 2 µl of the solution was loaded into the needle. The needle was mounted onto an Intracel LTD bevelling machine (Sutter Instrument Co., England) and bevelled on a fine (2.0-20 micron tip size) diamond abrasive plate (Intracel LTD, England).

The bevelled needle was fixed to the microinjection apparatus shown in Figure 2.2, which consisted of a BA400 Motic light microscope (Motic®, USA), an MN-151 micromanipulator (Narishige, Japan), and an Eppendorf FemtoJet microinjector air-pump (Eppendorf, Germany). As embryos were

prepared for injection (Section 2.2.3), the needle was lowered onto a drop of injection oil on a cover slide.

Figure 2.2. Microinjection apparatus. The micromanipulator enables fine adjustments and control of the needle. A constant back-pressure or injection pressure supplied by the microinjector air-pump enables injection of the solution into the embryos.

2.2.3 Embryo collection and preparation for microinjection

Ae. aegypti cages for injection were established according to Section 2.1, each containing at least 100 females and 50 males. To induce oviposition, cages were positioned in a dark environment, typically under a cardboard box. Embryos were collected using a labelled Whatman filter paper laid on wet cotton wool using warm water in a 10 cm petri dish. During optimum

oviposition, typically in the afternoon, one hour was sufficient to collect over 1000 embryos ready for microinjection. Fresh egg papers were continuously replaced for a constant provision of embryos.

A needle was used to gently align approximately 100 lightly greyed eggs on the damp filter paper with posterior ends pointing in the same direction, Figure 2.3. The eggs were carefully transferred onto a 22 mm x 22 mm cover slip using double-sided sticky tape, allowed to desiccate slightly, and covered with a thin layer of semi-permeable halocarbon oil. The oil was prepared to a final ratio of 1:9 of halocarbon oils 27 and 700 (Sigma, USA), and stored under purified water.

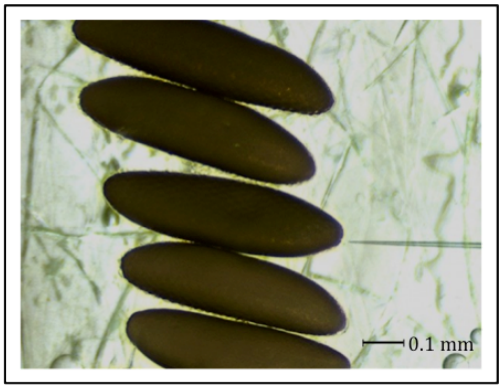

Figure 2.3. Eggs lined up with their posterior ends facing to the right and operculum to the left. Image source: Oxitec Ltd.

2.2.4 Microinjection and post-injection care of *Ae. aegypti* embryos

The prepared pre-blastoderm embryos were microinjected as described by Morris *et al.* (1989). To remove the oil, cover slips of the newly injected embryos were placed vertically in water for up to three hours, and then

placed in fresh water for four days, giving the embryos sufficient time to fully develop, before hatching them as described in Section 2.1.

2.2.5 Establishing transgenic lines

Surviving injected G0 insects were collected as pupae, sexed as described in Section 2.1, and crossed to the *Ae. aegypti* wild-type strain (AWT). If the transformation efficiency was not under investigation, injected females were pooled in batches of 15-20 with 10 virgin AWT males in cages, and offspring were collected. Injected males were placed in small pots with AWT virgin females at a 2:20 male:female ratio for three days to reduce the competition and ensure all males had the opportunity to mate. Insects from six to eight pots were then transferred into cages, blood fed, and offspring were collected.

The next generation (G1) were hatched in individual batches from corresponding cages and screened for the transformation marker, Section 2.3. Any fluorescent individuals were separated and crossed to virgin AWT of the opposite sex; individuals from different G0 pools were assumed to carry independent insertions and given alphabetic line names, for example OX[plasmid number]A. Genetically distinct lines were molecularly confirmed when necessary by obtaining the inserted transgenes' flanking sequences using the adaptor-mediated PCR method described in Section 2.10.

2.3 Transformation markers and fluorescence microscopy

Phenotype markers based on the following fluorescent proteins were used as markers of transgenesis: blue AmCyan (Matz *et al.*, 1999); enhanced blue ECFPaf (Horn and Wimmer, 2000); enhanced green EGFP (Horn *et al.*, 2000); faster maturing green ZsGreen (Matz *et al.*, 1999); and red DsRed2 (Matz *et al.*, 1999).

Expression of these markers were controlled by the eye-specific artificial promoter 3xP3, containing three tandem repeats of an optimised photoreceptor transcriptional activator binding site, P3, in front of a TATA box (Kokoza *et al.*, 2001), or the body-specific *hr5 enhancer*-driven baculovirus *ie1 promoter,* Hr5ie1 (Rodems and Friesen, 1995). Examples are shown in Figure 2.4.

To identify transgenic insects, third instar larvae (L3) or pupae were screened for the presence of fluorescent markers using a Leica MZFLIII or Olympus SZX12 fluorescence microscope in a darkened room. This was achieved by placing the immature mosquitoes in a 100 ml weighing boat, removing the water using pastettes, and temporarily immobilising the mosquito for more accurate screening by placing the weighing boat on ice water. A paintbrush was used to gently separate the transgenic and non-transgenic individuals.

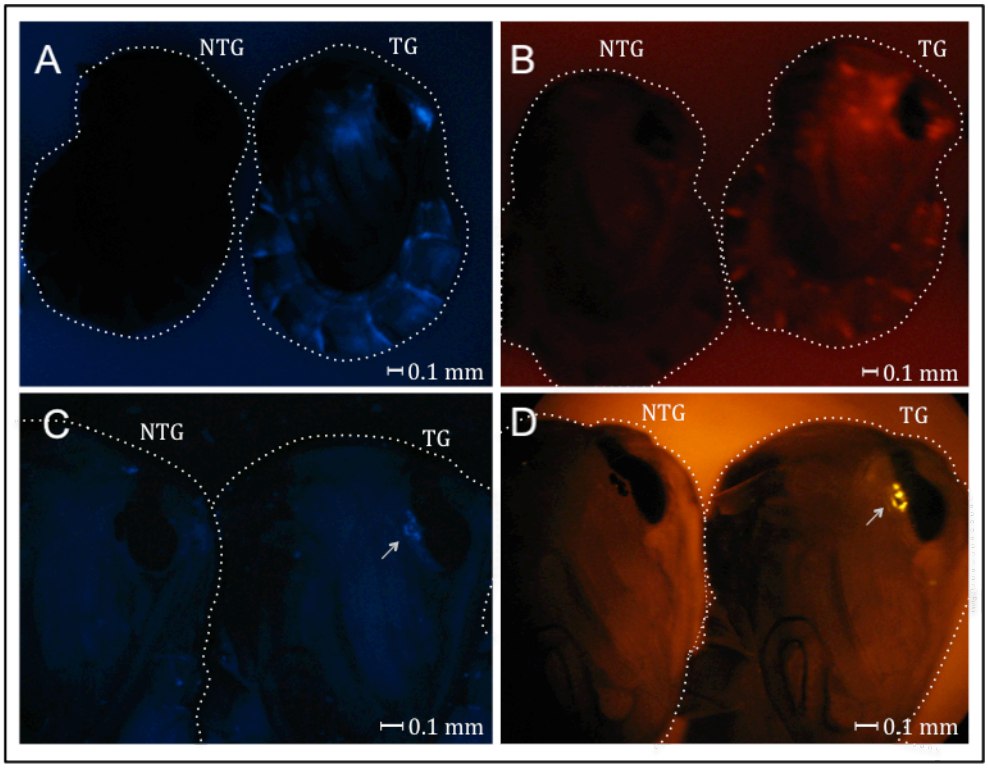

Figure 2.4 Examples of fluorescent transformation markers in *Ae. aegypti* pupae. In each image non-transgenic (NTG) pupae are to the left and transgenic (TG) pupae are to the right. Images show AmCyan expression driven by ubiquitous Hr5ie1 (A) or eye-specific 3xP3 (C) promoters, and DsRed2 expression driven by Hr5ie1 (B) or 3xP3 (B) Hr5ie1 promoters. Transgenic pupae are on the right side of each panel, and non-transgenic on the left. Dashed white lines outline pupae and arrows indicate fluorescence where the markers are difficult to see.

2.4 Nucleic acid isolation

2.4.1 Plasmid DNA isolation

Up to 5 µg of plasmid DNA was prepared using the GeneJET Plasmid Miniprep Kit (Fermentas, Lithuania) according to the manufacturer's protocol. Up to 100 µg and 500 µg of plasmid DNA was purified using the

QIAfilter Endofree plasmid Midi and Maxiprep kits respectively (Qiagen, Germany) in accordance with the manufacturer's instructions.

2.4.2 Genomic DNA extraction

Genomic DNA was isolated from single insects using the Machery-Nagel Nucleospin Kit (Machery-Nagel, Germany) according to the manufacturer's protocol and eluted in 50 µl endonuclease free water.

2.4.3 Total RNA extraction

Total RNA was isolated from separate sets of pooled tissue, four individual insects per sample and n=3. Using the RNeasy Mini Kit (Qiagen, Germany), 600 µl B-mercaptoethanol (B-ME) and Buffer RLT at a 0.1:1 ratio respectively were added to each sample, and insects were homogenised with a pestle. Samples were centrifuged for one minute, and the supernatant was transferred to a Qiashredder column (Qiagen, Germany) and centrifuged for two minutes at 13,000 x *g*. The flow through was centrifuged for a further three minutes at 13,000 x *g,* and the supernatant was transferred to a clean microcentrifuge tube. One volume of 70% ethanol was added and mixed by pipetting. The sample, including any precipitate, was transferred to an RNeasy spin column (Qiagen, Germany) and centrifuged for 15 seconds; the flow through was discarded. The column was washed and processed according to the manufacturers protocol; Qiagen RNase-Free DNase I (Qiagen, Germany) was added to the column membrane and incubated at room temperature for 15 minutes

between the two Buffer RW1 wash steps. RNA was eluted in 30 µl endonuclease free water.

2.5 Nucleic acid quantification

DNA and RNA were quantified on a spectrophotometer using GeneQuant II RNA/DNA Calculator (Amersham Pharmacia Biotech Inc., USA) at an absorbance value of 260 nm, using a conversion factor of 50 and 40 respectively; 1 µl of the sample was diluted in endonuclease free water, n=2, and readings were calibrated with the same batch of endonuclease free water. The ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) was also determined to investigate the purity of the extracted nucleic acids. An $A260/A_{280}$ ratio above 1.8 indicated clean DNA and RNA.

When available for use, 1 µl of RNA was quantified using P 300 NanoPhotometer UV/Vis spectrophotometer (Spectra, USA), using Lid 10 according to the manufacturer's protocol. The elution buffer used for the RNA extraction — buffered endonuclease free water — was used to calibrate the readings.

2.6 Oligonucleotide synthesis and standard PCR

Custom oligonucleotides were purchased from Invitrogen (Life Technologies, USA) on a 25 nM scale and were provided as lypholised pellets. These were re-suspended in TE buffer (10 mM Tris, pH 8.0, 0.1nm EDTA) to produce 100 µM primer stock solutions.

DNA was amplified using the polymerase chain reactions (PCRs) with DreamTaq DNA polymerase (Fermentas, Lithuania), according to the manufacturer's protocol. The final concentration of each primer was 0.5 μM μl^{-1}. The thermal cycling conditions were typically as follows: (i) an initial two minute denaturation step at 94°C, (ii) 35 cycles of: 94°C for 15 seconds, an annealing step with a primer-dependent temperature, typically 55-60°C, and an extension step of 72°C for 30 seconds per kilobase of DNA, (iii) and a final extension step of 72°C for 7 minutes.

2.7 Agarose gel electrophoresis

The 1X TAE buffer had a final concentration of 40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.4). DNA and RNA fragments were separated by size on a 1% agarose (in a TAE buffer) gel in a 1X TAE running buffer using electrophoresis at 120 V for 25 mins. Ethidium bromide was added to the gel at a final concentration of 0.5 μg μl^{-1}.

The DNA fragment sizes were determined by comparing the position of the fragment to a DNA ladder, Smartladder (Eurogentec, UK).

2.8 Gel extraction of DNA and purification of PCR products

DNA bands were visualised on the agarose gel using a 312 nm UV transilluminator, and excised using a scalpal. The DNA was extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Samples were eluted in 30 μl

elution buffer for fragments over 4 kb and 10 µl for fragments less than 4kb.

PCR products were purified using the Qiaquick Spin PCR Purification Kit (Qiagen, Germany), according to the manufacturer's instructions. DNA was eluted in 30 µl elution buffer.

2.9 DNA sequencing

Purified DNA samples were sequenced by GATC Biotech (Germany) with forward and reverse primers spaced 500-750 bp apart throughout the sequence of interest to ensure complete coverage.

2.10 Sequencing flanking genomic ends of *piggyBac* insertion

To obtain the genomic nucleotide sequences flanking the transposed *piggyBac*-based construct, an adaptor-based amplification technique shown in Figure 2.5 was performed using primers specific for the adaptors and *piggyBac* sequence.

Approximately 500-1000 ng of extracted and purified genomic DNA was digested with one unit of restriction enzymes predicted to cut approximately every 500 bp to 5 kb: *DpnII* and or *MspI* (New England Biolabs Inc., USA) according to manufacturer's protocol. Oligonucleotide dimers or 'adaptors' listed in Table 2.3 were made by mixing 2 µl of 100 µM 'MspI(TaqI) _short' or 'DpnII_short' oligonucleotides with 2 µl of 100 µM 'Adaptor_long' oligonucleotide in 100 µl annealing buffer (10 mM Tris

(pH 7.5), 1 mM EDTA, 50 mM NaCl), and by heating the mixture up to 65°C and cooling slowly to room temperature. 1μl adaptor, 2 μl T4 DNA ligase buffer and 0.5 μl T4 DNA ligase (New England Biolabs, UK) were added to each digest, and left to ligate at room temperature overnight.

The ligated genomic DNA samples were used as a template for PCR amplification of 5' and 3' flanking sequences. Adaptor-specific primer ('PRIMER'), and primers specific to *piggyBac*'s internal 5' sequence ('PB2') or 3' sequence ('PB3') were used at a PRIMER:PB2 ratio of 1:10 to favour *piggyBac*-specific amplification; primers are listed in Table 2.3. *Taq* polymerase (New England Biolabs, UK) was used in 25 μl reactions with the following cycling conditions: denaturation at 94°C for 2 min; 35 cycles of denature for 20 sec at 95°C, anneal for 45 sec at 55°C, extend for 1 min 30 sec at 68°C; and a final 9 min elongation step at 68°C. A second PCR using nested adaptor primer ('MID') and nested primers specific to *piggyBac*'s internal 5' sequence ('PB1') or 3' sequence ('PB4') was performed as before, with a MID:PB1/PB4 ratio of 1:1.

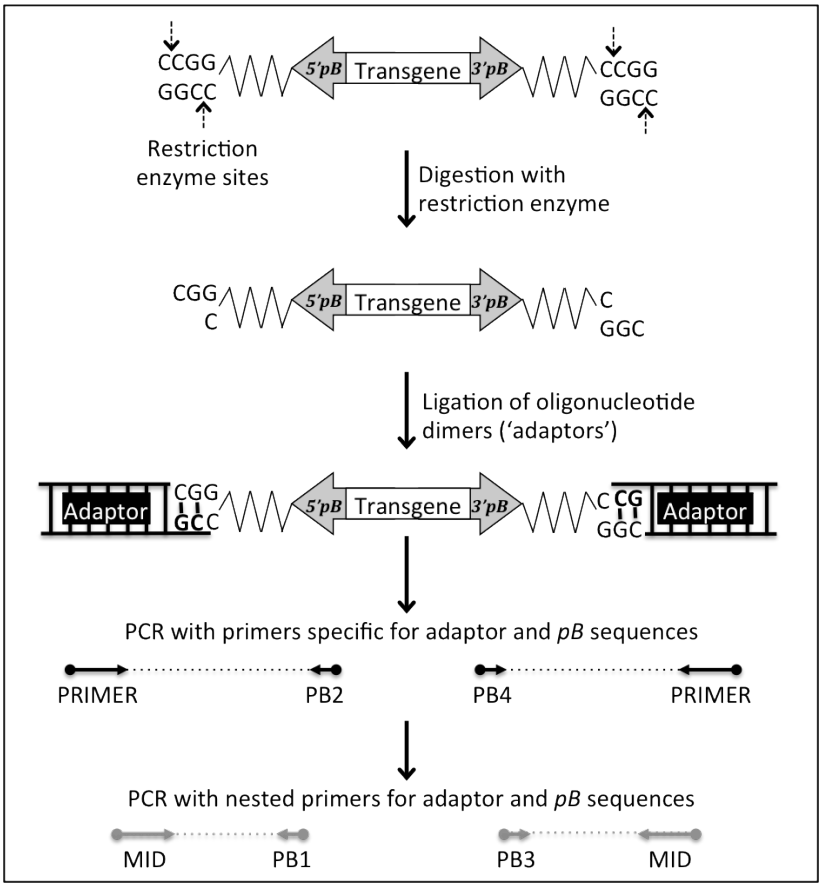

Figure 2.5 Adaptor-based PCR amplification to obtain genomic nucleotide sequences flanking the *piggyBac* insertion site. *pB= piggyBac;* primer sequences (PRIMER, PB1, PB2, PB3, PB4 and MID) are listed in Table 2.3. Figure adapted from Martins (2011).

Amplicons were gel extracted and column-purified (Sections 2.7 and 2.8), ligated into pJet plasmid vectors with the GeneJET PCR Cloning Kit (Fermentas, Lithuania) and transformed into XL_{-10} competent cells (Stratagene, USA) according to the manufacturer's protocol.

Colonies were selected using a pipette tip and mixed into 10 µl of PCR mix, which consisted of 0.2 µl of each 10 µM primer, 7.5 µl of water, 2 µl PCR Bio Buffer, and 0.1 µl PCR Bio Taq (Bioline Reagents Ltd, UK). In

parallel, selected colonies were transferred to corresponding wells of a 96-well culture plate containing 100 µl LB broth (containing 100 µg ml^{-1} ampicillin) and placed into a shaking incubator at 200 rpm at 37°C for a minimum of one hour.

DNA was amplified using the following thermal cycling conditions: 95°C for 1 min; two cycles of 95°C for 15 sec, 55°C for 40 sec and 72°C for 1 min; 29 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 15 sec per kilobase of DNA; and a final extension step of 72°C for 5 min. DNA fragments were visualised on a 0.8% agarose (in a TAE buffer) gel as described in Section 2.7.

Positive clones were prepared and purified using the GeneJET Plasmid Miniprep kit (Fermentas, Lithuania) and sequenced as in Section 2.9 using the pJET primers shown in Table 2.3.

Table 2.3 Oligonucleotides used for adaptor-based PCR amplification; each primer's order names are included.

Name	Description	Specificity	Sequence 5'-3'
Adaptor_long	Adaptor		GTGTAGCGTGAAGACGACAG AAAGGGCGTGGTGCGGAGG GCGGTG
MspI(TaqI)_ short	Adaptor		CGCACCGCCCTCCG
DpnII_short	Adaptor		GATCCACCGCCCTCCG
PRIMER	Primer	Adaptor	GTGTAGCGTGAAGACGACAG AA
PB1	Reverse primer	*piggyBac* 5' end	GGCGACTGAGATGTCCTAAA TGCAC
PB2	Reverse primer	*piggyBac* 5' end	CAGTGACACTTACCGCATTG ACAAG
PB3	Forward primer	*piggyBac* 3' end	CAGACCGATAAAACACATGC GTCA
PB4	Forward primer	*piggyBac* 3' end	GTGCCAAAGTTGTTTCTGAC TGACTA
MID	Primer	Adaptor	GACGACAGAAAGGGCGTGG TG
pJETFP2	Forward primer	pJET vector	ATCAACTGCTTTAACACTTG TGC
pJETRP2	Reverse primer	pJET vector	AAAGAAGAACATCGATTTTC CATG

2.11 Reverse transcription and quantitative real-time PCR

RNA was extracted from headless adults as the endogenous eye pigments were thought to interfere with the PCR reaction in adults (Eckhart *et al.*, 2000; Bhalla, 1968; Hawkins, 2008). RNA was extracted as described in Section 2.4.3, and 0.5 µg RNA was used for the reverse transcription reaction, which was carried out using Superscript® III RT kit (Invitrogen, USA) in 25 µl reactions according to the manufacturer's protocol. The reactions were incubated in a PCR block at 25°C for 5 min, followed by 50°C for 45 min, and 70°C for 15 min. Subsequently, real-time PCR was carried out on 1 µl cDNA in a 25 µl reaction using Taqman Gene Expression MasterMix (Applied Biosystems, UK). Primers and fluorescently-labelled probes were purchased from Invitrogen (Life Technologies, USA) and Eurofins (UK) respectively, and are listed in relevant chapter methods.

Amplification and detection was performed using an Mx3005P real-time PCR machine (Stratagene, USA) with the following thermal cycler conditions: 50°C for 2 min; 95°C for 10 min; and forty cycles of 95°C for 15 seconds, and 60°C for 1 min. Each reaction was carried out in triplicate. Differences in gene expression, expressed as fold change with respect to the appropriate control samples, were calculated using the ΔΔCt method (Livak and Schmittgen, 2001), with 18S as the endogenous control.

Chapter 3

Engineering gene drive in *Aedes aegypti*

3.1 Introduction

3.1.1 Working towards an ideal gene drive mechanism

Insect-borne diseases such as malaria, dengue fever, West Nile virus, trypanasomiasis, and many others threaten and claim the lives of millions, particularly in the developing world. Reducing the vector population has been a long-standing objective of many disease control programs; in some cases reducing the vector population may only provide temporary relief as the vacant niche may be filled by another invasive vector species. A different approach to the problem is to genetically engineer insect vectors such that they are unable to transmit the pathogen to the host. This is known as the population replacement strategy, and the insects referred to as 'refractory' (*e.g.* Franz *et al.*, 2006; Ito *et al.*, 2002; Moreira *et al.*, 2002). There is great potential for the use of genetically refractory insects to modulate vector competence and so diminish the detrimental effect on

human health and the concomitant societal burden (Boëte and Koella, 2002; Medlock *et al.*, 2009).

Overall, engineered refractoriness may impose a fitness penalty on the insect. This may be due to an evolutionary cost of mounting an immune response (Koella, 2003), or due to positional effects of the transgene(s) within the insect's genome. Consequently, this strategy relies on the use of an additional genetic mechanism to spread, or at least maintain, the refractory gene in the target population despite this selective pressure. These so-called 'gene drive' systems are designed to spread, or 'drive', tightly linked refractory genes ('cargo') through the target population at greater-than Mendelian ('super-Mendelian') rates; they overcome fitness costs of the refractory genes to rapidly increase the anti-pathogen property in the target population.

For future deployment of this technology, it is imperative that the gene drive mechanism also satisfies regulatory requirements. With this in mind, it is desirable for the engineered system to be 'recallable' in the event of any undesirable or deleterious consequences following a field release, and to prevent its spread to areas that have not officially permitted its use (Braig and Yan, 2001; James, 2005). The current suite of gene drive mechanisms is shown in Table 3.1. A gene drive system based on engineered underdominance has many of the properties of a desirable gene drive system.

Table 3.1 Summary of emerging gene drive technologies in insects. T= theoretical potential, D= developed, U= unsuccessful, F= field-testing.

Gene driver	Mode of Action	Development
Autonomous transposons	Encoded transposases move transgene(s) to new locations increasing their copy numbers within the genome	*Drosophila:* D= *P*-element[1] *Aedes aegypti:* U= no germline remobilisation[2]
Natural meiotic drive	Altered meiotic process results in preferential production of certain gametes	*Aedes aegypti:* U= naturally occurring allele discovered resulting in male-biased sex ratio, but not all wild-strains sensitive[3]
Maternal-effect-dominant embryonic arrest (*medea*)	Encodes maternally-expressed (ME) toxin and zygotically-expressed antidote; viable gametes from *medea*-carrying female must carry *medea*	*Drosophila:* D= using microRNA silencing of ME genes essential for embryogenesis[4]
Homing endonuclease genes (HEGs)	Induces targeted double strand break in homologous chromosome; cell's recombination repair mechanism uses the HEG as template	*Anopheles gambiae:* D= HEG against engineered GFP reporter gene[5] T= if HEG targets endogenous vector competence sequences
Wolbachia	Intracellular parasite inducing cytoplasmic incompatibility (CI); infected male mosquitoes are unable to fertilise uninfected eggs; population replacement occurs if infected males and females released	*Aedes aegypti:* D= CI and refractoriness[6] F= successful establishment of *Wolbachia*-infected *Ae. aegypti* in open field trial[7] *Anopheles stephensi:* D= CI and refractoriness to *Plasmodium*[8]
Engineered underdominance	Two mutually suppressing dominant lethal constructs inserted separately; offspring inheriting both or no constructs survive	*Drosophila:* D= related system based on maternal lethal effect and zygotic rescue[9]

[1] (Carareto *et al.*, 1997)
[2] (Sethuraman *et al.*, 2007; StJohn, 2012; Palavesam *et al.*, 2013)
[3] (Mori *et al.*, 2004)
[4] (Chen *et al.*, 2007)
[5] (Windbichler *et al.*, 2011)
[6] (Moreira *et al.*, 2009; McMeniman *et al.*, 2009; Kambris *et al.*, 2009; Bian *et al.*, 2010)
[7] (Hoffmann *et al.*, 2011)
[8] (Bian *et al.*, 2013)
[9] (Akbari *et al.*, 2013)

3.1.2 Gene drive based on underdominance

Underdominance describes a situation in which the heterozygous trait is less fit than either of the parent's homozygous traits. Therefore natural selection favours the homozygous traits, and if they are of equal fitness in a large randomly interbreeding population, the allele that is initially prevalent will become rapidly fixed, Figure 3.1.

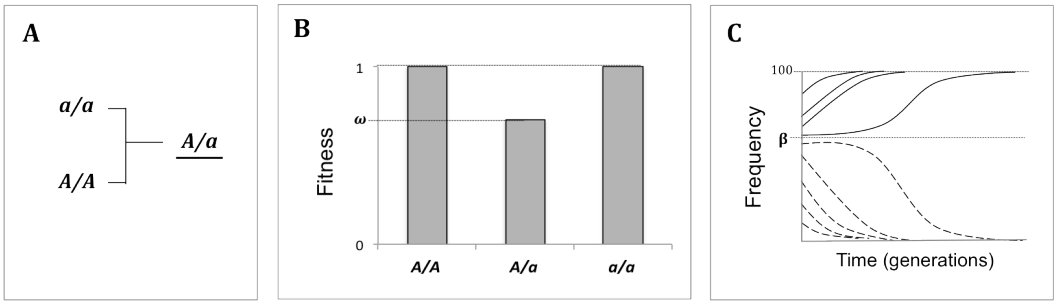

Figure 3.1 Classical underdominance. (A) In classical underdominance, when two alleles *A* and *a* appear at a single-locus in a diploid organism, heterozygous genotype *Aa* (underlined) is less fit (or inviable in extreme underdominance) than either homozygous genotypes. (B) Genotype configuration of underdominance when homozygous fitness= 1, and reduced fitness of heterozygous= ω ($1 > \omega \geq 0$); figure adapted from Altrock *et al.*, (2010). (C) Underdominance acts as a frequency-dependent drive system that has an unstable equilibrium frequency, β, above which the frequency tends towards fixation (100% prevalent) or an alternative equilibrium short of fixation, and below which the allele is lost from the population (Crow, 1986); figure adapted from Alphey, (2014). The strength of the tendency to fix alleles depends on the system's parameters, such as the fitness costs, which can be used to predict the threshold frequencies required to reverse the predominant allele in the wild.

Engineered underdominance based on reciprocal chromosomal translocations was first proposed in the 1960s as a method of vector control (Curtis, 1968). However, due to technical impracticalities, such as the inability to isolate reciprocal translocation strains, and limited success

with laboratory rearing of such strains due to low viability of offspring (Robinson, 1976), the system was not pursued.

More recently, Davis *et al.* (2001) proposed an engineered underdominance system based on a pair of constructs inserted at independently segregating loci. Each construct carries a lethal transgene and its promoter, a *trans*-acting suppressor, and the independently regulated refractoriness gene(s), Figure 3.2 (Davis *et al.*, 2001). The construct on the first chromosome only suppresses expression from the promoter on the second construct, and the reciprocal is also true. The same lethal gene can be used in both constructs. Insects carrying at least one copy of each construct will survive as both suppressors are present, and the independently regulated refractoriness gene(s) will be expressed. In a two-locus system, when a strain homozygous for both constructs mates with the wild strain, their offspring will be hemizygous[10] for both constructs. When these hemizygous insects mate with the wild strain, 25% of their progeny will carry both constructs and 25% will carry neither; both are viable genotypes. The remaining half of the progeny will carry only one of the constructs, and are consequently inviable. If inter-mating occurs with double homozygotes or double hemizygotes, a higher proportion of the progeny carry both constructs, and are viable assuming only one copy of each construct is sufficient to suppress two copies of the lethal effector.

Mathematical models predict that, assuming equal fitness of double homozygous traits, complete lethality of unsuppressed constructs, and

[10] Hemizygous insects have the transgene on one chromosome and do not have a corresponding allele on the other chromosome.

stable population size, a release ratio of one engineered insect per two native insects will lead to fixation of both constructs (Davis *et al.*, 2001; Gould *et al.*, 2008). If there is a fitness cost associated with the engineered strains, or if there is migration of engineered strains out of the area or wild-type immigrants into the local area, the release ratio required for fixation increases substantially (Gould *et al.*, 2008; Akbari *et al.*, 2013). In these circumstances, release over multiple generations permits a lower release ratio to achieve fixation (Magori and Gould, 2006; Akbari *et al.*, 2013).

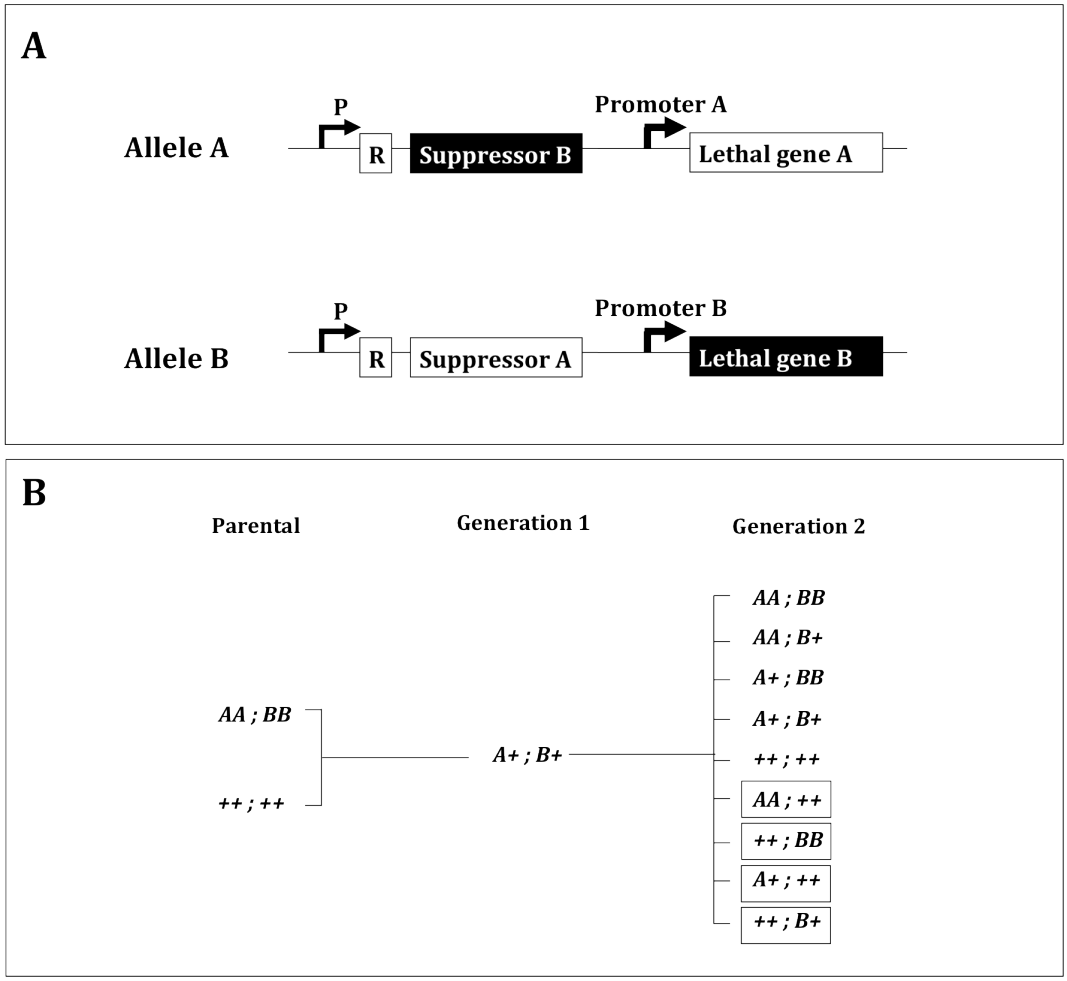

Figure 3.2 Engineered underdominance proposed in 2001 by Davis *et al.* (A) Two engineered constructs, A and B, are inserted at independently segregating loci; each construct carries a refractory genetic element 'R' driven by an independent promoter 'P', a lethal gene driven by a specific promoter, and a *trans*-acting suppressor. (B) Genotypes produced from double homozygous parents, either carrying engineered alleles A and B inserted at two unlinked loci (AA;BB), or wild-type alleles (++;++)*;* boxed genotypes are not viable due to the absence of the corresponding *trans*-suppressor, assuming that one suppressor element is adequate to suppress two copies of a lethal gene.

The high release ratio required for an underdominance system makes it more suitable for the control of local populations, such as for the control of *Ae. aegypti* in urban areas, where limited spread of the transgene is preferred (Sinkins and Gould, 2006). It is desirable for the insects to

establish themselves in the targeted area, but not for them to spread beyond this boundary. This is also beneficial from a regulatory perspective because accidental release of the transgenic mosquito would not result in population replacement (Magori and Gould, 2006; Sinkins and Gould, 2006). In addition, the system could be engineered to spread more than one refractory gene ideally with different modes of action that would make the evolution of resistant pathogens more challenging (Magori and Gould, 2006; Sinkins and Gould, 2006).

To date, the only existing synthetic underdominance-like system is known as maternal-effect lethal underdominance (UD^{MEL}). Unlike the proposed purely zygotic system (Davis *et al.*, 2001), UD^{MEL} consists of maternally expressed toxins and zygotically expressed antidote, and was successfully demonstrated in *Drosophila* (Akbari *et al.*, 2013). The system used embryogenesis-specific and maternally-expressed genes, tightly controlled maternal and early zygotic promoters, and engineered microRNAs – components that have been well characterised in *Drosophila* (Akbari *et al.*, 2013) but not yet in *Ae. aegypti*. The main difficulty with translating this approach to mosquitoes is the lack of suitable well-defined promoters to drive expression of each component of this complex system.

A similar system based on the expression of independently suppressible toxins driven by the same promoter is possible using current technology. Furthermore, although a bi-sex lethal underdominance system would be more efficient than female-specific lethality (Gould *et al.*, 2008), there are some benefits to using toxins that are only active in females, and passed from males to viable offspring at normal Mendelian ratios (Gould *et al.*, 2008; Akbari *et al.*, 2013). As only female mosquitoes blood feed and transmit disease, if a lethal effector gene under the control of a blood-meal

induced promoter is used, the resulting strain is expected to kill only females in the absence of the cross-suppressor. Female-specific lethality is likely to have lower fitness costs due to time- and tissue-specific expression (Gould *et al.*, 2008; Sinkins and Gould, 2006). Surviving females would carry the refractoriness gene essential for preventing the transmission of the virus, and males do not require the refractoriness because they do not feed on blood.

Therefore, identification of targeted promoters, and effective lethal genes, are key components of developing a synthetic underdominance system.

3.1.3 Dual use of RNAi as the suppressor and refractoriness element

I attempted to engineer underdominance in *Ae. aegypti* by integrating refractoriness into the gene drive itself, thereby overcoming potential loss of linkage between the drive mechanism and its refractoriness cargo, known as the 'linkage problem' (Curtis *et al.*, 2006). This pursuit was centred on the use of anti-pathogen RNA interference (RNAi), and the inclusion of the target sequence for the RNAi in a suitable region of the lethal expression system, Figures 3.3 and 3.4. RNAi against the pathogen's genome would knockdown the viral transcripts, preventing transmission of the pathogen, and would also knockdown the lethal transcripts containing the same target sequence, preventing lethal expression of the effector. Loss of refractoriness precludes rescue from the killer. The ability to spread multiple anti-pathogen microRNAs in both constructs (*e.g.* Chen *et al.*, 2007) is another valuable characteristic to counteract mutational

inactivation of the lethal effector, or the development of resistance or evasion by the pathogen.

For RNAi to be an effective refractoriness element, it is essential for the system to come into contact with the pathogen's RNAs. In *Ae. aegypti*, early experiments using anti-dengue RNAi in adult mosquitoes were ineffective when the anti-viral RNAs were not produced in relevant tissues (Olson *et al.*, 1996; Travanty *et al.*, 2004). Therefore, understanding the transmission pathway of the pathogen is important for identifying appropriate tissue- and time-specific promoters for use in our proposed system. Furthermore, it is essential that the lethal effector's mRNA is also available to the RNAi concurrently. This is potentially achievable by using the same promoter for the lethal as for the RNAi element. The RNAi molecule would be in tissues where it could stop viral replication and also in tissues where the lethal effectors are expressed for the underdominance gene drive system.

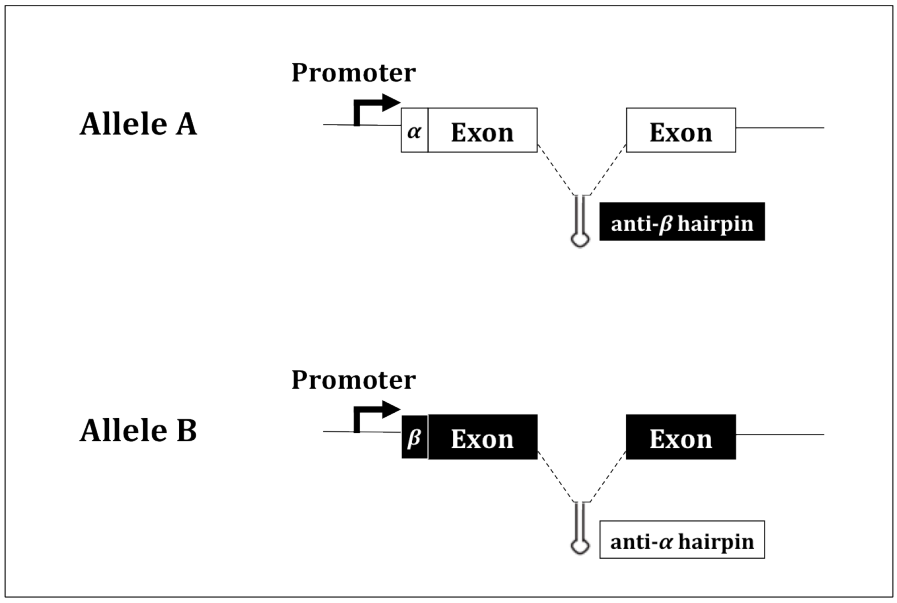

Figure 3.3 Proposed underdominance system combining refractoriness and suppressor elements using RNA interference (RNAi). Two engineered constructs, A and B, inserted at independently segregating loci. Allele A carries a lethal gene whose intron (dashed lines) includes the anti-α hairpin, which is spliced from the pre-mRNA and forms RNA-Induced Silencing Complex targeting the α sequence on Allele B (and vice versa); α and β target sequences are essential regions of the pathogen's genomic sequence, so RNA interference is against the pathogen and is the *trans*-acting suppressor against the lethal fusion mRNA. Promoters (black arrows) are the same on both constructs. In principle it is possible to use a bidirectional promoter and re-position or add hairpin sequences accordingly.

Transgenic *Ae. aegypti* RNAi lines are available that resist infection by the dengue serotype 2 virus (DENV2) when the endogenous anti-DENV2 RNAi pathway is triggered in the mosquito midgut (Franz *et al.*, 2006), fat-body (Franz, A.W.E., Sanchez-Vargas, I., and Olson, K.E., unpublished data), or salivary glands (Mathur *et al.*, 2010). The RNAi targets the DENV2 pre-membrane transcript, which is essential for virion assembly (Chambers *et al.*, 1990; Murray *et al.*, 1993). Expression of this anti-DENV2 target sequence (referred to as DEN2) is driven by the midgut-specific *carboxypeptidase A* promoter *(carb)* in strain CarbA, the fat-body-specific *vitellogenin 1 (vit)* promoter in strain Vg40, and salivary-

gland-specific *30K* promoter in strain 30Kb. These three promoters are induced by a blood meal, with peak expression at approximately 24 hours post blood meal (Cho and Raikhel, 1992; Edwards *et al.*, 2000; Hamblin *et al.*, 1987; Romans *et al.*, 1995; Wyatt, 1980; Yoshida and Watanabe, 2006; Adelman *et al.*, 2001; Franz *et al.*, 2006; Gaines *et al.*, 1996; Isoe *et al.*, 2009).

As an extension of this work, I focused on using these tissue- and time-specific promoters in the lethal systems described below.

The *30K* promoter was not investigated as expressing an effector molecule in the salivary gland tissues to produce a mosquito-killing effect seemed particularly problematic. The presumed need to use non-cell-autonomous (*i.e.* secreted) effectors to distribute the lethal proteins to more sensitive tissues and organs raised concerns in limiting secretion exclusively to the haemolymph and the likely presence of such effectors in the engineered mosquitoes saliva.

DENV2 titre was not reduced to the same extent in Vg40 females' salivary glands as in CarbA and 30Kb (Franz, A.W.E., Sanchez-Vargas, I., and Olson, K.E., unpublished data). This is not surprising, given that DENV2 disseminates to the female fat-body 48 hours after a blood meal (Salazar *et al.*, 2007), which is 24 hours after the vitellogenin promoter's peak activity. Nevertheless, Vg40 and CarbA lines and their respective promoters were used to investigate the use of anti-DENV2 RNAi against lethal transcripts as components of the proposed underdominance system.

The lethal systems in this chapter consisted of a two-component tetracycline-repressible system (Gossen and Bujard, 1992) shown to be highly penetrant in *Ae. aegypti* (Fu *et al.*, 2007), with these two parts on separate constructs and insertions. The first part consisted of a specific

promoter (*carb* or *vit*) controlling the tetracycline-repressible *trans*-activator (tTAV), and the second part contained the effector gene under the control of the tTAV response element (tetO). In this tTAV-tetO expression system, upregulation of the tetO-effector, induced by tTAV expression, is repressed in the presence of tetracycline (tet). Tet binds to the tTAV preventing its interaction with tetO. Relative to the use of simple promoter-effector constructs, this facilitates more efficient testing of large quantities of promoter-tTAV and tetO-effector combinations. In addition, the tet-repressibility of the lethal expression system facilitates generation of viable lethal strains.

To enable RNAi-mediated knockdown of the lethal transcripts, the DEN2 target sequence (Franz *et al.*, 2006) was fused as an RNAi target to the lethal constructs' tTAV, creating the dual-purpose for the RNAi construct. Specifically, the RNAi is a targeted suppressor against the lethal system and is the anti-dengue refractoriness element, shown in Figure 3.4.

A variety of lethal effector molecules were considered, each with different modes of action such as secreted excitatory neurotoxins (Higgs *et al.*, 1995; Bosmans and Tytgat, 2007; Moran *et al.*, 2007), secreted proteolytic enzymes (Barillas-Mury and Wells, 1993; Edwards *et al.*, 2000), inducers of apoptosis (Zhou *et al.*, 2005; Olson, 2003a and 2003b; Igaki *et al.*, 2002), an inhibitor of RNA processing, transcription and cell cycle progression (Van Eynde *et al.*, 2004; Beullens *et al.*, 1992; Parker *et al.*, 2002*;* Brand *et al.*, 1994), and the regulator of metabolic processes, insulin-like protein-2 (Riehle *et al.*, 2006). The secreted effector molecules were expected to cause additional damage to neighbouring tissues, amplifying any lethal effects, whereas non-secreted effectors were expected to cause fitness-reducing damage in the targeted tissues.

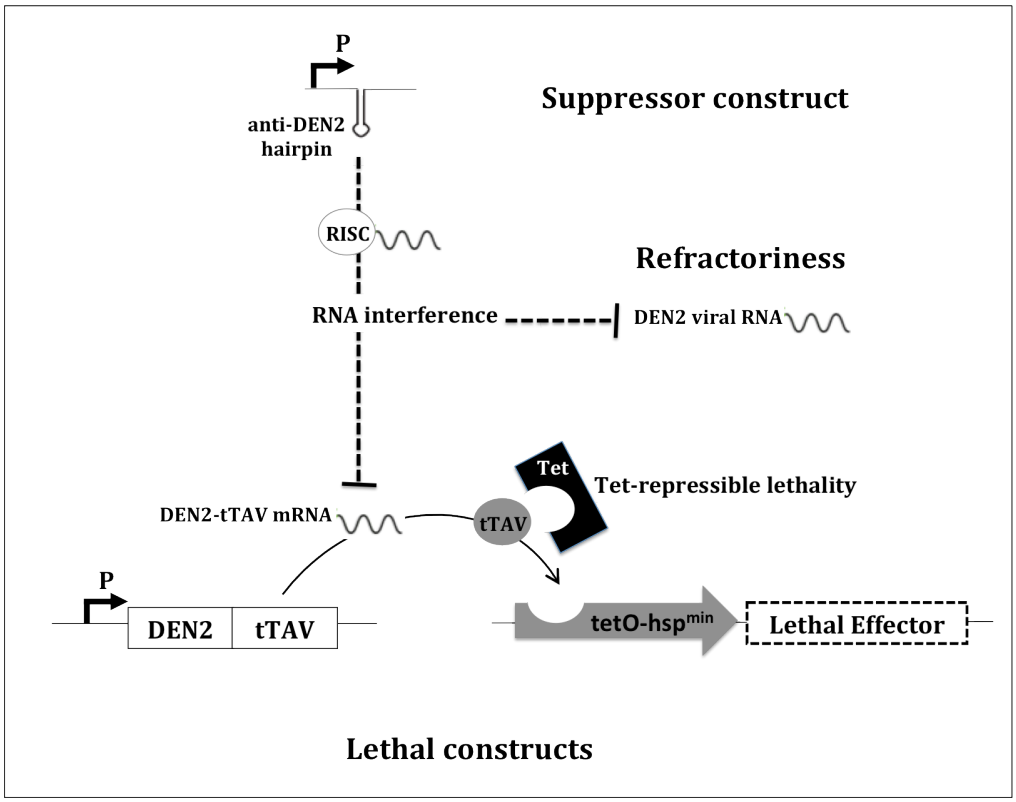

Figure 3.4 Dual use of RNA interference (RNAi) as a targeted suppressor of a two-component lethal system, and as an anti-dengue refractoriness element. The suppressor construct, a double stranded RNA hairpin consisting of an inverted DEN2 repeat sequence regulated by promoter (P), forms RNA-Induced Silencing Complex (RISC) targeting the DEN2 RNA of the virus and of the DEN2-tTAV fusion mRNA. The lethal constructs consist of the DEN2-tagged tetracycline transactivator (tTAV) also under the control of P. The tTAV binds to the tetracycline responsive operator (tetO) that drives expression of the lethal effector. This *trans*-activation is repressed in the presence of tetracycline (tet).

The work in this chapter examines the ability of stage- and tissue-specific promoters, *vit* and *carb*, and a panel of effectors, linked *via* the tTAV-tetO system, to induce a lethal phenotype in *Ae. aegypti*, and whether these effects can be suppressed by RNAi-mediated knockdown when expressed under the control of the same promoter.

3.1.4 Experimental aims

The central goal of this project was to assemble and test the components of an underdominance gene drive system based on RNAi in *Ae. aegypti*. Primarily, novel and existing effector strains were screened for their suitability by ensuring they induced lethality under the control of the strong, early-acting *hex*[11] promoter. Once identified, fitness-reducing effects in *Ae. aegypti* under the control of the *vit*[12] or *carb*[13] (the 'killer') were assessed and rescue of these fitness effects by RNAi was evaluated. Efforts were focused on *vit* and its ability to drive tissue- and time-specific expression of tTAV, and its ability to induce a fitness-reducing phenotype when used to drive expression of an effector.

Experiments were designed to test the following six hypotheses

(i) *Upregulation of the DEN2-tTAV*[14] *driven by* vit *is induced by a blood meal*

(ii) *Under the control of* vit*, RNAi knocks down the blood-meal induced upregulation of DEN2-tTAV mRNA in transgenic females*

(iii) *The tetO-effector construct alone imposes a significant fitness penalty on* Ae. aegypti *(for each effector)*

(iv) *Upregulation of the effectors driven by the strong, early-acting promoter,* hex*, results in larval lethality off tet*[15]

(v) *Upregulation of the effectors driven by vit and carb results in lethality in insects carrying both promoter-effector constructs off tet*

(vi) *The RNAi construct directed against the DEN2-tTAV suppresses the phenotype of lethal constructs under the control of the same promoter*

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[11] *Hex= Aedes altropalpus hexamerin 1.2* promoter
[12] *Vit= Ae. aegypti vitellogenin 1* promoter
[13] *Carb= Ae. aegypti carboxypeptidase A* promoter
[14] DEN2-tTAV= tTAV fused to the anti-dengue2 RNAi target sequence (DEN2); tTAV is the tetracycline repressed *trans*-activator of the lethal expression system
[15] Tet= tetracycline

3.2 Methods

3.2.1 Mosquito rearing

Ae. aegypti strains were reared according to standard operating procedures described in Chapter 2. They were kept at 26°C and 80% humidity with a 12:12 hour light:dark cycle and constant air circulation. Larvae were fed powdered TetraMin® Ornamental Fish Flakes (Tetra GmbH, Germany) *ad libitum* unless a feeding regimen was adhered to as stated in following sections. When in use, the feeding regimen per larva was as follows: 0.08 mg on day 1, 0.48 mg on day 3, 0.32 mg on days 5-10, and 0.06 mg on day 12.

3.2.2 On or off tetracycline experiments

Chlortetracycline hydrochloride (Sigma-Aldrich, Gillingham, UK) is referred to herein as tetracycline or tet. To ensure that any differences in the phenotypes of individuals reared either on or off tet were not due to the differences between the treatment and storage of the eggs, each egg paper was halved and one half was reared using each rearing condition. For those reared on tet, larval rearing water contained 30 μg ml^{-1} tetracycline. On-tet adult females were also reared on sugar water made using 30 μg ml^{-1} tetracycline, and provided with blood containing 30 μg ml^{-1} tetracycline.

3.2.3 Transformation markers

To identify transgenic insects, third instar larvae (L3) or pupae were screened for the presence of fluorescent markers as described in Section 2.3.

3.2.4 Background of *Ae. aegypti* transgenic promoter-tTAV strains

The molecular constructs OX4438 and OX4414 are *piggyBac*-based constructs containing a DEN2-tagged tetracycline-repressible *trans*-activator (DEN2-tTAV) gene under the control of the *vit* or *carb* respectively, and the transformation marker Hr5ie1-AmCyan. The OX4531 construct is a *piggyBac*-based construct containing *tTAV* under the control of the *Aedes altropalpus hexamerin 1.2 (hex)*, with the Hr5ie1-AmCyan transformation marker. Sarah Scaife cloned constructs according to standard molecular techniques, and Pamela Gray developed transgenic *Ae. aegypti* strains OX4438 Line A2, OX4414 Line F, and OX4531 Line C1.M3 according to standard germline transformation techniques described in Section 2.2.

3.2.5 Background of transgenic *Ae. aegypti* promoter-RNAi strains

The *Ae. aegypti* lines Carb109 and Vg40 were provided by project partner labs (James, A.A., University of California, Irvine; Olson, K.E., Colorado State University, Fort Collins). These lines carry the DEN2 hairpin under the control of the *carb* and *vit* respectively, and the transformation marker 3xP3-EGFP (Franz *et al.*, 2006; Olson, K.E., unpublished data).

3.2.6 Quantification of tTAV transcripts under the control of *vit*

Both quantitative real-time PCRs described below were carried out as described in Section 2.11, using the same primers and probes purchased from Invitrogen (Life Technologies, USA) and Eurofins (UK) respectively. DEN2-tTAV primers: 5'-GGGCCTTGATTTTCATCTTACTGACA-3' and 5'-GTCTTGCCGGTCAGGGTCTTG-3', DEN2 Probe: 5'-VIC-GTCGCTCCTTCAATGACAATTGCGTTGC-BHQ. The 18S ribosomal gene was used as an endogenous control; 18S primers: 5'- ACGCGAGAGG TGAAATTCTTG and 5'-GAAAACATCTTTGGCAAATGCTT, 18S probe: 6-Fam-CCGTCGTAAGACTAAC-MGB. Each biological sample (n=3) contained four pooled headless hemizygous insects. RNA was extracted from headless adults because the fluorescence emitted by endogenous eye pigments were thought to interfere with the PCR reaction in adults (Eckhart *et al.*, 2000). There were three technical repeats of each PCR reaction per biological sample.

To test whether *vit* was able to upregulate the expression of tTAV, relative levels of tTAV mRNA were calculated in hemizygous OX4438 transgenic females before, and up to 48 hours after a blood meal. Hemizygous transgenic OX4438 males were also included for comparison.

To test the ability of the RNAi to silence this upregulation, OX4438 males were crossed to RNAi line Vg40. To minimise any environmental inconsistencies between repeats, rearing conditions were standardised; three repeat trays were set up, each with larvae reared at 0.5 larvae ml^{-1}, and the feeding regimen described in Section 3.2.1 was adhered to. Females of each genotype (wild-type, OX4438, Vg40, and OX4438-Vg40) were pooled into separate cages. To test RNA interference of tTAV expression,

the levels of tTAV mRNA in these females were quantified before a blood meal, 24 hours after, and 48 hours after a blood meal.

3.2.7 Developing a panel of suitable effectors

3.2.7.1 Background of existing *Ae. aegypti* transgenic tetO-effector strains

Existing single-insertion effector lines developed at Oxitec Ltd, listed in Table 3.2, were obtained from enriched stocks. These were outcrossed to wild-type to obtain hemizygous insects, which were used for subsequent experiments.

All *attB* lines were generated by injection into an *attP* acceptor strain generated by Nimmo *et al.* (2006). Each carries a 21-tetO repeat and open reading frame for the listed effector. The remaining three are *piggyBac* insertion lines carrying the reaper^{KR} effector's open reading frame with variations in the number of tetO repeats and the minimal promoter used.

All effector lines carry the DsRed2 transformation marker regulated by the 3xP3 or Hr5Ie1 promoter.

Table 3.2 Panel of existing tetO-effector lines tested; *pB*= *piggyBac*-based transposon; *attB*= ΦC31 attachment site; tetO(x)= the number of tetO repeats; hsp= *Drosophila melanogaster* heat shock protein minimal promoter regions; adh= short intron from the *D. melanogaster* alcohol dehydrogenase gene included to improve expression levels; ubi= short coding sequence from *D. melanogaster* ubiquitin sequence added to allow N-terminal fusions to tTAV.

Line	Details	Description
OX3547[1]	*attB*-3xP3-DsRed2-tetO21-hsp70-adh-NIPP1	TetO-NIPP1
OX3582[1]	*attB* -3xP3-DsRed2-tetO21-hsp70-adh-*Ae.*michx	TetO-michelob_x
OX4230[1]	*attB*-3xP3-DsRed2-tetO21-hsp70-adh-*Ae.*trypsin-secreted	TetO-trypsin
OX4291[1]	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-carbA	TetO-carboxypeptidase
OX4292[1]	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-*Ae.*ILP2g	TetO-ILP
OX4293[1]	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-*Ae.*eiger	TetO-eiger
OX4393[1]	*pB* Hr5IE1-DsRed2-tetO14-hsp70-adh-reaper^{KR}	TetO-reaper^{KR}
OX4423[2]	*pB* Hr5IE1-DsRed2-tetO7-hsp83-ubi-reaper^{KR}	TetO-reaper^{KR}
OX4536[2]	*pB* Hr5IE1-DsRed2-tetO7-hsp70-ubi-reaper^{KR}	TetO-reaper^{KR}

[1]Developed by Pamela Gray and Sarah Scaife
[2]Developed by Oliver StJohn and Sarah Scaife

3.2.7.2 Generation of new effector lines OX4509, OX4711, and OX4712

Standard germline transformation techniques described in Section 2.2 were used to generate additional *Ae. aegypti* effector lines listed in Table 3.3.

Table 3.3 AaHIT Scorpion- and Av3 sea-anemone-toxin effector constructs injected; *pB*= *piggyBac*-based transposon; tetO(x)= the number of tetO repeats; hsp= *Drosophila melanogaster* heat shock protein minimal promoter regions; gp67 =secretory signal; adh= short intron from the *D. melanogaster* alcohol dehydrogenase gene included to improve expression levels.

Line	Details	Description
OX4509[1]	*pB* Hr5IE1-DsRed2-tetO14-hsp70-adh-gp67-AaHIT	TetO-scorpion Toxin
OX4711[1,2]	*pB* Hr5IE1-DsRed2-totO14-hsp70-adh-gp67-Av3	TetO-sea anemone toxin
OX4712[1]	*pB* Hr5IE1-DsRed2-tetO14-hsp70-adh-gp67-Av3P25A	TetO-mutant sea anemone toxin

[1]Sarah Scaife built constructs according to standard molecular techniques
[2]Kelly Matzen performed injections of this construct

3.2.7.3 Investigating the lethality of tetO-effectors under the control of *hex*

Hemizygous OX4531 males (line C1 M3) were crossed to the hemizygous single-insertion effector lines selected in Section 3.3.2.2.

Offspring were hatched and tetracycline was added to on-tet trays two hours later. Larvae were aliquoted and reared at 1 larvae ml^{-1} (n= 3 trays per treatment). Seven days later, L4 larvae were screened for the fluorescent transformation markers to identify the proportion of each genotype, Figure 3.5.

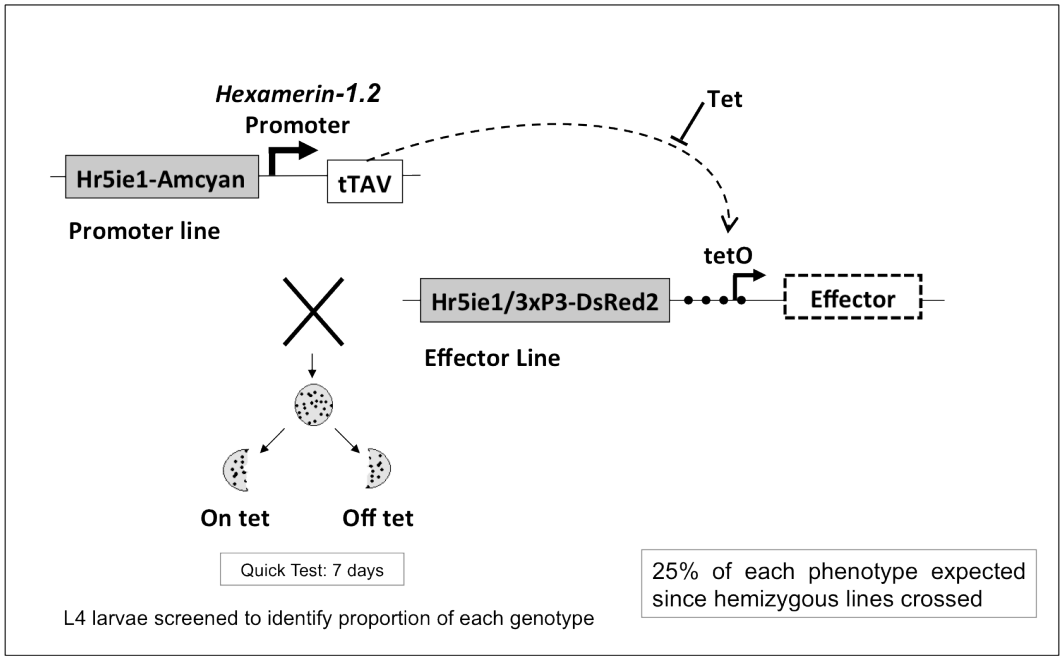

Figure 3.5 Diagram to show the *hex*-effector crosses. Fluorescence markers are in grey boxes. The hex promoter line carries the transformation marker Hr5ie1-AmCyan. Effector lines carry Hr5ie1- or 3xP3-DsRed2 transformation markers, a tetracycline responsive element that contains repeats of the tetracycline operator (tetO) sequence, to which tTAV binds, a minimal promoter (thin black arrow), and under its control, the effector gene (dashed box). The promoter and effector lines were crossed (the X means 'crossed to'), and their eggs (circular dotted disks that represent eggs laid onto a filter paper, known as 'egg-papers') were used for on or off tetracycline experiments; each egg paper was cut in half as depicted by half-circular dotted disks, and each half was reared either on or off tetracycline.

3.2.8 Investigating the lethality of tetO-effectors under the control of *vit* and *carb*

3.2.8.1 Pilot study: Effects of NIPP1 or michelob_x under the control of *vit*

The *vit* line, OX4438 A1, was crossed to (i) the NIPP1 effector line, OX3547, and (ii) the michelob_x effector line, OX3582. For each line, fluorescent individuals were selected from a population containing

homozygous, hemizygous and wild-type, and so may have been homozygous or hemizygous. Egg papers were collected and halved. Each half was used for either on or off tetracycline conditions, described previously.

Three hundred L3 larvae of each genotype (carrying either OX4438 only, the effector construct only, both OX4438 and the effector constructs, or neither) were screened and then reared separately at 0.3 larvae ml^{-1}. Percentage pupation from L3 larvae, and the percentage of these pupae that eclosed as female adults were recorded. Adult females were blood fed three days after the last adult eclosed, and fully engorged females were removed and placed into fresh cages. The number of dead adults was recorded daily for up to ten days and survival analysis (Section 3.2.10) was performed.

To investigate the fecundity of females, thirty females of each genotype were blood fed in pools, n= 3. Virgin wild-type males, two to five days of age, were added to all cages, with one male per three females. Eggs were collected for three gonotrophic cycles. Before each egg collection, dead females were counted, removed from cages, and excluded from the analysis.

3.2.8.2 Revised experimental design: lethality under the control of *vit* and *carb*

The OX4438 *vit* hemizygous individuals were crossed to the following hemizygous effector strains: line OX3582 (michelob_x), OX4711 (Av3) line D5, OX4509 (AaHIT) line H2, line OX4230 (trypsin), and OX4393 line 12 ($reaper^{KR}$). The hemizygous OX4414 *carb* line F was crossed to the following hemizygous effector lines: OX4711D5, OX4509H2, and OX4230.

As before, egg papers were halved. These were hatched separately in 200 ml of filtered water. Three hours later, tet was added to one half to a final concentration of 30 μg ml^{-1}. 1000 L1 larvae were aliquoted into trays (n= 3 per treatment), and reared at 1 larvae ml^{-1}. Pupae, instead of larvae, were picked daily to control development time and to minimise handling. Pupae were screened for both promoter and effector transformation markers, blue (AmCyan) and red (DsRed2) fluorescence respectively, and sexed. In parallel, wild-type larvae were hatched and aliquoted into separate trays and reared at the same density with no tet, and males were kept for crosses. Larvae were fed powdered TetraMin® Ornamental Fish Flakes (Tetra GmbH, Germany) according to the feeding regimen described in Section 3.2.1.

Male pupae were placed in small pots to eclose, and the females placed in separate cages; adult eclosion rates were compared. Female adults were blood fed three days after the last adult eclosed, and to enable synchronous blood feeding, sugar was removed from the females' cages the day before. To prevent damage to engorged females, insects that had not taken a blood meal were removed from the cages and were excluded from the analysis.

On the same day, wild-type males were added to cages at a 0.5:1 male:female ratio. Dead females were counted and removed from cages daily until the end of the experiment, with particular attention paid to 20-28 hours after a blood meal, which corresponds to the promoters' peak activities.

Egg papers were placed in cages and left overnight for females to lay Females were blood fed and progeny collected for a total of three gonotrophic cycles; non-engorged females were separated from cages after

each blood meal. Female fecundity (number of eggs per female) and fertility (egg hatch rates) in the first gonotrophic cycles were compared to investigate any secondary effects of the effectors.

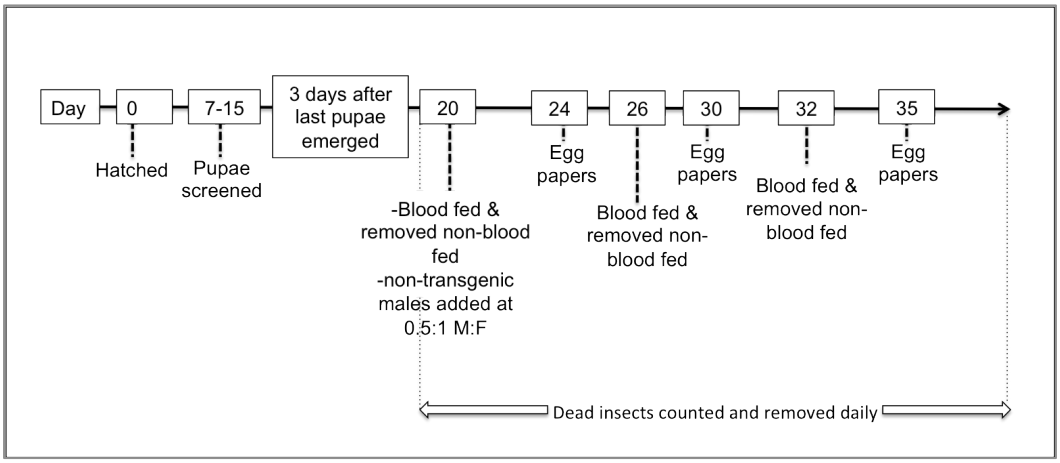

Figure 3.6 Summary of 35-day experiment to investigate *vit*- and *carb*-induced lethality

3.2.9 Investigating the ability of RNAi to suppress *vit*-induced fitness costs

Crosses were conducted as shown diagrammatically in Figure 3.7 to obtain individuals carrying lethal and RNAi suppressor constructs.

The promoter (P+) and RNAi (R+) lines were crossed; absent transgenic alleles are denoted by '+'. Progeny were screened and males carrying both constructs (P+;R+), as distinguished by their fluorescence patterns, were crossed to hemizygous females of the effector line (E+);. Progeny were reared in the absence of tet; 2000 larvae were aliquoted in trays, n= 3. Larvae were fed the same feeding regimen as before (Section 3.2.1). Pupae were screened for fluorescent markers and the presence of lethal phenotypes was investigated as described in Section 3.2.8.2.

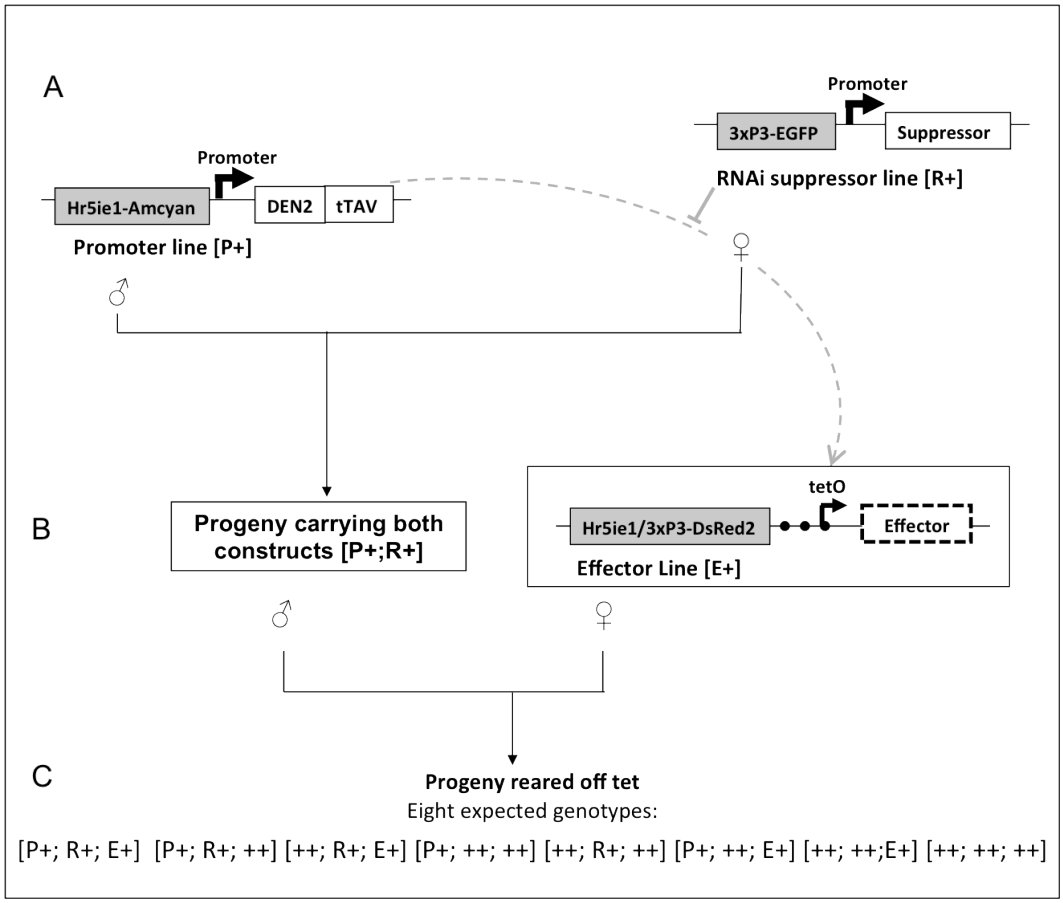

Figure 3.7 Schematic of crosses to investigate the ability of RNAi to rescue lethal effects of constructs. Absent transgenic alleles are denoted by '+'. Fluorescent markers are in grey boxes. The hemizygous promoter line (P+) carries Hr5ie1-AmCyan and *vit* (thick arrow), which directs the expression of tTAV with the DEN2 tag after the start codon (dashed grey arrow). The hemizygous effector line (E+) carries Hr5ie1-DsRed2, a tetracycline responsive element that contains repeats of the tetracycline operator (tetO) sequence -to which tTAV binds- and minimal promoter (thin black arrow); under its control, the effector gene (dashed box). The RNAi line (R+) carries the 3xP3-EGFP, and the DEN2 RNAi construct (suppressor) under the control of *vit*. The RNAi line rescues the lethal phenotype by degrading the DEN2-tTAV transcripts, thus preventing binding of the mRNA to the tetO sequence. To test this: (A) P+ males and R+ females were crossed; (B) P+;R+ male progeny were crossed to hemizygous E+ females and (C) progeny were aliquoted and investigated for lethal effects of the eight genotypes.

3.2.10 Statistical analysis

The Pearson's chi-squared (χ^2) analysis was used to compare observed and expected genotype ratios (Pearson, 1900).

When variables met the assumption of normality as assessed by the Shapiro-Wilk's test, pupation and eclosion rates were compared using one-way ANOVA between more than two groups, or the t-test between two groups (*e.g.* between binary sex variable) using the IBM SPSS Statistics program (version 21.0; SPSS Inc., Chicago, USA). Percentages and rates were converted to arcsine square root values before the analysis. When necessary, if variances between groups were equal and homogeneity of variance was met as assessed by the Levene's test, the Tukey's HSD *post-hoc* test was used. Welch's ANOVA was used for unequal variances and when required the Games-Howell *post-hoc* test was used to identify differences between groups.

Kaplan Meier survival analysis was carried out using the 'Rcmdrplugin.survival' package in the statistical program 'R for Mac OS X Version 1', available publicly from http://cran.r-project.org.

3.3 Results and discussion

3.3.1 Expression of tTAV under the control of *vit*

Hypothesis (i): Upregulation of the DEN2-tTAV driven by vit *is induced by a blood meal*

Hypothesis (ii): Under the control of vit*, RNAi knocks down the blood-meal induced upregulation of DEN2-tTAV mRNA in transgenic females*

To quantify upregulation of tTAV driven by *vit* in OX4438, the changes in tTAV mRNA levels in transgenic females before a blood meal and up to 48 hours later was investigated, Figure 3.8. Peak expression levels of tTAV in females occurred 24 hours after a blood meal, consistent with previous results (Cho and Raikhel, 1992). Low levels of basal expression in males and non-blood-fed females were also observed. Therefore the null hypothesis stating that there is no significant upregulation of tTAV under the control of Vg in females 24 hours after a blood meal was rejected.

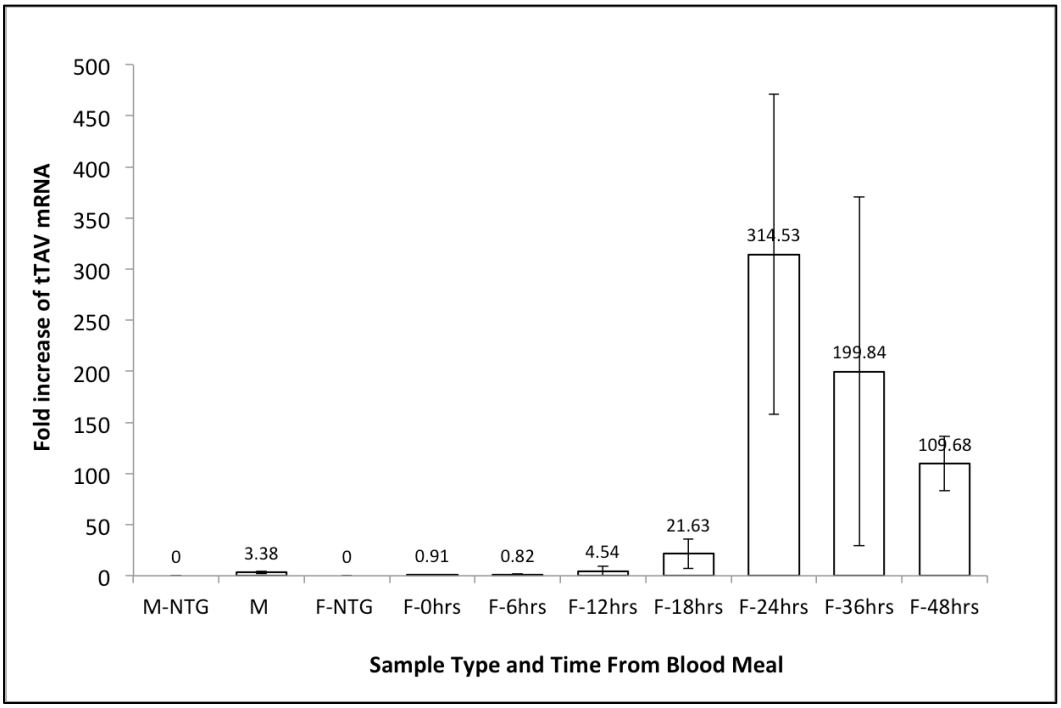

Figure 3.8 Fold change of tTAV mRNA under the control of *vit* in OX4438 insects. M= male, F= female, NTG= non-transgenic (wild-type), hrs= hours post blood feeding, 0hrs= before blood feeding. NTG samples were negative controls. There was baseline expression of the transgenic construct in males and females and upregulation in females 24 hours after a blood meal. Error bars show the standard error of the mean of three biological replicates of RNA extracted from pools of four individuals; real-time PCR reactions were performed in triplicate per biological replicate.

This experiment confirmed the peak activity of the vitellogenin promoter in line OX4438. To test the ability of RNAi silencing of this upregulation, levels of DEN2-tTAV mRNA before and 24 hours after a blood meal in female progeny of OX4438 crossed to RNAi line Vg40 were compared.

As shown in Figure 3.9, there was significant upregulation of the tTAV mRNA in OX4438 females 24 hours after a blood meal, and this upregulation was suppressed, or knocked down, in the presence of the RNAi construct regulated by the same promoter. Therefore the null

hypothesis stating no difference between the levels of tTAV mRNA in the presence and absence of the RNAi construct driven by the same promoter was rejected.

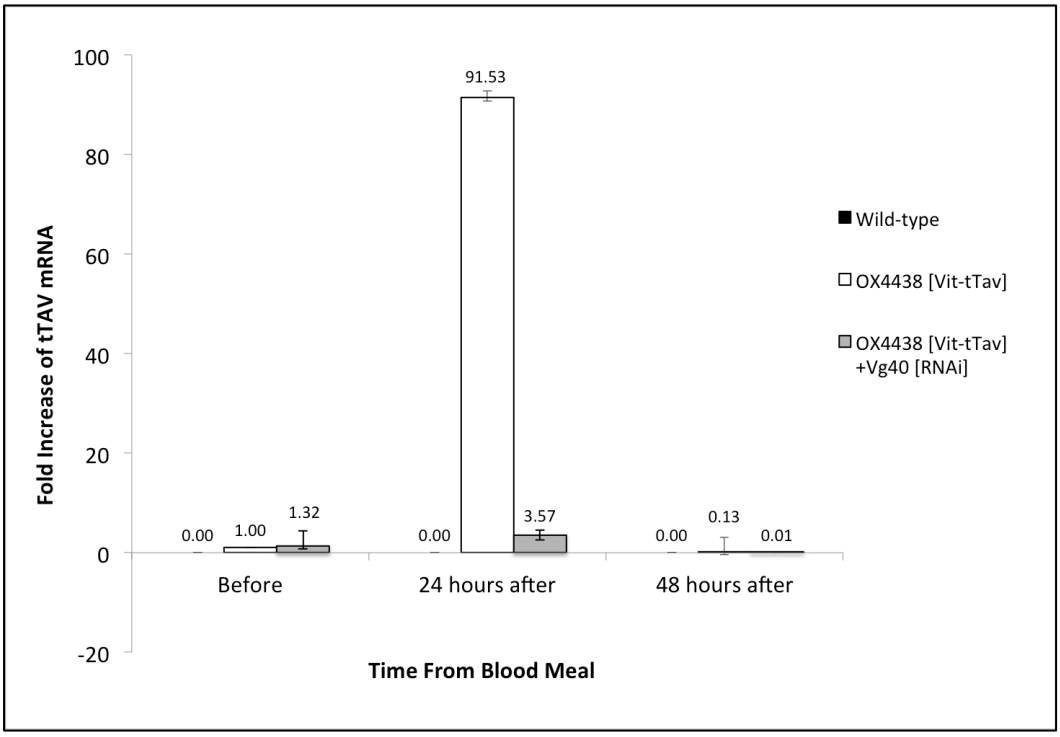

Figure 3.9 Fold change of tTAV mRNA under the control of *vit* in OX4438 and OX4438-Vg40 individuals before a blood meal, 24 hours after, and 48 hours after a blood meal. Expression levels are reported relative to the expression in females of the OX4438 line before blood feeding and normalised against the 18S gene. Error bars show the standard error of the mean of three biological replicates on RNA extracted from pools of four individuals; real-time PCR reactions were performed in triplicate per biological replicate. Non-transgenic samples (wild-type) were used as negative controls of the DEN2-tTAV fusion protein's gene, and are shown at each time point for comparison.

These results suggest that other transcripts with the same DEN2 tag, regulated by *vit*, may be suppressed by the same anti-DEN2 RNAi construct, and Vg40 was considered a good suppressor candidate for the proposed system.

3.3.2 Developing a panel of effectors

3.3.2.1 Generation of new effector lines OX4509 (tetO-AaHIT), OX4711 (tetO-Av3), and OX4712 (TetO-Av3P25A)

Initially, 1897 embryos were injected with 300 ng/µl OX4712 plasmid DNA and 700 ng/µl *piggyBac* transposase mRNA, and 889 embryos were injected with the same concentration of OX4711 plasmid DNA and *piggyBac* transposase mRNA. Unexpectedly, there was only 4% survival of injected OX4711 G0s, and no surviving OX4712 G0 progeny. Given the high volume of injections carried out simultaneously for Chapter 4 with approximately 10% survival of injected G0s to adulthood, the unusual embryo lethality was likely due to transient expression of the sea anemone toxins in the embryos, causing lethality. This was also suggested for low survival of OX4509 injected G0s experienced by StJohn (2012) when injected at the same DNA and mRNA concentrations.

To facilitate the generation of OX4712 and OX4509 lines, plasmid concentrations in the injection mixes were halved, using endonuclease free water as the diluent. Table 3.4 summarises the concentrations of plasmid DNA used, the number of embryos injected with OX4711, OX4712, and OX4509 and surviving G0s.

The percentage of survived G0s was still lower than usual, but adequate to establish transgenic lines. G0s were not screened for transient expression of the fluorescent marker to minimise stress endured by survivors. In total three, three and five independent lines of OX4711, OX4712 and OX4509 respectively were established, each originating from separate G0 pools.

Table 3.4 Summary of OX4509, OX4711, and OX4712 injections

Construct	DNA Conc.	Embryos injected	Survived G0s	TG pools/total G0 pools	Lines established[2]
OX4711[1]	300 ng/µl	1897	77	6/10	3
OX4712	300 ng/µl	889	0	0	0
OX4712	150 ng/µl	1540	66 (4.3%)	4/9	3
OX4509	150 ng/µl	980	97 (9.9%)	6/9	5

[1]Kelly Matzen performed injections of this construct.
[2]Each line originated from a separate G0 pool.

3.3.2.2 Mendelian inheritance of effector transgenes

Hypothesis (iii): The tetO-effector construct alone imposes a significant fitness penalty on Ae. aegypti *(for each effector)*

Prior to testing for lethality, hemizygous effector lines, including the new OX4509, OX4711 and OX4712 lines and those listed in Table 3.2, were crossed to the wild-type. The Mendelian inheritance of the fluorescent markers was investigated to ensure that the lines carried a single insertion, and that there was no significant fitness cost of carrying the construct alone.

The results shown in Table 3.5 indicated that the transgenic (TG): non-transgenic (NTG) ratios produced by existing stock lines — OX3547, OX3582, OX4230, OX4291, OX4292, OX4293, OX4393, OX4423, OX4536 — were not significantly different from the expected 1:1 ratio. These lines were previously selected for stock due to their desirable characteristics

(Gray, P., unpublished data), such as Mendelian inheritance patterns and strength of fluorescent markers, and so this outcome was expected.

Table 3.5 Mendelian inheritance ratios of effector transgenes. χ^2 was used to quantify the significance of the difference between observed transgenic (TG):non-transgenic (NTG) ratio and the expected 1:1 ratio according to Mendelian inheritance predictions, where $p<0.05$ represents a significant difference (*). OX3547= tetO-NIPP1; OX3582= tetO-michelob_x; OX4230= tetO-trypsin; OX4291= tetO-carboxypeptidase; OX4292= tetO-ILP; OX4293= tetO-eiger; OX4393, OX4423 and OX4536= tetO-reaperKR; OX4509= tetO-AaHIT scorpion toxin; OX4711= tetO-Av3 sea anemone toxin.

Effector lines		TG: NTG	χ^2 (df= 1)	p-value	Effect size (Φ)
OX3547[1]		238:221	0.63	0.73	
OX3582[1]		232:233	0	0.999	
OX4230[1]		123:139	0.98	0.614	
OX4291[1]		167:157	0.31	0.857	
OX4292[1]		191:185	0.1	0.953	
OX4293[1]		187: 215	1.95	0.377	
OX4393[1]	12	125:130	0.1	0.952	
OX4423	2	264:292	1.41	0.494	
	3	256:292	2.36	0.307	
OX4536[1]	A	203:217	0.47	0.792	
OX4509	B2	40:109	31.95	<0.001*	0.463
	D5	135:148	0.6	0.742	
	F1	120:129	0.33	0.85	
	H2	85:107	2.52	0.284	
	I6	73:180	42.25	<0.001*	0.681
OX4711	B	27:230	160.35	<0.001*	0.790
	D5[2]	540:610	4.26	0.119	
	D9[2]	149:135	0.69	0.708	
	C1	99:363	150.86	<0.001*	0.571
OX4712	F3	29:248	173.14	<0.001*	0.791
	G2	83:781	563.89	<0.001*	0.808
	H1	44:149	57.12	<0.001*	0.544

[1]Only one line available for testing

[2]OX4711 lines D5 and D9 originated from the same G0 pool but showed different phenotypic characteristics in the fluorescent markers; line D5 was brighter

The transgenic:non-transgenic ratios of scorpion toxin OX4509 lines B2 and I6, sea anemone toxin OX4711 lines B and C1, and all OX4712 lines significantly differed from the expected 1:1 ratio, therefore a fitness cost — possibly due to the genomic insertion site and/or intolerable levels of basal expression from the minimal promoter of respective transgenes — was indicated, and these lines were discarded. In addition, all OX4712 lines, which carried the more potent variant of the Av3-encoding gene than OX4711 (Bosmans and Tytgat, 2007; Moran *et al.*, 2007), suffered severe fitness penalties where few offspring were transgenic; these were also discarded.

Effector lines were selected for further analysis if the transgenic:non-transgenic ratio was not significantly different from the expected 1:1 ratio at the 5% level, shown in Table 3.5. In these instances, the null hypothesis stating no statistically significant deviation from the expected transgenic:non-transgenic ratio was accepted; indicating healthy and hemizygous test lines.

3.3.2.3 Investigating the lethality of tetO-effectors under the control of *hex*

Hypothesis (iv): Upregulation of the effectors driven by a strong, early-acting promoter, hex, *results in larval lethality off tet*

To determine the various effectors' potential for lethality, their transcripts were overexpressed in the early developmental stages by crossing selected lines to the *hexamerin*-tTAV promoter line OX4531. As OX4531 upregulates ubiquitous expression of tetO-effectors early in L1 larvae (Labbé, 2011; Gray, P., and Neira, M., unpublished data), this test was

more efficient than crossing a total of fifteen effector lines to the adult-specific promoter lines induced by a blood meal.

Hemizygous OX4531 and effector lines were crossed; progeny were either reared in the presence of tetracycline (on tet) or its absence (off tet) and results are summarised in Figure 3.10. The difference between the observed genotype proportions of controls — progeny carrying OX4531 only, or effector construct only, or non-transgenic progeny, all reared on or off tet — and the expected 0.25 Mendelian ratio were not statistically significant, Appendix 1.2. Fewer insects carried both lethal constructs in the absence of tet in all crosses (apart from OX4291), and these differences were statistically significant (Appendix 1.2), indicating lethality due to the expression of the effector. However, occasionally only partial lethality was observed (OX4292, OX4293, OX4423, lines 2 and 3, OX4711 lines D5 and D9), or the lethality was not repressed on tet (OX4509 lines D5, F1, and H2), as shown in Figure 3.10.

Effector lines were selected for further testing based on the following criteria: (i) if the mean survival percentage of progeny carrying both promoter-tTAV and tetO-effector constructs in the absence of tet was below 5%, indicating highly penetrant lethality (ii) and if over 20% of progeny carrying both constructs survived in the presence of tet, indicating repressibility of the lethal effect.

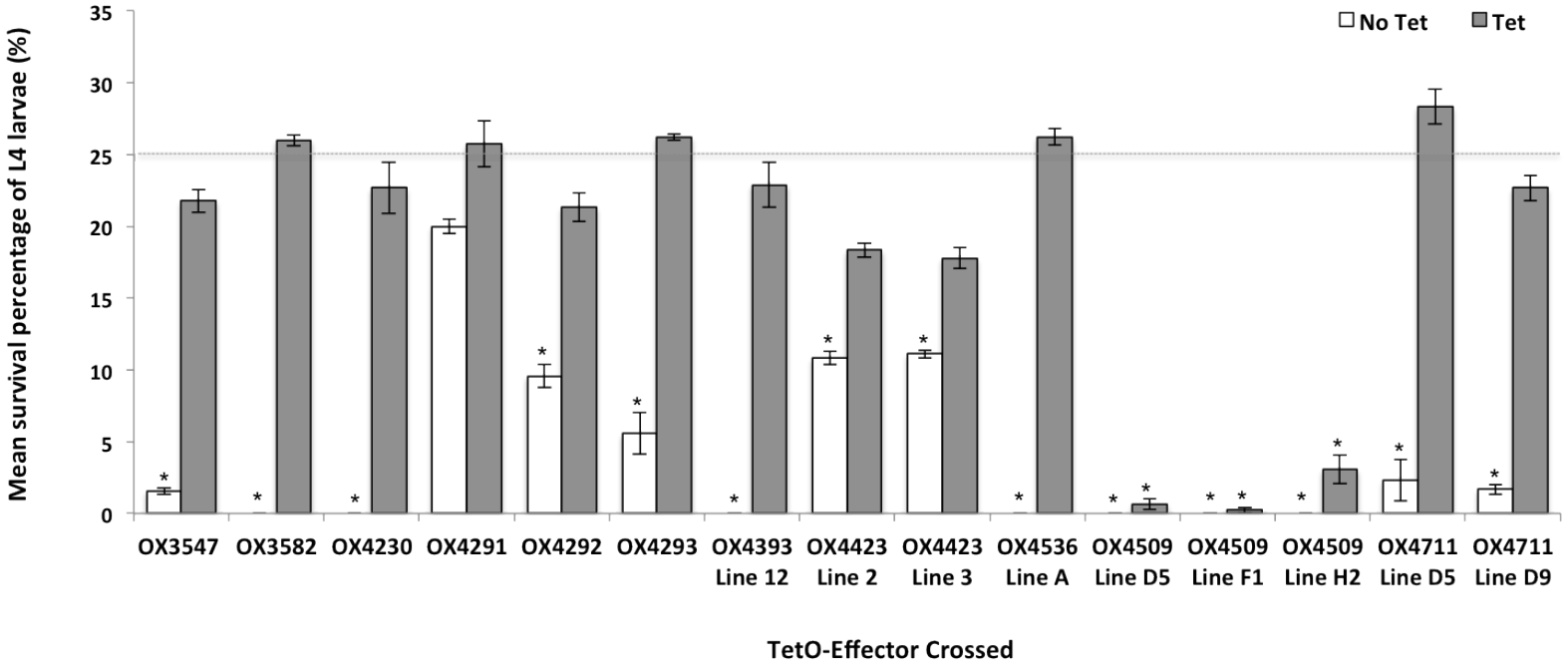

Figure 3.10 Survival percentage (%) of progeny from *hex*-effector crosses carrying both constructs, reared on or off tet for each test cross. Open bars are off-tet the shaded bars are on-tet. χ^2 analysis was used to quantify the significance of the difference between observed mean survival ratio of insects reared on tet: off tet and the expected 25:25 ratio according to Mendelian inheritance predictions (grey line), where $p<0.05$ represents a significant difference (*); error bars denote the standard error of the mean.

OX3547= tetO-NIPP1; OX3582= tetO-michelob_x; OX4230= tetO-trypsin; OX4291= tetO-carboxypeptidase; OX4292= tetO-ILP; OX4293= tetO-eiger; OX4393, OX4423 and OX4536= tetO-reaper^{KR}; OX4509= tetO-AaHIT scorpion toxin; OX4711= tetO-Av3 sea anemone toxin.

The most promising and useful effector lines selected for further investigations were: michelob_x (line OX3582), an antagonist of the inhibitor of apoptotic proteins that regulates cell death in mosquitoes (Zhou *et al.*, 2005); proteolytic enzyme, trypsin (line OX4230) (Barillas-Mury and Wells, 1993); line OX3547, carrying *Drosophila*'s nuclear inhibitor of protein phosphatase 1 (NIPP1) important in RNA processing, transcription, and cell cycle progression (Van Eynde *et al.*, 2004; Beullens *et al.*, 1992; Parker *et al.*, 2002; Brand *et al.*, 1994); one line (OX4393 line 12) carrying reaper^{KR}, a *D. melanogaster* inducer of apoptosis, driven by fourteen copies of the tetracycline operator (tetO) sequence instead of the seven in OX4536 line A (Olson, 2003a and 2003b); and one line (OX4711 line D5) carrying the arthropod-specific Av3 neuronal toxin of the *Anemonia virisidis* sea anemone (Moran *et al.*, 2007; Bosmans and Tytgat, 2007), which originated from the same G0 pool as line D9 but its fluorescent marker was easier to score. In addition, given previous successes with OX4509, when the effector caused temporary paralysis in females when crossed to a *vit*-tTAV line and blood fed (StJohn, 2012), OX4509 H2 was also selected, despite the inability to completely suppress its lethal effects with tetracycline when crossed to the *hex*-tTAV line. The OX4509 construct carries the insect-specific excitatory neurotoxin AaHIT, from the scorpion *Androctonus australis* (Higgs *et al.*, 1995; De Dianous *et al.*, 1987).

3.3.3 Identifying lethal effectors under the control of *vit* or *carb*

Hypothesis (v): Upregulation of the effectors driven by vit *and* carb *results in lethality in insects carrying both promoter-effector constructs off tet*

3.3.3.1 Pilot study: Effects of NIPP1 or michelob_x under the control of *vit*

To investigate the effects of NIPP1 (OX3547) and michelob_x (OX3582) under the control of *vit*, fitness parameters of progeny from effector lines crossed to *vit* were tested in a pilot study.

Fewer female pupae carrying both *vit* and michelob_x constructs were observed off tet than the expected proportion (χ^2=28.17, df= 1, p<0.001, Φ= 0.195), whereas males were not adversely affected (χ^2=1.50, df=1, p=0.472). Genotype, sex and tet status did not affect the pupation of all remaining progeny from both NIPP1 and michelob_x effector crosses, Appendix 1.2. The lethality observed in *vit*-michelob_x insects pre-pupation was also reflected in the eclosion of adult females. Fewer females eclosed than expected (χ^2=6.78, df= 1, p=0.034, Φ= 0.099). Genotype and tet status did not impact eclosion of all remaining progeny from both NIPP1 and michelob_x effector crosses, Appendix 1.2.

The number of dead females per time point after a blood meal was recorded, and used to produce survival curves; the number of blood fed females used in the survival analysis is listed in Table 3.6. Any survival effect was expected to occur in females approximately 24 hours after a blood meal, corresponding to the vitellogenin promoter's peak activity. To

give enough time for expression of potentially lethal quantities of the effectors, insects were observed for ten days.

Table 3.6 Total number of blood fed females (n) used to test survival of progeny from *vit*-michelob_x and -NIPP1 crosses. Tet= tetracycline.

Effector crossed	Genotype	Treatment	n	% Blood fed females
OX3582 Michelob_x	Promoter	Off Tet	52	43
	NTG	Off Tet	82	63
	Effector	Off Tet	73	61
	Promoter-Effector	Off Tet	54	89
	Promoter Effector	Tet	57	43
	NTG	Tet	54	44
OX3547 NIIPP1	Promoter	Off Tet	116	97
	NTG	Off Tet	115	98
	Effector	Off Tet	110	92
	Promoter-Effector	Off Tet	102	99
	Promoter Effector	Tet	108	81
	NTG	Tet	105	78

The survival curves of females from both crosses are shown in Figure 3.11. There was no difference between all survival curves of progeny from the NIPP1 effector cross (χ^2= 5, df= 5, p= 0.41). In contrast, the survival of females carrying both *vit*-michelob_x constructs off tet was adversely affected compared to the same genotype reared on tet and compared to all other genotypes (χ^2= 20.1, df= 5, p<0.001). This indicated that the lethality observed after a blood meal was tet-repressible. There was only a small decrease in the survival of females by approximately 20%, seen in Figure 3.11B, which was notable within the first five days after a blood meal. The genotype and tet status did not affect the survival of control

groups, *i.e.* females carrying: effector only, or *vit* only constructs, or non-transgenic progeny reared on or off tet (χ^2= 1, df= 3, p= 0.80). This suggested that the effect was caused by michelob_x because lethality did not occur in the absence of tetO-michelob_x, and the effect was tet-repressible.

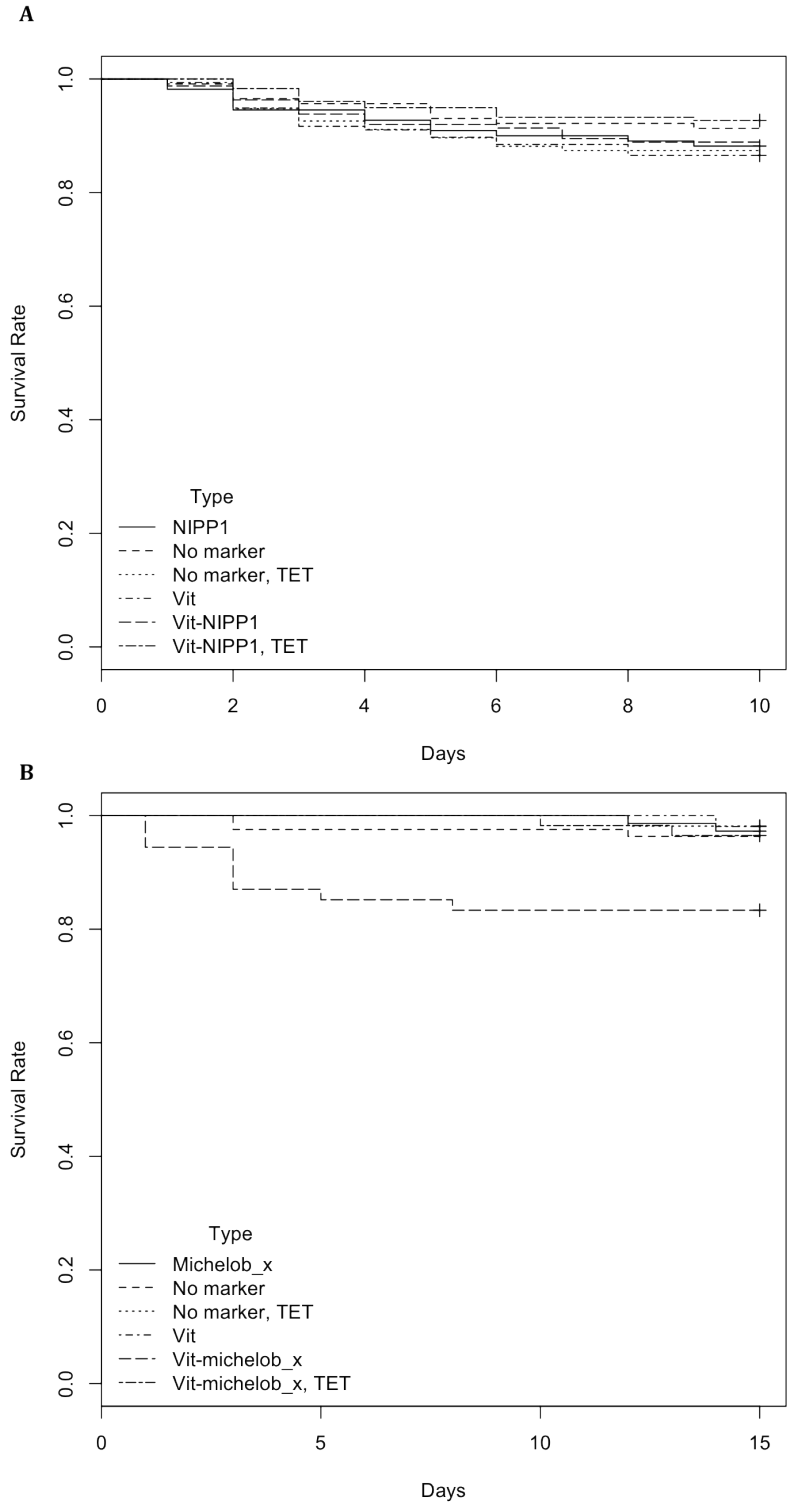

Figure 3.11 Survival of female progeny from crosses between *vit* and tetO- (A) NIPP1, and (B) michelob_x. TET= reared on tet, Mich= michelob_x.

In a separate study, the effects of NIPP1 on the female fecundity (eggs laid per female) and fertility (egg hatch rates) were investigated for three gonotrophic cycles as a measure of fitness, (n= 3 cages; 30 females in each); the number of blood fed females carrying *vit* and michelob_x constructs was too low to investigate. These parameters were of particular interest for *vit* crosses, since vitellogenin, the major egg yolk protein precursor and source of amino acids, is required for embryogenesis and upregulation of effectors in the fat-body, where the vitellogenins are synthesised (Wyatt, 1980). Therefore, expression of effectors in this tissue might affect the egg laying ability of the female. If damage was caused to the female's offspring, reduced egg hatch rate could result as well.

Genotype and tet status of the females did not impact egg production in the first ($F_{(5,\ 5.528)}$= 1.561, p= 0.308), second ($F_{(5,\ 5.294)}$= 0.156, p= 0.969) and third ($F_{(5,\ 5.304)}$= 0.209, p= 0.945) gonotrophic cycles, Figure 3.12.

Egg-papers collected from all three gonotrophic cycles were used to compare fertility rates, Figure 3.13. Genotype or tet status of the female did not have affect the fertility of females in their first ($F_{5,\ 12)}$= 0.487, p= 0.780) and second ($F_{(5,\ 12)}$= 0.950, p= 0.484) gonotrophic cycles.

In the third gonotrophic cycle, fewer eggs hatched from females carrying *vit*-NIPP1 constructs compared to *vit*-only females, and this difference was statistically significant ($F_{(5,\ 12)}$= 4.808, p= 0.012, ω^2= 0.514). However there was no difference between *vit*-NIPP1 progeny hatch rates compared to remaining on-tet and non-transgenic controls. Therefore the null hypothesis stating no effect on the fecundity of insects carrying both *vit* and NIPP1 lethal constructs was accepted.

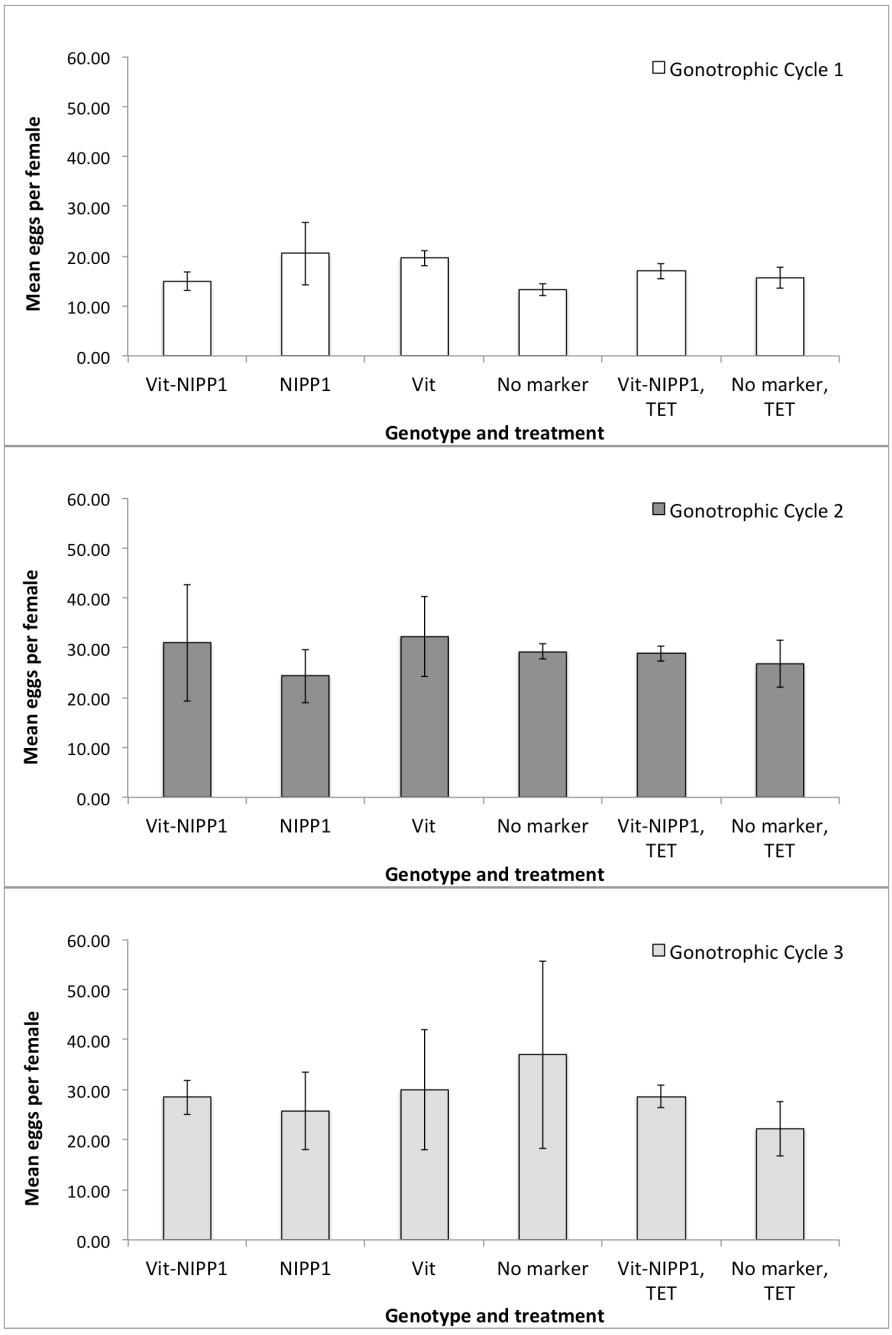

Figure 3.12 Fecundity (eggs per female) over three gonotrophic cycles of progeny from *vit*-NIPP1 crosses. Cages were set up with 30 female progeny from the *vit*-NIPP1 cross, and 20 wild-type males, n= 3. Error bars denote the standard error of the mean. Genotype and tet status did not impact the mean number of eggs per female.

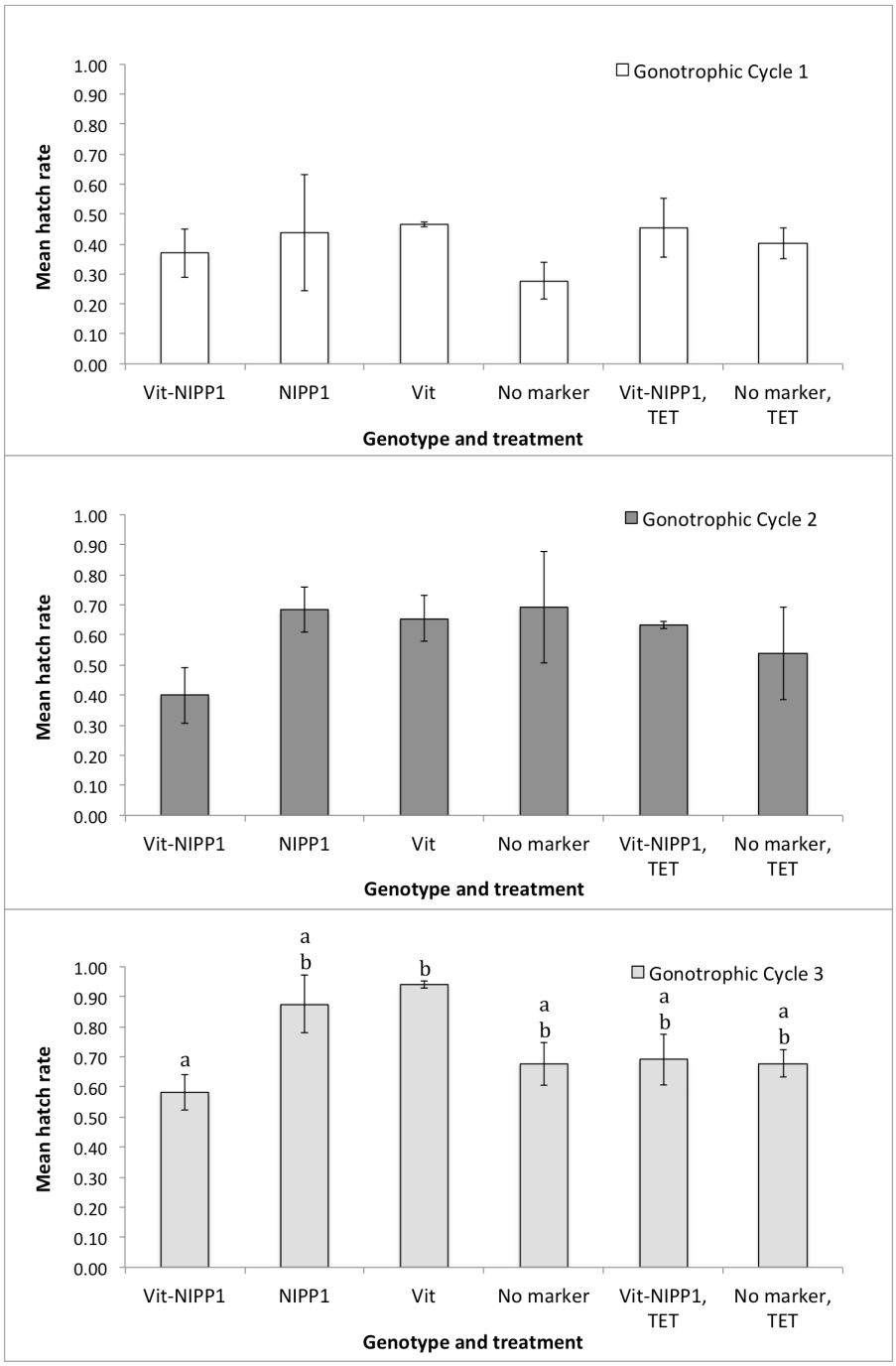

Figure 3.13 Fertility (egg hatch rate) of females from *vit*-NIPP1 crosses over three gonotrophic cycles. Genotype and tet status did not impact the eggs per female in gonotrophic cycle 1 and 2; groups with different letters in cycle 3 are significantly different from each other at the 5% level; error bars show the standard error of the mean.

The results of these pilot studies indicated that michelob_x could be used as the lethal element in the proposed gene drive system, since tet-repressible lethality was observed in 20% of females after a blood meal, whereas NIPP1 did not cause a reduction in survivorship.

To verify the magnitude of the observed effects of michelob_x, progeny of the *vit*-michelob_x cross were tested using the revised experimental design; the aim was to increase any lethality induced by a blood meal.

3.3.3.2 Revision of experimental design

The experimental design was modified based on findings from the pilot study. Progeny from hemizygous crosses were aliquoted at the L1 larval stage instead of L3, and reared at low density (1 larvae ml^{-1}) to reduce larval competition that is shown to have a negative effect on longevity (Bargielowski *et al.*, 2011). The percentage pupation and adult eclosion of each genotype in both sexes were determined for comparison. The proportion of each genotype was used to enable investigation of the Mendelian inheritance of constructs. These off-target effects were of interest because if they were not repressed by the RNAi 'rescue' component, death could result from the corresponding non-RNAi-repressible fitness penalty.

To enhance the lethality of effector transgenes and to simulate the availability of a blood meal in the wild, females were blood fed and their survival rates were investigated for three gonotrophic cycles instead of one.

As shown in Figure 3.12, although the mean number of eggs per female varied between each gonotrophic cycle, this was predictable, and results between classes in each cycle were consistent. Therefore, tests for longevity

and female fecundity were combined. Female fecundity was calculated from the first gonotrophic cycle of female cages used for the longevity experiment (n= 3 cages).

Previously, tet in blood was not shown to affect female feeding behaviour, fecundity or hatch rate in a different transgenic tet-repressible lethal strain not used in this study, *Ae. aegypti* OX513A (Gray, P., personal communication). Given these findings, tet in blood was not expected to compromise any differences between treatment groups. Therefore, tet was provided to on-tet females in this study to improve the repressibility of the system by supplementing the blood and sugar water.

3.3.3.3 Investigating lethal phenotypes in adults

3.3.3.3.1 Off-target effects of transgenes in immature progeny from test crosses

Hypothesis (v): Upregulation of the effectors driven by vit *and* carb *results in lethality in insects carrying both promoter-effector constructs off tet*

Michelob_x: OX3582

Genotype and tet status did not affect the pupation of progeny from *vit*-michelob_x test crosses ($F_{(7, 16)}$= 2.412, p= 0.069), shown as a percentage of aliquoted larvae in Figure 3.14A. Fewer *vit*-michelob_x pupae reared off-tet eclosed to adults compared to the same genotype reared on tet and all other genotypes ($F_{(7, 16)}$= 229.351, p<0.001, ω^2= 0.985), Figure 3.14B.

This suggests that the negative effect was not attributable to a fitness penalty imposed by the presence of either construct (*vit* or michelob_x) separately, but implied that leaky expression of the effector under the

control of the *vit* was sufficient for michelob_x-induced lethality in pupae. To test whether this was sex-specific, total adult eclosion was compared between male (33±15) and female (36±7) progeny carrying both constructs; sex did not impact the number of eclosed adults carrying both *vit* and michelob_x lethal constructs ($t_{(4)}$= 0.277, p= 0.795).

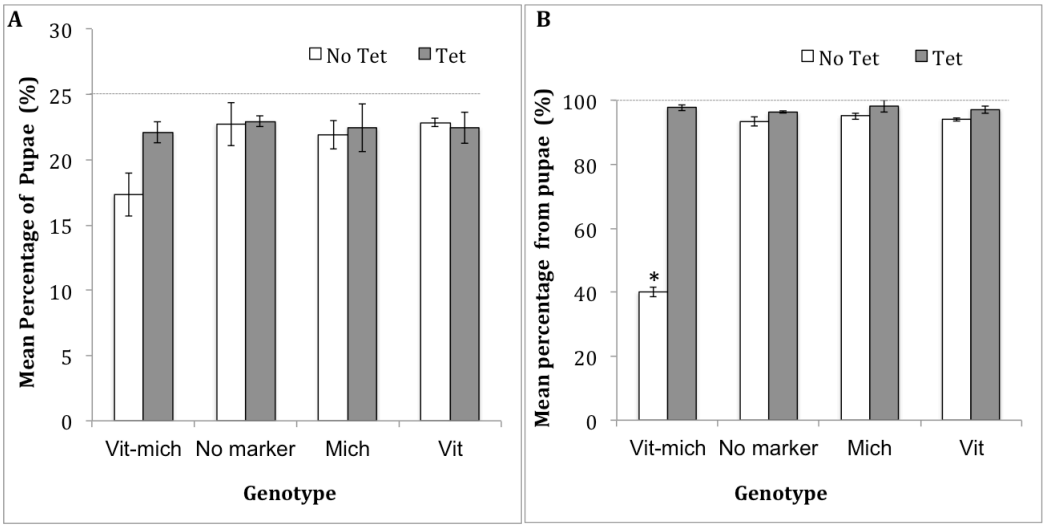

Figure 3.14 Percentage pupation (A) and eclosion of adults from pupae (B), in *vit*-michelob_x progeny reared on or off tet. Mich= michelob_x, no marker= non-transgenic**.** Pupation is shown as a percentage from 1000 aliquoted larvae, 25% of each genotype was expected. Adult eclosion was calculated as a percentage from pupae, with 100% expected if all pupae eclosed. Error bars show the standard error of the mean. Genotype and tet status did not affect pupation (A), except that significantly fewer *vit*-michelob_x insects eclosed off tet at the 5% level (B) denoted with (*).

This effector was not crossed to the *carboxypeptidase* promoter line since expressing this effector into the midgut cells was not expected to induce a more lethal effect than expressing it in the female's fat-body, an essential organ for reproduction.

Av3 sea anemone toxin: OX4711

Early lethality was observed in insects carrying *vit* and Av3-effector constructs, both on and off tet ($F_{(7,\ 16)}$= 39.342, p<0.001, ω^2= 0.918), regardless of their sex (on tet :$t_{(4)}$= 0.811, p= 0.463; off tet: $t_{(4)}$= 2.692, p= 0.055). This indicated that the lethal effect occurred before tet could repress the effects, probably during the L1 or L2 larval stage as there was no visible lethality in trays. It is unlikely that this off tet lethality occurred during the embryonic stages since the expected Mendelian proportion (25%) of each remaining genotype was represented in late pupae counts, Figure 3.15A. Genotype and tet status did not affect adult eclosion of *vit*-michelob_x progeny, Figure 3.15B ($F_{(7,\ 6.750)}$=2.809, p= 0.102).

In progeny from the *carb*-Av3 test cross, genotype did not affect pupation off tet ($F_{(3,\ 8)}$= 2.281, p= 0.156) or on tet ($F_{(3,8)}$= 0.042, p= 0.988), Figure 3.15C. However, there were differences between on and off tet groups ($F_{(7,\ 16)}$= 5.101, p= 0.003, ω^2= 0.545), suggesting a problem with tet-treated trays independent of the genotype, which was likely an effect of their environment.

The presence of the Av3 transgenic constructs on tet had a small effect on adult eclosion in some insects ($F_{(7,\ 16)}$= 4.315, p= 0.007, ω^2= 0.492), Figure 3.15D. The VP16 domain of the tTAV protein is known to be toxic to mosquitoes (Fu *et al.*, 2010; Lin *et al.*, 2007), and Av3 is a known sea anemone toxin, so any leaky expression may have made already vulnerable insects susceptible to the harmful effects of the environment.

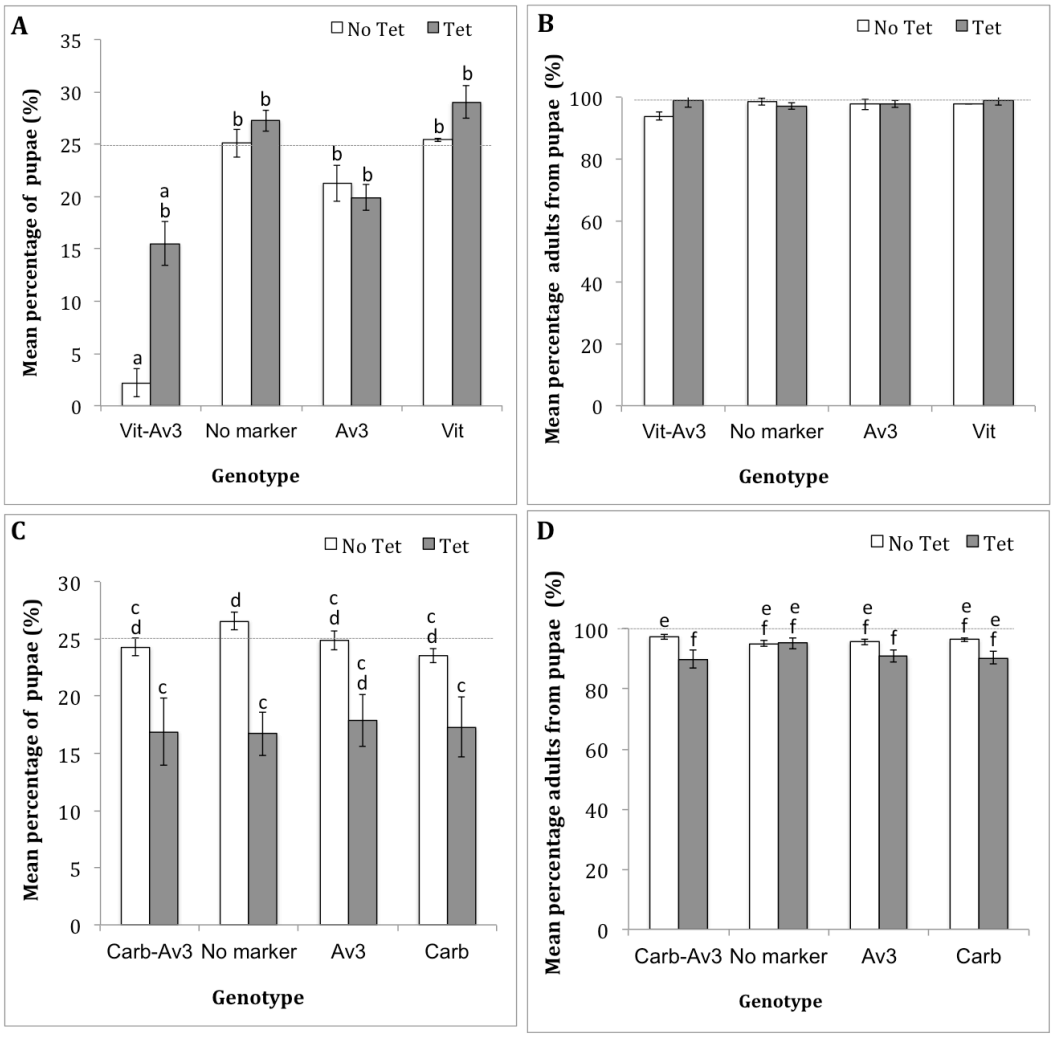

Figure 3.15 Percentage pupation and adult eclosion in progeny from Av3 crosses reared on or off tet; (A) mean percentage of pupae and (B) adult eclosion of progeny from *vit* cross; (C) mean percentage of pupae and (D) adult eclosion of progeny from *carb* cross. Pupation is shown as a percentage from 1000 aliquoted larvae, 25% of each genotype was expected. Adult eclosion was calculated as a percentage from pupae, with 100% expected if all pupae eclosed. Error bars show the standard error of the mean. Genotype and tet status affected variables in panels A, C, and D, indicated above bars where groups with different letters are significantly different from each other at the 5% level; but not in panel B.

AaHIT scorpion toxin: OX4509

Some lethality was observed in insects carrying both *vit*-AaHIT constructs off tet ($F_{(7,\ 16)}$= 2.835, p=0.040, ω^2= 0.349), Figure 3.16A, however this was not a big effect, and was possibly due to low numbers or high variability within the data. Nevertheless the observed lethality may be due to leaky expression of the effector in the earlier stages, or due to handling stress. Genotype and tet status did not affect eclosion of *vit*-AaHIT progeny ($F_{(7,\ 6.739)}$= 1.270, p=0.383), Figure 3.16B.

Furthermore, the genotype and tet status did not affect the pupation ($F_{(7,\ 16)}$= 1.218, p= 0.249) and adult eclosion ($F_{(7,\ 16)}$= 0.758, p= 0.629) of progeny from the *carb*-AaHIT test cross, Figures 3.16C and 3.16D.

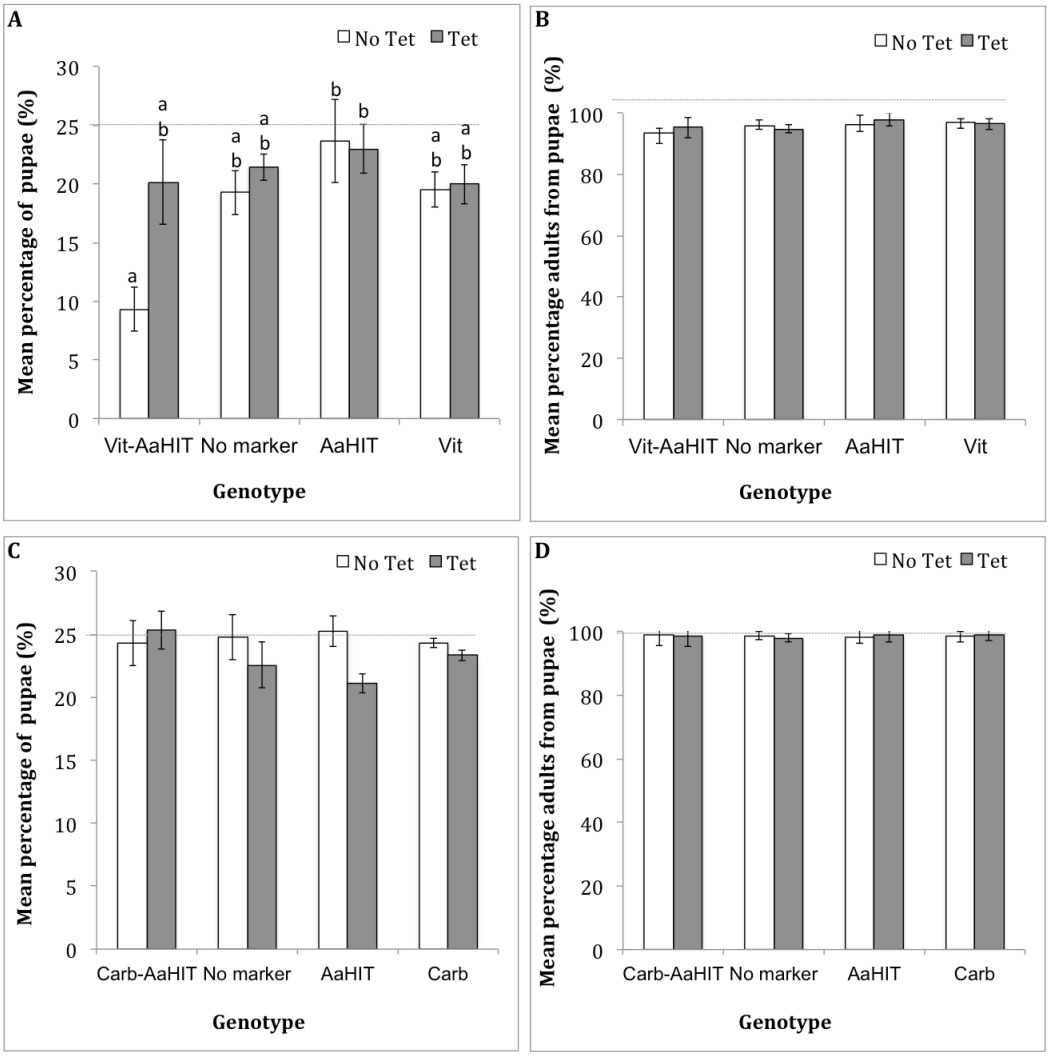

Figure 3.16 Percentage pupation and mean adult eclosion of progeny from AaHIT (OX4509) crosses reared on or off tet. (A) mean percentage of pupae and (B) adult eclosion of progeny from vit-AaHIT cross; (C) mean percentage of pupae and (D) adult eclosion of progeny from carb-AaHIT cross. Pupation is shown as a percentage from 1000 aliquoted larvae, 25% of each genotype was expected. Adult eclosion was calculated as a percentage from pupae, with 100% expected if all pupae eclosed. Error bars show the standard error of the mean. There was a significant difference between groups in A, indicated above bars where groups with different letters are significantly different from each other at the 5% level, but not in B, C, or D.

Reaper^{KR}: OX4393

Pupation and eclosion of progeny carrying the lethal *vit* and reaper^{KR} constructs were not affected; although there were fewer reaper^{KR}-only pupae ($F_{(7,\ 6.313)}$= 7.857, p= 0.010, ω^2= 0.667) and eclosed adults ($F_{(7,\ 6.438)}$= 9.468, p= 0.006, ω^2= 0.7118) in the presence of tet, Figure 3.17. This was probably phenotypic variance within this group due to handling, as there was not a consistent significant difference between insects carrying reaper^{KR} and other control groups.

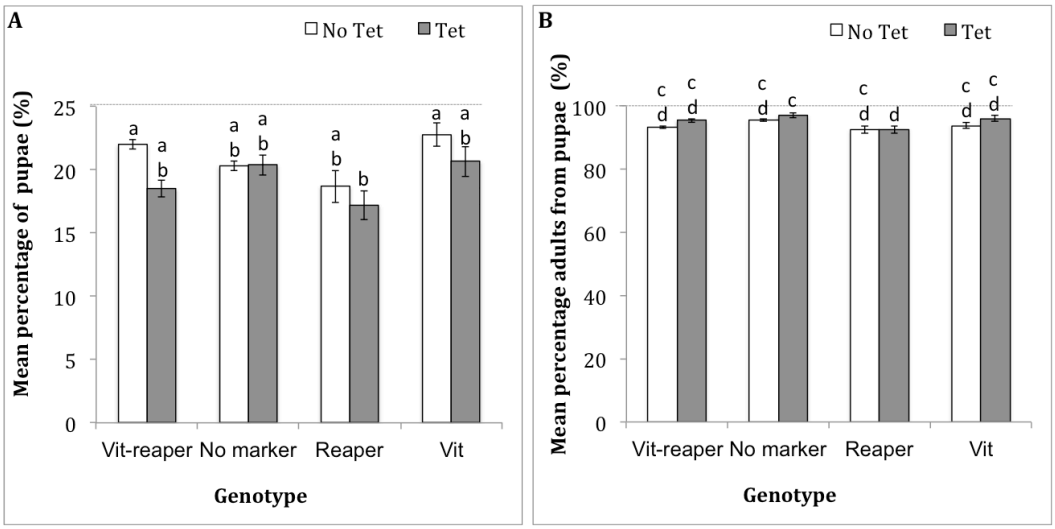

Figure 3.17 Percentage pupation (A), and eclosion of adults (B), in *vit*-reaper^{KR} progeny reared on or off tet. Significant differences between groups are indicated above bars where groups with different letters are significantly different from each other at the 5% level. Pupation is shown as a percentage from 1000 aliquoted larvae, 25% of each genotype was expected. Adult eclosion was calculated as a percentage from pupae, with 100% expected if all pupae eclosed.

Trypsin: OX4230

Early lethality was observed in insects carrying *vit*-trypsin constructs, in both tet treated and untreated trays, Figure 3.18A ($F_{(7,\ 6.493)}$= 20.900, p= 0.001, ω^2= 0.853). As seen in progeny of the Av3 sea anemone toxin, this indicates that the lethal effect occurred before tetracycline could repress the effects, probably during the L1 or L2 larval stage because no visible lethality was observed in trays. Genotype and tet status did not have an affect adult eclosion, Figure 3.18B, ($F_{(7,\ 16)}$= 0.688, p= 0.681); vulnerable insects were probably already dead.

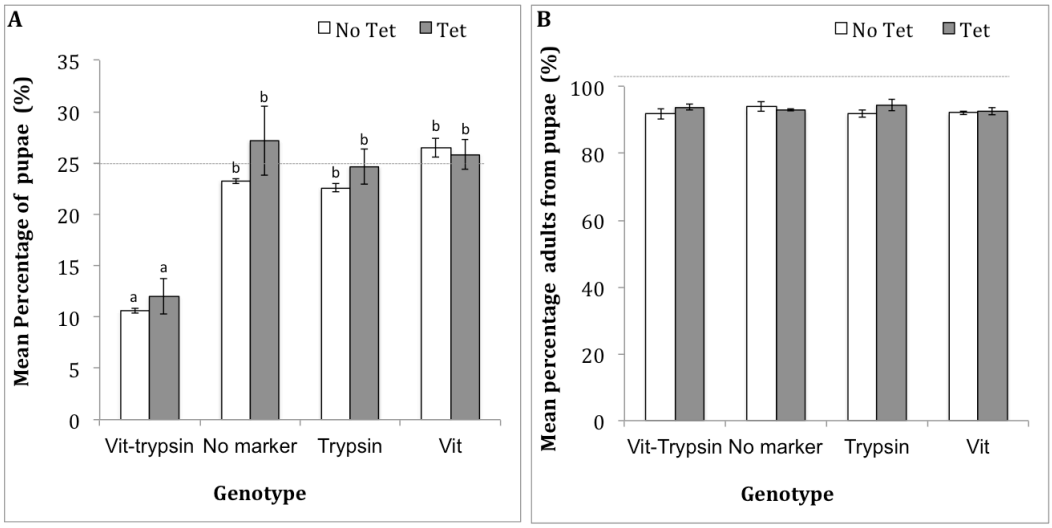

Figure 3.18 Percentage pupation (A), and eclosion of adults (B), in *vit*-trypsin progeny reared on or off tet. Significant differences between groups are indicated above bars where groups with different letters are significantly different from each other at the 5% level. Pupation is shown as a percentage from 1000 aliquoted larvae, 25% of each genotype was expected. Adult eclosion was calculated as a percentage from pupae, with 100% expected if all pupae eclosed.

3.3.3.3.2 Survival of blood fed female progeny from test crosses

To investigate the lethality of promoter-effector constructs, survival analyses were performed on female progeny of test crosses for three gonotrophic cycles. Primarily, the survival curves of the three experimental repeats per cross were compared using the log rank χ^2 analysis (Table 3.7, 'Between Repeats').

There was a significant difference between experimental repeats from the *vit*-AaHIT cross when paralysed insects (see pages 106-107) were counted as dead (χ^2= 25, df= 2, p<0.001). Therefore survival analyses between genotypes were carried out on the three repeats separately, Appendix 1.3. However, as results from each analysis and the pooled analysis suggested the same conclusions, survival data were pooled. Repeats were not significantly different from each other in all other crosses, shown in Table 3.7 ('Between repeats'). Therefore data were pooled and survival analysis carried out on pooled data.

Kaplan-Meier analysis showed that genotype and tet status did not have a significant effect on the survival of females from (i) *vit*- michelob_x, reaper^{KR}, and trypsin crosses, or (ii) *carb*- AaHIT and Av3 crosses, shown in Table 3.7; survival curves are shown in Appendix 1.4.

The survival of non-transgenic females reared on tet from the *vit*-Av3 test cross was adversely affected compared to the same genotype off tet (χ^2= 5, df= 1, p= 0.026.) However this did not affect the hypothesis since there was no difference between the survival of transgenic and non-transgenic females. Therefore the null hypothesis stating no effect of the Av3 driven by the vitellogenin promoter was accepted.

Table 3.7. Log rank χ^2 analyses of differences between survival curves of female progeny after a blood meal from each test cross. Significant differences (*) between groups, where $p<0.05$. Genotype are shown in brackets where df= degrees of freedom; Promoter-Effector= insects carrying both promoter and effector constructs; NTG= non-transgenic insects; Effector= insects carrying the effector construct only; Promoter= insects carrying the promoter construct only.

				Log rank χ^2 tests using pooled data from three repeats									
						Induced lethallity		Tet repressibility		Controls			
		Between repeats		Between all groups; pooled repeats		Between (NTG) and (Promoter-Effector)		Between (Promoter-Effector) tet and no tet		Between (NTG) tet and no tet		Between (NTG), (Promoter), and (Effector)	
Promoter	Effector	χ^2 (df=2)	p-value	χ^2 (df=5)	p-value	χ^2 (df=1)	p-value	χ^2 (df=1)	p-value	χ^2 (df=1)	p-value	χ^2 (df=2)	p-value
Vit	Michelob_x	1.4	0.509	3.5	0.616	2.9	0.087	1.1	0.289	0.5	0.478	2.0	0.4
	Av3	1.9	0.384	18.3	0.003*	1.8	0.178	0.2	0.664	5	0.026*	0.8	0.66
	Trypsin	3.6	0.161	3.8	0.575	0.5	0.487	0.0	0.865	3.5	0.061	0.9	0.653
	Reaper^{KR}	1.2	0.537	3.2	0.677	0.2	0.680	1.6	0.200	0.6	0.439	1.4	0.506
	AaHIT[1]	25.0	<0.001*[2]	406	<0.001*	162	<0.001*	164	<0.001*	1.3	0.245	2.0	0.4
Carb	Av3	0.0	0.988	8.5	0.133	0.0	0.934	2.5	0.117	1.0	0.314	3.6	0.169
	AaHIT	0.3	0.850	1.7	0.891	1.1	0.287	0.3	0.592	0.2	0.617	1.2	0.547

[1]Survival curves shown for *vit*-AaHIT progeny where paralysed insects were counted as dead (see pages 106-107 for details).
[2]The significant difference between the three experimental repeats was investigated and due to the similarity between survival curves of genotypes between each repeat, data were pooled and tested in the same way as for other test crosses.

Interestingly, females carrying *vit*-AaHIT constructs exhibited a striking sub-lethal phenotype approximately 24-30 hours after a blood meal; this was not observed in any other genotype. The females lay paralysed on the bottom of the cage, often on their dorsal thorax, with their legs and wings occasionally convulsing asynchronously; see supplementary videos at http://bit.ly/19zwZML. The frequency of paralysis, calculated as a proportion of the total number of females per cage is shown in Figure 3.19.

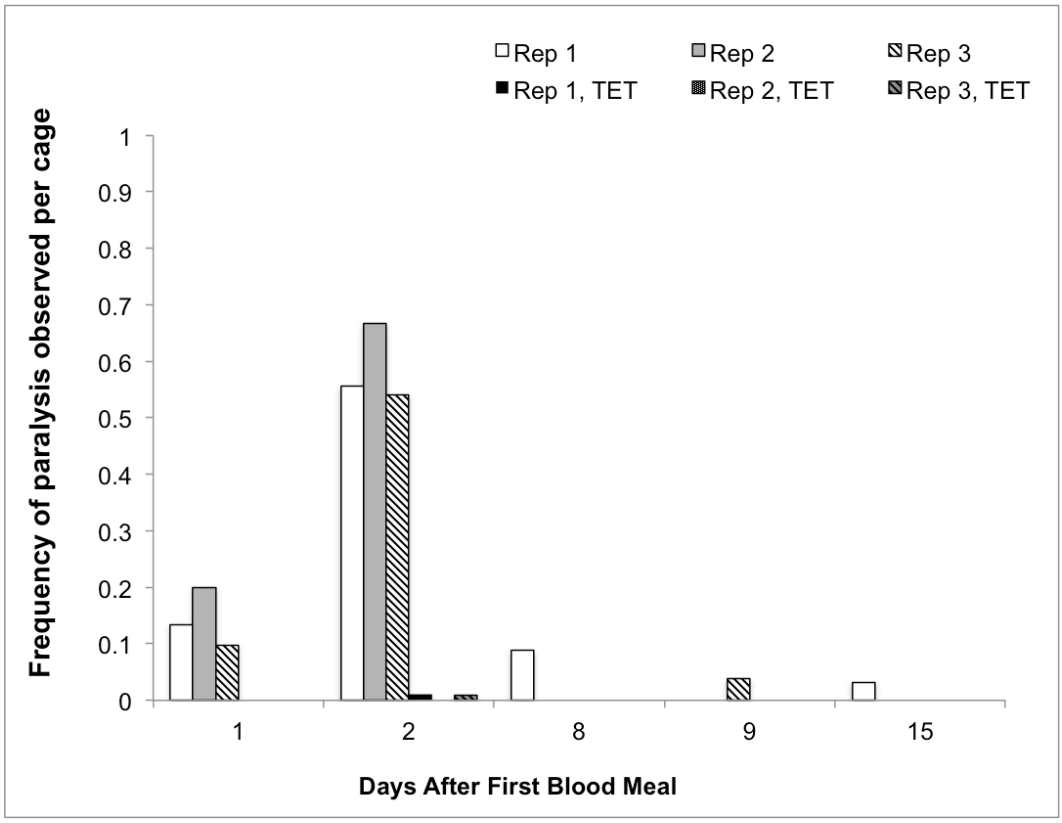

Figure 3.19 Frequency of paralysis observed per cage in females carrying *vit*-AaHIT constructs. Rep= experimental repeat. Paralysis was only observed in females carrying both *vit* and AaHIT constructs. On tet, paralysis was observed on the second day after their initial blood meal in 1/86 females in 'Rep 1, TET' and 1/112 females in 'Rep 3, TET'. Insects were provided with blood on days zero, seven and fourteen.

Over half of *vit*-AaHIT genotypes reared off tet were paralysed 48 hours post blood meal in all three experimental repeats. This was considered an important fitness cost for females, as they would be exposed to predators in the wild. Therefore, paralysed females were counted as dead and were removed and placed into separate cages, Figure 3.20. There was a significant difference between females carrying both *vit*-AaHIT off tet and all other genotypes and treatments (Table 3.7).

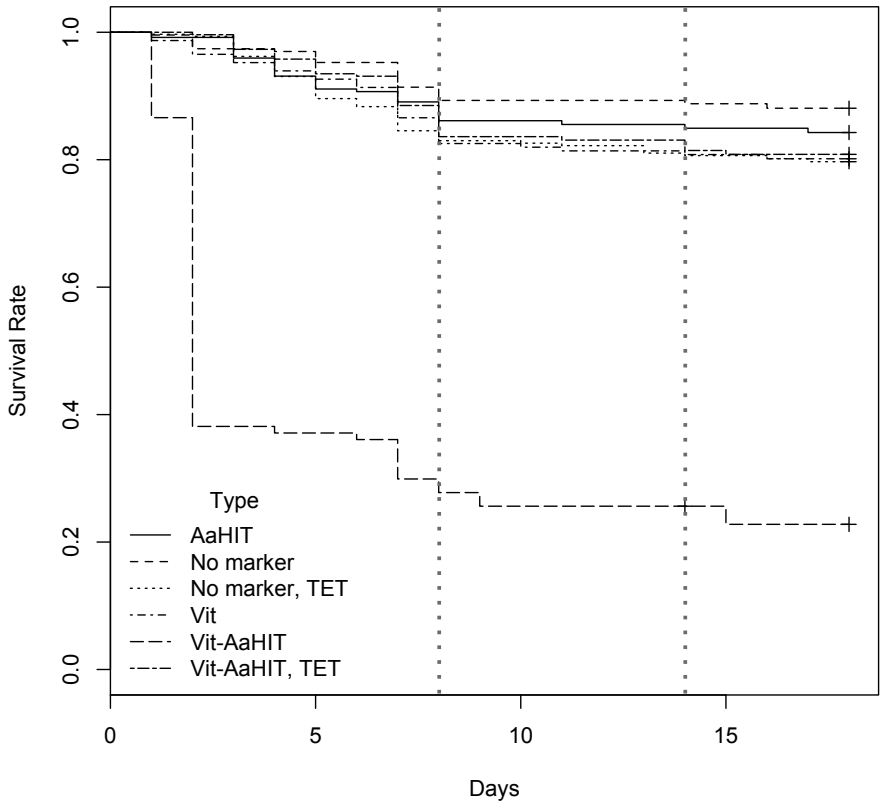

Figure 3.20 Survival of blood-fed female progeny of *vit*-AaHIT cross; results are pooled from three experimental repeats. No_marker= non-transgenic, TET= on tet. Second and third blood meals are denoted by vertical dotted lines, where non-blood fed females were counted and removed from cages. Paralysed females were removed from cages and counted as dead for the survival analysis. See Table 3.7 for statistical analysis.

For comparison, paralysed females were removed from the analysis and Kaplein Meier survival analysis carried out on remaining insects, Appendix 1.5; the genotype and tet status had no impact on the survival of blood fed females (χ^2= 8.3, df=5, p= 0.138).

Paralysed females that had been removed from cages were observed for two more gonotrophic cycles; most females recovered and were flying approximately 12 hours after paralysis, Figure 3.21.

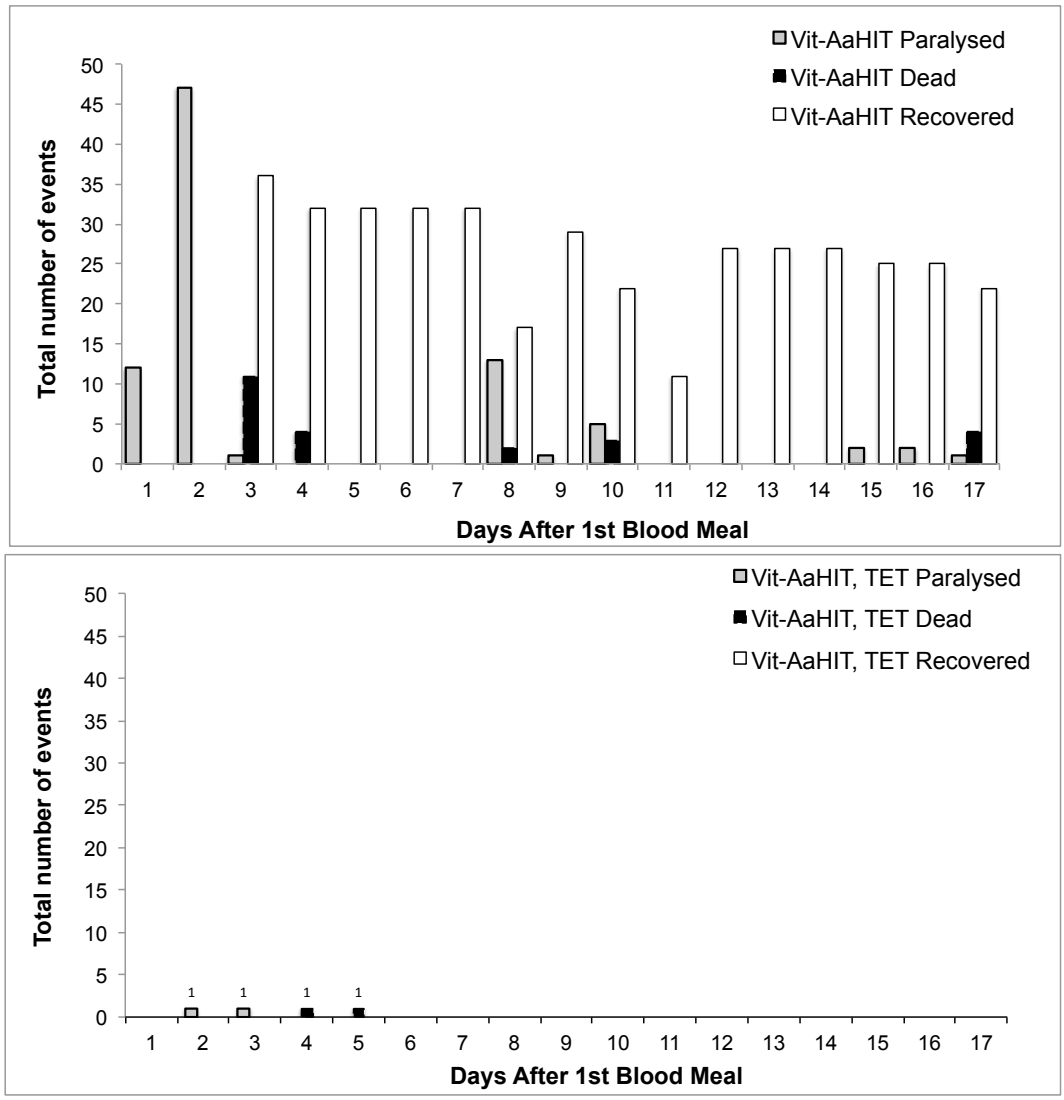

Figure 3.21 Phenotypes observed in *Vit*-AaHIT females post-paralysis; results pooled from three experimental repeats. (A) Off tet. (B) On tet. Females were provided with blood on days zero, seven and fourteen.

The sub-lethality was almost entirely (apart from two individuals in total) repressed in the presence of 30 μg ml^{-1} tet. Tet was used to supplement the diet in three ways: in distilled larval water added two hours after hatching, in the sugar used supplied to treated cages, and in blood used to feed females. It was unclear as to which one, or combination,

of tet provisions was essential for the suppression of the gene expression system.

The timing of paralysis observed in *Ae aegypti* corresponded to the peak activity of the vitellogenin promoter, at which time up to 70% of insects exhibited the phenotype. Previously (Figure 3.9), upregulation of the tTAV transcript under the control of *vit* was shown to be up to 90-fold higher 24 hours after a blood meal than before a blood meal, which may not be enough to cause irreversible damage in adult tissues. The results in this study were consistent with observational findings from a similar experiment carried out by StJohn (2012) using different *vit* promoter and AaHIT effector strains where reversible paralysis was observed.

3.3.3.3.3 Fecundity of female progeny from test crosses

Ideally, any effects on the fitness of females would occur after a blood meal since hypothetically the rescue element is expressed at its highest levels at that point. Nevertheless, it was interesting to examine whether there were any consequences of expression soon after a blood meal on the fecundity of females carrying lethal constructs compared to non-transgenic females. Eggs were collected from the first gonotrophic cycle from cages used for the survival analysis. Using survival data, the number of eggs per female was estimated and compared. Egg numbers produced by female progeny from the test crosses were not significantly different to each other regardless of genotype and tet status at the 5% level, although variation amongst the three repeats of each genotype was quite high, shown in Appendix 1.6. Individual egg laying of females would provide more accurate information on fecundity, but this was used as a rough test to

identify any obvious effects on fecundity. If there had been any indication of this, an experiment looking at individual female egg laying would have been carried out.

3.3.3.3.4 Summary of results

Results are summarised in Table 3.8. When expressed under the control of the *carb* promoter, the OX4711 (sea anemone toxin Av3) and OX4509 (scorpion toxin AaHIT) effectors were not proven toxic. This may be due to lower expression of the *carb*-tTAV in OX4414. Isoe *et al.* (2009) showed a 25-fold increase of the endogenous protein in females following a blood meal relative to non-blood-fed females, however only a 6.6-fold increase in transgenic tTAV mRNA was reported in hemizygous OX4414 (StJohn, 2012) relative to non-blood-fed females. As discussed by the author, missing regulatory elements of the selected *carb* promoter, or positional effects due to the *piggyBac* insertion site may have contributed to the transgene's lower expression levels. In addition, the toxin may have been secreted into the lumen of the gut or retained within the cell rather than secreted into haemolymph, or differential effects of secretion from different tissues may have reduced the lethality of the toxin.

Despite significant upregulation of DEN2-tTAV in blood fed *vit* females — up to 91-fold compared to non-blood fed females (Figure 3.9) — michelob_x, Av3, trypsin, and reaper^{KR} were not lethal, and AaHIT induced only temporary sub-lethality under the control of this promoter construct. Nevertheless, as this 'fainting' phenotype was easy to score, it was used to investigate the ability of the RNAi construct to knockdown the frequency of paralysis.

Table 3.8 Summary of results from all *vit*- and *carb*-effector crosses

Promoter Line	Effector Line	Leaky expression in immature stages	Survival analysis of adults
Vit	OX3582 tetO-michelob_x	Tet repressible partial lethality	No blood meal induced effects
	OX4711 tetO-Av3	Partial lethality; not tet repressible	No blood meal induced effects
	OX4509 tetO-AaHIT	Partial lethality observed in promoter-effector genotype and controls in off tet group, not seen in tet groups	Tet repressible blood meal induced paralysis; 'fainting phenotype'; most insects recover
	OX4230 tetO-trypsin	Partial lethality; not tet repressible	No blood meal induced effects
	OX4393 tetO-reaper^{KR}	Partial lethality in most insects carrying the effector construct; indicates toxic effect of effector construct alone	No blood meal induced effects
Carb	OX4711 tetO-Av3	Partial lethality observed in all genotypes on reared on tet; indicates unknown environmental factor negatively affected pupation rates in tet crosses independently of transgene	No blood meal induced effects
	OX4509 tetO-AaHIT	No lethality observed in all genotypes in both treatment groups	No blood meal induced effects

3.3.4 Investigating the ability of RNAi to suppress lethal phenotypes

Hypothesis (vi): The RNAi construct directed against the DEN2-tTAV suppresses the phenotype of lethal constructs under the control of the same promoter

To evaluate the ability of RNA interference to suppress any observed fitness costs, males carrying both *vit* promoter and RNAi constructs were crossed to females carrying the Av3 or AaHIT effector constructs. Progeny were tested for the knockdown of two phenotypes: sub-lethality in the immature stages due to Av3, or partial and reversible paralysis caused by AaHIT.

The RNAi in line Vg40 targets the DEN2-tTAV fusion mRNA under the control of *vit* in the promoter construct. The 90-fold upregulation of the DEN2-tTAV mRNA in *vit* line OX4438 from before a blood meal was knocked down significantly in the presence of Vg40, driven by the same promoter, Figure 3.9. Therefore, this RNAi system was expected to suppress paralysis in *vit*-AaHIT females after a blood meal.

In addition, as both RNAi and DEN2-tTAV elements were driven by the same *vit* promoter with identical sequences, unless leaky expression was due to the insertion site of the transgenes, the RNAi was expected to knock down lethal transcripts enough to suppress the sub-lethality observed in immature insect progeny from the Av3 cross. Suppression of the sub-lethality observed in immature progeny from the *vit*-michelob_x cross was not investigated due to an overlap of the transgenic constructs' fluorescent markers; the michelob_x construct carries a 3xP3-AmCyan

blue marker that masks the Vg40 RNAi construct's 3xP3-EGFP green marker.

3.3.4.1 RNAi against leaky sub-lethality caused by *vit*-Av3

Surprisingly, in offspring from the *vit*-Av3-Vg40 test cross, genotype did not affect pupation ($F_{(7,\ 16)}$= 1.277, p= 0.322), or eclosion ($F_{(7,\ 16)}$= 0.373, p= 0.905), Figure 3.22. This contradicts previous results where partial lethality pre-pupation was observed in the *vit*-Av3 genotype off tet. The likely explanation is that the sub-lethality observed in immature *vit*-Av3 mosquitoes may have been due to the rearing conditions and/or handling stress specific to the experiment, which may have had a greater effect on the more vulnerable insects.

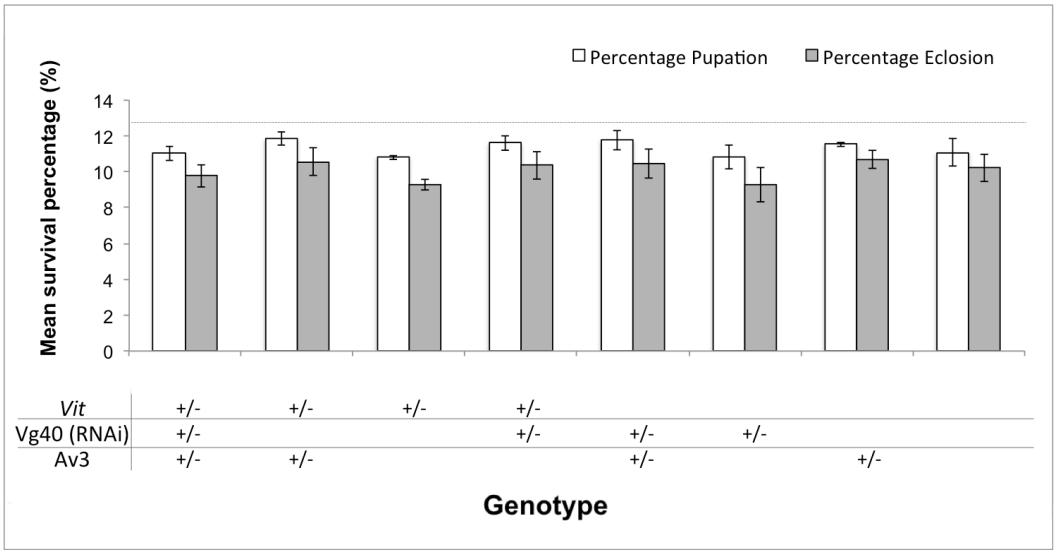

Figure 3.22 Percentage pupation and adult eclosion of progeny from *vit*-Vg40-Av3 cross. Pupation is shown as a percentage from 1000 aliquoted larvae, 12.5% of each genotype was expected, indicated by the grey line (n= 3). Adult eclosion was calculated as a percentage from aliquoted larvae.

In addition, genotype did not affect the survival of female progeny following a blood meal (χ^2= 4.5, df=2, p= 0.107); there was no significant difference between results from three experimental repeats (χ^2= 0.5, df= 2, p= 0.798), so survival analysis was performed on pooled data.

3.3.4.2 RNAi against the 'fainting' phenotype caused by *vit*-AaHIT

Fewer pupae carrying both *vit and* AaHIT lethal constructs survived regardless of whether they carried the Vg40 RNAi construct compared to other genotypes ($F_{(7, 16)}$=62.217, p<0.001, ω^2= 0.947), whereas genotype did affect adult eclosion ($F_{(7, 16)}$= 1.613, p= 0.202), Figure 23.

As seen in progeny from the *vit*-Av3 effector cross, this indicates that the lethal effect in insects carrying *vit*-AaHIT constructs probably occurred early during larval development because there was no visible lethality in trays. Perhaps there was a fitness cost associated with the presence of two or more constructs, either due to leaky expression of the constructs, or effects of flanking genomic regions due to the insertion sites. Furthermore, perhaps the RNAi machinery was not assembled at a suitable time and quantity for suppression of the leaky lethal constructs, resulting in the secretion of the lethal protein, if they indeed were secreted outside of the fat-body.

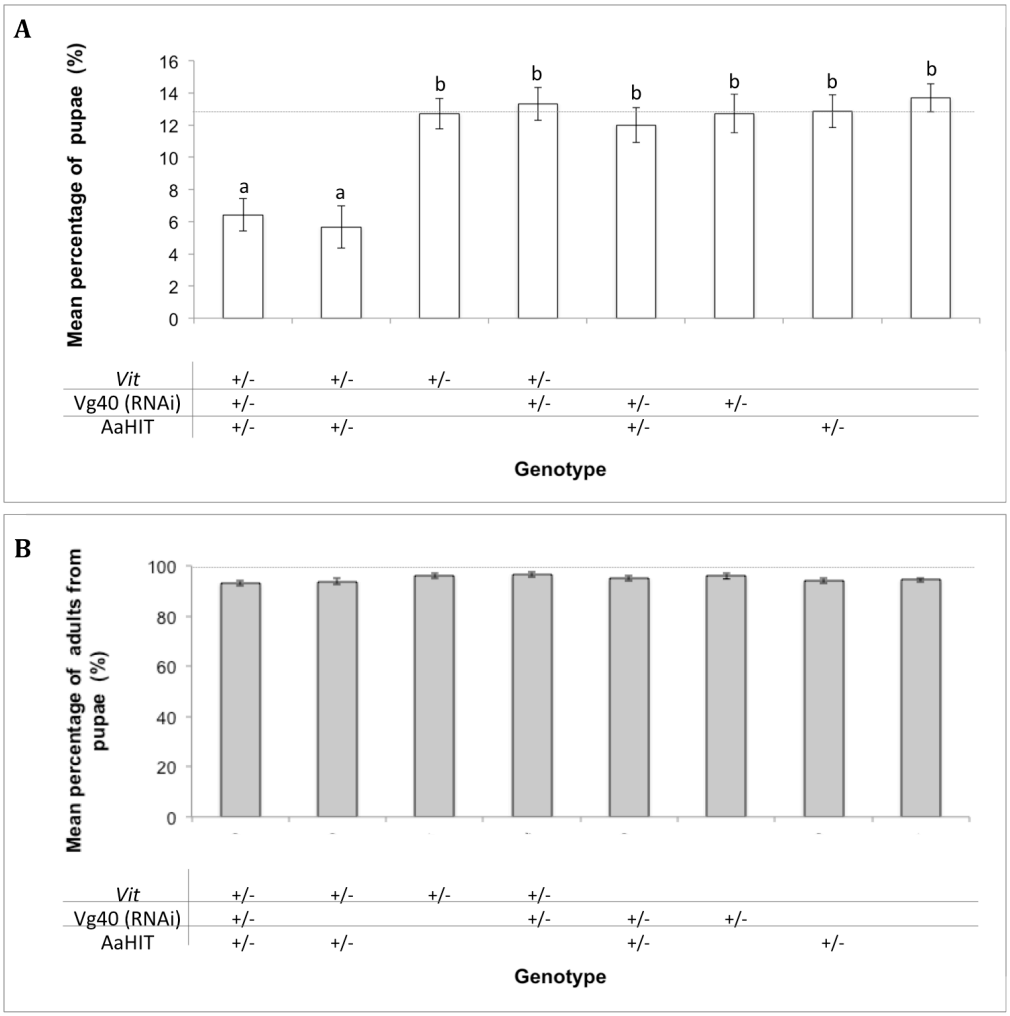

Figure 3.23 Percentage pupation (A) and adult eclosion (B) of progeny from *Vit*-Vg40-AaHIT test cross. Pupation is shown as a percentage from 1000 aliquoted larvae, 12.5% of each genotype was expected, indicated by the grey line (n= 3). Adult eclosion was calculated as a percentage from pupae, with 100% expected if all pupae eclosed.

Paralysis was observed in females carrying the *vit*-AaHIT constructs regardless of whether they also carried the RNAi construct, Figure 3.24. The time to recovery was consistent with previous results, at approximately 24 hours after paralysis.

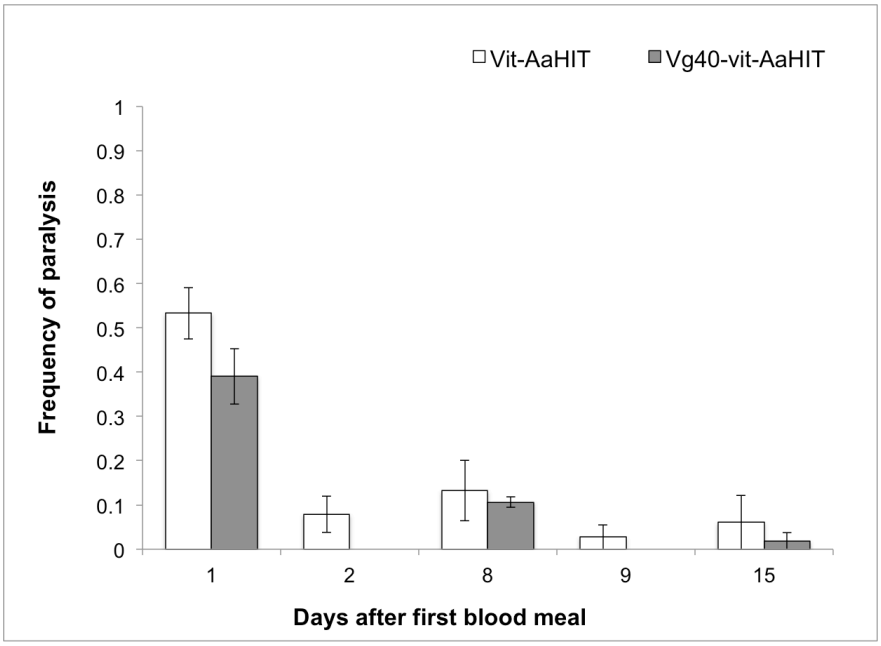

Figure 3.24 Frequency of paralysis in females carrying *vit*-AaHIT constructs either with the Vg40 RNAi construct or without. Insects were provided with blood on days zero, seven and fourteen; error bars show standard error of the mean

All survival curves where paralysed insects were classified as dead are shown in Appendix 1.7. It was difficult to conclude whether there was any suppression of the lethal constructs in the presence of RNAi. Therefore the paralysis frequency data was converted to Kaplan Meier survival estimates, where paralysis was defined as an event, and the likelihood of this event occurring in living females was calculated as a proportion of live insects at any time point. Dead insects were removed from the analysis at the specific time death had occurred, and therefore only the frequency of paralysis was compared. There was no statistically significant difference between the three repeats (χ^2= 1.9, df= 2, p= 0.393), so results were pooled for this analysis, Figure 3.25.

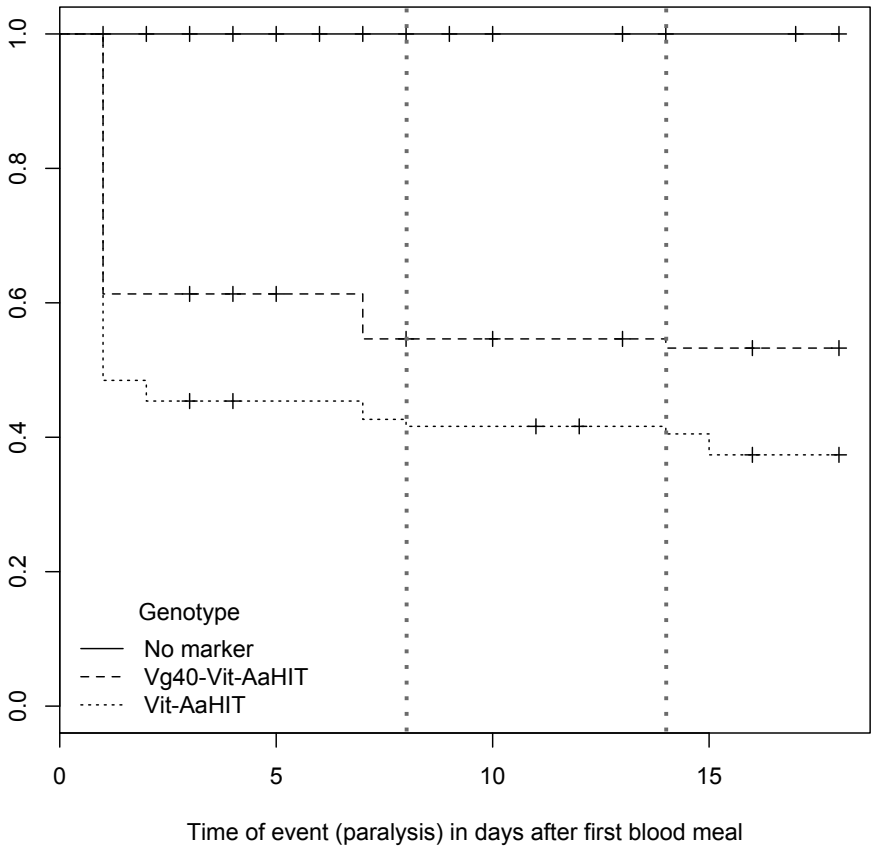

Figure 3.25 Kaplein Meier analysis of paralysis in females carrying *vit*-AaHIT construct with or without the Vg40 RNAi construct. Results are pooled from three experimental repeats. No_marker= non-transgenic; insects were provided with blood on days zero, seven and fourteen, shown by horizontal lines; event= paralysis; dead insects were removed from the analysis, and therefore this only compares the likelihood of paralysis among live insects at any time point.

Consistent with previous experiments, a significant number of females carrying the *vit*-AaHIT constructs exhibited paralysis after a blood meal compared to non-transgenic females (χ^2= 275, df= 1, p<0.001). In addition, approximately 20% more *vit*-AaHIT females exhibited paralysis compared to Vg40-*vit*-AaHIT carrying the RNAi construct (χ^2= 4.7, df= 1, p= 0.029). This implied a partially rescued phenotype, which is visible

in Figure 3.25, however, rescue by RNAi was not near levels that were achieved using tet.

Fully recovered females removed from cages due to paralysis were crossed to wild-type males reared at the same density, and were placed in separate tubes to lay. Twelve recovered *vit*-AaHIT females and ten Vg40-*vit*-AaHIT females laid eggs. Too few were laid for any analysis to be carried out. This may be due to the damage caused to females when handling them at such a fragile stage, or it may be due to a non-repressible detrimental effect of the two component lethal system.

3.4 Conclusions and recommendations

This ambitious project set out to provide a proof of principle for a component of an underdominance system combining refractory and rescue mechanisms into a single element. The proposed system exploited the RNA interference system directed against the dengue virus-2 envelope protein mRNA's target sequence (DEN2), whereby a two component lethal system was designed to include a fusion DEN2-tTAV *trans*-activator. Therefore any lethality induced by the killing component and the dengue virus would be knocked down simultaneously.

The lethal potential of each effector line was tested under the control of *hex*[16], and suitable lines were then examined under the control of *vit*[17] and *carb*[18]. AaHIT was the only effector able to induce a recognisable phenotype after a blood meal (24-hour paralysis under the control of *vit*)

[16] *Hex= Aedes altropalpus hexamerin 1.2* promoter
[17] *Vit= Ae. aegypti vitellogenin 1* promoter.
[18] *Carb= Ae. aegypti carboxypeptidase A promoter.*

with reasonable penetrance. Furthermore, paralysis was suppressed in the presence of tetracycline (tet).

In a separate experiment, the RNAi construct was able to knock down the upregulation of DEN2-tTAV transcripts after a blood meal to significantly low levels; this gave good grounds to test the ability of the RNAi to rescue the *vit*-AaHIT phenotype. Furthermore, the phenotype was easy to score and was enough to test suppression. Temporary paralysis was only partially rescued by the RNAi.

One explanation for the limited RNAi suppression is that there is a very low sensitivity threshold in adult *Aedes aegypti* towards AaHIT. This is plausible because we know that the upregulation of DEN2-tTAV mRNA after a blood meal is knocked down by over 96% in the presence of the anti-DEN2 RNAi construct (Figure 3.9). Crucially, this could be confirmed by setting up an experiment to quantify the transcript levels of the AaHIT and tTAV mRNAs in females carrying constructs at several stages: (i) before a blood meal, (ii) paralysed 24 hours after a blood meal, (iii) flying females that were not paralysed 24 hours after a blood meal, (iv) recovered females 48 hours after a blood meal, (v) females that were never paralysed taken 48 hours after a blood meal, and (vi) paralysed females 48 hours after a blood meal.

The phenotype was almost entirely suppressed in the presence of tet, provided throughout the life cycle, suggesting that the ineffectiveness of the RNAi was due to the tTAV mRNA levels. The tTAV mRNA levels in insects carrying the RNAi and the lethal transcripts were shown to be approximately 3.57-fold more in blood fed females than before a blood meal, compared to 91.52-fold more in females lacking the RNAi construct. The lower mRNA levels seen in females carrying RNAi could therefore be adequate to produce enough protein to induce toxic expression of the

effector. This proposed sensitivity-threshold of *Ae. aegypti* towards levels of AaHIT is consistent with outcomes from Wu *et al.* (2008); cotton bollworm (*Heliothis armigera)* larvae were more susceptible to the toxic effects of AaHIT when fed on transformed cotton plants expressing high levels of the AaHIT protein, and were able to survive effects when fed on plants expressing levels below a minimum-threshold.

One potential option to improve the RNAi-mediated knockdown of the lethal constructs is to put the target DEN2 sequence into the effector sequence as well as into the tTAV, providing the RNAi with additional targets. One possible negative aspect of this approach would be the need for duplication of the DEN2 sequence within the lethal construct if the tTAV and effector were in the same construct, leading to hotspots where recombination may occur.

It is also important to note that the heritable dengue-2 resistance phenotype in *Ae. aegypti* strain carb77 became notably weaker after just thirteen generations, and was completely lost after seventeen generations; the transgene no longer expressed the DEN2 hairpin, despite the absence of detectable mutations (Franz *et al.*, 2009). This was likely due to a fitness load of the RNAi transgene due to its genomic position, overcome by chromatin rearrangements that allowed the insect to silence the RNAi effector (Franz *et al.*, 2009; Adelman *et al.*, 2004). Given that the *vit*-Vg40-AaHIT cross was carried out a few generations after the initial DEN2-tTAV knockdown experiment, this phenomenon may be responsible for the inability of the RNAi to suppress DEN2-tTAV transcripts, though this would be apparent in the aforementioned recommended experiment.

We were limited by the need to express the RNAi in virally important tissues for refractoriness. This was especially important to maintain

linkage between refractoriness and the gene drive system. It would be interesting to determine whether provision of the larvae with tet is enough to suppress the 'fainting' phenotype in adults. This could be examined by carrying out a similar survival analysis in which the test treatment types would be (i) tet supplied in the larval water only, and (ii) tet provided at the adults stage in blood and sugar only compared to control groups: (i) no tet supplied and (ii) tet supplied through all stages. This would allow us to identify the extent to which stage-specific saturation of tissues with the antidote is important for the phenotype to be suppressed, and results could inform us as to whether using a slightly earlier-acting or more broadly-expressing promoter to regulate the rescue element might be more useful. Advances in *Ae. aegypti* transcriptomics will assist identification of more suitable promoters and regulatory elements to tailor expression of transgenes (Harker *et al.*, 2013).

Suppression could be achieved through other means. The transcription factor *groucho* could potentially be used (Fisher and Caudy, 1998), and transcriptional supression by the protein is under investigation (Chen and Courey, 2000; Nibu *et al.*, 2001). Homologous *groucho* proteins have already been identified in *Ae. aegypti* (Gene ID: AAEL010433) and *Culex quinquefasciatus* (Gene ID: AAEL010433), indicating that this strategy may be transferable to mosquito species.

In adition, multimers of synthetic microRNAs were designed to silence genes as the lethal component of an engineered underdominance-based gene-driver in *Drosophila* (Akbari *et al.*, 2013). Multimerisation of small interfering RNAs has shown enhanced gene-silencing efficiencies (Mok *et al.*, 2010), and is also an attractive alternative to the RNAi system used in this chapter.

Another challenge was ensuring that the lethal phenotype was fully penetrant in adults. Of all effectors examined, only the AaHIT gave replicable fitness-reducing phenotypes, but was only up to 50-68% penetrant 24 hours after the first blood meal, and up to 80% by the third. Therefore, penetrance of the lethal constructs was incomplete because up to 20% of individuals failed to exhibit paralysis despite carrying the constructs, as determined by the presence of the corresponding fluorescent markers. Temporary paralysis was considered an important fitness cost for the female mosquitoes because they would be exposed to predators in the wild. Therefore, even partial penetrance is sufficient to bring about population replacement. However extreme underdominance (*i.e.* 100% penetrance of the lethal genes) is preferred to avoid a strong selection pressure towards insect resistance to the lethal effects of the transgenes. Moreover, partial penetrance would reduce the ability of the system to spread, may facilitate re-invasion of wild-type alleles after apparent success, and could also complicate or slow recalling the constructs.

In invertebrates, the fat-body is the functional analogue of the liver (Wyatt, 1980), and is essential for lipid synthesis for hormonal regulation and egg development (Hagedorn and Fallon, 1973; Ziegler, 1997). Nevertheless it was not a sensitive tissue to many of the effectors used, and any effects shown from the Av3 and AaHIT neurotoxins were most likely due to the secretion of the proteins rather than specific targeting of the fat-body. This may be because adults do not undergo any significant developmental and metamorphic stages that leave the insect vulnerable to the effects of otherwise disruptive proteins.

Lethality is perhaps easier to induce in the more sensitive immature stages, such as the mitotic embryonic, early larval and pupal stages. The earlier-acting *hex* used in this chapter, may be a good choice to drive

expression of the lethal constructs since lethality induced under the control of this promoter was often between 90-100% penetrant. This level of penetrance is far more desirable to avoid the rapid emergence of resistant insect strains in the field. However this requires unlinking refractoriness from the gene drive system since anti-pathogen RNAi is unlikely to be effective when driven by *hex*. Unlinking refractoriness from the gene drive mechanism would therefore facilitate a wider choice of promoters, which highlights the importance of developing a range of tissue- and stage-specific regulators and a panel of toxins for finely tailoring phenotypes.

An important limitation of this study was the use of only one line for each effector. Efforts were focussed on the development of the new secreted effectors (AaHIT and Av3) rather than re-injecting lines that were shown to induce lethality in insects. Furthermore, in some cases the effector lines had been selected previously based on their phenotypic characteristics (Gray, P., unpublished data). Nevertheless, as a result of testing only one line per effector, comparative analysis of the different lethal proteins was not possible because transgenes were inserted at different chromosomal loci. Even effector lines transformed using the site-specific ΦC31-*att* integration system (Thorpe and Smith, 1998) were not comparable because lines were integrated into *attP* target sites at different chromosomal loci. Therefore, phenotypic differences were due to an unspecified combination of the transgenes' intrinsic properties and their insertion sites (Wilson *et al.*, 1990). Consequently, it was also difficult to attribute any phenotypes to the specific feature of the architecture of the constructs. For example, the number of tetO repeats was different in some constructs (Table 3.2 and Table 3.3). More tetO repeats are thought to result in stronger expression of the effector when driven by the tTAV-tetO expression

system (Scaife, S., personal communication), so it may be possible to improve penetrance of the AaHIT phenotype by increasing the number of tetO repeats. However, it would be important to test this, and other construct features of interest, in a reliable and replicable way rather than rely on random variation due to unspecified positional effects.

Future work should therefore focus on using pre-characterised site-specific docking strains such as those developed in Chapter 4 to compare a panel of effectors, promoters, and suppressors directly to each other. Ideally, comparisons between effectors inserted into more than one docking strain are preferred, *i.e.* all constructs should be inserted into each site-specific docking line, because the expression and phenotype may vary by integration site. This comparative analysis would enable fine-tuning of components for assembly of a gene drive system in mosquitoes.

The recent development of a working engineered underdominance-based system in the well-studied *Drosophila* (Akbari *et al.*, 2013) is encouraging, and with comparable in-depth analysis of mosquito genomes in progress, this may soon be possible in *Ae. aegypti*.

Chapter 4

Engineering site-specific cassette exchange in *Aedes aegypti*

4.1 Introduction

4.1.1 Improving the existing tool-set for genetic engineering of *Ae. aegypti*

With the advent of genetic control strategies as an appealing alternative to traditional methods, efficient transgenesis of the target vector is essential. Non-autonomous transposable elements are now widely used for germ-line transformation of insects (Fraser, 2012). One of the most efficient transposon systems used to integrate transgenes into the genome is *piggyBac,* which was originally identified in a baculovirus from the cabbage looper moth, *Trichoplusia ni* (Cary *et al.*, 1989). This transposon inserts specifically into TTAA tetranucleotide target sites (Fraser *et al.*, 1983; Mitra *et al.*, 2008), and is commonly used to transform *Ae. aegypti*. The TTAA target site exists at many locations in the *Ae. aegypti* genome. Integration of the *piggyBac*-based constructs at some sites may cause positional effects that prevent the correct functioning of the transgene due to the presence of silencing or enhancing elements in the neighbouring

sequences (Wilson *et al.*, 1990). In addition, possible gene disruptions of the host DNA may induce a fitness penalty (Wilson *et al.*, 1990). Since phenotypic differences are due to an unspecified combination of the transgenes' intrinsic properties and their loci, this complicates comparative analyses of different transgenes.

Site-specific systems have been engineered to allow integration at highly specific target sequences that do not occur naturally in insect genomes. Examples of such engineered systems include ΦC31-*att* (Thorpe and Smith, 1998), Cre-*loxP* (Sauer and Henderson, 1988) and FLP-*FRT* (O'Gorman *et al.*, 1991), which require the initial integration of the target-sequence into the host's genome as a 'docking site' using a transposon-based vector. Once a transgenic line has been established, transgenes of interest can be inserted into the docking site in the presence of the appropriate integrase (Groth *et al.*, 2004). If construct architectures are kept consistent, these can be directly compared and differences attributed to the transgenic modifications since the positional effects remain constant.

The ΦC31 system, from a *Streptomyces* bacteriophage, involves recombination between the specific phage and bacterial attachment sites *attP* and *attB* in the presence of the ΦC31 integrase. Integration of the *attB* into *attP* produces hybrid sites *attL* and *attR* (Thorpe and Smith, 1998), Figure 4.1. These hybrid sites are no longer recognised by the integrase, making ΦC31 integration stable and efficient for transgenesis.

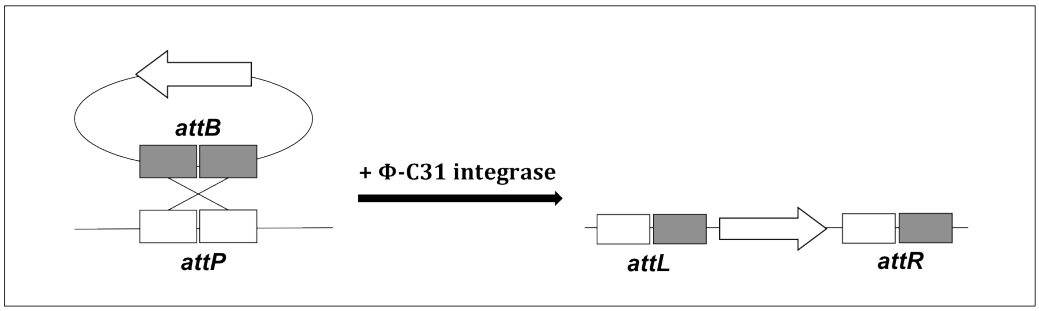

Figure 4.1 Schematic of ΦC31 site-specific integration. Recombination of the *attB* (grey) and *attP* (white) sites in the presence of ΦC31 integrase results in the crossover of half-sites, forming hybrid *attL* and *attR* sites. These are no longer recognised by the integrase, and so the integration of the donor plasmid's transgene (white arrow) is stable.

Standard molecular techniques require the use of a selectable marker, such as ampicillin resistance, to allow cloning of constructs for injection. The site-specific integration of an *attB* plasmid, due to recombination at a single site, introduces these antibiotic resistance genes — as well as other backbone sequences such as the bacterial origin of replication, *ori* — into the mosquito genome. From a regulatory and public perception perspective this is not ideal for any field release as it represents the introduction of extraneous sequences, some of which code for resistance genes. Concerns of this nature have been more prominent in plant transformation, where despite no known harmful effects, public perceptions of possible risks have limited the acceptance of transgenic products in some British and other international markets (Matthews *et al.*, 2001).

There are also concerns of resistance-gene transfer to microorganisms in the environment, potentially leading to resistant pathogens (Macer, 2003; Tiedje *et al.*, 1989; Hoy, 1995; Nuffield Council on Bioethics, 1999). In mosquitoes, there is some evidence of horizontal gene transfer from

Wolbachia into *Aedes,* presumably because the mosquito harboured the bacteria at some point in its evolutionary history (Klasson *et al.*, 2009), but generally speaking the risks have been considered low (Murphy *et al.*, 2010). To overcome the risks of horizontal gene transfer in plants, a number of techniques have been developed to remove resistance genes of the introduced plasmid's backbone (Yoder and Goldsbrough, 1994). These include:

(i) co-transformation of separate constructs into independently segregating loci, one construct carrying the gene of interest and the other carrying the selectable marker gene, and then allowing effective separation in subsequent generations. This is a complicated procedure that is impractical for use in *Ae. aegypti* as transformation efficiencies are low, and chemical-based selectable markers are not currently suitable for use in mosquitoes since drug resistance can be problematic.

(ii) intra-genomic relocation of transgenes via transposable elements, allowing subsequent separation as before. This is a strategy that could not be applied to *Ae. aegypti,* since transposons are not efficiently remobilised in germline cells (Palavesam *et al.*, 2013; StJohn, 2012; Sethuraman *et al.*, 2007).

(iii) site-specific recombination systems to excise the desired sequences from the transgenic construct using systems such as CRE-*loxP* or FLP-*FRT*.

The CRE-*loxP* system was originally described in the bacteriophage P1 found in *Escherichia coli,* where the Cre (causes recombination) recombinase excises DNA flanked by two identical *loxP* (locus of crossing over (x), P1) target sites (Austin *et al.*, 1981). The FLP-*FRT* system comes from the 2 μm plasmid of *Saccharomyces cerevisiae*, where FLP

(flippase recombinase) excises intervening DNA between identical *FRT* (flippase recognition target) sites in *cis* (McLeod *et al.*, 1986). Both recombination events create a synaptic complex if the target sequences are present as direct repeats, which excises the intervening DNA, Figure 4.2.

These systems have been used to manipulate transgenes in diverse organisms. Excision has been shown using both CRE-*loxP* and FLP-*FRT* systems in *Drosophila melanogaster* (Siegal and Hartl, 1996; Golic *et al.*, 1997; Gong and Golic, 2003; Groth *et al.*, 2004), and *Ae. aegypti* (Jasinskiene *et al.*, 2003; Morris *et al.*, 1991).

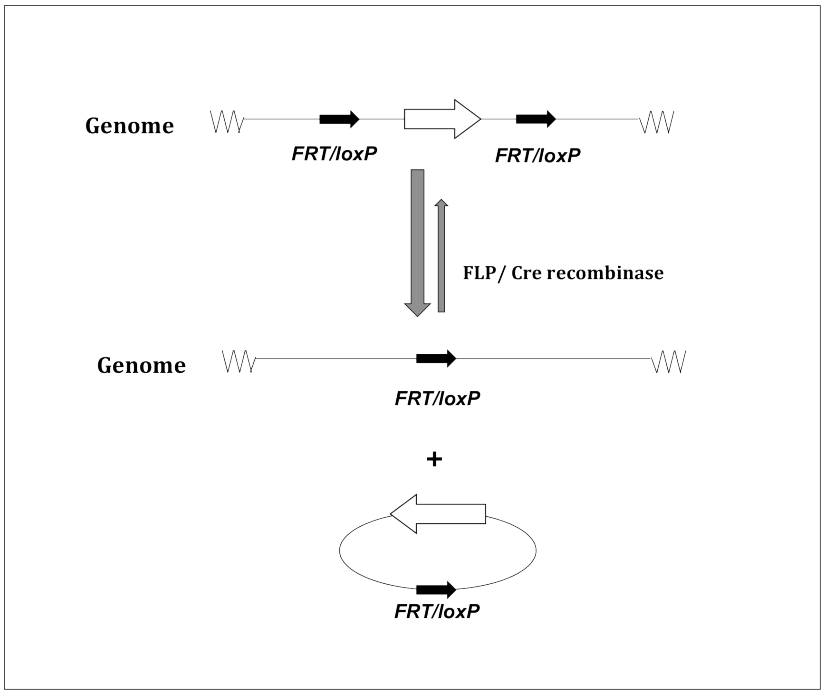

Figure 4.2 Cre- and FLP-recombinase mediated excision. Recombination between tandem repeats of *loxP* or *FRT* sites in *cis* (black arrows) causes excision of flanked DNA (white arrow). Recombination between two sites in *trans* results in insertion of the transgene; this reaction is less favoured (grey arrows).

Recombinase-mediated cassette exchange (RMCE) is when a target sequence, pre-inserted into the host's genome, is completely exchanged with a donor cassette. RMCE enables the integration of engineered plasmid constructs without the prokaryotic vector backbone if both the target and donor sequences are flanked by inverted (or 'heterospecific') recombinase attachment sites (Schlake and Bode, 1994).

In *D. melanogaster*, RMCE systems have been successfully shown using a single round of injections mediated by FLP (Horn and Handler, 2005), Cre (Oberstein *et al.*, 2005) and ΦC31 (Bateman *et al.*, 2006) recombinases, and with the ΦC31 recombinase in *Ae. aegypti* (StJohn, 2012). The use of a single round of injections makes these systems less laborious for engineering transgenic mosquitoes. However, integration of transgenes in the first round, and subsequent excision of extraneous sequences in a separate step is an attractive tool. Carefully designed donor cassettes could be used to compare transgenes in the presence or absence of additional elements that can later be excised from the specific recombination sites. Furthermore, multiple transgenes of interest can be inserted into the genome at the remaining *FRT* or *loxP* recombinase docking-site, although these reactions would be reversible (Figure 4.2).

This chapter examines the ability of the ΦC31-*att*, Cre-*loxP* and FLP/FRT systems to work in *cis* and allow the exchange of marker genes in *Ae. aegypti,* and provides a proof of principle for the site-specific cassette exchange system shown in Figure 4.3. It consists of three phases.

The first phase requires the pre-insertion of one *piggyBac*-based molecular construct carrying the *attP* docking site and one each of FRT- and Cre-recognition sites into the *Ae. aegypti* genome. More than one

transgenic *attP* docking line is required because the efficiency of the cassette exchange system may vary by genomic integration site and some docking lines may work better than others. Therefore the general properties and fitness of each docking line should be characterised for any subsequent use.

The second phase requires the integration of an *attB* donor plasmid that also carries an FRT- and a Cre-recognition site, into the *attP* docking site. Successfully integrated transgenic lines carry the *attB* transgenes that include the plasmid backbone flanked by *loxP* and *FRT* sites on either side.

The third phase requires the provision of Cre- or FLP-recombinase for the excision of the plasmid backbone, flanked by the two recognition sites (*loxP* or *FRT* respectively), from the genomic DNA. This completes the RMCE system resulting in (i) integration of the *attB* construct and (ii) the excision of plasmid backbone sequences.

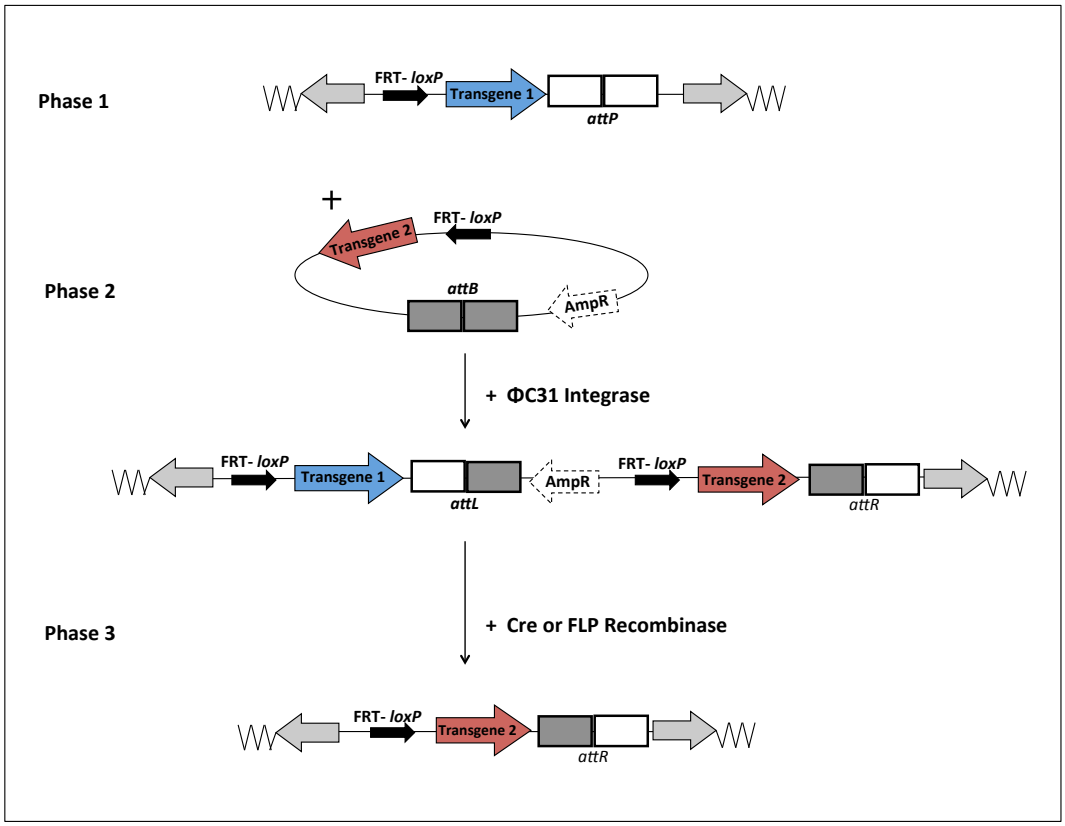

Figure 4.3 Schematic of the proposed recombinase-mediated cassette exchange system. Small black arrows indicate engineered *FRT* and *loxP* sites present in donor and acceptor constructs; AmpR represents the plasmid backbone sequences, which includes the ampicillin-resistance gene. The Transgene 1 cassette (blue arrow) is exchanged for Transgene 2 (red arrow) in three phases. Phase 1: The *attP* docking site is inserted into the genome using the *piggyBac*-based system; grey arrows indicate *piggyBac* ends. Phase 2: ΦC31 integration of an engineered *attB* donor plasmid occurs. Phase 3: In the presence of FLP or Cre recombinase, the flanked region is excised leaving one intact *FRT* and one *loxP* site, and the *attR.* Subsequently, genes of interest can be integrated at the specific *FRT* or *loxP* site that remains in the genome, using FLP or Cre recombinases, respectively.

4.1.2 Experimental aims

The work in this chapter intended to combine the ΦC31-*att* integration system with the Cre-*loxP* and FLP/FRT excision systems to develop a recombinase-mediated cassette exchange system, shown in Figure 4.3, resulting in: (i) the integration of exogenous genetic material into the *Ae. aegypti* genome, and (ii) the excision of plasmid backbone sequences in a separate step.

4.2 Methods

4.2.1 Mosquito rearing

Ae. aegypti strains were reared according to standard operating procedures described in Chapter 2. They were reared at 26°C (±1°C) and 80% (±10%) humidity with a 12:12 hour light:dark cycle and constant air circulation.

4.2.2 Background of existing *Ae. aegypti* docking strain Keele2, and molecular constructs[19] used in this study

Construct maps are shown in Figure 4.4. The *attP* docking strain called '2', carrying the OX3860 *pBac*[3xP3-ECFPaf]-*attP* construct, was developed at Keele University (Nimmo *et al.*, 2006). Hereafter, this *attP* '2' docking strain is referred to as Keele2.

The *piggyBac*-based docking construct OX4476 carrying *pBac*[FRT-*loxP*-3xP3-AmCyan-a*ttP*], is based on the OX3860 plasmid *pBac*[3xP3-ECFPaf]-*attP* (Nimmo *et al.*, 2006) with a different fluorescent marker gene. The donor construct OX4494 carries an *attB, FRT*, and *loxP* site, and two markers: 3xP3-DsRed2 and Hr5ie1-ZsGreen. Donor constructs OX4580 and OX4712 are based on OX4494, the only difference being the transformation markers; OX4580 carries the Hr5ie1-ZsGreen marker only, and OX4712 carries the 3xP3-DsRed2 marker only, summarised in Table 4.1.

Table 4.1 *attB* donor constructs injected

Construct	Details	Transformation markers
OX4494	*attB* 3xP3-DsRed2-*FRT-loxP*-Hr5iE1-ZsGreen	Red eyes and green body
OX4580	*attB FRT-loxP*-Hr5iE1-ZsGreen	Green body only
OX4714	*attB* 3xP3-DsRed2-*FRT-loxP*	Red eyes only

[19] Sarah Scaife built constructs according to standard molecular techniques.

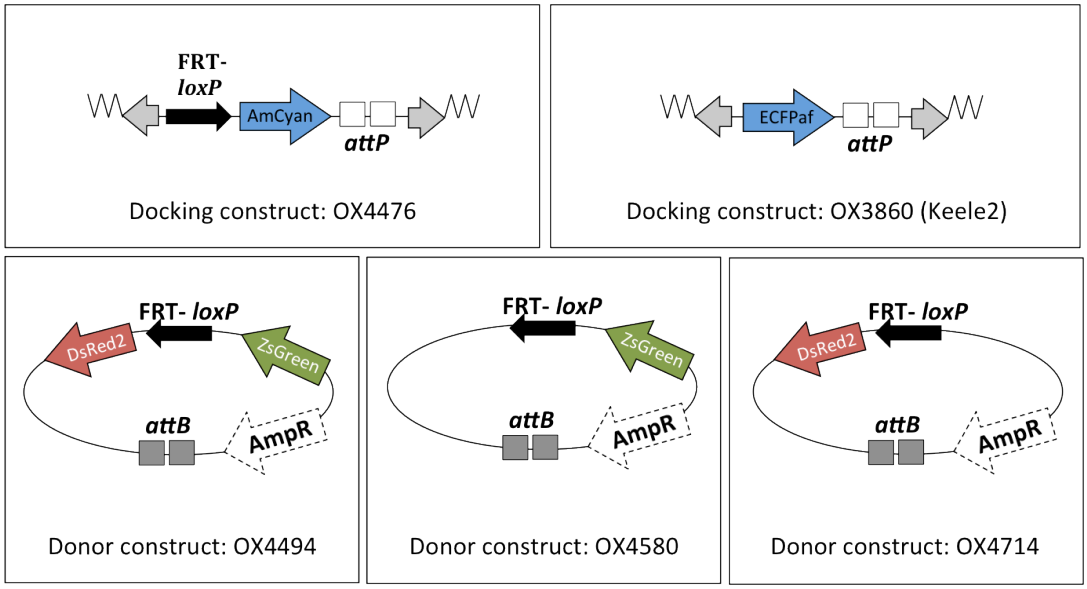

Figure 4.4 Construct maps showing linearised *attP* docking constructs (upper panels) and the donor *attB* constructs (lower panels). The DsRed2, AmCyan, and ECFPaf markers are driven by 3xP3, and Hr5ie1 drives expression of the ZsGreen.

4.2.3 Microinjection of *Ae. aegypti*

Injection solutions were prepared and *Ae. aegypti* embryos were transformed as described in Section 2.2. Injection solutions contained: 300 ng μl^{-1} DNA and 700 ng μl^{-1} *piggyBac* transposase mRNA for *piggyBac* transformation; 300 ng μl^{-1} DNA and 730 ng μl^{-1} ΦC31 integrase mRNA for ΦC31 integration; and 700 ng μl^{-1} of recombinase mRNA for either Cre and flippase (FLP) excision. For ΦC31-*att* integration and Cre/FLP excision, G0s that did not express the transformation marker for the *attP*-docking construct (3xP3-AmCyan) were discarded.

4.2.4 Molecular genotyping of integration and excision events

PCRs were carried out as described in Section 2.6; primers were purchased from MGB (Applied Biosystems, UK), and primer pairs are shown in Table 4.2. Genomic DNA from *Ae. aegypti* line OX3582 [3xP3-AmCyan-*attL*-3xP3-DsRed2-teto21-hsp-adh-AeMichxc-*attR*] was used as a positive control for PCR amplification of ΦC31 integration junctions' *attL* and *attR*; OX3582 is referred to as michelob_x in text.

Table 4.2 PCR primer pairs used and genomic regions amplified; primers' order names and numbers are included. 'P' numbers in the first column are primer pair numbers referred to in Figure 4.7 and Figure 4.9.

Amplicon [size]	Forward Primers	Reverse Primers
P1: 3xP3- ECFP [755 bp]	ECFPDiagR (TD1342): GTACAGCTCGTCCATG CCGAGAG	Diag-3xP3 (TD876): GGTACCGCTAGAGTCGAC GG
P2: *loxP/FRT*-DsRed2 [500 bp]	FRTAscF (SS.1136): TCGAAGAAGTTCCTAT TCCGAAGTTCC	RedSeqF (SS.20): AAGGGCGAGACCCACAAG G
P3: Hr5iE1-ZsGreen [511 bp]	Diag2-ie1 (TD548): GCAGTATAAATTGACG TTCATGTTGG	Diag-green (TD223): GAACAGGAAGGAGCGGTC CCAGG
P4: *attL* [491 bp]	Diag-recomb (TD635): CGACTCACTATAGGGC GAATTGG	Diag3pb-xho (TD936): TCATCTGATGTACCAGGC ACTTC
P5: *attR* [394 bp]	AttPPstF(SS.1046): GTTCTGTGATGACCTG CAGCCCG	AttBXhoR (SS.1090): GGTGTGCTCGAGAAGCTT ATCGATACCGTCGACATG
P6: OX4476C 5' genomic-*pBac* junction [1084 bp]	AttPFlF1 (RHK.F1): TGGGATTCGATTCGAA CTCATG	PB1 (flanking): GGCGACTGAGATGTCCTA AATGCAC
P7: OX4476F 5' genomic-*pBac* junction [498 bp]	AttPFlF3 (RHK.F3): GTTGATGGCGGTGCGA CAATATAAC	PB1 (flanking): GGCGACTGAGATGTCCTA AATGCAC
pBac-DsRed2: across excision junction if excision: [845 bp] if not: [5556 bp]	Seq-transp-3 (TD.61) CCTCATGATGTGTGAC AGTGGTAC	RedseqF (SS.20) AAGGGCGAGACCCACAAG G

4.2.5 Alignment of *attP* attachment-site sequences

The plasmid DNA of OX3860 (resulting in the Keele2 strain) and OX4476 constructs were sent to GATC Biotech for sequencing. ClustalX version 2.1, which is available freely from www.clustal.org, was used to align the OX3860 and OX4476 *attP* sites with the bacteriophage *attP* attachment site sequence available from Genbank (accession: AJ006589.2).

4.2.6 Mendelian inheritance of the OX4476 construct

The progeny of hemizygous OX4476 lines were crossed to the wild-type, and the Pearson's chi-squared (χ^2) analysis was used to compare observed and expected genotype ratios (Pearson, 1900). In addition, progeny of the OX4476 hemizygous lines where crossed to hemizygotes of the same line, and the same statistical analysis was used to compare the observed and expected genotype ratios (Pearson, 1900).

4.3 Results and Discussion

4.3.1 Phase I: Development of OX4476 acceptor *attP* strains

Of the 2000 eggs injected with the *attP* docking construct OX4476, 400 larvae successfully hatched (20% survival). This is equivalent to the percentage survival routinely obtained in *Ae. aegypti* (*e.g.* Kokoza *et al.*, 2001; Nimmo *et al.*, 2006). Of the hatched larvae, 25% survived to adulthood (total= 101; 48 females, 53 males), but no transient expression was observed in the G0 larvae. In total, six independent lines of OX4476

were produced, each originating from separate G0 pools. Mendelian inheritance data are shown in Table 4.3.

The progeny of OX4476 lines B, C, F, H and J, when crossed to the wild-type, showed a transgenic versus wild-type ratio statistically indistinct from the expected 1:1 transgenic:non-transgenic ratio, indicative of typical Mendelian inheritance of a single copy of the transgene. For line I, the significantly lower proportion of transgenic progeny indicated (i) a severe fitness cost associated with the integration of the construct, or (ii) that the transformation marker was not expressed adequately to distinguish transformants from non-transgenic insects; line I was discarded. No obvious variation in the intensity or pattern of the 3xP3-AmCyan transformation marker was observed between remaining lines.

Table 4.3 Inheritance of the OX4476 construct in progeny from hemizygous to wild-type crosses. Chi squared analysis (χ^2) was used to quantify the significance of the difference between the observed number of transgenic:non-transgenic offspring (TG:NTG) and the expected 1:1 ratio according to Mendelian inheritance predictions, where $p<0.05$ represents a significant difference (*).

Effector lines	TG: NTG	% Transgenic	χ^2 (df= 1)	p-value	Effect size (Φ)
OX4476B	1036:1065	49	0.40	0.82	
OX4476C	1114:1141	49	0.32	0.85	
OX4476F	1819:1830	50	0.03	0.98	
OX4476H	67:71	49	0.12	0.94	
OX4476I	24:124	16	67.57	<0.001*	0.676
OX4476J	84:90	48	0.21	0.90	

To check the integrity of the docking site, the *attP* sequences of the new OX4476 docking lines and the Keele2 strain, as well as the bacteriophage ΦC31 *attP* site (GenBank AJ006589.2), were aligned in ClustalX.

One mismatched nucleotide, the transversion of a guanine (G) residue in the original bacteriophage *attP* attachment site for thymine (T), was observed in Keele2 and OX4476 *attP* sites, Figure 4.5. The OX4476 plasmid was derived from the Keele2 construct and so carries the same mutation. This mutation is outside the 39 bp region thought to be essential for ΦC31-*att* integration (Groth *et al.*, 2000). The Keele2 strain carries a functional *attP* site, as shown by integration of *attB*-marker constructs into this docking strain by Nimmo *et al.* (2006), and by integration of *attB*-effector constructs at Oxitec Ltd (Fu *et al.*, 2010; Gray, P., personal communication). Therefore, the sequence was also expected to work in OX4476 docking lines, although it is possible that the point mutation makes integration into these sites less efficient.

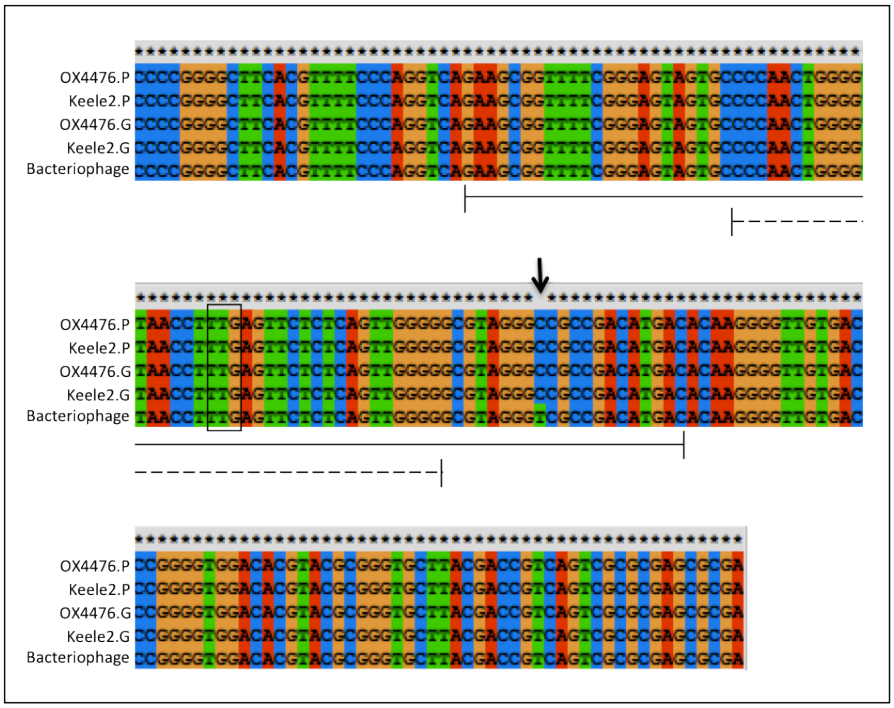

Figure 4.5 ClustalX alignment of *attP* sequences from OX4476, Keele2 and bacteriophage ΦC31 DNA. P= plasmid DNA, G= genomic DNA, stars indicate matched nucleotides. The continuous line below sequences indicates the 84 bp *attP* attachment site identified by Thorpe and Smith (1998). Within this region there is one mismatched nucleotide (black arrow). The dashed line indicates the minimum required 39 bp region thought to contain critical sequences for ΦC31 integration (Groth *et al.*, 2000). The *attP* crossover with the *attB* site occurs within the boxed TTG nucleotides (Thorpe *et al.*, 2000). The OX4476 *attP* sequences were identical in all lines, so only one genomic sequence is shown.

4.3.2 Phase II: Site-specific integration of donor *attB* constructs

As the Keele2 strain's docking site had been shown to work despite the point mutation (Nimmo *et al.*, 2006; Fu *et al.*, 2010; Gray, P., personal communication), and its *attP* docking-site sequence is identical to the new OX4476 lines, Keele2 was used as a positive control.

To test the functionality of the *attB* donor construct OX4494 — which carries the 3xP3-Ds-Red and Hr5ie1-ZsGreen markers and the two

additional recombination sites *FRT* and *loxP,* see Figure 4.4 for construct map — over 2000 Keele2 embryos were injected with OX4494 and ΦC31 integrase mRNA. Approximately 20% survived post-injection, and of these 40% survived to adulthood (total= 121; 53 females, 68 males). There was transient expression of the OX4494 markers in 33.2% of the G0 larvae. All G0 survivors were crossed to wild-type in sixteen pooled G0 cages, and their offspring were screened for fluorescence. G1s expressing the donor construct's 3xP3-DsRed2 and Hr5ie1-ZsGreen in addition to the docking construct's 3xP3-AmCyan (Figure 4.6) indicated one successful integration event. This line was called Keele2[OX4494]. Precise transformation efficiency was not calculated, as there were too few G0 pooled cages; assuming 50% G0 fertility, the minimum calculated transformation efficiency was 1.65%.

The eye-specific 3xP3-DsRed2 marker was also expressed in the anal papillae, previously shown in Keele2 integrations with the 3xP3 promoter (Nimmo *et al.*, 2006). The green body-specific marker, Hr5ie1-ZsGreen, was almost indistinguishable from the green autofluorescence, with fluorescence prominent in the anal papillae and siphon, Figures 4.6C and 4.6G. These characteristics may be due to the genomic position of the docking site in the Keele2 strain.

PCR analysis of Keele2[OX4494] indicated ΦC31 integration, Figure 4.7. The *attL* and *attR* junctions in Keele2[OX4494] and the positive control for integration (michelob_x) were successfully amplified. The Hr5ie1-ZsGreen marker unique to the donor OX4494 *attB* construct was amplified in Keele2[OX4494] and the positive control (OX4494 plasmid DNA). The Keele2[OX4494] *attL* and *attR* junction amplicons were

sequenced and confirmed ΦC31-*att* integration (data not shown). These results confirmed that the Keele2 strain, carrying an identical docking site to OX4476 test lines, carries a functional *attP* recognition sequence, and that the *attB* donor construct OX4494 is able to recombine with an *attP* docking site.

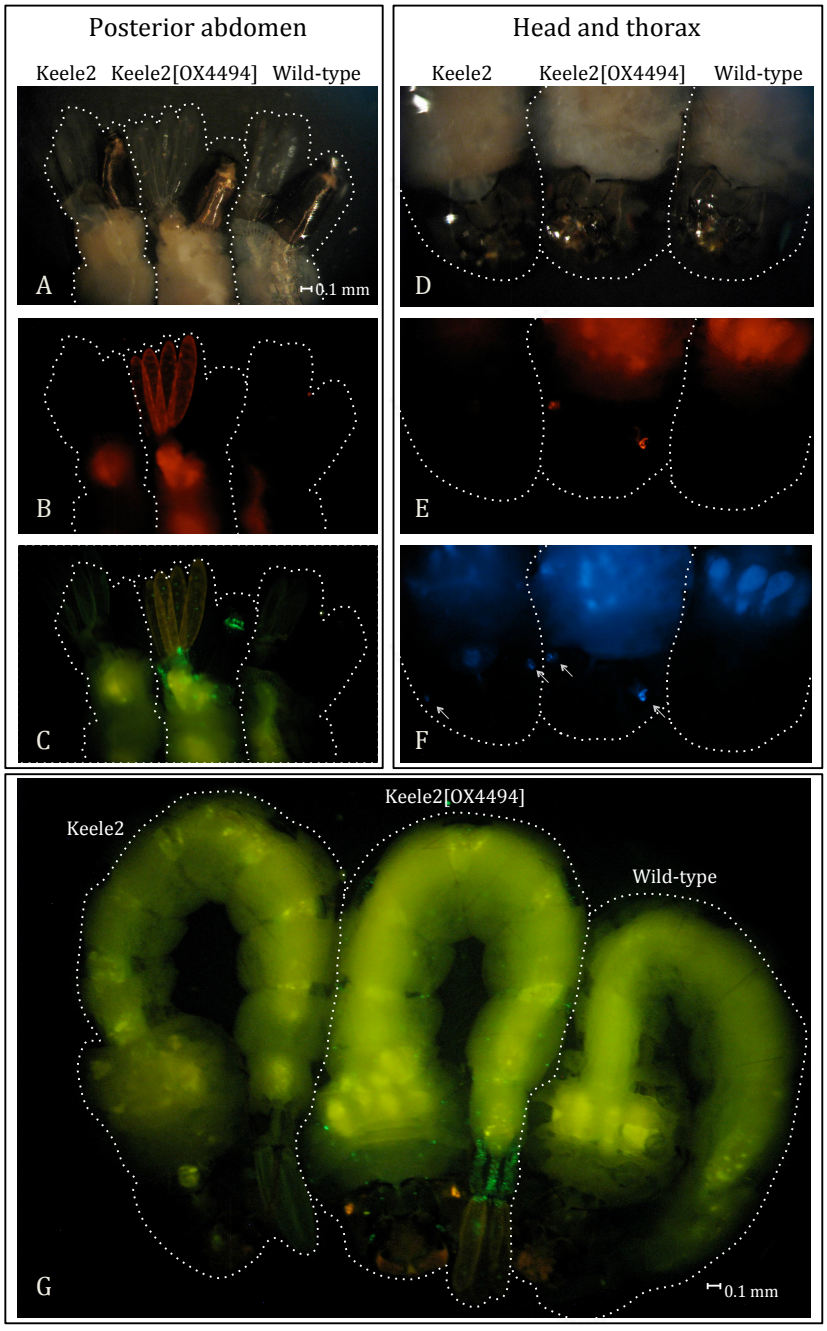

Figure 4.6 Fluorescent phenotypes of Keele2, Keele2[OX4494], and wild-type larvae; under white light (A and D), and red (B and E), green (C and G) and cyan (F) excitation light and filters. Arrows indicate fluorescence where the markers are difficult to see and white `dashed lines outline larvae. Images A-F were taken under the same magnification.

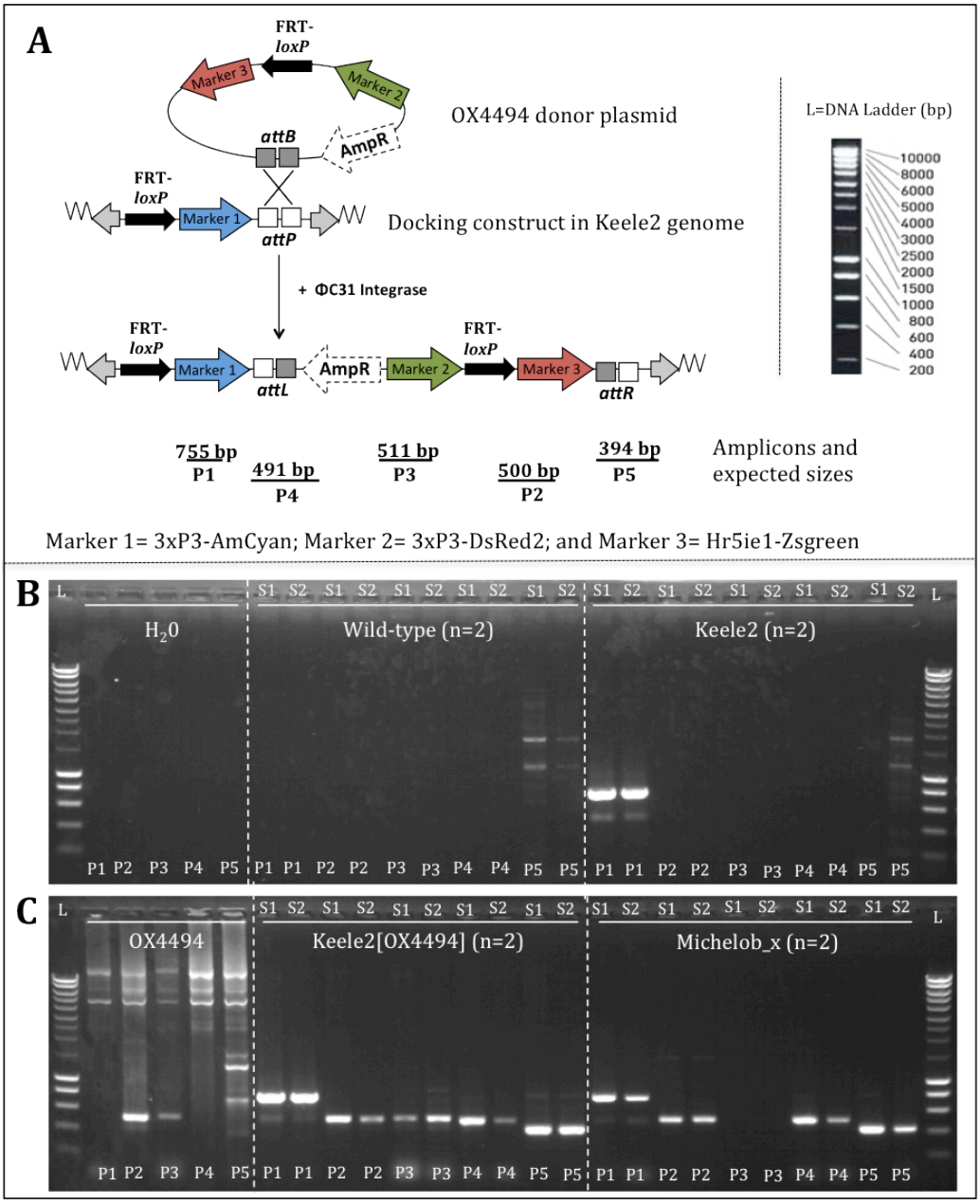

Figure 4.7 PCR analysis showing ΦC31-mediated integration of OX4494 into Keele2; **(A)** Schematic of ΦC31 integration and amplicons. **(B)** Two biological replicates (samples 'S1' and 'S2') were tested per PCR. H_20 and wild-type were negative controls for all primers; Keele2 was a negative control for ΦC31-*att* integration. **(C)** Diluted OX4494 plasmid DNA was a positive control for P2 and P3; michelob_x (a Keele2 strain carrying a different ΦC31 integrated plasmid than OX4494) was a positive control for ΦC31-*att* integration (P4 and P2) and also for the Keele2 construct's marker (P1). Canonical ΦC31-*att* integration was shown in Keele2[OX4494] samples as all reactions were positive, including the P1 and P2 *att*-integration junctions.

In the diamondback moth, *Plutella xylostella,* integration of a different *attB* donor construct, OX4580 — based on OX4494 but lacking the 3xP3-DsRed2 — showed successful integration into an *attP* acceptor strain OX4540[20]. Furthermore, ΦC31 integration efficiency is known to depend on the *attP* genomic location in a range of organisms as chromatin configuration or some flanking sequences at the target site may prevent the integrase from locating or attaching to *attP* (*e.g.* Bischof *et al.*, 2007; Labbé *et al.*, 2010; StJohn, 2012; Martins, 2011). Therefore, to test the new donor plasmid (OX4580) in *Ae. aegypti,* three independent OX4476 test lines, each originating from separate G0 crosses, and the Keele2 strain as a positive control, were injected with OX4494 and OX4580 donor plasmids in parallel.

There was no integration of OX4580 or OX4494 into the control Keele2 docking line and the OX4476 test lines, Table 4.4.

[20] OX4540, *pBac*[FRT-*loxP*-Opie2-DsRed2-a*ttP*], was derived from the OX4476 construct, (Martins, 2011). OX4494 donor plasmid was injected into *P. xylostella* OX4540, but no integration was observed (Martins, S., personal communication).

Table 4.4 Summary of OX4494 and OX4580 parallel injections. Integration efficiency in all lines was 0%.

attB donor	*attP* docking	Embryos injected	Survived G0 adults	Integration events
OX4494	OX4476B	1441	98 [6.80 %]	0
	OX4476C	932	88 [9.44 %]	0
	OX4476F	776	78 [10.05 %]	0
	Keele2	528	44 [8.33 %]	0
OX4580	OX4476B	1100	89 [8.09 %]	0
	OX4476C	1100	115 [10.45 %]	0
	OX4476F	1100	122 [11.09 %]	0
	Keele2	1005	85 [8.46 %]	0

The number of Keele2 G0 adults injected with OX4494 was relatively low, making this an inadequate control for the experiment. The number of fertile OX4476 G0s was comparable to those observed in *Ae. aegypti* (*e.g.* Nimmo *et al.*, 2006) and *Ae. albopictus* (*e.g.* Labbé *et al.*, 2010) using constructs with identical *attB* and *attP* target sequences.

The negative results from OX4580 plasmid injections in Keele2, and OX4476 test lines, indicated either (i) an error in the injection methodology or the quality of the components used for injection, or (ii) interference of the integration system by components of OX4580, the docking constructs, or genomic locations of the *attP* sites, or (iii) autofluorescence observed under green excitation and light filters masked any weak expression of OX4580's Hr5ie1-ZsGreen marker, see Figure 4.6, so integration events were not identified by fluorescence.

The OX4494 and OX4580 *attB* construct architectures (Figure 4.4) were compared to different *attB* constructs successfully integrated by others into the *Ae. aegypti* Keele2 strain (Fu *et al.*, 2010; Nimmo *et al.*, 2006; Gray, P., personal communication) and an equivalent *Ae. albopictus* docking strain (Labbé *et al.*, 2010). All constructs that successfully integrated into the *attP* site carried only 3xP3-DsRed2 as the transformation marker. Therefore, a new construct, OX4714, was designed based on OX4494 but lacking the Hr5ie1-ZsGreen transformation marker (Figure 4.4). This construct was smaller in size, which is not always associated with better integration efficiency but is unlikely to be detrimental, and has fewer enhancers that are likely to interact with one another and elements in the genome.

OX4714 was injected in parallel with OX4494, which was previously shown to integrate at a very low frequency (Table 4.5). To improve the reliability of negative results, each donor construct was injected into a minimum of 2000 embryos per docking line. Due to the availability of lines, OX4476H was tested instead of OX4476B.

Table 4.5 Summary of OX4494 and OX4714 parallel injections. Integration events are defined as the number of transgenic pools. Efficiency= minimum calculated integration efficiency assuming 50% G0 fertility.

attB donor	attP docking	Embryos injected	Survived G0 adults	Integration events [efficiency]
OX4494	OX4476H	2585	214 [8.27 %]	0
	OX4476C	2780	248 [8.28 %]	0
	OX4476F	2544	280 [11.01 %]	0
	Keele2	4090	253 [6.19 %]	1 [0.79%]
OX4714	OX4476H	2507	230 [9.17%]	2 [1.74 %]
	OX4476C	2968	280 [9.43%]	2 [1.43 %]
	OX4476F	2698	273 [10.12]	4 [2.93 %]
	Keele2	2632	245 [9.31%]	0

G0s were not screened for transient expression to avoid stress due to handling, and G0s were pooled, so minimum transformation efficiency was calculated assuming 50% G0 fertility, Table 4.5. Only one integration event was observed with the OX4494 donor construct into the Keele2 strain, showing the same expression profiles as before, but the construct did not integrate into any OX4476 lines. This integration event was confirmed by PCR (data not shown).

The results from OX4714 injections were interesting. Transient expression of OX4714's 3xP3-DsRed2 marker was observed in G1 larvae of all docking lines regardless of whether they carried the acceptor construct; fluorescence was only observed in one eye, at a frequency of approximately 5% per G0 male or female pool, Figure 4.8. Although this phenomenon has not been reported in the literature, this has been observed in *Ae. aegypti* G1 offspring of other *attB* injections into Keele2 (Gray, P., personal

communication). The transgene expression may have been silenced in response to a strong fitness load caused by the genomic integration site (Adelman *et al.*, 2004), an unstable genomic pseudo-integration may have been lost, or G0 reproductive tissues may have transferred residual donor plasmid to G1 offspring. This could be investigated further by PCR analysis. If silencing followed integration, components of the donor OX4714 construct would still be amplified in subsequent generations, whereas residual donor plasmid would not. The integrity of the *attB* site could also be investigated in the G1s exhibiting transient expression if the *attL* and *attR* sequences are not amplified, which may indicate pseudo-integration if the *attB* sequence is no longer intact.

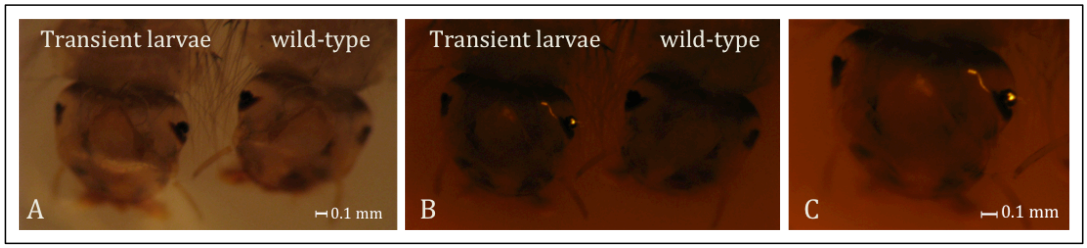

Figure 4.8 Fluorescent phenotype of transiently expressed 3xP3-DsRed2 in G1 progeny of OX4714 injections. Images were taken under white light (A), and red, excitation light and filter (B and C). Images A and B were taken at the same magnification.

Pseudo-integration of the new OX4714 donor cassette was indicated phenotypically in all OX4476 lines (H, C and F) by the presence of the *attB* constructs' red fluorescent marker in both eyes despite the absence of the *attP* docking construct's blue marker. This phenotype was not observed in offspring from OX4494 injections. In pseudo-integration indicated G1 insects, there was variable expression of the DsRed2 and

there was no positive amplification of the *attL* and *attR* junctions that are characteristic of ΦC31 integration. Segregation of the DsRed2 marker and the OX4476 AmCyan marker was observed in G3 insects, which confirmed the absence of stable ΦC31 integration. Recombination of the donor construct's *attB* site with an endogenous site that has approximately 25% sequence identity with *attP* has been observed in *Ae. aegypti*, *D. melanogaster*, and mammalian cells (Nimmo *et al.*, 2006; Thyagarajan *et al.*, 2001). This pseudo-integration complicates analysis.

Canonical ΦC31 integration of OX4714 into OX4476C and OX4476CF was indicated by positive amplification of *attL* and *attR* junctions, Figure 4.9, and was confirmed by sequencing analysis (Appendix 2.1). ΦC31 integration of OX4714 into OX4476H was also confirmed (data not shown), however the integrated line was lost due to a suspected recessive fitness cost.

Consequently, Mendelian inheritance of the OX4476 construct was investigated from hemizygous to hemizygous crosses in each line. This was carried out to test the new hypothesis, which states that OX4476H has a recessive fitness cost affecting inheritance of the transgenic construct, and to ensure that there was no recessive fitness cost associated with the OX4476 docking construct in remaining lines. As predicted, fewer progeny than the expected 0.75 Mendelian ratio inherited the transgene from OX4476H, Table 4.6, so line H was discarded. For OX4476J, the higher proportion of transgenic progeny suggested that there could be a second insertion in the genome.

Given that no recessive fitness cost was identified in either OX4476C or OX4476F (Table 4.6), and ΦC31 integration of OX4714 with no obvious

fitness load was confirmed in both (Figure 4.9), lines OX4476C[OX4714] and OX4476F[OX4714] were reared in preparation for injections with Cre and FLP.

In addition, to assess the utility of the new functional docking strains for routine site-specific transformation of *Ae. aegypti*, OX4476C and OX4476F were assessed more rigorously in Chapter 5. Assessments were carried out using a variety of fitness measures such as size, longevity, male mating competiveness as well as female fecundity and fertility.

Table 4.6 Inheritance of the OX4476 construct in progeny from hemizygous to hemizygous crosses. Chi squared analysis (χ^2) was used to quantify the significance of the difference between the observed number of transgenic:non-transgenic offspring (TG:NTG) and the expected 75:25 ratio according to Mendelian inheritance predictions, where $p<0.05$ represents a significant difference (*).

Effector lines	TG: NTG	% Transgenic	χ^2 (df= 1)	p-value	Effect size (Φ)
OX4476B	702:254	73	1.26	0.53	
OX4476C	568:214	73	2.33	0.31	
OX4476F	709:256	73	1.20	0.55	
OX4476H	583:264	69	17.19	<0.001*	0.143
OX4476J	792:156	84	36.91	<0.001*	0.197

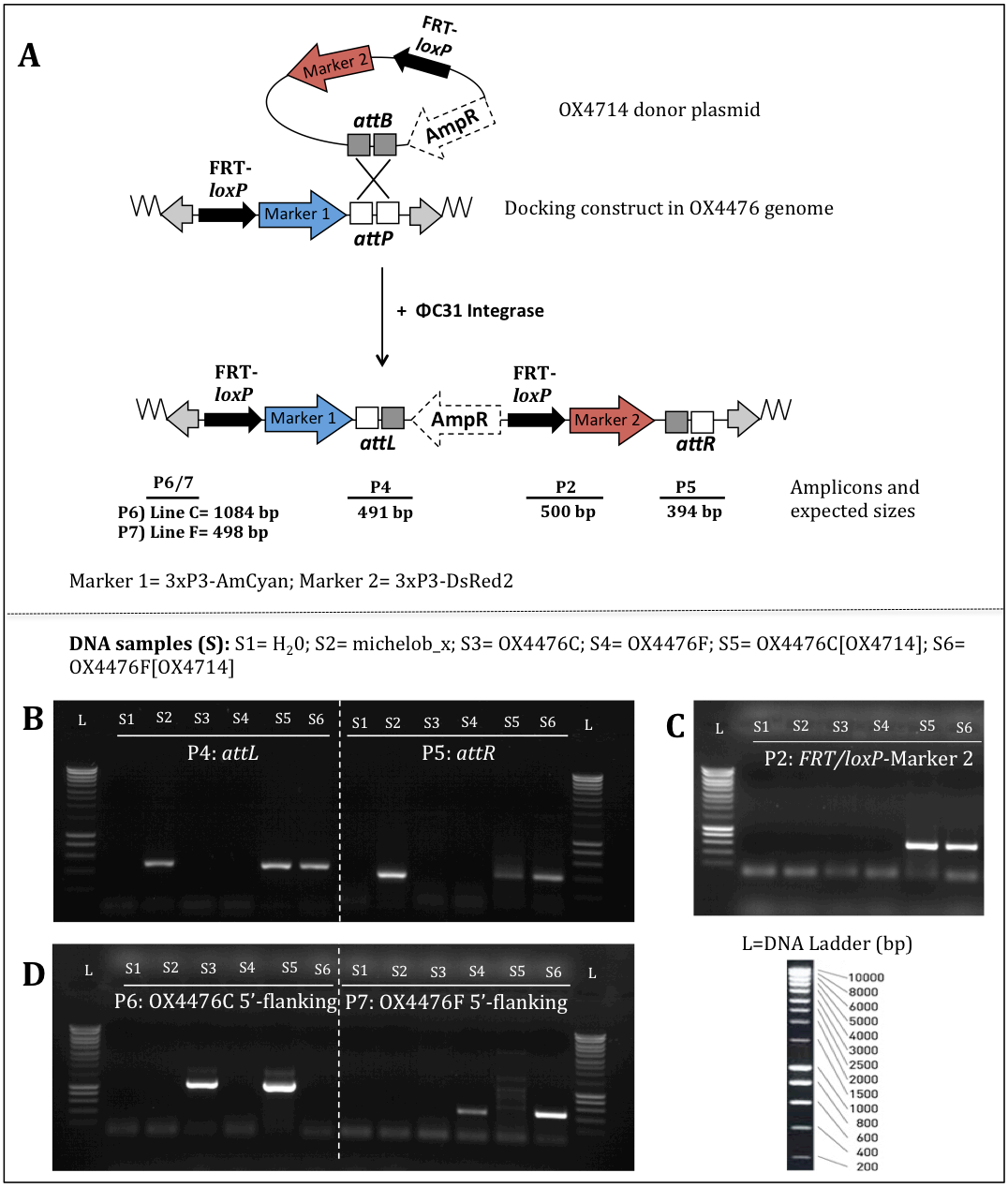

Figure 4.9 PCR analysis showing ΦC31-mediated integration of OX4714 into OX4476C and OX4476F. (A) Schematic of ΦC31 integration and amplicons. Samples: S1 (H_20) was a negative control for all primers; S2 (michelob_x; a different strain carrying a ΦC31-*att* integrated plasmid) was a positive control for ΦC31-*att* integration (P4 and P5); OX4476C and OX4476F were negative controls for ΦC31-*att* integration but positive controls for genomic flanking regions (P6 and P7 respectively). **(B)** Canonical ΦC31-*att* integration was shown in S5 (OX4476C[OX4714]) and S6 (OX4476F[OX4714]) as the P1 and P2 *att*-integration junctions in,and in **(C)** the donor OX4714 marker P2 were amplified in both. **(D)** Different OX4476 flanking sequences were amplified in S5 and S6.

Observational analysis of OX4476C[OX4714] and OX4476F[OX4714] revealed that ΦC31-*att* integration resulted in reduced expression of the docking cassette's 3xP3-AmCyan marker, shown in Figure 4.10. This was consistent with findings in *Ae. albopictus* (Labbé *et al.*, 2010). This partial repression of the marker may be due to transcriptional interference by (i) 'promoter occlusion', (Adhya and Gottesman, 1982), in which RNA polymerase initiating 3xP3-DsRed2 blocks access to adjacent *cis* 3xP3-AmCyan, or (ii) titration of promoter specific transcription factors (Kennett *et al.*, 2002). The partial repression could also be due to changes in chromatin structure resulting in polymerase interference (Callen *et al.*, 2004; Arimbasseri and Bhargava, 2008). The reduced AmCyan expression made the identification of excision events (characterised by the loss of AmCyan fluorescence) challenging.

4.3.3 Phase III: FLP- and Cre-mediated excision

In the OX4476[OX4714] lines, sequences including the eye-specific AmCyan marker and the donor plasmid's backbone are flanked by *loxP* and *FRT* sites in the same orientation. In the presence of Cre or FLP respectively, excision of the flanked sequences was expected. To test excision efficiencies, OX4476C[OX4714] and OX4476F[OX4714] lines were injected with FLP and Cre in parallel and transgenic survivors were crossed to wild-type in smaller pools of 2-5 injected G0s.

Individuals bearing excision events were identified through loss of the blue AmCyan marker (Figure 4.10), and excision did not improve DsRed2 expression.

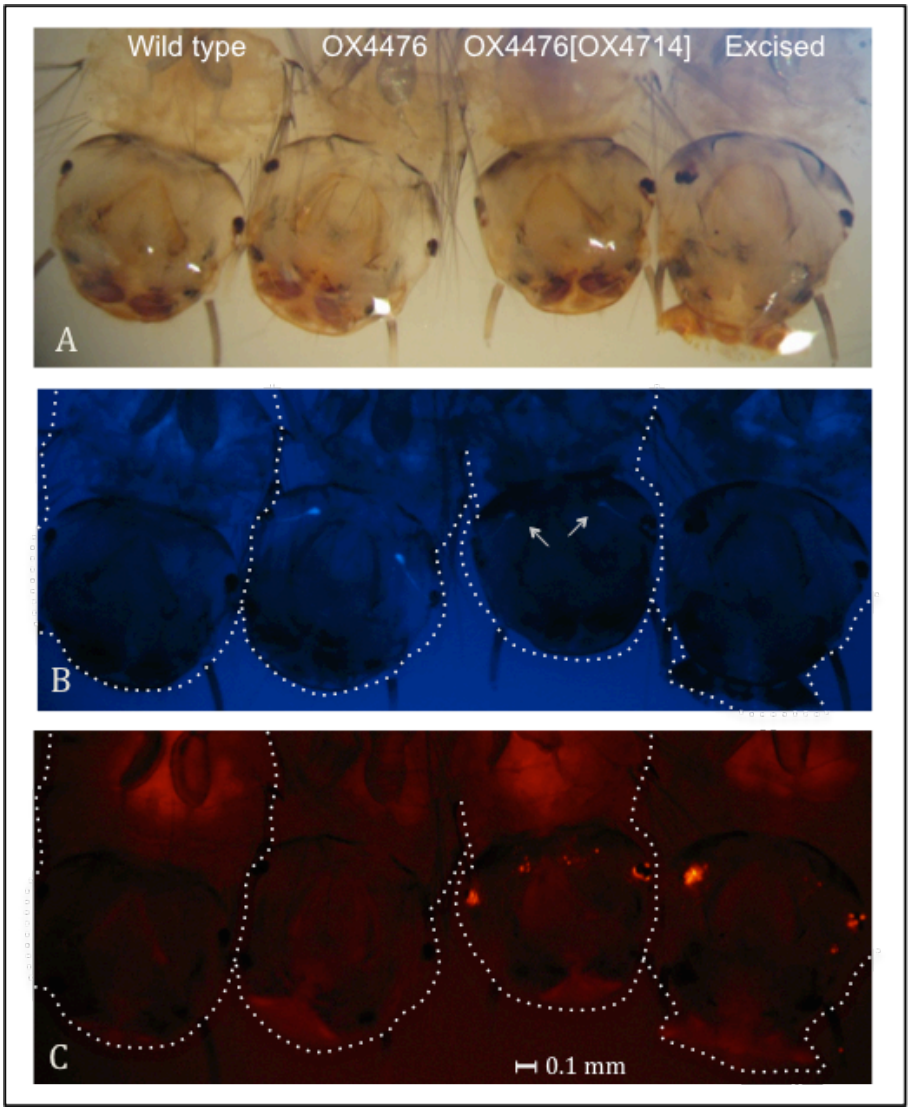

Figure 4.10 Fluorescent phenotypes showing complete cassette exchange in OX4476F. L4 larvae are shown under (A) white light, (B) cyan, and (C) DsRed2 excitation light and filters. Images show complete exchange of the 3xP3-AmCyan cassette (OX4476), by integration of 3xP3-DsRed2 (seen in OX4476[OX4714]), and excision of the 3xP3-AmCyan marker ('excised' larva). The integration of OX4714 resulted in lower expression of blue AmCyan in OX4476[OX4714] compared to OX4476 (white arrows). Images A-C were taken under the same magnification. White dashed lines outline larvae.

Next, PCR amplification across the excision junction (Figure 4.11) and sequencing of products (Appendix 2.2) confirmed these excision events.

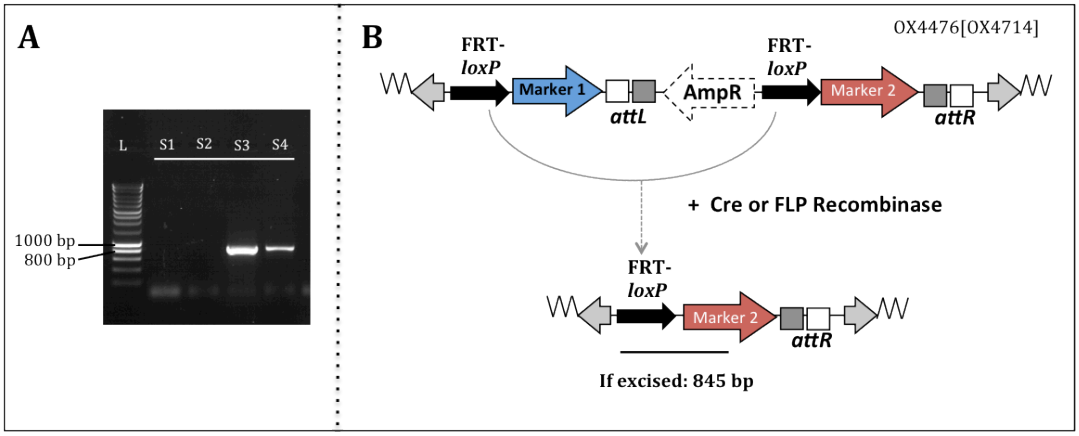

Figure 4.11, PCR amplification (A) across the excision junction shown diagrammatically in (B). S1-4 are samples: S1= H20; S2= OX4476F[OX4714]; S3= OX4476F[OX4714] injected with Cre; and S4= OX4476F[OX4714] injected with FLP. L= DNA ladder, Marker 1= 3xP3-AmCyan; Marker 2= 3xP3-DsRed2. Excision was indicated in samples S3 and S4 because the expected 845 bp sequence across the excision junction was positively amplified.

As anticipated, the number of false-positive excision events identified by fluorescence was high due to difficulty in correctly identifying AmCyan-positive individuals. Excision was confirmed using both Cre and FLP in line OX4476F[OX4714] but there was no excision in line OX4476C[OX4714], Table 4.7.

Table 4.7 Summary of Cre and FLP parallel injections. The number of pools with progeny lacking the AmCyan marker is shown ('marker loss'). Excision was confirmed by PCR and sequencing ('excision confirmed') and the minimum calculated excision frequencies (shown as a percentage) assumed 50% G0 fertility.

	Line	Embryos injected	Survived G0 adults	G0 pools	Marker loss	Excision confirmed
Cre	OX4476F[OX4714]	2520	205 [8.1%]	164	23	4 [4.88%]
	OX4476C[OX4714]	2570	312 [12.1 %]	307	18	0
FLP	OX4476F[OX4714]	2650	247 [9.3%]	241	42	3 [2.49 %]
	OX4476C[OX4714]	2535	263 [10.37 %]	251	18	0

The estimated Cre excision frequency was 4.88%. This was lower than approximately 90% excision in *P. xylostella* using the same Cre recombinase mRNA source, which was modified to contain the *D. melanogaster vasa gene's* T7 3'UTR (Martins, 2011) to assist delivery of the mRNA to these cells (Sano *et al.*, 2002).

Similarly, the FLP recombinase used in this study was fused to the T7 3'UTR (Sano *et al.*, 2002), and is the first demonstration of genomic FLP-mediated excision in the *Ae. aegypti* genome. Jasinskiene *et al.* (2003) was previously unsuccessful at inducing FLP-mediated excision in *Ae. aegypti* using a helper plasmid containing FLP under the control of the *D. melanogaster hsp70* gene's promoter. This method has been used to induce Cre-mediated excision at high excision frequency by heat-shocking injected insect embryos; excision frequencies were 90% in *D. melanogaster* (Siegal and Hartl, 1996), and 33% in *Ae. aegypti* (Jasinskiene *et al.*, 2003). It is unclear whether the T7 3'UTR or the use of FLP mRNA aided the successful FLP-mediated excision in this study. The utility of using the

heat-shock-inducible approach with these constructs should be investigated to improve our understanding of factors that influence excision frequencies of both Cre and FLP.

4.4 Conclusions and recommendations

This chapter demonstrates the first cassette exchange system combining the use of the ΦC31-*att* integration system alongside the Cre-*loxP* or FLP-*FRT* excision systems in *Ae. aegypti*. The cassette exchange system provides a useful tool for engineering mosquitoes for field release since it minimises extraneous sequences that may have regulatory implications. The system can also be utilised for comparative analysis of genes to learn their function in relation to sequences in *cis,* which can subsequently be excised and differences directly compared. This casette exchange system may also be transferable to other organisms since ΦC31-*att*, Cre-*loxP* and FLP-*FRT* systems have been used successfully in a number of species.

In *Ae. aegypti,* the survival rate of injected G0 adults was consistent with previous experiments, indicating that injection of ΦC31, Cre and FLP recombinases did not affect the G0 survival of transgenic *Ae. aegypti* lines. However, the efficiency of these systems was low, and only one of three tested lines, OX4476F, carried functional docking sites required for complete cassette exchange, without an imposed fitness load.

No degradation of the ΦC31, Cre and FLP mRNA was detected before injections, so the quality of injection solutions was not considered a reason for low efficiencies.

Codon optimisation of the ΦC31 integrase sequence, and addition of a nuclear localisation signal to the C-terminal (NLS-C) to enhance integration frequency has had mixed success. Improved integration efficiency of ΦC31 integrase with the addition of NLS-C was achieved in mammalian cells (Andreas *et al.*, 2002) and *D. melanogaster* (Bischof *et al.*, 2007). In *P. xylostella*, Martins (2011) unsuccessfully attempted ΦC31 integration with a nuclear localisation signal added to the N- and C-terminals of the integrase. Furthermore, Martins (2011) did not observe integration using a codon-optimised ΦC31 integrase in the same species.

The integrase used for experiments in this chapter was unmodified except that it included the *vasa* gene's T7 3'UTR, which was expected to assist mRNA delivary to the embryo's pole cells (Sano *et al.*, 2002). This version of the ΦC31 integrase (carrying the T7-3'UTR) has been used successfully, albeit at low integration efficiencies, to integrate plasmids into *P. xylostella* (Martins, 2011) and *Ae. aegypti* (StJohn, 2012). Nevertheless, it may be useful to investigate the use of codon-optimisation and nuclear localised integrases to improve the efficiency and reliability of the system in *Ae. aegypti.*

Alternative methods of improving the efficiency of the cassette system could include optimising the source of the ΦC31, Cre and FLP enzymes. Alternatives to injecting the helper mRNA are available that should also be evaluated for use with this system. For example, co-injecting a helper plasmid containing gene sequence under the control of the *D. melanogaster hsp70* gene's promoter and heat-shocking embryos is an efficient method used for Cre excision in *Ae. aegypti*, though this did not work for FLP

(Jasinskiene *et al.*, 2003). In addition, transgenic 'self-docking' strains have been developed in *Anopheles gambiae* that express the ΦC31 integrase in the posterior oocyte (Meredith *et al.*, 2013). These strains drastically improved ΦC31 integration efficiencies and could potentially improve the excision frequency of Cre and FLP if such lines were developed. Future work on this system should therefore include the direct comparison of alternative methods to supply the oocyte with the relevant recombinases.

Although integration efficiencies were too low to draw any firm conclusions, removing the Hr5ie1-ZsGreen from the OX4494 donor cassette improved efficiency of the ΦC31 integration in *Ae. aegypti* in this chapter, whereas removing the 3xP3-DsRed2 from OX4494 improved ΦC31 efficiency in *P. xylostella* (Martins, S., personal communication). This may suggest interaction of the two markers when in the same construct, albeit unpredictably, affecting the ability of the integrase to facilitate recombination between its target sites. One possible reason is due to the repetitive Hr5 and 3xP3 sequences forming their own secondary structures. For future experiments it would be useful to take this into consideration, as different combinations of donor and docking cassettes may be more efficient than others.

The variation in ΦC31 integration and FLP/Cre-mediated excision frequencies between docking lines suggested that the structure of flanking genomic sequences may affect the efficiency of these systems, which is consistent with previous findings (Bischof *et al.*, 2007; Monetti *et al.*, 2011; Labbé *et al.*, 2010; StJohn, 2012; Martins, 2011). Perhaps neighbouring sequence structures obstruct the formation of relevant complexes or prevent access of the relevant enzyme to specific genomic sites.

Nevertheless, integration and excision were at such low frequencies that significant differences between the different strains were difficult to assess.

The weakened expression of AmCyan in the eyes of insects carrying an integrated plasmid made screening for loss of the marker less efficient. This demonstrates the need for more adequate markers in this system. A promoter driving expression in a larger organ than the eyes would facilitate large scale screening for integration and excision, and would minimise the probability of false positives. For example, the ubiquitous Hr5ie1 could be used to drive expression of AmCyan instead of 3xP3, though it is important to note that the integration of OX4714 occurred once the Hr5ie1-ZsGreen sequences were removed.

Further refinements to the system may include strategies to stabilise the *piggyBac*-based docking construct for species in which remobilisation is a possibility. Established methods include the preferential remobilisation of smaller vectors within the larger vector and would require the addition of shorter flanking internal transposons around the docking transgene (Handler, 2004; Dafa'alla *et al.*, 2006). Alternatively, double-strand break repair could be utilised by redesigning the donor plasmid to contain rare cutting I-SceI and I-CreI homing endonucleases and segments of DNA homologous to the docking construct's flanking sequences (Tkachuk *et al.*, 2011). This is possible with the *Ae. aegypti* OX4476C and OX4476F lines developed in this chapter because the genomic sequences flanking the transgenic integration site were successfully obtained.

In addition, insulated *piggyBac*–based transposons are thought to minimise position effects through (i) enhancer-blocking, which prevents interactions between neighbouring enhancers and the transgenic promoter, or (ii) barrier activity, which inhibits euchromatic expression (Kuhn and

Geyer, 2003; Bell *et al.*, 2001). Therefore genetic insulators are another potential refinement to the system and could be considered for use. Recently, Carballar-Lejarazú *et al.* (2013) reported that the exogenous *gypsy* insulator sequence derived from *D. melanogaster* increases and stabilises transgene expression in *Anopheles stephensi.* This could be tested in *Ae. aegypti* by inserting two donor *attB* constructs into the same OX4476 acceptor strain in parallel, one carrying a promoter-marker cassette, and one carrying the same but with the expression cassette flanked by the *gypsy* insulator sequences. Expression of the markers in the donor cassettes and also in the acceptor's AmCyan cassette could then be quantified and compared to evaluate any differences between transcript levels in the transgenic insects.

The work in this chapter showed that lines OX4476C and OX4476F both carry functional and accessible *attP*-docking sites that can be used to integrate a panel of genes into the *Ae. aegypti* genome. Given that no obvious fitness cost was identified in lines OX4476C and OX4476F based on Mendelian inheritance data, they may also be suitable for developing strains for field release, particularly OX4476F, in which full cassette exchange was shown. Therefore, the fitness of these lines, and the positional effects of the integrated plasmid was investigated in greater depth in Chapter 5 to determine their utility for further study. OX4476 lines B and J remain to be tested with the new donor construct OX4714, whereas OX4476 lines H and I were discarded as both exhibited fitness penalties.

In conclusion, the development of site-specific cassette exchange docking strains is a key development for the field that aids direct comparative analysis of transgenes and facilitates the elimination of

extraneous sequences that may have regulatory implications for field release. Assuring the utility of the new docking lines engineered in this chapter, as ultimately their functionality was proven, was an important immediate step. Therefore subsequent work in Chapter 5 evaluates the utility of new OX4476C and OX4476F lines for further study.

Chapter 5

Characterisation of new transgenic *attP* docking lines

5.1 Introduction

5.1.1 Effects of transgenesis on the insect and the transgenes

Chapter 4 reported the development of two functional *Aedes aegypti attP* docking strains OX4476F and OX4476C, one of which was proven viable for cassette exchange (OX4476F). These strains may be suitable for use as tools for creating new transgenic field strains, or to develop strains carrying a panel of transgenes at the specific *attP* site for research purposes. To evaluate their utility it is important to characterise the docking strains and any associated positional effects.

In OX4476 lines, the *attP* docking-site was inserted into the *Ae. aegypti* genome using an engineered *piggyBac* transposon. *piggyBac* integration occurs at specific, albeit randomly interspersed, TTAA tetranucleotide target sites (Fraser *et al.*, 1983). In *Drosophila melanogaster*, 26 chromosomal hot spot regions were identified for

piggyBac integration, although integration into any of these is random (Thibault *et al.*, 2004). The genomic environment of the integration leaves transgenes susceptible to positional effects that may prevent their correct functioning and regulation (Esnault *et al.*, 2011).

These effects are attributed to heterochromatin (Wallrath and Elgin, 1995) and enhancers or silencing elements in flanking DNA. The size and arrangement of the *Ae. aegypti* genome is also thought to contribute to more marked position effects than in other insect species such as *Drosophila* or *Tribolium* because it has a shorter interspersed repeat pattern (Sethuraman *et al.*, 2007).

Moreover, unpredictable gene disruptions of the host DNA may induce a fitness penalty on the insect. This could be due to interference with coding or regulatory sequences at the integration site, or regulatory elements within the construct that may impact expression of endogenous genes in *cis* and *trans* (Wilson *et al.*, 1990; Mellert and Truman, 2012).

The success of any genetic vector control strategy relies on the performance of the organism released. However, transgenes in mosquitoes are often associated with a fitness cost (*e.g.* Catteruccia *et al.*, 2003; Irvin *et al.*, 2004; Moreira *et al.*, 2004). Thus assessing fitness parameters, specifically the ability of insects to survive and reproduce, is of great practical importance.

For transgenic field releases, homozygous strains are used to maximise the transgene frequency in the wild and for more efficient mass rearing. Inbreeding and harmful effects of homozygous recessive genes have been suggested as causes for reduced fitness (Marrelli *et al.*, 2006). However, making transgenic strains homozygous is not always deleterious if inbreeding is minimised (Moreira *et al.*, 2004; Allen *et al.*, 2004). For example, insects could be interbred from several geographically distinct

populations, known as a genetically diverse laboratory strain or GDLS (Wise de Valdez *et al.*, 2010).

In Chapter 4, experiments investigating the Mendelian inheritance of the OX4476 transgene in lines OX4476C and OX4476F did not detect any dominant or recessive fitness costs. Due to the time-consuming and difficult nature of generating homozygous strains in *Ae. aegypti,* hemizygous[21] lines were investigated in this study. Therefore, the work in this chapter provides baseline life-history data. In the future, assessing fitness parameters of homozygous strains developed from the OX4476 *attP* docking lines is important, especially prior to any field release.

The following critical parameters were considered for investigation:

(i) the desired transgenic phenotype should be highly penetrant, with minimal positional effects from flanking regions (McGraw and O'Neill, 2013),

(ii) the males' mating competitiveness should not be demonstrably less than the background comparator strain in a laboratory setting (Benedict *et al.*, 2009; Bush *et al.*, 1976), and

(iii) the fecundity of females should be adequate for maintaining transgenic strains, and potentially for mass production (Benedict *et al.*, 2009*;* Collado A., unpublished data).

The experiments presented in this chapter aimed to measure the quality of OX4476 lines in relation to these critical parameters.

[21] Hemizygous insects have the transgene on one chromosome and do not have a corresponding allele on the other chromosome.

5.1.2 Experimental aims

The work reported in this chapter aimed to characterise and provide life history traits of the two new functional *attP* docking lines developed in Chapter 4, OX4476C and OX4476F. The position effects of the OX4476 transgene were investigated in both lines. Furthermore, fitness effects were evaluated by comparing the OX4476 transgenic lines to the *Aedes aegypti* background wild-type line used to create the transgenic strains.

Experiments were designed to test the following hypotheses:

Hypotheses (i): The position of the OX4476 within the Ae. aegypti *genome affects the expression of the transgene.*

Hypothesis (ii): The transgenic Ae. aegypti *OX4476 lines have lower fitness compared to the wild-type strain (for each measure of fitness):*

a) *pupation and eclosion rates,*

b) *adult survival,*

c) *male mating competitiveness,*

d) *size, and*

e) *female fecundity and fertility.*

5.2 Methods

5.2.1 Analysis of sequences flanking OX4476 insertions

Genomic DNA was extracted from individual pupae of OX4476C and OX4476F strains as described in Section 2.42. Sequences flanking *piggyBac*-based construct OX4476 were obtained using the adaptor-based method described in Section 2.10.

Obtained sequences were used for bioinformatics analysis. Nucleotide basic local alignment search tool (BLASTN) and VectorBase sequence repository were used to find any similarity to sequences deposited into GenBank. TFSEARCH (Heinemeyer *et al.*, 1998) was used to identify any putative transcription factor binding sites.

5.2.2 Quantification of expression levels of the transgenic marker

Quantitative real-time PCRs were carried out using primers and probes — listed in Table 5.1 — purchased from Invitrogen (Life Technologies, USA) and Eurofins (UK) respectively. These were carried out as described in Section 2.11. The 18S ribosomal gene was used as an endogenous control. To test whether levels of AmCyan mRNA were different between OX4476C and OX4476F strains, relative levels of AmCyan mRNA were calculated in hemizygous insects. Each biological sample (n=3) contained four pooled hemizygous insects. L3 larvae, one-day-old pupae, and newly eclosed adults were used. Heads were removed from the adults to avoid inhibition of the PCR by eye pigments (Eckhart *et al.*, 2000). There were three technical repeats of each PCR reaction per biological sample.

Table 5.1 PCR primer pairs used and the genomic regions amplified

Probe	Forward Primers	Reverse Primers
Amcyan Taq (1145) 5'-VIC-GCGTGAACGGC CACTACTTCACCGTGA-BHQ	AmCyTaqF(1146) 5'-GGCGACGACATGA AGATGACCTAC	AmCyTaqR(1147) 5'- GTCACCTTGAAG GTGGAGGTCTGG
18S ribosomal probe: 6-Fam-CCGTCGTAAGAC TAAC-MGB	18S Forward 5'-ACGCGAGAGGTGA AATTCTTG	18S Reverse: 5'-GAAAACATCTTT GGCAAATGCTT

5.2.3 Hemizygous viability: fitness

Hemizygous OX4476C and OX4476F lines, and the background *Ae. aegypti* wild-type strain, were used for the following analyses. Larvae reared at 1 larva ml^{-1} were hatched and aliquoted on the same day (day 0) and fed powdered TetraMinff Ornamental Fish Flakes [Tetra GmbH, Germany] per larva as follows: 0.08 mg on day 1, 0.48 mg on day 3, 0.32 mg on days 5-10, and 0.06 mg on day 12. Unless otherwise stated, a 10% sucrose solution was available to adults *ad libitum* throughout the following experiments.

5.2.3.1 Longevity

Genotypes were reared separately. Wild-type, OX4476F, and OX4476C pupae were picked daily and all were screened for fluorescence to minimise handling differences (n=3).

Surviving adults were distributed into small 17.5 x 17.5 x 17.5 cm cages (Bugdorm, Taichung, Taiwan); three female cages and three male

cages were set up per genotype. Dead insects were recorded and removed daily.

5.2.3.2 Mating competitiveness

Two experiments, the first comparing wild-type males to OX4476C males, and the second comparing wild-type males to OX4476F males, were performed in the same way.

Genotypes were reared separately (n=5), and males from the first three days of pupation were collected. All insects were screened for fluorescence to keep handling consistent between the two strains. To ensure virginity, pupae of the same sex and genotype were placed into separate cages to eclose. Four days after the last pupae eclosed, ten OX4476 and ten wild-type males were placed into two separate pots. These two genotypes were released into large 30 x 30 x 30 cm cages (Bugdorm, Taichung, Taiwan) simultaneously to prevent any favourable setting for either strain (n=20). The distal ends of most wings from insects used in the experiments were damaged and unusable, so fifty remaining virgin males were used to measure wing-lengths using the protocol described in Section 5.2.3.3. Ten wild-type females of the same age were added to the cage the following day, and fifty remaining females were used to measure wing-lengths as before (Section 5.2.3.3). Adults were recovered 48 hrs later, females were blood-fed, and single females were transferred to tubes for egg laying.

Offspring were hatched five days after eggs were laid and screened for the OX4476 transformation marker 3xP3-AmCyan (blue-eyes) to determine the parental genotype. The number of transgenic:non-transgenic offspring was compared to the expected Mendelian ratios to determine whether transgenic and wild-type double-mating had occurred. Offspring

batches containing fewer than five larvae were removed from the analysis, as the sample was not large enough to have greater than 95% confidence of paternity.

5.2.3.3 Wing lengths

Genotypes were reared separately. The wings of fifty males and fifty females per genotype — wild-type, OX4476C and OX4476F — were removed and mounted onto slides using 70% ethanol. Wings were photographed alongside a graticule, and measured from the distal end of vein 3 to the distal end of the allula excluding wing as shown in Figure 5.1 using ImageJ software, available publicly from http://rsbweb.nih.gov/ij/ (National Institutes of Health, USA).

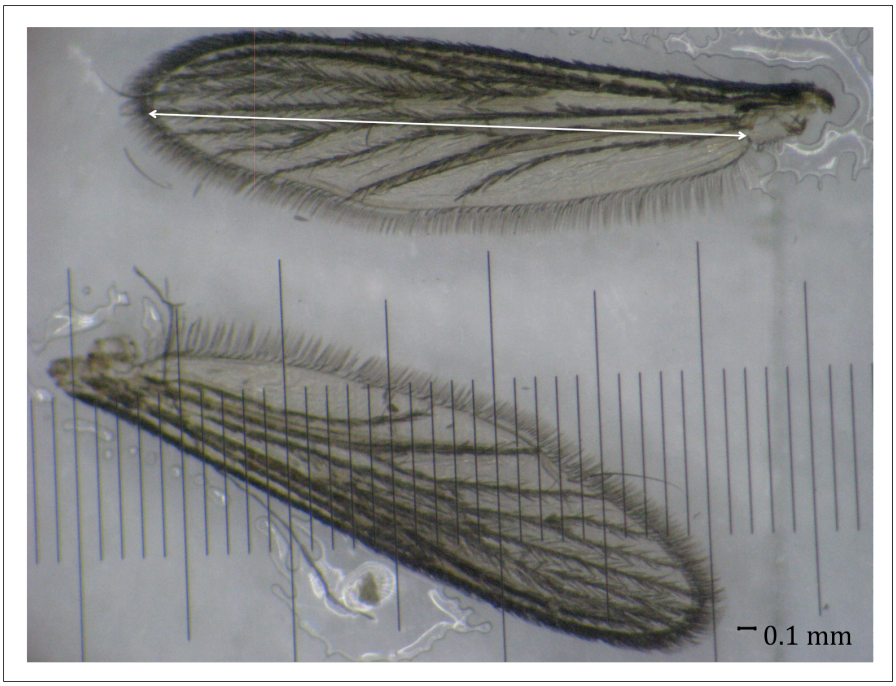

Figure 5.1 Mosquito wing lengths. Measurements were taken from the distal end of vein R3 to the distal end of the allula (white arrow) excluding any wing fringes. Anatomical references from Christophers (1960).

5.2.3.4 Female fecundity and fertility

Genotypes were reared separately. Fifty females of each genotype — wild-type, OX4476C and OX4476F — were crossed to 25 wild-type males. Cages were not disturbed for one week to allow access to females, after which, sucrose feeders were removed from cages. Cages were blood fed simultaneously 24 hrs later, and the 10% sucrose solution was returned.

Females were placed in separate tubes for egg collection three days after their blood meal, and returned to cages with sugar feeders 48 hrs later. Four-day-old eggs were hatched, and the number of unhatched eggs was counted four hours later.

Surviving females were blood fed following a 24-hour starvation period for two more gonotrophic cycles.

5.2.3.5 Statistical analyses

Kaplan Meier survival analysis was carried out using the "Rcmdrplugin.survival" package in the statistical program 'R for Mac OS X Version 1', which is publicly from http://cran.r-project.org/.

Mean pupation and eclosion data met the assumption of normality as assessed by the Shapiro-Wilk's test. These were compared using one-way ANOVA between more than two groups, or the t-test between two groups (*e.g.* between binary sex variable) using the IBM SPSS Statistics program (version 21.0; SPSS Inc., Chicago, USA). Percentages and rates were converted to arcsine square root values before the analysis.

There were outliers in the wing length, fecundity and fertility data, and normality was not achieved by transformation. Therefore the Kruskal-Wallis H test was used to compare the distribution of the data between

genotypes with the same IBM SPSS Statistics program. If groups were statistically different from each other, pairwise comparisons were performed using Dunn's 1964 procedure with a Bonferroni correction for multiple comparisons.

Paternity was determined using Pearson's chi-squared (χ^2) analysis on progeny to compare observed and expected genotype ratios (Pearson, 1900). The Yates' correction was applied if offspring genotypes contained fewer than ten larvae (Yates, 1934). Mating competitiveness was determined using Pearson's chi-squared (χ^2) analysis (Pearson, 1900).

5.3 Results and discussion

5.3.1 Positional effects of the transgene

Hypothesis (i): The position of the OX4476 within the Ae. aegypti *genome affects the expression of the transgene.*

5.3.1.1 Integration site of *piggyBac*-based transposon

The genomic flanking sequences of the OX4476 construct were identified in both OX4476C and OX447F lines, Table 5.2. This was to confirm whether the OX4476 *piggyBac*-based transposon had inserted into genetically distinct sites in OX4476C and OX4476F. In addition, sequences were used to identify any structures in the flanking sequences that may interfere with the correct functioning of the transgenes (Wittkopp, 2006). All sequences carried the TTAA tetranucleotide sequence at the construct-genomic junctions, as expected from *piggyBac*-mediated transposition (Fraser *et al.*,

1995), and the integration sites in both lines were within highly repetitive genomic regions.

Table 5.2 Sequences flanking the OX4476 construct in lines OX4476C and OX4476F. The duplicated TTAA *piggyBac*-insertion site was present in all 5' and 3' insertion boundaries (underlined); full sequences are listed in Appendix 3.1.

Line	5'-genomic flanking sequence	3'-genomic flanking sequence
OX4476C	ATGTTTTCCATACAGACTTAA CATTTAA	TTAAAAAAGGCCATGTTTTAG TGCTGAT
OX4476F	GGTTGAGCGATGTTAAAGTTT GTTTTAA	TTAATTAGTGTCTGATTCTGG GAATGAC

Obtained sequences were used to determine similarity to protein coding or regulatory genes to give some insight into the function of the local DNA. All sequences aligned to regions of the *Ae. aegypti* Liverpool strain not known to contain functional genes. There were more putative transcription factor binding sites per 100 bp in the full 5'- and 3'-flanking genomic sequences in OX4476C than OX4476F, Table 5.3. This suggested that the genomic region flanking the transgenes in OX4476C may be more transcriptionally active and therefore more susceptible to positional effects.

Table 5.3 Summary of analyses carried out on sequences flanking the OX4476 construct in lines OX4476C and OX4476F

Query Sequence	Length (bp)	Total TFs [1]	TFs[1] per 100 bp	BLASTN sequence similarity[2]
OX4476C 5'	982	62	6	Contig1.11142
OX4476C 3'	602	23	4	Contig1.8891
OX4476F 5'	387	11	3	Contig1.14216
OX4476F 3'	644	28	4	Contig1.8464

[1]High scoring (>80% identity) transcription factor binding sites (TFs) were found using TFSEARCH.
[2]The nucleotide basic local alignment search tool (BLASTN) was used to align query sequences to the *Ae. aegypti* Liverpool strain; full alignments are shown in Appendix 3.2.

5.3.1.2 Transgene expression levels

There was no difference between expression levels of the AmCyan transgene between OX4476C and OX4476F, Figure 5.2. A higher level of AmCyan mRNA was detected in pupae compared to larvae in both strains.

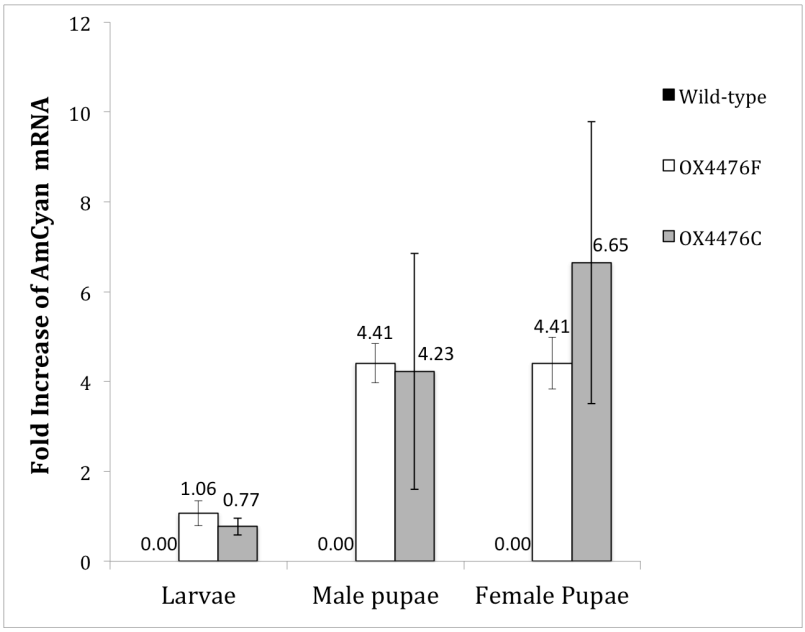

Figure 5.2 Fold change in AmCyan mRNA relative to OX4476F larvae in OX4476C and OX4476F insects. Wild-type samples were negative controls. There was no difference in AmCyan expression between lines. Error bars show the standard error of the mean of three biological replicates of RNA extracted from three four-pooled individuals; real-time PCR reactions were performed in triplicate per biological replicate.

In both lines the AmCyan transcript was not detected in adults. This was not surprising given that the 3xP3 promoter drove expression of AmCyan in the eyes and the RNA was extracted from headless adults. Headless adults were used because the endogenous eye pigments were thought to interfere with the PCR reaction in adults (Eckhart *et al.*, 2000; Bhalla, 1968; Hawkins, 2008). Nevertheless, an undetectable level of AmCyan in headless adults suggests that there was no leaky expression of the transgene outside of targeted tissues in OX4476C and OX4476F.

It was difficult to identify any phenotypic differences between OX4476C and OX4476F transgenic adults because the adults' eye pigmentation completely quenched fluorescence and masked any dissimilarity (Kokoza *et al.*, 2001).

5.3.2 Hemizygous viability: fitness

5.3.2.1 Longevity

Hypothesis ii(a): The transgenic Ae. aegypti *OX4476 lines have lower pupation and eclosion rates compared to the wild-type strain*

Hypothesis ii(b): The transgenic Ae. aegypti *OX4476 lines have reduced adult survival compared to the wild-type strain*

Consistent with findings in Chapter 4, the number of transgenic:non-transgenic offspring produced by OX4476C (χ^2=1.31, df= 1, p= 0.520) and OX4476F (χ^2= 0.08, df= 1, p= 0.96) were not significantly different from the expected Mendelian ratios. Furthermore, genotype did not have an impact on the proportion of male and female pupae in progeny of OX4476C ($t_{(4)}$= 1.488, p= 0.211), OX4476F ($t_{(4)}$= 0.250, p= 0.815), and the wild-type strain($t_{(4)}$= 2.218, p= 0.091), indicating no sex linkage of the integrated transgenes (Figure 5.3A). Sex and genotype did not have an impact on the eclosion of adults ($F_{(5, 12)}$= 0.636, p= 0.677), Figure 5.3B.

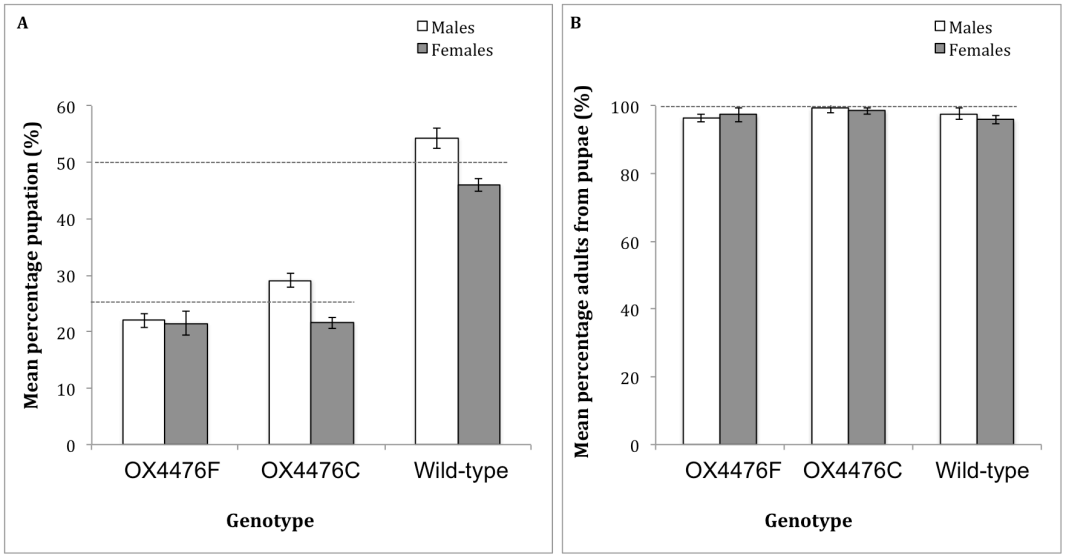

Figure 5.3 Percentage pupation (A) and eclosion from pupae (B) of hemizygous OX4476F and OX4476C, and the wild-type strain. Pupation is shown as the percentage from aliquoted larvae (n=3), 25% of each sex was expected in hemizygous OX4476 lines, and 50% of each sex was expected in the wild-type strain. The eclosion was calculated as a percentage of adults from pupae, with 100% expected if all pupae emerged (n=3). Error bars show the standard error of the mean.

There was no significant difference between adult survival data between experimental repeats (χ^2= 1.3, df= 2, p= 0.531), and so the data were pooled. There was also no significant difference between OX4476C and OX4476F survival curves in adult males (χ^2= 0.3, df= 1, p=0.611) and females (χ^2= 1.5, df= 1, p= 0.217). However, the adult survival of the wild-type strain was compromised in comparison (males: χ^2= 24.9, df= 2, p<0.001; females: χ^2= 53.7, df= 2, p<0.001), Table 5.4 and Figure 5.4.

Table 5.4 Statistical analysis of of wild-type, OX4476C and OX4476F adult survivorship. Data from three experimental repeats were pooled for the analysis. n= number of pooled insects per group; for comparison the mean number of insects per replicate cage is also included below ('mean (n) per cage'). Groups denoted by the same letter are not significantly different from each other at the 5% level.

Sex	Genotype	n	Median survival (days)	Statistical Group	Mean (n) per cage
Male	OX4476F	144	40	A	51
	OX4476C	120	40	A	40
	Wild-type	213	33	B	75
Female	OX4476F	156	52	C	52
	OX4476C	91	50	C	31
	Wild-type	199	39	A	63

Rearing immature mosquitoes at high density is known to generate longer development time as well as smaller size and lower fitness of adults (*e.g.* Macia, 2006; Moore and Whitacre, 1972). The immature insects used in this chapter were reared at the same density. However, the lower survival of wild-type adults may be due to more crowded cages, with approximately fifteen more insects per cage than transgenic cages, shown in Table 5.4. There may be a threshold adult density, above which the survival rate of adults is adversely affected. This was indicated since OX4476F cages were more densely populated than OX4476C cages, however there was no difference between adult survival rates, whereas wild-type cages were more densely populated than OX4476F and the wild-type survival rates were adversely affected.

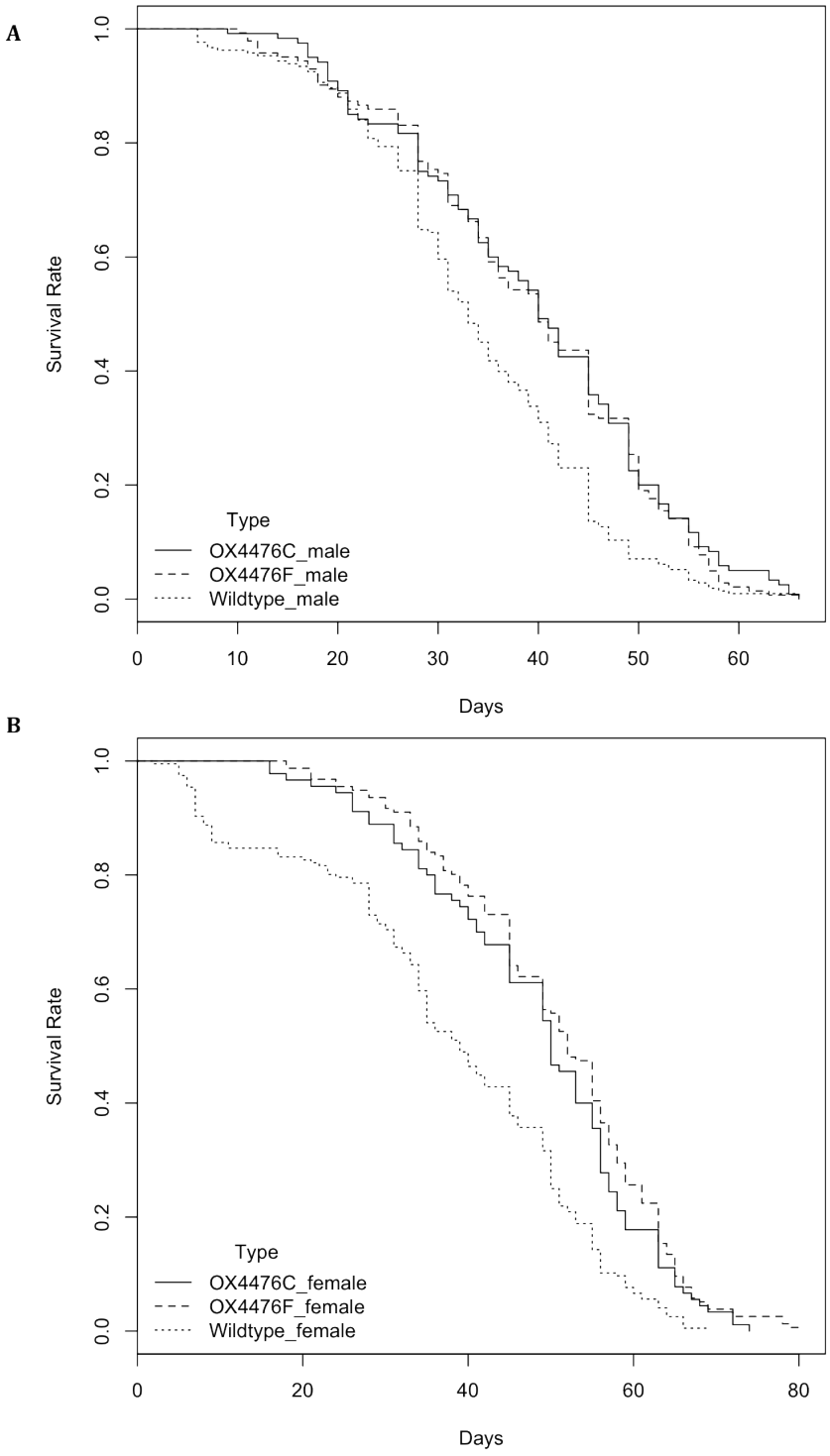

Figure 5.4 Survival of the wild-type strain and hemizygous OX4476C and OX4476F adults: (A) males, (B) females. Results are pooled from three experimental repeats.

5.3.2.2 Mating competitiveness

Hypothesis ii(c): The transgenic Ae. aegypti *OX4476 lines have lower male mating competitiveness compared to the wild-type strain*

Experiments were designed to ensure that the OX4476 docking plasmid did not compromise the mating competitiveness of either OX4476C males or the OX4476F males. In the laboratory, male size and age are known to be vital determinants of mating success in *Ae. aegypti* (Ponlawat Ponlawat and Harrington, 2009). In an attempt to exclude any confounding effects in these experiments, larval rearing densities, feeding regimen, insect age, and handling of all genotypes were kept consistent.

Since transgenic lines were hemizygous, some degree of competition was expected because OX4476 transgenic and non-transgenic siblings were reared together in a 1:1 ratio until pupation. Thus wild-type males and females were taken from independently reared wild-type trays to ensure that the quality of this strain was consistent between the two experiments. In addition the wing lengths of insects, which is an indicator of body size, were measured and compared within and between the two experiments (Figure 5.5). This was to ensure that the size of competing wild-type and OX4476 transgenic males was consistent within each experiment, and that the quality of all insects between the two experiments was also consistent so that the two experiments were comparable.

There was no significant difference between the mating competitiveness of OX4476C males and the wild-type background strain, however OX4476F males mated with more wild-type females than the background wild-type strain; test statistics are shown in Table 5.5.

Table 5.5 Male mating competitiveness between OX4476 transgenic strains and the wild-type strain. CI= Confidence interval. Results show the proportion of transgenic matings excluding or including multiple matings (Excl Multiple or Inc Mutiple, respectively); $p<0.05$ (*) represents a significant difference between the expected proportion of transgenic mating successes (0.5) and the observed transgenic mating successes. The full dataset is shown in Appendix 5.3.

	Proportion OX4476C mating		Proportion OX4476F mating	
	Excl Multiple	Incl Multiple	Excl Multiple	Incl Multiple
Mean	0.47	0.52	0.59	0.62
χ^2	0.68	0.44	6.50	12.83
p-value	0.41	0.51	0.01*	0.00*
Lower 95% CI	0.39	0.45	0.52	0.56
Upper 95% CI	0.54	0.59	0.66	0.69
Effect size (Φ)			0.57	0.80

There was no size difference between OX4476F males and the competing wild-type males (H= 0.033, df= 1, p= 1). OX4476C males were larger than the competing wild-type males (H= 3.207, df= 1, p= 0.020, η^2= 0.032), however no significant difference was observed in mating success between OX4476C and wild-type males.

Table 5.6 Statistical analysis of wing lengths (mm) from OX4476C and OX4476F male mating competitiveness tests. M= male; F= female; SEM= standard error of the mean. The average of both wings per insects were used in this analysis. Groups denoted by the same letter are not significantly different from each other at the 5% level.

Mating Test	Genotype	n	Mean ± SEM (mm)	Median (mm)	Statistical Group		
OX4476C	OX4476C M	1	2.1921 ± 0.00838	2.1955	A		
	Wild-type M	52	2.0959 ± 0.03362	2.1240		B	
	Wild-type F	50	2.8297 ± 0.01921	2.8143			C
OX4476F	OX4476F M	50	2.1178 ± 0.00895	2.1093		B	
	Wild-type M	50	2.1279 ± 0.01759	2.1140		B	
	Wild-type F	50	2.8233 ± 0.01635	2.8210			C

There was no significant size difference between the wild-type males used for the two mating competitiveness tests (H= 0.633, df= 1, p= 1). In addition, there was no size difference between the wild-type females used in the two studies (H= 0.027, df= 1, p= 1). Therefore the experiments were comparable since transgenic strains competed against insects of the same size, and competed for females of equivalent sizes. The results of the two studies indicated that overall OX4476F males were more competitive against the wild-type than OX4476C.

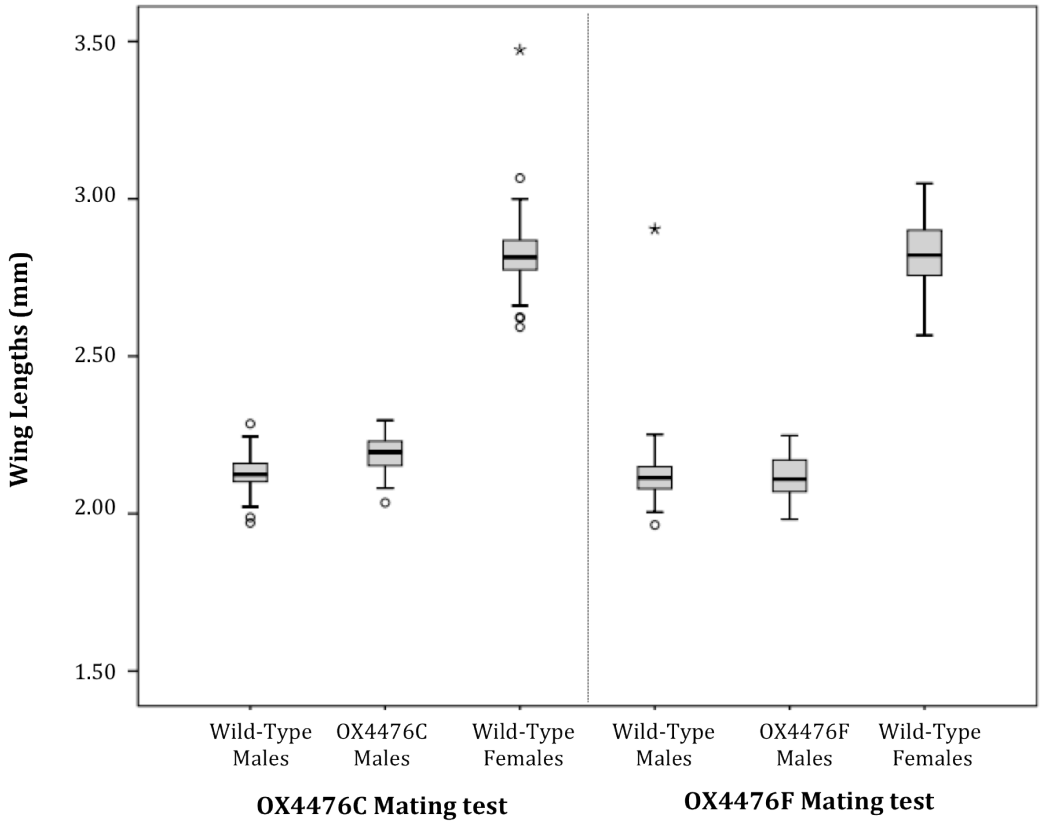

Figure 5.5 Wing lengths (mm) of insects used in OX4476C and OX4476F male mating competitiveness tests. The average of both wings per insect was used in this analysis. Small circles represent outliers, and stars represent extreme outliers.

5.3.2.3 Wing lengths

Hypothesis ii(d): The transgenic Ae. aegypti *OX4476 lines have different wing lengths compared to the wild-type strain*

Research has shown that adult body size is associated with key life-history parameters (Ng'habi *et al.*, 2005; Ng'habi *et al.*, 2008; Ponlawat and Harrington, 2009; Briegel, 1990; Collado, A., unpublished data). To assess

this in OX4476 lines, wing lengths of transgenic and the background wild-type strain were compared.

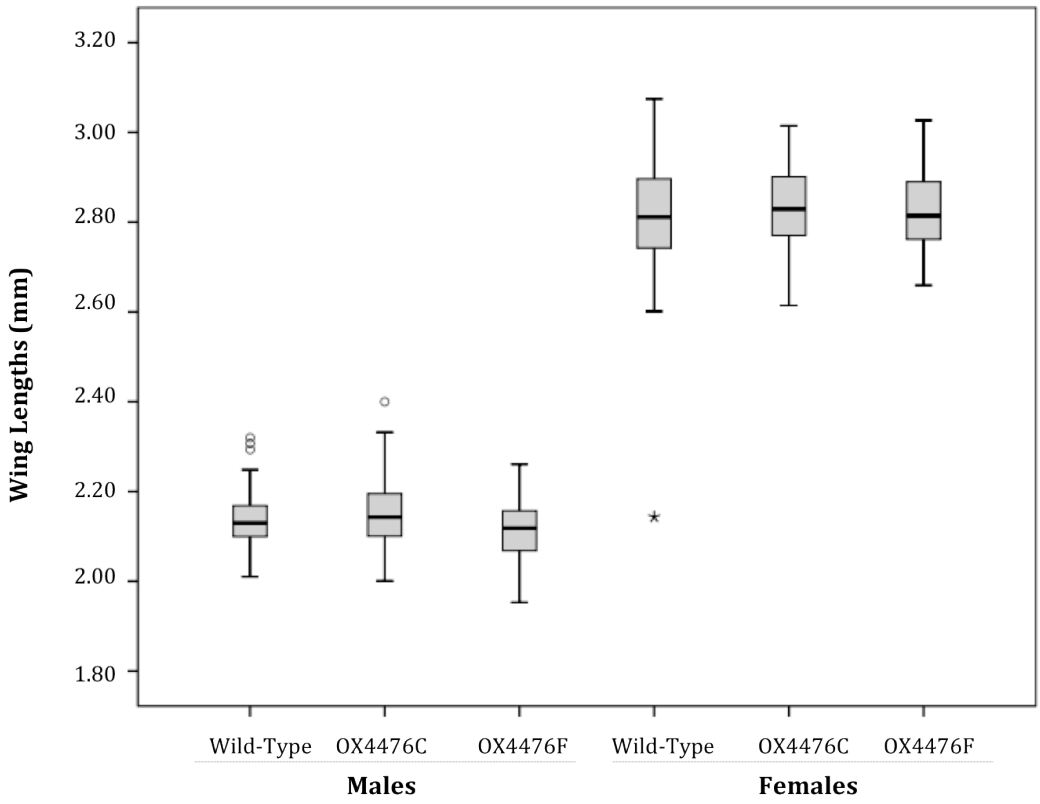

Figure 5.6 Wing lengths (mm) of hemizygous OX4476C and OX4476F, and the wild-type strain. The average of both wings per insect was used in this analysis. Small circles represent outliers, and stars represent extreme outliers.

As expected (Andrew and Bar, 2013), females were larger than males (H= 223.773, df= 5, p<0.001, η^2= 0.746). Genotype did not affect wing lengths in males (H= 4, df= 2, p= 0.135), or females (H= 0.250, df= 2, p= 0.883), Table 5.7.

Table 5.7 Statistical analysis of wing lengths (mm) from OX4476 and wild-type insects. The average of both wings per insect was used in this analysis; M= male, F=female, SEM= Standard error of the mean. Groups denoted by the same letter are not statistically different from each other at the 5% level.

Mating Test	Genotype	n	Mean ± SEM (mm)	Median	Statistical Group
Males	OX4476C	50	2.1498 ± 0.01115	2.1428	A
	OX4476F	50	2.1165 ± 0.00940	2.1185	A
	Wild-type	50	2.1396 ± 0.00977	2.1298	A
Females	OX4476C	50	2.8302 ± 0.01563	2.8293	B
	OX4476F	50	2.8268 ± 0.01271	2.8150	B
	Wild-type	50	2.8065 ± 0.02077	2.8113	B

Therefore, no significant size differences were found between wild-type and OX4476 strains of the same sex.

5.3.2.4 Female fecundity and fertility

Hypothesis ii(e): Females of the transgenic Ae. aegypti *OX4476 lines have reduced fecundity and fertility compared to the background non-transgenic wild-type strain*

The fecundity (eggs laid per female[22]) and fertility (egg hatch rate) were investigated. Genotype did not affect female fecundity in the first (H= 5.081, df= 2, p= 0.079) gonotrophic cycle. However, wild-type females laid fewer eggs in the second (H= 22.100, df= 2, p<0.001, η^2= 0.174), and third (H= 3.630, df= 2, p= 0.163) gonotrophic cycles, though the latter was not statistically significant, Figure 5.7. Genotype did not affect female fertility in the first (H= 3.091, df= 2, p= 0.213), second (H= 1.014, df= 2, p= 0.602), or third (H= 2.547, df= 2, p= 0.280) gonotrophic cycles.

[22] Egg batches were collected from individual females.

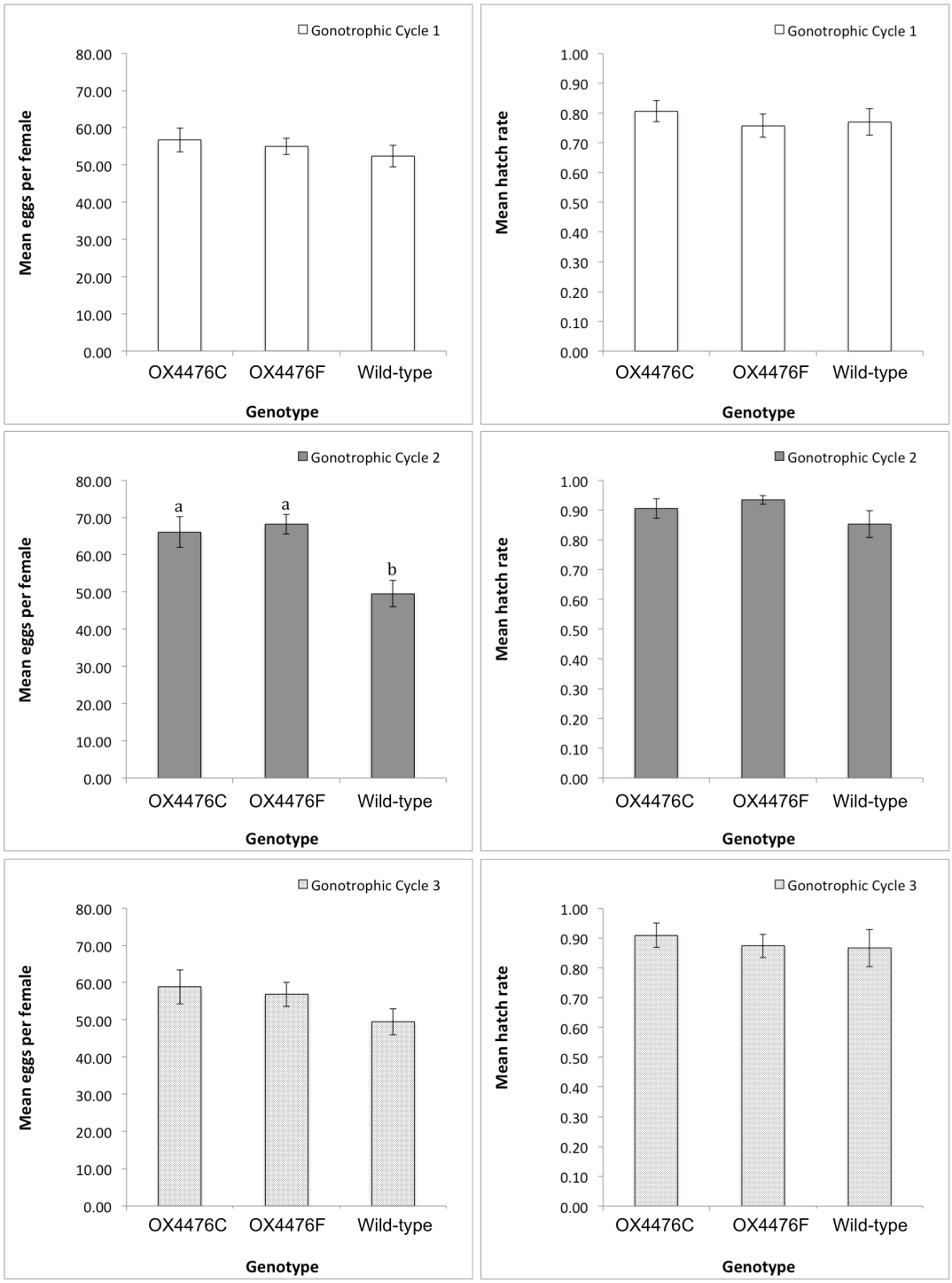

Figure 5.7 Female fecundity (eggs per female) and fertility (egg hatch) of hemizygous OX4476 lines and the wild-type over three gonotrophic cycles. Eggs were collected from individual females. Error bars denote the standard error of the mean; different letters above bars denote significant differences at the 5% level between genotypes.

Therefore the null hypothesis stating no difference between transgenic and wild-type strains was rejected; OX4476C and OX4476F laid more eggs per female than the background wild-type strain.

5.4 Conclusions and recommendations

The work carried out in this chapter evaluated the fitness of two *Aedes aegypti* transgenic docking strains developed in Chapter 4 using the *piggyBac* transformation system. The experimental design facilitated assessment of reproductive success and associated factors that are important for any field release and the characterisation of any positional effects that could have implications for research.

Adult size is an important parameter that has been linked to factors associated with male-mating success as well as the fecundity (eggs laid per female) and fertility (proportion of eggs that hatched to larvae) of females (Ng'habi *et al.*, 2005; Ng'habi *et al.*, 2008; Ponlawat and Harrington, 2009; Briegel, 1990; Collado, A., unpublished data).

The results presented in this chapter showed no difference between the size of the wild-type strain, hemizygous OX4476C and OX4476F. In addition, there was no significant difference between the male mating competitiveness of hemizygous OX4476C and the wild-type. However, more OX4476F males successfully mated with females than the wild-type males.

Furthermore, the wild-type strain showed reduced longevity — perhaps somewhat due to more overcrowded adult cages — and reduced fecundity compared to both transgenic strains, even though the insects were reared at the same time and in the same conditions prior to each experiment.

This was surprising given that competition between transgenic and wild-type larvae from hemizygous OX4476 parents was expected to adversely affect the size of transgenic strains (Labbé, 2011).

This indicates that either (i) male mating competitiveness, longevity, and fecundity are more sensitive assays for fitness and the wild-type strain is less fit than the transgenic strains, (ii) the insertion of the OX4476 transgene at the specific genomic sites was beneficial to the insects' fecundity and adult survival, or (iii) parental rearing conditions between transgenic and wild-type strains had an effect on these parameters. As differences observed were small, and the transgene is unlikely to confer a fitness benefit, especially in two separate insertion lines, we can conclude that there are no gross negative fitness effects conferred by this transgene at either integration locus.

Parental rearing conditions are known to affect offspring life history (Grech *et al.*, 2007), and were likely to be partly responsible for the lower longevity and fecundity of wild-type observed in this study. Parents of the OX4476C and OX4476F individuals used in these experiments were reared in favourable conditions. They were well fed and reared at low larval densities. Parents of the wild-type insects used in this study were from stock cages, where insects were reared at much higher densities at larval and adult stages (Sulston, E., personal communication). Repeating longevity and fecundity experiments using offspring from transgenic and wild-type parents reared under the same conditions could test this, and may be an invaluable assessment of how subsequent fitness tests should be performed. Since the transgenic lines tested in this chapter were hemizygous, this could have been achieved by using the non-transgenic siblings. In a well-known study, Marrelli *et al.* (2007) optimistically

reported a fitness advantage of transgenic malaria-resistant mosquitoes compared to non-transgenic mosquitoes. As discussed by the authors, the key feature leading to a fitness advantage of the *Plasmodium*-refractory mosquitoes may be the unrealistically high exposure to *Plasmodium* oocytes during a blood meal, although it would be interesting to know the parental rearing conditions of each strain.

Furthermore, transgenic insects are made homozygous prior to any field release to maximise the transgene frequency in the wild and for more efficient mass rearing. In Chapter 4, the frequency of the OX4476 construct was tracked from hemizygous to hemizygous crosses and the transgenic:non-transgenic proportions were not significantly different to the expected Mendelian proportions in OX4476C and OX4476F, indicating no obvious recessive fitness penalty in these lines. Future work should include more rigorous assays such as using cage-invasion experiments in which the transgenic allele is introduced into a wild-type population and the allele frequency is tracked over multiple generations (*e.g.* Moreira *et al.*, 2004; Catteruccia *et al.*, 2003; Marrelli *et al.*, 2007). This could be useful for the identification of lines that are adequate for making healthy homozygous strains.

In addition to assessing the fitness of transgenic mosquitoes, sequences flanking the genomic site of the OX4476 integration in both transgenic lines were identified and confirmed that the two strains are genetically distinct. In addition, primers that amplify the flanking sequences were designed and could be used to genotype whether strains carry the wild-type allele. This has potential use for making strains homozygous prior to any field release.

The positional effects of the transgenes were also assessed. Analysis of the transgene's AmCyan transcripts revealed no difference between

OX4476C and OX4476F expression levels. This indicated no substantial interference from genomic *cis*- or *trans*-regulatory elements. Differences between AmCyan mRNA in OX4476C and OX4476F may not have been detected because expression was limited to a small organ, the eyes, under the control of 3xP3 promoter. Nevertheless, the equivalent expression during immature stages and healthy life history characteristics of the transgenic strains make these lines suitable for downstream use, their biggest shortcoming being inefficiency of the integration system.

In conclusion, the ability to both survive and reproduce is critical for the success of all genetic control strategies. The work presented in this chapter showed that there was no significant fitness penalty in OX4476C and OX4476F lines when compared to the background wild-type strain. In addition, the absence of obvious positional effects of the transgene make these lines good candidates for research and the generation of new transgenic strains for genetic control of *Ae. aegypti.*

Chapter 6

Overview and conclusions

6.1 Personal contributions to the genetic control of *Aedes aegypti*

The public health burden of mosquito-borne diseases continues to motivate research into developing improved and novel strategies for their control. Annually, *Ae. aegypti* alone infects up to 300 million people with the dengue virus (Bhatt *et al.*, 2013), 200,000 with the yellow fever virus (WHO, 2011b), and 200-250,000 with the chikungunya virus (Staples *et al.*, 2009).

As discussed in Chapter 1, the aim of all control strategies is to disrupt the chain of infection by targeting closely interlinked factors: the pathogen, the host's susceptibility, and the vector. Drugs and vaccines are widely used where available, but are often too expensive for the medical systems of resource-poor countries, and pathogen resistance to drugs may evolve, especially in areas of extensive use (*e.g.* McGraw and O'Neill, 2013).

Vectors provide an attractive target toward which control methods can be directed, and have been extensively targeted using conventional environmental, chemical, and biological methods. (Chan, 1985; WHO,

2011a; Goldberg and Margalit, 1977; Bailey *et al.*, 1983; Schreiber, 2007). The success of insecticide use, elimination of aquatic breeding sites, and biological control methods rely on the ability of humans to seek out and access the vectors' niche and habitats. Some breeding sites are inaccessible, cover a wide area, and/or are subject to repeated flooding. Identifying a sufficient proportion of these sites is often an impossible task, and has been cited as a reason for the poor control of *Ae. aegypti* using conventional methods (*e.g.* Chadee, 1985; Hudson, 1986; Perich *et al.*, 1990; Perich *et al.*, 2000).

The use of genetically engineered mosquitoes represents an additional tool to decrease the burden of disease and shows great promise. The male mosquito's naturally evolved mate-seeking behaviour can be exploited to introduce desirable genetic traits for disease control. These traits could either render his progeny unable to transmit disease so that the wild mosquito population can be changed to be refractory to disease (population replacement strategy), or cause his progeny to die so that the population can be reduced over time, also breaking the cycle of disease (population suppression strategy).

The success of these types of efforts, known collectively as genetic control, rely on the ability to reliably and safely transform the target mosquito species to express the desired phenotype, and the ability to drive the desired phenotype into the wild populations. The work in this thesis contributes to this field by exploring components of an engineered system to drive refractoriness into target *Ae. aegypti* populations, and by developing an improved site-specific integration system for the safe, reliable, and targeted transformation of this species for field release.

6.2 Gene drive

Research presented in Chapter 3 discusses attempts to assemble and investigate components of an underdominance-based gene drive system in *Ae. aegypti*. The high release ratio required for an underdominance system makes it desirable where limited spread of the transgene is preferred (Sinkins and Gould, 2006). This is also beneficial from a regulatory perspective since accidental release would not result in population replacement (Magori and Gould, 2006; Sinkins and Gould, 2006).

During the course of my studies, a rather different synthetic underdominance-like system known as maternal-effect lethal underdominance (UD^{MEL}) was successfully demonstrated in *Drosophila melanogaster* (Akbari *et al.*, 2013). This system used tightly controlled maternal and early zygotic promoters and effectors, alongside engineered microRNAs (Akbari *et al.*, 2013). The main difficulty with translating this approach to mosquitoes is the lack of suitable well-defined promoters to drive expression of each component of this complex system.

Chapter 3 proposed the use of independently-suppressible toxins driven by the same promoter, thought to be possible using current technology. A feature of the system was the integration of refractoriness into the gene drive itself, thereby overcoming potential loss of linkage between the drive mechanism and the engineered refractoriness, known as the 'linkage problem' (Curtis *et al.*, 2006). The integration of refractoriness into the gene drive itself was based on the use of RNA interference (RNAi) directed against a region of the dengue virus' mRNA that is essential for the transmission of the pathogen to humans.

Transgenic *Ae. aegypti* anti-dengue RNAi lines are available that resist infection by the dengue virus serotype 2 (DENV2). In these lines, the anti-DENV2 RNAi pathway is triggered in the mosquito midgut (Franz *et al*, 2006), fat-body (Franz, A.W.E., Sanchez-Vargas, I., and Olson, K.E., unpublished data), or salivary glands (Mathur *et al.*, 2010). To enable RNAi-mediated knockdown of lethal transcripts, the DEN2 target sequence (Franz *et al.*, 2006) was fused as an RNAi target to the lethal construct. This created the dual-purpose for the RNAi construct. Specifically, the RNAi was a targeted suppressor against the lethal system of the gene driver, and the anti-dengue refractoriness element, as shown in Figure 3.4, Chapter 3. It was therefore essential for the RNAi molecule to be available in tissues where it could stop viral replication, and also in the tissues where the lethal effectors were expressed for gene drive. This was attempted by using the same promoter for the lethal as for the RNAi element, with targeted expression in the midgut or the fat-body.

Novel and existing effector strains were screened for their suitability. Once identified (Section 3.3.2; Chapter 3), their fitness-reducing effects in *Ae. aegypti* under the control of the specific blood-meal inducible promoters (i.e. the 'killer') were assessed (Section 3.3.3; Chapter 3).

Lethality was difficult to induce in adults, and the only observed fitness-reducing phenotype was 24-hour paralysis of females expressing the scorpion toxin AaHIT driven by the *vitellogenin* promoter. Nevertheless, this was considered an important fitness cost for the female mosquitoes because they would be exposed to predators in the wild.

Up to 70% of the females exhibited paralysis after the first blood meal, and by the third blood meal up to 80% had exhibited the phenotype. The penetrance of the lethal-inducing transgenes was therefore incomplete

because up to 20% of individuals failed to express the trait despite carrying the constructs, as determined by the presence of the corresponding fluorescent markers. Even low penetrance is enough to bring about population replacement provided the frequency of the introduced alleles is sufficiently high and the fitness penalty of the constructs (including refractory genes, if any) is not too high. However, extreme underdominance (*i.e.* 100% penetrance of the lethal genes) is preferred because partial penetrance would produce a strong selective pressure towards insects resistant to the lethal effects of the transgenes. This would further reduce the ability of the system to spread, and might allow wild-type alleles to re-invade even after a period of apparent success. In principle it might also complicate or slow recalling the constructs, if the fitness cost of carrying only one lethal-suppressor construct became so low as to be near-neutral. Furthermore, an engineered underdominance system with incomplete lethality has not been considered for replacement of insect populations in the scientific community. Therefore the theoretical behaviour of such a system has not yet been investigated.

To try to maximise the chance of finding a good killer, a variety of lethal effector molecules were considered. Each effector had different modes of action, from secreted digestion enzymes and excitatory neurotoxins, to disrupting cellular and metabolic processes. The secreted effector molecules (carboxypeptidase, trypsin, AaHIT scorpion toxin, and the Av3 sea anemone toxin) were expected to cause additional damage to neighbouring tissues, amplifying any lethal effects. Non-secreted effectors (NIPP1[23],

[23] NIPP1= Nuclear inhibitor of protein phosphatase 1

eiger, ILP[24], reaper[KR] and michelob_x) were expected to cause cell death in targeted tissues, leading to fitness-reducing damage. In addition, efforts were focused on expressing these effectors in the fat-body of females driven by the *vitellogenin* promoter. This is because the fat-body is the functional analogue of the vertebrate liver and is essential for lipid synthesis for hormonal regulation and egg development (Hagedorn and Fallon, 1973; Ziegler, 1997). Therefore the fat-body was considered a more sensitive tissue to target than the midgut.

Despite this, lethality proved difficult to induce in adults. Effects shown from Av3 and AaHIT, as neurotoxins, were probably not targeting the fat-body. The difficulty in obtaining a lethal effect may be because adults do not undergo any significant developmental and metamorphic stages that leave the insect vulnerable to the effects of otherwise disruptive proteins. Lethality is probably easier to induce in the more sensitive immature stages, such as the mitotic embryonic, early larval and pupal stages. This was shown when the same effectors induced lethality in the early larval stages when overexpressed by the earlier-acting *hexamerin* promoter.

It may be possible to improve lethality by using dual effectors, although there is a fine tipping point between increasing lethality and the ability to knockdown lethal transcripts to rescue the insect from these effects.

One critical limitation of this study was that only one line for each effector was investigated. This was because only one line of each was

[24] ILP= Insulin-like protein 2

available at the time. Efforts were focused on the development of new secreted toxic effectors (AaHIT and AV3) instead of re-injecting lines that had in some cases previously been selected based on their optimal phenotypic characteristics. Furthermore, some of the effector lines were shown to induce lethality in insects in the earlier stages so had at least some confirmation of functionality.

Effector lines were developed using *piggyBac*- or ΦC31-*att*-based integration systems. *piggyBac* integrates specifically into TTAA tetranucleotide target sites that exist at many locations in the *Ae. aegypti* genome. (Fraser *et al.*, 1983; Mitra *et al.*, 2008). In addition, the site-specific ΦC31-*att*-integrated effector lines were not developed using a single integration site; a number of *attP* docking lines were used (Gray, P., personal communication). Therefore as a result of testing only one line per effector, comparative analysis of the different lethal proteins was not possible since transgenes were inserted at different chromosomal loci.

This limitation also had an impact on the ability to attribute phenotypes to specific features of the architecture of the constructs. Constructs were adapted to facilitate the development of transgenic strains, or to maximise the likelihood of inducing a highly penetrant lethal phenotype, Table 6.1. For example, in most effector lines the *D. melanogaster* minimal promoter hsp70 was used for the repressible lethal systems. However, one reaper^{KR} construct (OX4423) contained the *D. melanogaster* hsp83 minimal promoter because the hsp70 version of the construct did not result in transformants, so was considered too toxic (Gray, P., personal communication); the hsp70 promoter was thought to result in weaker expression (Xiao and Lis, 1989). In addition, the number of tetO repeats was different in some constructs. More repeats were

thought to result in stronger expression of the effector when driven by the tTAV-tetO expression system (Scaife, S., personal communication). Therefore, it may be possible to improve penetrance of the AaHIT phenotype by increasing the number of tetO repeats. However, it is important to test this in a reliable and replicable way rather than rely on random variation due to unspecified positional effects.

Table 6.1 Selected features of tetO-effector lines used in Chapter 3. *pB*= *piggyBac*-based transposon; *attB*= ΦC31 attachment site; tetO(x)= the number of tetO repeats; hsp= the minimal promoter region from the *Drosophila melanogaster* heat shock protein's regulatory elements; gp67 and sec =secretory signals; adh= short intron from the *D. melanogaster* alcohol dehydrogenase gene included to improve expression levels; ubi= short coding sequence from *D. melanogaster* ubiquitin sequence added to allow N-terminal fusions to tTAV.

Line	Details	Description
OX3547	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-NIPP1	TetO-NIPP1
OX3582	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-*Ae*michxc	TetO-michelob_x
OX4230	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-*Ae*trypsin-sec	TetO-trypsin
OX4291	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-carbA	TetO-carboxypeptidase
OX4292	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-*Ae*ILP2g	TetO-ILP
OX4293	*attB* 3xP3-DsRed2-tetO21-hsp70-adh-*Ae*eiger	TetO-eiger
OX4393	*pB* Hr5IE1-DsRed2-tetO14-hsp70-adh-reaper^{KR}	TetO-reaper^{KR}
OX4423	*pB* Hr5IE1-DsRed2-tetO7-hsp83-ubi-reaper^{KR}	TetO-reaper^{KR}
OX4536	*pB* Hr5IE1-DsRed2-tetO7-hsp70-ubi-reaper^{KR}	TetO-reaper^{KR}
OX4509	*pB* Hr5IE1-DsRed2-tetO14-hsp70-adh-gp67-AaHIT	TetO-scorpion toxin
OX4711	*pB* Hr5IE1-DsRed2-totO14-hsp70-adh-gp67-Av3	TetO-sea anemone toxin
OX4712	*pB* Hr5IE1-DsRed2-tetO14-hsp70-adh-gp67-Av3P25A	TetO-mutant sea anemone toxin

Future work in this area should therefore focus on using a pre-characterised site-specific docking strain (such as those developed in

Chapter 4) to compare a panel of effectors directly to each other. Ideally, more than one docking strain would be used with all constructs inserted in each, because the expression and phenotype may vary by integration site. This would enable fine-tuning of components such as the minimal promoters used for the tTAV-tetO expression system, or the 3'UTR of the effector sequences.

A similar limitation was that only one *vitellogenin* promoter-tTAV line and one *carboxypeptidase* promoter-tTAV line were investigated. The ability to upregulate the lethal tTAV transcripts in the *vitellogenin* promoter line was shown in this study, and StJohn (2012) previously showed upregulation of tTAV driven by the *carboxypeptidase* promoter in the same line used here. Therefore, although testing multiple lines (or one line of each promoter construct inserted at the same chromosomal loci and with the same construct architecture) is preferred, this was not considered a priority. Given the high tTAV expression levels observed in the *vitellogenin* promoter lines, efforts were focused on testing effectors with this line.

In addition, the central goal of this work was not necessarily to make a final system. The aim was to assemble and test the components of an underdominance gene drive system based on RNAi in *Ae. aegypti*. Therefore, despite only partial penetrance of the AaHIT-induced paralysis, the 'fainting' phenotype was easy to score and so incomplete penetrance was still enough to test suppression. Therefore, the rescue of temporary paralysis by RNAi was subsequently evaluated.

A major finding of this chapter, shown in Figure 3.9 (Chapter 3), was that anti-dengue RNAi driven by the *vitellogenin* promoter suppressed *trans*-activating tTAV transcripts fused to the anti-dengue RNAi target

sequence by up to 96%. Unfortunately though, rescue of the temporary paralysis by AaHIT was only partial, and paralysis was observed in insects carrying AaHIT killer and RNAi constructs, perhaps due to a low sensitivity threshold of *Ae. aegypti* to AaHIT. These results suggest that RNAi could potentially be used as the suppressor in a gene drive system, however using the same promoter for the RNAi and lethal transcripts it targets, though elegant in design, might not be optimal.

We were limited by the need to express the RNAi molecule in virally important tissues for refractoriness, and by the sensitivity of these tissues to lethal proteins. This was especially relevant to maintain linkage between the RNAi refractoriness and the gene drive system. However, as the results in Chapter 3 demonstrate, toxins are not particularly effective in these tissues, and knockdown of the scorable phenotype was also incomplete.

It is possible that ubiquitous or multi-tissue expression of the RNAi molecule could improve RNAi suppression. Using a slightly earlier-acting promoter to drive the anti-dengue RNAi in addition to the *vitellogenin* promoter may also provide a solution. In this case, the RNAi molecule would still be necessary for survival of the mosquito in the underdominance gene drive system, and refractoriness is still maintained. One potential limitation of this method is the identification of promoters that upregulate expression of its protein product before a blood meal. Pre-vitellogenin promoters are likely to be ovarian and therefore unsuitable for this purpose.

A separate possibility would be to put the RNAi target sequence in both the tTAV and the effector sequence, thereby potentially increasing the overall degree of suppression. One possible limitation of this approach would be the need for duplication of the DEN2 sequence within the lethal

construct if the tTAV and effector were in the same construct, leading to hotspots for where recombination may occur.

Another viable option for improving the underdominance system is unlinking the refractoriness from the underdominance gene drive mechanism, giving a much wider choice of promoters, and consequently more chance of achieving death. The hexamerin promoter was shown to induce a fully penetrant tetracycline-repressible phenotype with some of the effectors and may be suitable for this type of system.

Construct design for this study highlighted the limited range of characterised control elements (*e.g.* promoters, 5' and 3' UTRs) available for rational genetic engineering of *Ae. aegypti*. Therefore, another important area for future research would be to systematically characterise a wider range of transcriptional control elements and promoters that could be used to control the expression and efficiency of effectors more specifically.

Suppression could be achieved through other means. The transcription factor *groucho* could be used to form active complexes that repress the transcription of targeted genes in the absence of its DNA binding domain (Fisher and Caudy, 1998). Otherwise, multimerisation of small interfering RNAs has shown enhanced gene-silencing efficiencies (Mok *et al.*, 2010), and is another attractive alternative to the longer RNAi system used in this chapter. These areas could be investigated to facilitate progression in this field.

It is important to note that in a previous study, the heritable dengue-2 resistance phenotype in the *Ae. aegypti* strain carb77 became markedly

weaker after thirteen generations, and was completely lost after seventeen generations (Franz *et al.*, 2009). Given that the *vit*-Vg40-AaHIT[25] test cross (Section 3.3.4.2) was carried out a few generations after the initial DEN2-tTAV knockdown experiment (Section 3.3.1), perhaps this phenomenon was responsible for the inability of the RNAi to suppress DEN2-tTAV transcripts. Future work should test the current ability of the Vg40 line to knockdown expression of its target sequence.

In conclusion, the success of gene drive mechanisms in mosquitoes remains an important and challenging goal for genetic control strategies. Future work on *Ae. aegypti* should include the direct comparison of lethal effector and suppressor molecules and their different regulatory regions to optimise highly penetrant lethal genes and the complete rescue of their lethal effects. Furthermore, this work should be undertaken using site-specific docking lines, such as the new docking lines developed in Chapter 4, so that direct comparison of transgenes is possible.

6.3 Improving transformation technologies in *Ae. aegypti*

As discussed, comparative analysis of different transgenes is a difficult task as phenotypic differences are due to an unspecified combination of the transgenes' intrinsic properties and their insertion sites (Wilson *et al.*, 1990). To overcome this in *Ae. aegypti*, Nimmo *et al.* (2006) developed docking strains carrying a highly specific ΦC31 target sequence (*attP*) that

[25] *Vit*-Vg40-AaHIT cross= the *vitellogenin* promoter line was crossed to the Vg40 RNAi line; offspring carrying both of these constructs were then crossed to the AaHIT effector line.

does not occur naturally in insect genomes. This allows integration of constructs bearing the attachment site (*attB*). Transgenes of interest can be inserted into the *attP* site and directly compared because the positional effects remain constant, especially if construct architectures are kept consistent. Based on these results, optimum transgenic cassettes can be selected and developed further in preparation for field release.

A drawback of site-specific integration of cloned plasmids using the ΦC31-*att* integration system is the introduction of plasmid backbone sequences into the mosquito genome. These nearly universally contain bacterial antibiotic resistance genes. From a regulatory and public perception perspective, this is not ideal for any field release as it represents the introduction of extraneous sequences that invites concerns.

To overcome this, Chapter 4 provides a proof of principle for a novel cassette exchange system that combines the use of ΦC31-*att* integration and the excision mechanisms of Cre-*loxP* or FLP-*FRT* systems in *Ae. aegypti*. The technique allows for the excision of extraneous sequences that may have regulatory implications for field release, and also provides an additional research tool for learning the function of genes. The system may also be transferable to other organisms as ΦC31-*att*, Cre-*loxP* and FLP-*FRT* have been used successfully in a number of species. In mosquitoes, ΦC31-*att* transformation technology has also been established in *Aedes albopictus* (Labbé *et al.*, 2010), *Anopheles gambiae* (Meredith *et al.*, 2011), and *Anopheles stephensi* (Isaacs *et al.*, 2012). However, excision using the Cre-*loxP* and FLP-*FRT* systems has not been transferred to other mosquito species.

One limitation of the novel cassette exchange system discussed in Chapter 4 is the low ΦC31 integrase and Cre or FLP recombinase efficiencies. Recommendations to improve the efficiency and reliability of the system in *Ae. aegypti* include optimising the recombinase source.

Improved integration efficiency of ΦC31 integrase with the addition of a nuclear localised signal to the C-terminal (NLS-C) was achieved in mammalian cells (Andreas *et al.*, 2002) and *D. melanogaster* (Bischof *et al.*, 2007). However, in *Plutella xylostella*, ΦC31 integration with a nuclear localisation signal added to the N- and C-terminals of the integrase was not successful (Martins, 2011). In addition, Martins (2011) did not observe integration using a codon-optimised ΦC31 integrase in the same species, *P. xylostella*. The integrase used for experiments in Chapter 4 was unmodified — except that it contained the *D. melanogaster vasa* gene's T7 3'UTR — and was used by others successfully, albeit at low integration efficiencies, to integrate plasmids into *P. xylostella* (Martins, 2011) and *Ae. aegypti* (StJohn, 2012). Nevertheless, future work should include the investigation of codon-optimisation and the addition of nuclear localised signals to terminal sequences to enhance the efficiency and reliability of the system in *Ae. aegypti.*

Another future area of research should include the use of ΦC31 'self-docking' strains. These self-docking strains express endogenous integrase in the posterior oocyte, and have been developed in *An. gambiae* (Meredith *et al.*, 2013). Using the *An. gambiae nanos* regulatory region, the ΦC31 integrase was expressed in the site of germline development. This drastically improved the efficiency and utility of the ΦC31-*att* system

(Meredith *et al.*, 2013). Future work in *Ae. aegypti* should therefore include the comparative analysis of 'self-docking' strains and co-injection of mRNA or plasmid intergrase helper. In addition, this could also be utilised to improve the excision frequencies of Cre and FLP recombinases.

Jasinskiene *et al.* (2003) was previously unsuccessful at inducing FLP-mediated excision in *Ae. aegypti* using a helper plasmid containing FLP under the control of the *D. melanogaster hsp70* gene's promoter. This method has been used to induce Cre-mediated excision at high excision frequency by heat-shocking injected insect embryos in *D. melanogaster* (Siegal and Hartl, 1996), and *Ae. aegypti* (Jasinskiene *et al.*, 2003).

In Chapter 4 the Cre and FLP genes were modified to contain the *D. melanogaster vasa* gene's T7 3'UTR (Martins, 2011) to assist delivery of the mRNA to these cells (Sano *et al.*, 2002). It was unclear whether the T7 3'UTR or the use of FLP mRNA aided the successful excision of sequences in this study. However, excision frequencies were low. Therefore the utility of using the heat-shock-inducible approach with Cre and FLP should be investigated to improve our understanding of factors that influence excision frequencies of both Cre and FLP.

Further refinements of the cassette exchange system could also include incorporating methods to stabilise the *piggyBac*-based docking construct to circumvent remobilisation risks for species in which remobilisation is known to occur, such as *An. stephensi* (O'Brochta *et al.*, 2011). Established methods include the preferential remobilisation of smaller vectors within the larger vector (Handler, 2004; Dafa'alla *et al.*, 2006), or the use of double-strand break-repair with I-SceI and I-CreI homing

endonucleases and segments of DNA homologous to the docking construct's flanking sequences (Tkachuk *et al.*, 2011).

Genetic insulators could also be investigated to minimise any positional effects (Kuhn and Geyer, 2003; Bell *et al.*, 2001), and potentially improve the system. For example, the exogenous *gypsy* insulator sequence derived from *D. melanogaster* was reported to increase and stabilise expression in *Anopheles stephensi* (Carballar-Lejarazú *et al.*, 2013).

Assuring the utility of the new docking lines engineered in Chapter 4, as ultimately their functionality was proven, was an important immediate step. Therefore Chapter 5 set out to investigate the quality of the two new transgenic docking strains that were shown to carry functional and accessible *attP*-docking sites, OX4476C and OX4476F. These strains could be used to integrate a panel of genes into the genome for direct comparison. They could also be suitable for developing strains for field release, especially OX4476F, in which complete cassette exchange was shown.

Life-history parameters such as longevity, male mating competitiveness and female fecundity were compared to the background wild-type strain, and there were no detectible fitness costs. In addition, there were no obvious positional effects of the transgene, making these lines good candidates for both research and the generation of new transgenic strains for genetic control of *Ae. aegypti.*

The development of site-specific cassette exchange docking lines is a key development for the field. The two healthy OX4476C and OX4476F lines could be used to integrate transgenes into pre-characterised docking sites for field release.

Furthermore, the OX4476 docking lines could also facilitate the engineering of gene drive systems in two ways. The ability to directly compare lethal, suppressor or refractory constructs facilitates the fine-tuning of expressed phenotypes. This minimises the time-consuming nature of transforming and characterising transgenic insects using *piggyBac*-based transposons since baseline properties of the docking strains are known and a panel of insertions do not need to be evaluated for each construct to understand the 'average' behaviour. Also, in *D. melanogaster* an underdominance-like gene drive system utilised pre-characterised *attP* docking strains to integrate lethal-*trans*-suppressor constructs into the same chromosomal loci (Akbari *et al.*, 2013). The characterised docking strains described in this thesis could therefore be used to develop a working underdominance system in *Ae. aegypti* and resulting strains could be adequate for release.

The flanking sequences for the insertion sites in OX4476C and OX4476F were also obtained, and primers designed that could assist making new transgenic strains homozygous for research or field release. In fact, strains could be made homozygous using the new docking strains in two related ways. PCR analysis across the integration site could facilitate identification of homozygous insects by the amplicon's size. These homozygotes could then be crossed to each other to obtain homozygous lines. Crucially, this method could be exploited to develop a more efficient method for making transgenic *Ae. aegypti* lines homozygous for future use. For example, site-specific integration of a reporter construct carrying a blue body marker would result in insects with blue eyes and blue bodies (the blue-eye phenotype is from the docking construct's marker). This strain could then be made homozygous using the previously mentioned

PCR method resulting in a 'homozygous reporter' strain. If, for example, a transgenic effector line carrying a red-eye marker was established using the *attP* docking line, the construct would be integrated into the same chromosomal loci. Transgenic insects could then be crossed to genetically diverse laboratory strains if necessary, and offspring could be crossed to the homozygous reporter strain. Offspring inheriting all three markers (red and blue eyes, and blue bodies) would be heterozygous for both constructs, yet homozygous for the *piggyBac*-based construct. If insects carrying all three markers are crossed to each other, 25% of their resulting offspring will carry the red and blue eyes only, and will be homozygous for the effector construct. This can be verified using PCR across the genomic flanking regions, and could be an efficient method for making transgenic homozygous strains.

Future work on these docking strains for such use should also include more rigorous assays to identify recessive fitness costs, such as the tracking of allele frequencies over multiple generations. This would allow the identification of strains that are not suitable for making transgenic homozygous strains, with important implications for research and especially for field release. Moreover, the current suite of fitness-assessing parameters is currently under investigation (Black, I., unpublished data), and should guide research priorities for further characterisation of these strains.

In addition, research focused on developing a proof of principle for the new cassette exchange system and so the first three docking lines available for use were prioritised for immediate evaluation. Consequently, characterisation of the three further *attP* docking lines that were not evaluated adequately in this thesis should also be pursued.

Finally, an exciting contribution of this work is that site-specific integration into the new OX4476 docking strains facilitates exploration into several different high-profile areas of current research. For example, Windbichler *et al.* (2011) recently published a report on a working homing endonuclease gene (HEG) drive system. This relied on the use of the targeted transformation of *An gambiae* using the ΦC31-*att* system (Windbichler *et al.*, 2011). A HEG construct and the reporter construct it targets were integrated at the same genomic loci using pre-characterised *attP* docking sites. This could be tested in *Ae. aegypti* using the new OX4476 lines.

6.4 Concluding statement

In conclusion, this thesis contributes to the goal of using transgenic mosquitoes to decrease the burden of disease by exploring mechanisms to drive refractoriness into target populations, and by developing tools for the safe, reliable, and targeted transformation of *Ae. aegypti* potentially suitable for field release. What remains is to optimise and improve site-specific transformation in a broader number of mosquito species, and to continue efforts to develop effective gene drivers. The success of the latter remains this field's biggest challenge.

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Appendix 1

Engineering gene drive in *Aedes aegypti*

1.1 Inheritance of transgenes in progeny of hemizygous *hex*-effector crosses

Chi squared analysis (χ^2) was used to quantify the significance of the difference between the observed pupal survival of each genotype proportion and the expected 0.25 ratio according to the Mendelian inheritance prediction, where a significant difference at the 5% level is indicated by (*). The effector line crossed to the *hexamerin* promoter line (*hex*) is indicated in the first column. The genotype of offspring from each test cross and the associated statistical analysis is shown for offspring reared either in the presence of tetracycline (tet) or without.

Table showing the Chi^2 (χ^2) test of observed mean survival and the expected 25% genotype proportions predicted by Mendelian inheritance; NR= not recorded, NTG=non-transgenic; df= degrees of freedom; a significant difference between observed and expected genotype proportions at the 5% level is indicated by (*).

		Treatment					
		No Tetracyline			Tetracyline		
Effector Line	Genotype	Mean survival %	χ^2 (df=1)	*p*-value	Mean survival %	χ^2 (df=1)	*p*-value
OX3547	Effector	29.78	1.59	0.452	23.11	5.52	0.063
NIPP1	*Hex*	24.44	4.55	0.103	22.67	5.87	0.053
	Hex-Effector	1.56	34.45	<0.001*	21.78	6.59	0.037
	NTG	22.22	6.22	0.045	22.44	6.04	0.049
OX3582	Effector	26.67	3.13	0.209	24.89	4.24	0.120
Michelob_x	*Hex*	22.00	6.41	0.041	22.89	5.69	0.058
	Hex-Effector	0.00	37.50	<0.001*	26.00	3.53	0.171
	NTG	28.67	2.08	0.353	23.11	5.52	0.063
OX4230	Effector	20.67	7.56	0.023	21.33	6.97	0.031
Trpsin	*Hex*	30.67	1.25	0.537	27.33	2.76	0.252
	Hex-Effector	0.00	37.50	<0.001*	22.67	5.87	0.053
	NTG	29.11	1.88	0.391	25.11	4.09	0.129
OX4291	Effector	21.82	6.56	0.038	28.79	2.02	0.363
Carboxypeptidase	*Hex*	25.15	4.07	0.131	26.67	3.13	0.209
	Hex-Effector	20.00	8.17	0.017	25.76	3.68	0.159
	NTG	18.18	9.95	0.007	28.79	2.02	0.363
OX4292	Effector	21.33	6.97	0.031	21.11	7.16	0.028
ILP	*Hex*	22.22	6.22	0.045	22.44	6.04	0.049
	Hex-Effector	9.56	20.82	<0.001*	21.33	6.97	0.031
	NTG	21.33	6.97	0.031	19.78	8.38	0.015
OX4293	Effector	18.22	9.91	0.007	23.33	5.35	0.069
Eiger	*Hex*	24.67	4.39	0.111	28.89	1.98	0.372
	Hex-Effector	5.56	27.21	<0.001*	26.22	3.39	0.183
	NTG	18.89	9.24	0.010	28.89	1.98	0.372
OX4393 12	Effector	24.22	4.70	0.095	25.11	4.09	0.129
Reaper^{KR}	*Hex*	21.11	7.16	0.028	26.89	3.00	0.223
	Hex-Effector	0.00	37.50	<0.001*	22.89	5.69	0.058
	NTG	20.00	8.17	0.017	21.11	7.16	0.028
OX4423 Line 2	Effector	21.11	2.63	0.268	NR		
Reaper^{KR}	*Hex*	16.67	5.93	0.052	NR		
	Hex-Effector	10.83	12.25	<0.001*	18.33	4.54	0.103
	NTG	21.67	2.31	0.314	20.28	3.15	0.207
OX4423 Line 3	Effector	21.39	2.47	0.291	NR		
Reaper^{KR}	*Hex*	17.50	5.21	0.074	NR		
	Hex-Effector	11.11	11.89	<0.001*	17.78	4.98	0.083
	NTG	20.56	2.97	0.226	20.28	3.15	0.207
OX4536 A	Effector	21.11	7.16	0.028	24.00	4.86	0.088
Reaper^{KR}	*Hex*	26.67	3.13	0.209	25.33	3.95	0.139
	Hex-Effector	0.00	37.50	<0.001*	26.22	3.39	0.183
	NTG	25.11	4.09	0.129	25.33	3.95	0.139

		Treatment					
		No Tetracyline			Tetracyline		
Effector Line	Genotype	Mean survival %	χ^2 (df=1)	*p*-value	Mean survival %	χ^2 (df=1)	*p*-value
OX4509 D5	Effector	32.00	0.81	0.668	30.00	1.50	0.472
AaHIT scorpion	*Hex*	31.33	1.01	0.602	33.78	0.37	0.831
toxin	*Hex*-Effector	0.00	37.50	<0.001*	0.67	36.18	<0.001*
	NTG	34.67	0.21	0.898	32.89	0.57	0.753
OX4509 F1	Effector	28.89	1.98	0.372	26.67	3.13	0.209
AaHIT scorpion	*Hex*	33.33	0.46	0.793	28.22	2.30	0.317
toxin	*Hex*-Effector	0.00	37.50	<0.001*	0.22	37.06	<0.001*
	NTG	28.22	2.30	0.317	28.67	2.08	0.353
OX4509 H2	Effector	32.22	0.16	0.921	23.61	1.36	0.506
AaHIT scorpion	*Hex*	24.17	1.13	0.567	30.83	0.02	0.988
toxin	*Hex*-Effector	0.00	30.00	<0.001*	3.06	24.20	<0.001*
	NTG	30.00	0.00	1.000	29.72	0.00	0.999
OX4711.D5	Effector	23.00	0.16	0.923	26.00	0.04	0.980
Av3 sea-anemone	*Hex*	30.00	1.00	0.607	26.67	0.11	0.946
toxin	*Hex*-Effector	2.33	20.55	<0.001*	28.33	0.44	0.801
	NTG	31.00	1.44	0.487	26.67	0.11	0.946
OX4711 D9	Effector	21.00	0.64	0.726	23.33	0.36	0.835
Av3 sea-anemone	*Hex*	22.00	0.36	0.835	25.00	0.00	1.000
toxin	*Hex*-Effector	1.67	21.78	<0.001*	22.67	0.16	0.923
	NTG	24.00	0.04	0.980	19.67	1.00	0.607

1.2 Pupation of progeny from *vit-* NIPP1 and *vit-* michelob_x crosses

Chi squared analysis (χ^2) was used to quantify the significance of the difference between observed pupae survival from the 300 aliquoted larvae, and the expected 100% survival in all genotypes. Note in this experiment that L3 larvae of each genotype (per test cross) were aliquoted into single repeats of 300 larvae. A significant difference at the 5% level is indicated by (*). The effector line crossed to the *vitellogenin* promoter line (*vit*) is indicated in the first column. The genotype of offspring from each test cross and the associated statistical analysis is shown for offspring reared either in the presence of tetracycline (tet) or without.

Table showing the Chi^2 (χ^2) test of observed (A) pupation numbers and (B) adult eclosion Rates. NTG=non-transgenic; df= degrees of freedom; a significant difference between observed and expected genotype proportions at the 5% level is indicated by (*).

				Males			Females		
A	Effector Crossed	Offspring Genotype	Treatment	No. pupated	χ^2 (df=1)	*p*-value	No. pupated	χ^2 (df=1)	*p*-value
	OX3582	Promoter	No Tetracyline	163	1.13	0.569	124	4.51	0.105
	Michelob_x	NTG	No Tetracyline	154	0.11	0.948	141	0.54	0.763
		Effector	No Tetracyline	177	4.86	0.088	121	5.61	0.061
		Promoter-Effector	No Tetracyline	135	1.50	0.472	85	28.17	<0.001*
		Promoter-Effector	Tetracyline	159	0.54	0.763	136	1.31	0.520
		NTG	Tetracyline	156	0.24	0.887	135	1.50	0.472
	OX3547	Promoter	No Tetracyline	157	0.33	0.849	121	0.33	0.849
	NIPP1	NTG	No Tetracyline	147	0.06	0.970	134	0.06	0.970
		Effector	No Tetracyline	140	0.67	0.717	120	0.67	0.717
		Promoter-Effector	No Tetracyline	142	0.43	0.808	130	0.43	0.808
		Promoter-Effector	Tetracyline	143	0.33	0.849	148	0.33	0.849
		NTG	Tetracyline	126	3.84	0.147	166	3.84	0.147

				Males			Females		
B	Effector Crossed	Offspring Genotype	Treatment	No. eclosed	χ^2 (df=1)	*p*-value	No. eclosed	χ^2 (df=1)	*p*-value
	OX3582	Promoter	No Tetracyline	158	0.15	0.926	121	0.07	0.964
	Michelob_x	NTG	No Tetracyline	143	0.79	0.675	130	0.86	0.651
		Effector	No Tetracyline	171	0.20	0.903	120	0.01	0.996
		Promoter-Effector	No Tetracyline	132	0.07	0.967	61	6.78	0.034*
		Promoter-Effector	Tetracyline	151	0.40	0.818	132	0.12	0.943
		NTG	Tetracyline	149	0.31	0.855	124	0.90	0.639
	OX3547	Promoter	No Tetracyline	145	0.92	0.632	120	0.01	0.996
	NIPP1	NTG	No Tetracyline	140	0.33	0.846	117	2.16	0.340
		Effector	No Tetracyline	137	0.06	0.968	119	0.01	0.996
		Promoter-Effector	No Tetracyline	139	0.06	0.969	123	0.38	0.828
		Promoter-Effector	Tetracyline	140	0.06	0.969	133	1.52	0.468
		NTG	Tetracyline	120	0.29	0.867	136	5.42	0.066

1.3 Survival analyses of blood fed females from *vit*-AaHIT crosses: testing the differences between experimental repeats

In the following analyses, paralysed females were counted as dead. Survival curves of three experimental repeats from the *vitellogenin*-promoter (*vit*) and scorpion toxin effector (AaHIT) cross were compared using the log rank χ^2–analysis. There was a significant difference between repeats (χ^2=

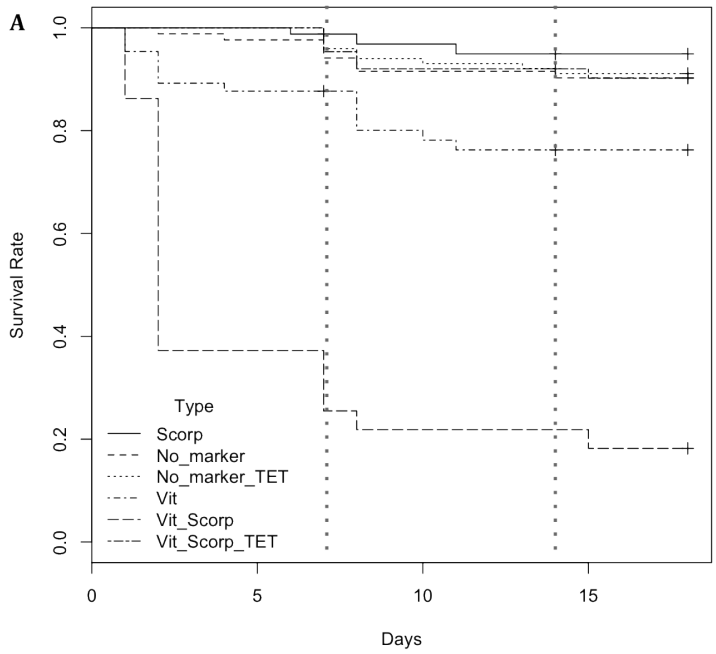
25, df= 2, $p<0.001$). Therefore survival analysis between genotypes was carried out on the three repeats separately.

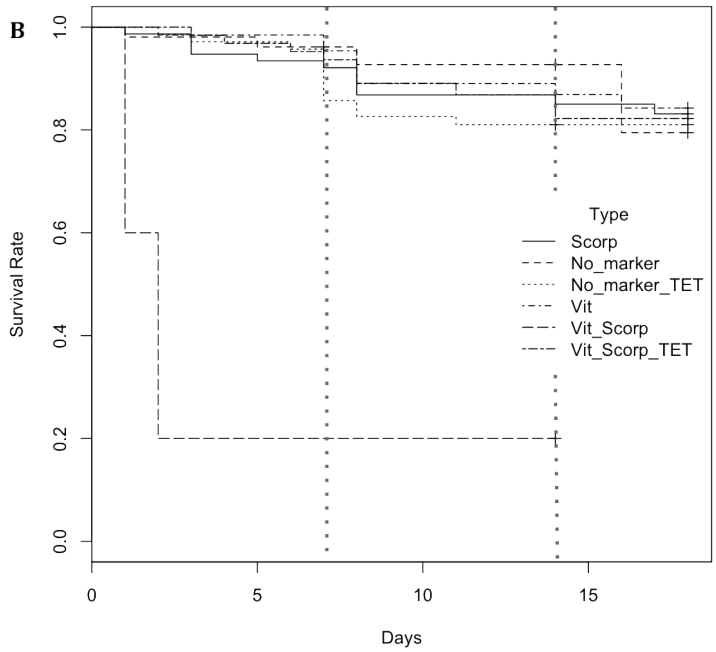
In all three instances, there was a significant difference between females carrying both *vit* and AaHIT constructs and control groups, and also when compared to the same genotype reared on tetracycline (repeat 1: $\chi^2= 281$, df= 5, $p<0.001$; repeat 2: $\chi^2= 61.7$, df= 5, $p<0.001$; repeat 3: $\chi^2= 93.7$, df= 5, $p<0.001$).

Survival curves were compared between the controls gentoypes, *i.e.* non-transgenic insects reared on tetracycline and non-transgenic, effector only, and promoter only genotypes reared in the absence of tetracycline. This revealed a problem in repeat 1 (rep1) where the survival rate of the *vit*- only genotype was lower than remaining control groups ($\chi^2= 14$, df= 3, $p<0.001$). Whether the rep1 data was removed from the analysis, or the *vit*-only genotype was removed from rep1, there was still a significant difference between the repeats (rep1 removed: $\chi^2= 17.4$, df= 1, $p<0.001$; *vit* removed from rep1: $\chi^2= 26.1$, df= 2, $p<0.001$).

When data from the three repeats were pooled, the reduced survival of the off-tet *vit*-only genotype seen in rep1 was masked when compared to other control groups, *i.e.* off tet non-transgenic and AaHIT-only genotypes ($\chi^2= 2$, df= 2, $p= 0.329$).

Nevertheless, survival curves from all analyses suggested the same conclusions: that insects carrying the *vit* and AaHIT lethal constructs had a significantly lower survival rate compared to remaining genotypes following a blood meal.


A
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Days
Type
Scorp
No_marker
No_marker_TET
Vit
Vit_Scorp
Vit_Scorp_TET


B
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Days
Type
Scorp
No_marker
No_marker_TET
Vit
Vit_Scorp
Vit_Scorp_TET

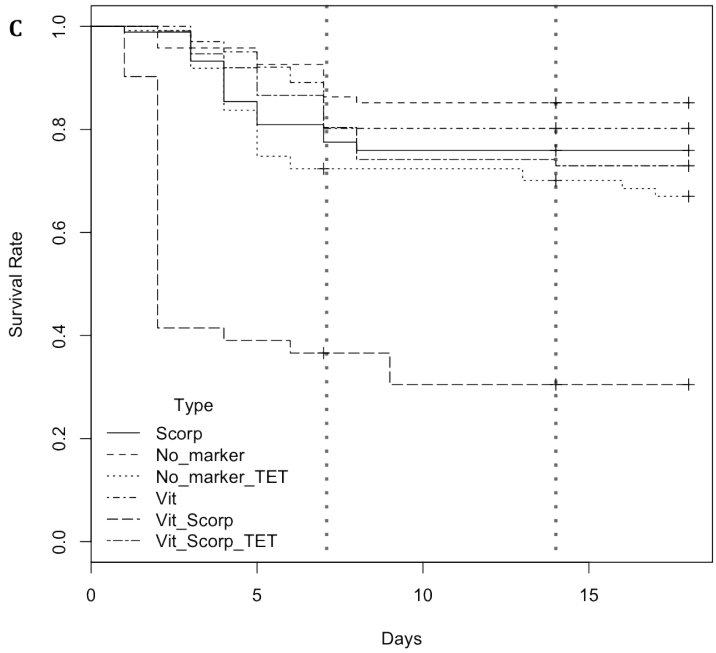
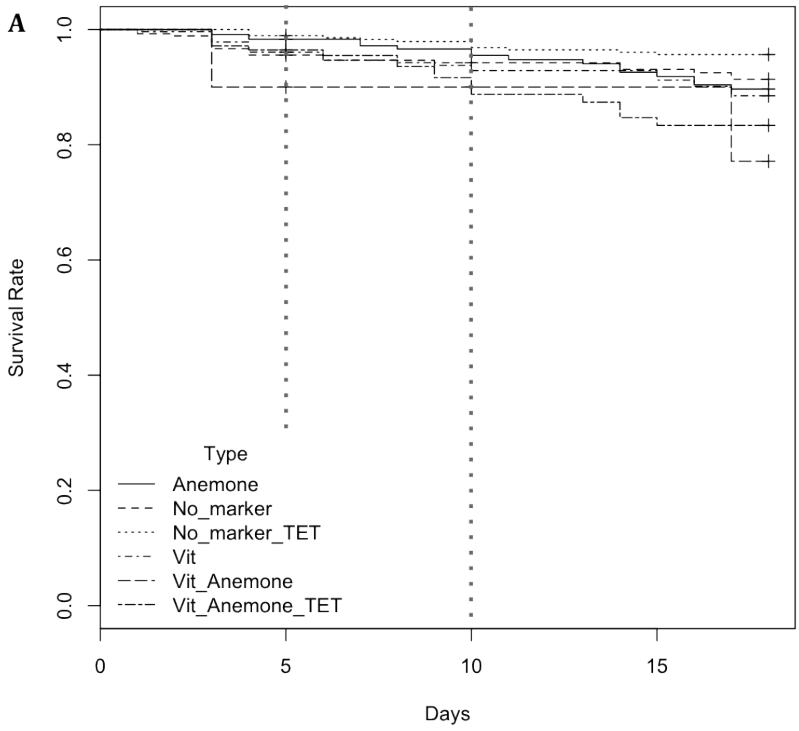
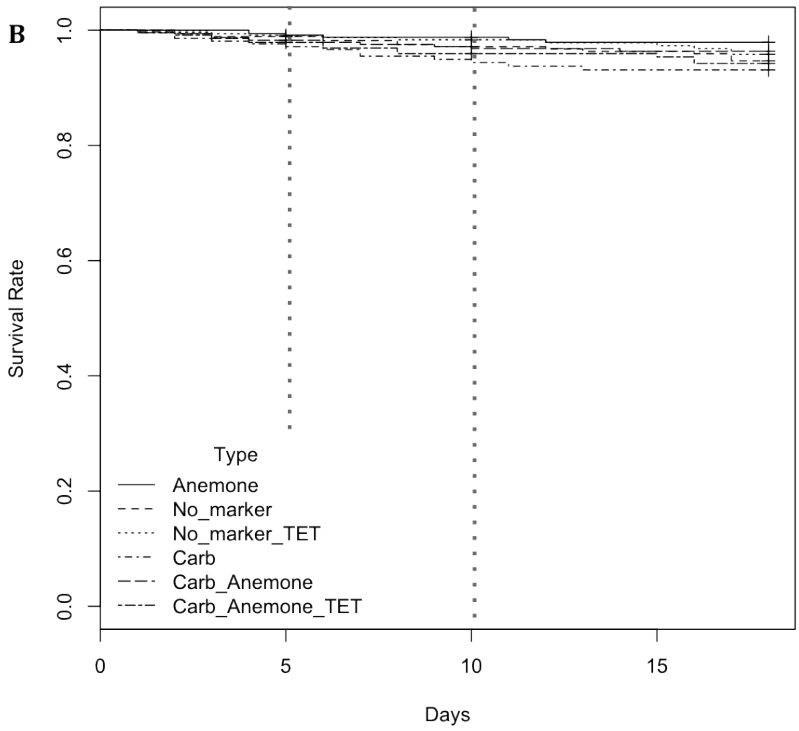


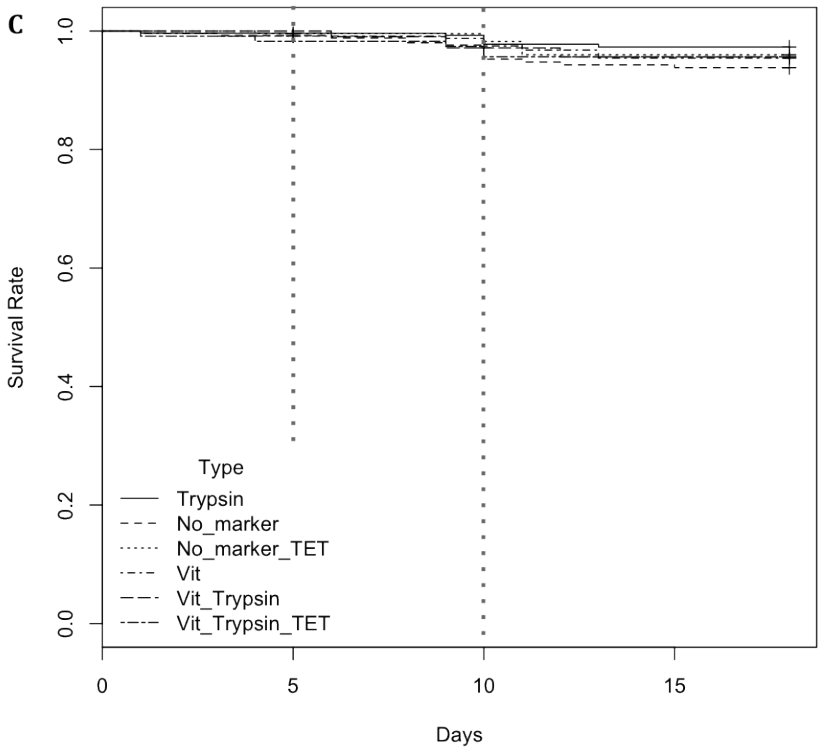
Figure showing the survival of blood-fed female progeny from *vit*-AaHIT crosses; experimental repeats shown separately (A) repeat 1, (B) repeat 2 and (C) repeat 3. Females were blood fed on days 0, 7 and 14, denoted by vertical dotted lines; Scorp=AaHIT.

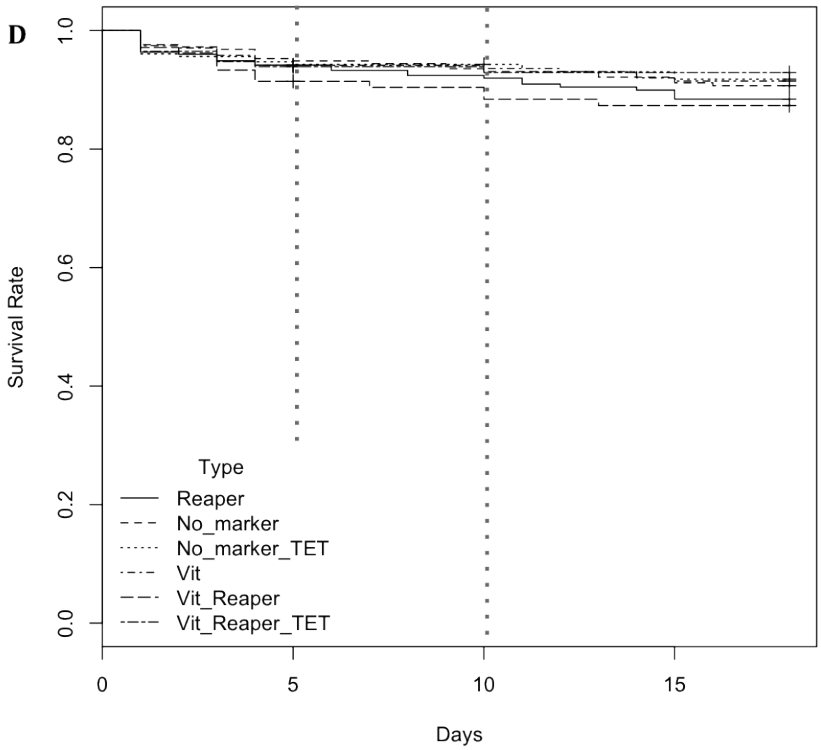
1.4 Survival analysis of adults from all effector crosses (except *vit*-AaHIT) using data pooled from three experimental repeats

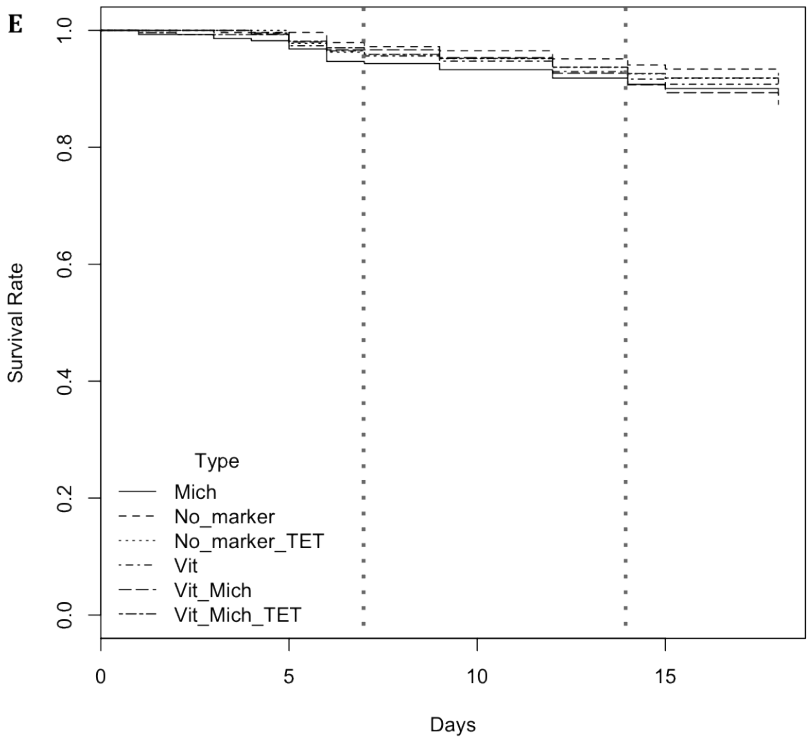
The survival analyses of blood fed female progeny from crosses between the *vitellogenin* and *carboxypeptidase* promoter lines (*vit* and *carb* respectively), and the tetO-effector lines are shown below. Females were analysed for three gonotrophic cycles, with second and third blood meals indicated by horizontal lines.

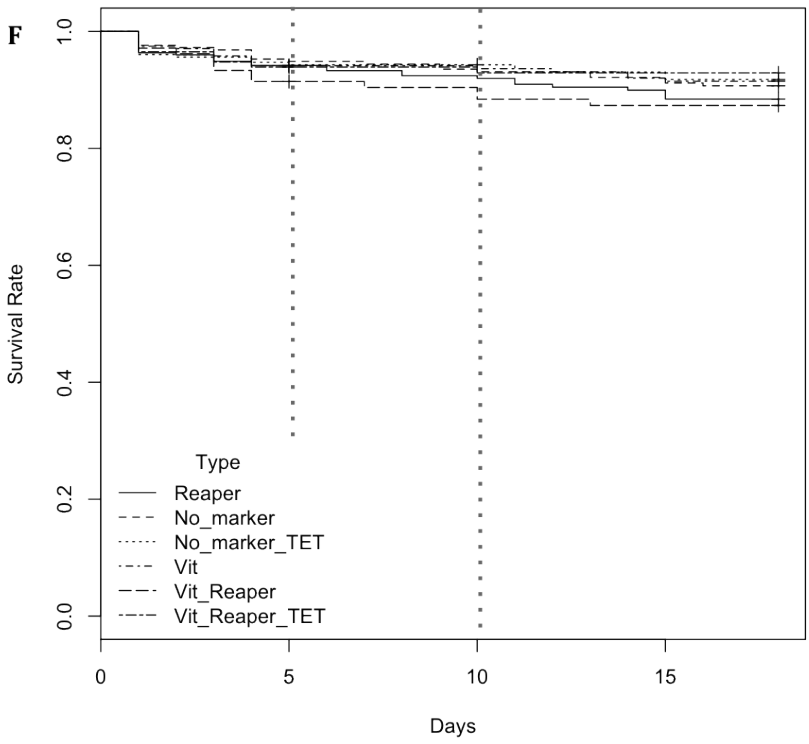

A
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Days
Type
Anemone
No_marker
No_marker_TET
Vit
Vit_Anemone
Vit_Anemone_TET


B
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Days
Type
Anemone
No_marker
No_marker_TET
Carb
Carb_Anemone
Carb_Anemone_TET


C
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
Type
Trypsin
No_marker
No_marker_TET
Vit
Vit_Trypsin
Vit_Trypsin_TET
0
5
10
15
Days


D
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
Type
Reaper
No_marker
No_marker_TET
Vit
Vit_Reaper
Vit_Reaper_TET
0
5
10
15
Days


E
Survival Rate
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
Days
Type
Mich
No_marker
No_marker_TET
Vit
Vit_Mich
Vit_Mich_TET


F
Survival Rate
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
Days
Type
Reaper
No_marker
No_marker_TET
Vit
Vit_Reaper
Vit_Reaper_TET

Figures showing the survival of blood-fed female progeny from (A) *vit*-Av3, (B) *carb*-Av3, (C) *vit*-trypsin (D) *vit*-reaper, (E) *vit*-michelob_x, and (F) *carb*-AaHIT crosses; results are pooled from three experimental repeats. Second and third blood meals are denoted by vertical dotted lines, where non-blood fed females were counted and removed from cages; Scorp=AaHIT, Anemone=Av3.

1.5 Survival analysis from *vit*-AaHIT test crosses using pooled data where paralysed insects were removed from the analysis

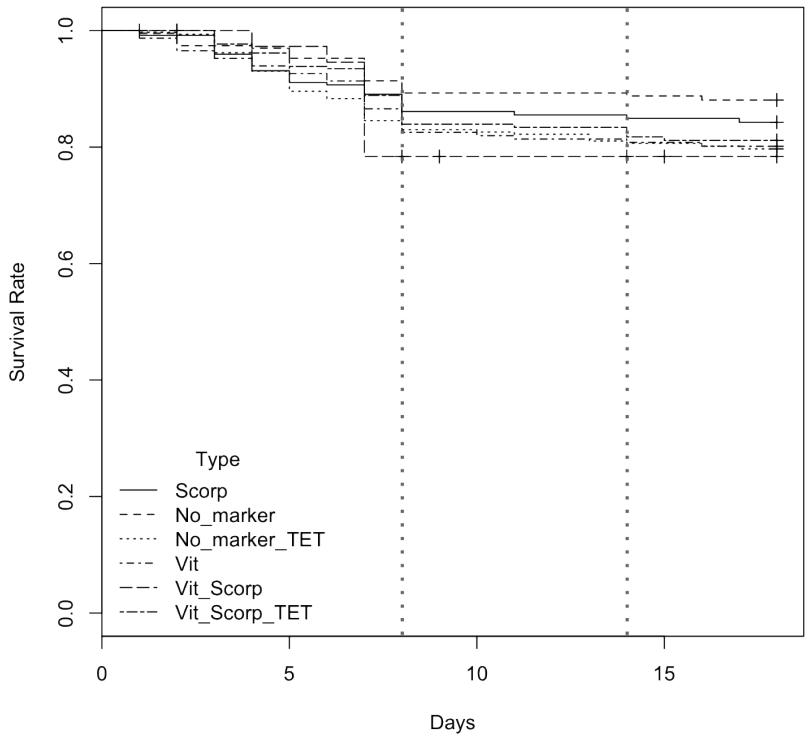


Figure showing the survival curves of blood-fed female progeny from Vit-AaHIT test cross where paralysed females were removed from analysis; results are pooled from three experimental repeats. Second and third blood meals are denoted by vertical dotted lines, where non-blood fed females were counted and removed from cages. Scorp=AaHIT.

1.6 Fecundity (egg laying) and fertility (egg hatch) of female progeny from test crosses

Fecundity was estimated by dividing the number of eggs produced by each cage by the number of surviving females; there were three replicate cages per genotype and treatment for each test cross. The data was extremely variable, which was expected given that there was no way of determining how many of the females laid eggs; individual egg laying of females would have provided a much more accurate estimate of fecundity.

Fertility was estimated as a proportion of hatched eggs from the total number of eggs per cage; statistical analysis was therefore carried out on arcsine square root values.

Genotype and tetracycline-status did not have an affect on the fecundity and fertility of females for each test cross.

Table 3.7. Statistical analyses of differences between (A) fecundity and (B) fertility of female progeny from each test cross. Both= females carrying both promoter and effector constructs; NTG= non-transgenic females; Effector= females carrying the effector construct only; Promoter= females carrying the effector construct only; TET= females reared in the presence of tetracycline.

A

		Mean Eggs per Female (± Standard Deviation); N=3						
		Both	NTG	Effector	Promoter	Both TET	NTG TET	One-way ANOVA analysis
Vit	Michelob_x	61 (±39)	63 (±38)	54 (±41)	69 (±44)	12 (±15)	46 (±25)	$F_{(5,\ 12)}= 1.025,\ p= 0.446$
	Av3	57 (±42)	36 (±34)	-	-	38 (±52)	37 (±23)	$F_{(3,\ 8)}= 0.202,\ p= 0.892$
	Trypsin	46 (±38)	40 (±41)	-	-	66 (±16)	48 (±32)	$F_{(3,\ 8)}= 0.319,\ p= 0.811$
	Reaper^{KR}	61 (±14)	65 (±20)	-	-	73 (±15)	70 (±15)	$F_{(3,\ 8)}= 0.337,\ p= 0.799$
	AaHIT[1]	69 (±14)	68 (±15)	-	-	69 (±17)	69 (±17)	$F_{(3,\ 8)}= 0.004,\ p= 1.000$
Carb	Av3	32 (±26)	34 (±23)	-	-	54 (±9)	41 (±13)	$F_{(3,\ 8)}= 0.861,\ p= 0.500$
	AaHIT	48 (40±)	43 (±40)	-	-	36 (±24)	38 (±23)	$F_{(3,\ 8)}= 0.110,\ p= 0.952$

B

		Mean Hatch Rate (± Standard Deviation); N=3						
		Both	NTG	Effector	Promoter	Both TET	NTG TET	One way ANOVA of arcsine
Vit	Michelob_x	0.98 (±0.01)	0.96 (±0.05)	0.99 (±0.01)	0.99 (±0.002)	0.99 (±0.14)	0.99 (±0.002)	$F_{(5,\ 5.342)}= 1.366,\ p= 0.364$
	Av3	0.74 (±0.22)	0.87 (±0.08)	-	-	0.53 (±0.28)	0.88 (±0.08)	$F_{(3,\ 8\ )}= 2.105,\ p= 0.178$
	Trypsin	0.91 (±0.04)	0.81 (±0.13)	-	-	0.87 (±0.04)	0.88 (±0.11)	$F_{(3,\ 8)}= 0.606,\ p= 0.629$
	Reaper^{KR}	0.95 (±0.04)	0.94 (±0.06)	-	-	0.90 (±0.09)	0.91 (±0.07)	$F_{(3,\ 8)}= 0.411,\ p= 0.750$
	AaHIT[1]	0.94 (±0.03)	0.94 (±0.03)	-	-	0.96 (±0.04)	0.87 (±0.05)	$F_{(3,\ 8)}= 2.987,\ p= 0.096$
Carb	Av3	0.80 (±0.05)	0.77 (±0.06)	-	-	0.91 (±0.06)	0.91 (±0.05)	$F_{(3,\ 8)}= 2.132,\ p= 0.174$
	AaHIT	0.85 (±0.06)	0.87 (±0.04)	-	-	0.88 (±0.08)	0.84 (±0.07)	$F_{(3,\ 8)}= 0.118,\ p= 0.947$

[1]The fecundity and fertility of females not exhibiting paralysis was investigated in this test.

1.7 Survival analyses of blood fed female progeny from *vit*-Vg40-AaHIT test cross

In the following analyses, paralysed insects were counted as dead and included in the analysis, and significant difference are indicated at the 5% level. Survival curves of three experimental replicates from progeny of the *vitellogenin*-promoter (*vit*), scorpion toxin effector (AaHIT) and Vg40 RNAi line crosses were compared using the log rank χ^2 analysis. There was a significant difference between experimental repeats (χ^2=29, df= 2, p<0.001). Therefore survival analysis between genotypes was carried out on the three repeats separately.

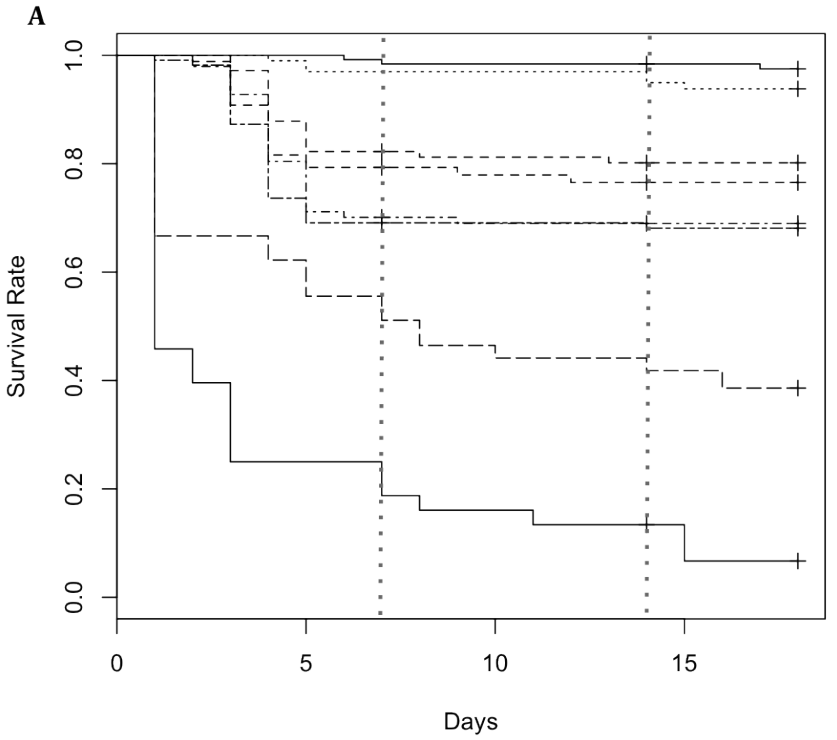
In all three instances, fewer females carrying both lethal constructs (*vit*-AaHIT) survived after a blood meal compared to the non-transgenic control (repeat 1: χ^2=176, df= 1, p<0.001; repeat 2: χ^2=122, df= 1, p<0.001; repeat 3: χ^2=29.2, df= 1, p<0.001). This was consistent with previous findings.

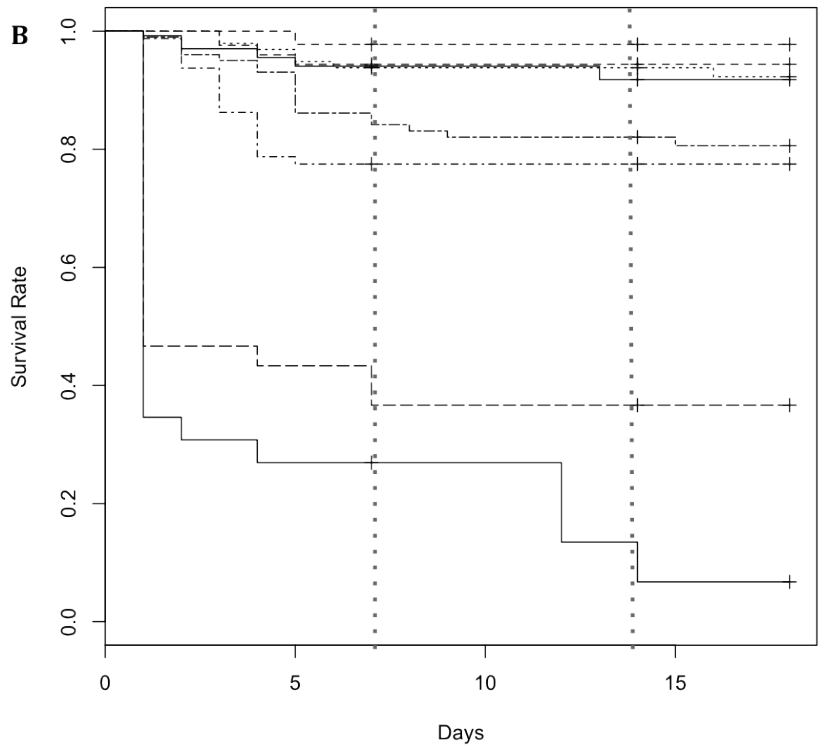
In addition, there were also fewer females carrying Vg40-*vit*-AaHIT (lethal constructs with the RNAi construct) than the non-transgenic control (repeat 1: χ^2= 176, df= 1, p<0.001; repeat 2: χ^2= 122, df= 1, p<0.001; repeat 3: χ^2= 41.7, df= 1, p<0.001).

To identify any effects of carrying transgenic constructs alone, the survival curves were compared between controls groups (*i.e.* all genotypes except *vit*-AaHIT and Vg40-*vit*-AaHIT). In all instances there were significant differences between control groups (repeat 1: χ^2= 58.3, df= 5, p<0.001; repeat 2: χ^2= 35.1, df= 5, p<0.001; repeat 3: χ^2= 6.2, df= 5,

$p<0.001$), indicating a fitness penalty associated with some transgenic constructs.

Finally, to test the ability of the Vg40 RNAi construct to knockdown the lethal effects of *vit*-AaHIT, the differences between the survival curves of *vit*-AaHIT and Vg40-*vit*-AaHIT were compared. Although the survival difference was small, fewer *vit*-AaHIT females survived compared to the Vg40-*vit*-AaHIT in repeat 1 ($\chi^2= 9.2$, df= 1, $p= 0.002$), Figure A. This indicated some rescue of the lethal effects. Though fewer *vit*-AaHIT females survived compared to the Vg40-*vit*-AaHIT in repeat 2, this difference was not statistically significant ($\chi^2= 3.2$, df= 1, $p= 0.072$), Figure B. There was no difference between the survival of *vit*-AaHIT and Vg40-*vit*-AaHIT in repeat 3 ($\chi^2= 1.2$, df= 1, $p= 0.268$), Figure C.


A
Survival Rate
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
Days


B
Survival Rate
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
Days

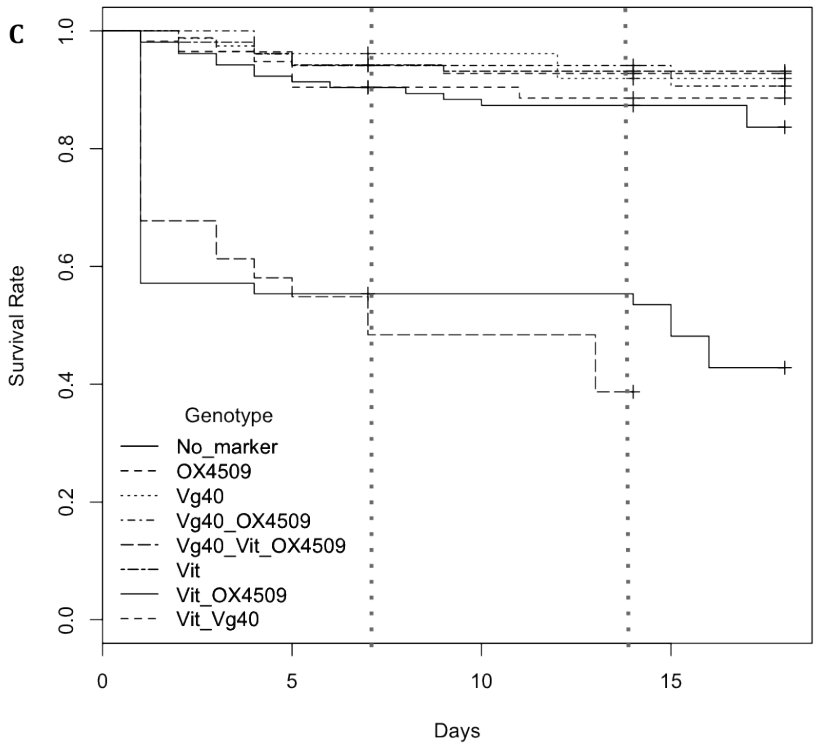


Figure showing the survival of blood-fed female progeny from the *vit*-Vg40-AaHIT cross, three experimental repeats shown separately: (A) repeat 1, (B) repeat 2 and (C) repeat 3. Females were blood fed on days 0, 7 and 14; denoted by vertical dotted lines; legend for all graphs is shown in (C). OX4509=AaHIT, No_marker= non-transgenic.

Appendix 2

Engineering site-specific cassette exchange in *Aedes aegypti*

2.1 Sequences across the *attL* and *attR* integration junctions

Table showing sequencing analysis of the *attL* and *attR* junctions. Sequences in grey show the OX4714 donor (5'-end= underlined) and the OX4476 acceptor (3'-end) sequences flanking the *att* junction (in bold). Boxed TTG sequence= recombination core.

Region	Sequence
attL	5'-GAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGATGT AGGTCACGGTCTCGAAGCCGCGGTGCGGGTGCCAGGGCGTG CCCTTGAGTTCTCTCAGTTGGGGGCGTAGGGCCGCCGACAT GACACAAGGGGTTGTGACCGGGGTGGACACGTACGCGGGT GCTTACGACCGTCAGTCGCGCGAGCGCGACTAGTTCTAGAG CGGCCGCCTGCAGTAGGAAGACGAATAGGTGGCCTATGGCA TTATTGTACGGAATGATAAACATTGCCTGCATAAATTCTTT TATTATATACAGCCATAATGTCAGTAGCAAGGGAGAAAAGG TCCAAAGTCGCAAAAAATTTATGAGAAACCTTTACATGA
attR	5'-GTTCTGTGATGACCTGCAGCCCGGGGGATCCACTAGTA CTGACGGACACACCGAAGCCCCGGCGGCAACCCTCAGCGGA TGCCCCGGGGCTTCACGTTTTCCCAGGTCAGAAGCGGTTTT CGGGAGTAGTGCCCCAACTGGGGTAACCTTTGGGCTCCCCG GGCGCGTACTCCACCTCACCCATCTGGTCCATCATGATGAA CGGGTCGAGGTGGCGGTAGTTGATCCCGGCGAACGCGCGG CGCACCGGGAAGCCCTCGCCCTCGAAACCGCTGGGCGCGGT GGTCACGGTGAGCACGGGACGTGCGACGGCGTCGGCGGGT GCGGATACGCGGGGCAGCGTCAGCGGGTTCTCGACGGTCA CGGCGGGCATGTCGACGGTATCGATAAGCTT

2.2 Sequences across the FLP and Cre excision junctions

Sequences obtained from PCR of the excision junction across the 5'-*piggyBac* inverted terminal repeats and the DsRed2 gene. Sequences from the DsRed2 gene are underlined.

Table showing sequencing analysis of the excision junctions. Sequences in grey show the OX4476 construct's 5'- *piggyBac* inverted terminal repeat (5'-end of sequences) and the OX4714 donor DsRed2 sequence (3'-end, underlined) flanking the excision junction (boxed sequences). *loxP*= white text on black; *FRT*= Black text on white.

	Sequence
Cre	5'-CCTGGTGGAGTTNNNTCCATCTNCATGGCCAAGAAGCCCGTG CAGCTGCCCGGCTACTACTACGTGGACGCCAAGCTGGACATCACCT CCCACAACGAGGACTACACCATCGTGGAGCAGTACGAGCGCACCG AGGGCCGCCACCACCTGTTCCTGAGATCTCGACCCAAGAAAAAGC GGAAGGTGGAGGACCCGTAAGATCCACCGGATCTAGATAACTGAT CATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAA AACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAA TTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATA AAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTG CATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAACGCG GGCGCGCCGCAGG**ATAACTTCGTATAGCATACATTATAC**GAA GTTATCCTAGGGAAGTTCCTATACTTTCTAGAGAATAGGAA CTTCGGAATAGGAACTTC**TTCGAGCTAGGCCGGCCGATCTCGA** ACTTATACGGTTCTTGTAGTAAGTTTTTTGCCAAAGGGATTGAGG TGAACCAATTGTCACACGTAATATTACGACAACTACCGTGCACAGG CTTTGATAACTCCTTCACGTAGTATTCACCGAGTGGTACTCCGTTG GTCTGTGTTCCTCTTCCCAAATAAGGCATTCCATTTATCATATACT TCGTACCACTGT
FLP	5'- TCCTTAGTCCAGGGGGGCCATACCCTGGGGAGGAGTTCAAG TCCATCTACCTGGCCAAGAAGCCCGTGCAGCTGCCCGGCTACTACT ACGTGGACGCCAAGCTGGACATCACCTCCCACAACGAGGACTACAC CATCGTGGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTT CCTGAGATCTCGACCCAAGAAAAAGCGGAAGGTGGAGGACCCGTA AGATCCACCGGATCTAGATAACTGATCATAATCAGCCATACCACAT TTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCT GAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTT ATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATT TCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTC CAAACTCATCAATGTATCTTAACGCGGGCGCGCCGCAGG**ATAACT** **TCGTATAGCATACATTATAC**GAAGTTATCCTAGGGAAGTTCCTATA CTTTCTAAAGAATAGGAACTTCGGAATAGGAACTTCTTC**GAGCTA** GGCCGGTCGATCTCGAACTTATACGGTTCTTGTAGTAAGTTTTTTG CCAAAGGGATTGAGGTGAACCAATTGTCACACGTAATATTACGAC AACTACCGTGCACAGGCTTTGATAACTCCTTCACGTAGTATTCACC

Appendix 3

Characterisation of new transgenic *attP* docking strains

3.1 OX4476 flanking genomic sequences in lines C and F

The duplicated *piggyBac* target sites are boxed and highlighted in grey.

OX4476C 5' sequence

5'-TGGGATTCGATTCGAACTCATGACCAGGGTTGCCATTATGCCAGATTAATC
TGGCACTGCAAGACTTTTGATCGAGGAAAAGATAAACTACTCATTTTGCAAGA
CTTTTTCAAATTCAGCCAGACTTGACAGACCGACGGTTAGCCATACTTACGCA
GGATTTAGAATTTTTCTTACGATTGGTAAATTCGCGTTTCAAAACAATAGTTT
GATGAGAGCCTGTTGTGTAGAAATTAAGCAAATTGTGGCAATCGTTTTTAAGT
AATGTGTATTCATGTGTTTGGTTCCACTCTGGCCGTAAAGAAATCTTGAAAAC
TTCGAAAAACATCATTTTGTAATTTTTAGTTTTCTTGAAGATAACTCAACCGA
TCTTTATATTTTTTTTTGAGAGAATTGCCTTAAAACCTGACATTGTTTTGCCC
ATGTTTTTTTTTTTTATAAATTGAATAAAAACAAAATGACGGGTAAAGGAGTT
TTATATGGGGAATGTAATTCCCCCCAAGAAATTCGGAATATTTTTAAAACCAT
ATAACCAATGATTCAAAAGCGATACAGATTTCAAAAACAAAAGAGTTTTTTTT
AGAGCTGCTAAACTATTGAAAAACAAAAAAAGTCATCGTGAACAAAATATTGT
TTTACGGTAGAAAAATGATGATGGCAGTAGAGGTTATCCTAGTGACTTTGTTT
ATTATGCGATGGTATTTTAGAATTTAAATCATATTTACCCTAGAATCTTCTGT
TCATTGAAAAAATGACGGTAGTAGTTTAAACTGTGTCCAGATCAACTCTACTA
GAGTGCAGTTCTCATTAATAAATTTATTTAGAGTTTTGAAAACATACATGGTG
AAACTTTTTCAGGGATTCATCATTTTGACTTTATAAAATCATAGCATCTTTGT
TCCATAATGTAAACAAACATAAAGGACTTATTTGATAAATACCATAGCTGACA
TACTAAATGTTTTCCATACAGACTTAACATTTAA

OX4476C 3' sequence

5'-TTAAAAAAGGCCATGTTTTAGTGCTGATGTGAATTTAGATTTTTTTTTTCT
ATGTTGTATATTTTTGTTACCGTTGAAATTCGAGTCTAGTAGTATACCTGCAC
AATGGTGTCTGTCAGACTTTGCAAGACTTTTTATTTTGAACTCGCCAGACATT

TTCAAAAATCTAGTGGCAACCCTGCTCATGACACTCAGCTTGGTCTTGATAAA
TAGGTGCGCGTCAAACGCTACGGCTGTTTGCCCCCCATACTTTTCTCATCCCC
TTCACTTGATTCTGATGGAATTCTTATTGGGTATCATTAAAAAATGACGTCCA
TCATTTGGGGGATGGGGAGGTCTACGAATGTGTCACATGTGCATGTATTTAG
TATTGGAAAAATTTCGGAGGACTCATACATAAAACGTAACATAGGGGGGAGG
GGGTCAAAAATGTTGAAATTTAGCGTGACATAATTTGTGTACTATCCCTGTGA
AGAAATTGGCTCTAAAGGCCGAATTCTTCACCTTCGCTTAAGCTCTAAACCAC
GTTTACCCATATGTTTAAACTAAGGTTTTGAAAAAAAAACCGCTTATAATTAT
GCTGAAGGTGTCTGGGTTCGATTCCCG

OX4476F 5' sequence

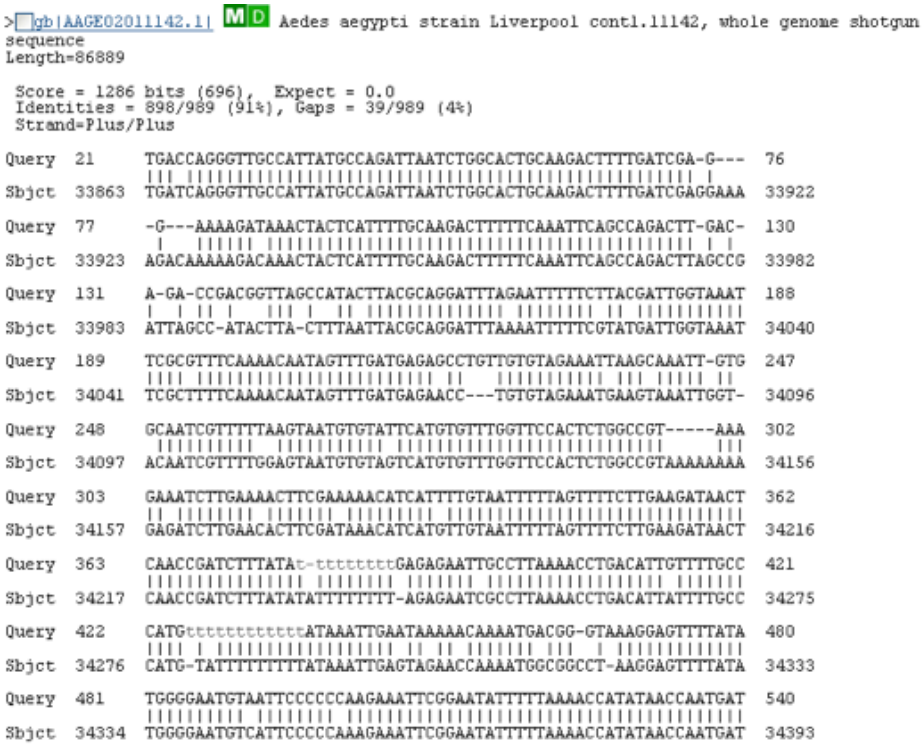
5'-CGGTTGATGGCGGTGCGACAATATAACAATAGTCCAGTTGTTAATTACACC
TATCGTTTGTCTGGTGAGTTGCAAGCTTACTGTCAATGATGATGTCACGCTAC
ACGATAAGTGTTCATCTGCAACAACCAGACAGCTCACCAATCCACCGCGAGGT
GGATCGTGCTGAGTTGTTGAGTTGAAGGAGAGCGAAAGAAGGAAGGCACAAA
AGTCTGTTTGACAGCTCTGCAGAAAGCGCGTGCGCACATACAGAGCGCGTGCG
CACGGAAAGAGCGCGTGCGCACAGAAAGAGCGCCAGTTCGGCTCGATTTGCA
ATGATCGGTTATTATTATTATTTTTTCGCTATTGGATATTCTATGGTTTGGTT
GAGCGATGTTAAAGTTTGTTTTAA

OX4476F 3' sequence

5'-TTAAATACGTTAGTGTCTGATTCTGGGAATGACGATCTGGTGCCATCGTTT
TCGTGTCCTTCATCATGTGAAATTACATTTTTTTTTCAATACATCTTCAAGAA
CACGTTTTTGTGTCGTTCCATCACTTACATCATGCATCATTCAGTCATAACGT
GAATTACGTCCACAGTAATTTTCATTATTTTTAAGAGTGTAGCTAGAAGATTT
ATTATAGAAGTATACTTCTAATTTGTCTCCATTCCAGTCAAGCCCTTTCCTAT
ACAAATTAGAGTGTATGAAACTCGAACAAAAATTTCCTTTAATATAACAACTG
GATAATCCGAATATTTGAACCATCAATTTTTTGTACGATTATTTGAAGTTCCT
AGAGCGTAGTAATTTTGAAGTCAGAGAAAGCAAATAATGTGAATCAAAATACA
GATATAGGTAAACTTTGAAAATTCCCATGCACAAAAGCAACTGTTTTTGTCGT
AAATAAATCAACCTTGCTCGTTAGTTTGTTTGCAAACAATCATTTTCATGGTC
GCGCATGCGTGTTTCCCCCAATTTTCACCCATTTATCATAATGTTTATAAACA
CAACGGTGTGTCGGAAAACATGTTTACCAACAAATCTCCAAAAGCGTCCAAAA
CAACGCCAAAACCG

3.2 BLAST nucleotide alignments

OX4476C 5' sequence



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Score = 1286 bits (696), Expect = 0.0
Identities = 898/989 (91%), Gaps = 39/989 (4%)
Strand=Plus/Plus

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Sbjct  33863  TGATCAGGGTTGCCATTATGCCAGATTAATCTGGCACTGCAAGACTTTTGATCGAGGAAA  33922

Query  77     -G---AAAAGATAAACTACTCATTTTGCAAGACTTTTTCAAATTCAGCCAGACTT-GAC-  130
               |   |||||| ||||||||||||||||||||||||||||||||||||||||||| | |
Sbjct  33923  AGACAAAAAGACAAACTACTCATTTTGCAAGACTTTTTCAAATTCAGCCAGACTTAGCCG  33982

Query  131    A-GA-CCGACGGTTAGCCATACTTACGCAGGATTTAGAATTTTTCTTACGATTGGTAAAT  188
              |  | || |   ||| |  || ||||||||||||||| |||||||| || ||||||||||
Sbjct  33983  ATTAGCC-ATACTTA-CTTTAATTACGCAGGATTTAAAATTTTTCGTATGATTGGTAAAT  34040

Query  189    TCGCGTTTCAAAACAATAGTTTGATGAGAGCCTGTTGTGTAGAAATTAAGCAAATT-GTG  247
              |||| |||||||||||||||||||||| ||   |||||||||| ||| ||||| ||
Sbjct  34041  TCGCTTTTCAAAACAATAGTTTGATGAGAACC---TGTGTAGAAATGAAGTAAATTGGT-  34096

Query  248    GCAATCGTTTTTAAGTAATGTGTATTCATGTGTTTGGTTCCACTCTGGCCGT-----AAA  302
               |||||||||  |||||||||| ||||||||||||||||||||||||||     |||
Sbjct  34097  ACAATCGTTTTGGAGTAATGTGTAGTCATGTGTTTGGTTCCACTCTGGCCGTAAAAAAAA  34156

Query  303    GAAATCTTGAAAACTTCGAAAAACATCATTTTGTAATTTTTAGTTTTCTTGAAGATAACT  362
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Sbjct  34157  GAGATCTTGAACACTTCGATAAACATCATGTTGTAATTTTTAGTTTTCTTGAAGATAACT  34216

Query  363    CAACCGATCTTTATAt-tttttttGAGAGAATTGCCTTAAAACCTGACATTGTTTTGCC  421
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Sbjct  34217  CAACCGATCTTTATATATTTTTTTT-AGAGAATCGCCTTAAAACCTGACATTATTTTGCC  34275

Query  422    CATGttttttttttttATAAATTGAATAAAAACAAAATGACGG-GTAAAGGAGTTTTATA  480
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Sbjct  34276  CATG-TATTTTTTTTTATAAATTGAGTAGAACCAAAATGGCGGCCT-AAGGAGTTTTATA  34333

Query  481    TGGGGAATGTAATTCCCCCCAAGAAATTCGGAATATTTTTAAAACCATATAACCAATGAT  540
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Sbjct  34334  TGGGGAATGTCATTCCCCCAAAGAAATTCGGAATATTTTTAAAACCATATAACCAATGAT  34393
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OX4476 C 3' sequence

>gb|AAGE02008891.1| Aedes aegypti strain Liverpool cont1.8891, whole genome shotgun sequence
Length=9780

Sort alignments for this subject sequence by:
E value Score Percent identity
Query start position Subject start position

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 Score =  533 bits (590),  Expect = 4e-147
 Identities = 331/351 (94%), Gaps = 3/351 (1%)
 Strand=Plus/Minus

Query  178   CTCATGACACTCAGCTTGGTCTTGATAAATAGGTGCGCGTCAAACGCTACGGCTGTTTGC  237
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Sbjct  9673  CTCATGACACTCAGCTTGGTCTTGATAAATAGGTGCGCGTTAAACGCTACGGCTATTTGC  9614

Query  238   CCCCCATACTTTTCTCATCCCCTTCACTTGATTCTGATGGAATTCTTATTGGGTATCATT  297
             |||| ||||||||||||||| || |||||| ||| |||| ||||||||| |||||||||
Sbjct  9613  CCCC-ATACTTTTCTCATCCCATTTACTTGACTCTTATGGGATTCTTATTAGGTATCATT  9555

Query  298   AAAAAATGACGTCCATCATTTGGGGGATGGGGAGGTCTACGAATGTGTCACATGTGCATG  357
              |||||||||||||||||||||| |||||| ||||||||||||||||||||||||| |||
Sbjct  9554  CAAAAATGACGTCCATCATTTGGAGGATGGAGAGGTCTACGAATGTGTCACATGTGTATG  9495

Query  358   TATTTAGTATTGGAAAAATTTCGGAGGACTCATACATAAAACGTAACATAgggggaggg  417
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Sbjct  9494  TATTTAGTATTGGAAAAATTTCGGAGGACTCATACAAAAAACGTGACATA-GGGGGAGGG  9436

Query  418   ggTCAAAAATGTTGAAATTTAGCGTGACATAATTTGTGTACTATCCCTGTGAAGAAATTG  477
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Sbjct  9435  GGTCAAAAATGTTGAAATTAAGCGTGACATAATTTGTGTACTATCCCTGTGAAGAAATTG  9376

Query  478   GCTCTAAAGGCCGAATTCTTCACCTTCGCTTAAGCTCTAAA-CCACGTTTA  527
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Sbjct  9375  GCTCTAAGGGCCGAATTCTTCACCTTCGCTTAAGCTCTAAATCCAGGTTTA  9325


 Score = 82.4 bits (90),  Expect = 2e-11
 Identities = 54/59 (92%), Gaps = 3/59 (5%)
 Strand=Plus/Minus

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Sbjct  9145  TCTAAACCACCTTTACCCATATGTTTAAACTAAGGTTTTG---AAGAAACCGCTTATAA  9090


 Score = 53.6 bits (58),  Expect = 0.011
 Identities = 29/29 (100%), Gaps = 0/29 (0%)
 Strand=Plus/Minus

Query  574   ATGCTGAAGGTGTCTGGGTTCGATTCCCG  602
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Sbjct  9043  ATGCTGAAGGTGTCTGGGTTCGATTCCCG  9015
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OX4476 F 5' sequence

>gb|AAGE02014216.1| Aedes aegypti strain Liverpool cont1.14216, whole genome shotgun sequence
Length=49496

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 Score =  645 bits (349),  Expect = 0.0
 Identities = 357/361 (99%), Gaps = 0/361 (0%)
 Strand=Plus/Plus

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Sbjct  20321  AACAAACTTTAACATCGATCAACCAAACCATAGAATATCCAATAGCGAAAAAATAATAAT  20380

Query  61     AATAACCGATCATTGCAAATCGAGCCGAACTGGCGCTCTTTCTGTGCGCACGCGCTCTTT  120
              |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  20381  AATAACCGATCATTGCAAATCGAGCCGAACTGGCGCTCTTTCCGTGCGCACGCGCTCTTT  20440

Query  121    CCGTGCGCACGCGCTCTGTATGTGCGCACGCGCTTTCTGCAGAGCTGTCAAACAGACTTT  180
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Sbjct  20441  CCGTGCGCACGCGCTCTGTCCGTGCGCACGCGCTTTCTGCAGAGCTGTCAAACAGACTTT  20500

Query  181    TGTGCCTTCCTTCTTTCGCTCTCCTTCAACTCAACAACTCAGCACGATCCACCTCGCGGT  240
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Sbjct  20501  TGTGCCTTCCTTCTTTCGCTCTCCTTCAACTCAACAACTCAGCACGATCCACCTCGCGGT  20560

Query  241    GGATTGGTGAGCTGTCTGGTTGTTGCAGATGAACACTTATCGTGTAGCGTGACATCATCA  300
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Sbjct  20561  GGATTGGTGAGCTGTCTGGTTGTTGCAGATGAACACTTATCGTGTAGCGTGACATCATCA  20620

Query  301    TTGACAGTAAGCTTGCAACTCACCAGACAAACGATAGGTGTAATTAACAACTGGACTATT  360
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Sbjct  20621  TTGACAGTAAGCTTGCAACTCACCAGACAAACGATAGGTGTAATTAACAACTGGACTATT  20680

Query  361    G  361
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Sbjct  20681  G  20681
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OX4476F 3’ sequence

>gb|AAGE02008464.1| Aedes aegypti strain Liverpool cont1.8464, whole genome shotgun sequence
Length=60689

Score = 1149 bits (622), Expect = 0.0
Identities = 634/640 (99%), Gaps = 0/640 (0%)
Strand=Plus/Plus

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Sbjct  4906  GACCATGAAAATGATTGTTTGCAAACAAACTAACGAGCAAGGTTGATTTATTTACGACAA  4965

Query  181   AAACAGTTGCTTTTGTGCATGGGAATTTTCAAAGTTTACCTATATCTGTATTTTGATTCA  240
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Sbjct  4966  AAACAGTTGCTTTTGTGCATGGGAATTTTCAAAGTTTACCTATATCTGTATTTTGATTCA  5025

Query  241   CATTATTTGCTTTCTCTGACTTCAAAATTACTACGCTCTAGGAACTTCAAATAATCGTAC  300
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Sbjct  5026  CATTATTTGCTTTCTCTGACTTCAAAATTACTACGCTCTAGGAACTTCAAATAATCGTAC  5085

Query  301   AAAAAATTGATGGTTCAAATATTCGGATTATCCAGTTGTTATATTAAAGGAAATTTTTGT  360
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Sbjct  5086  AAAAAATTGATGGTTCAAATATTCGGATTATCCAGTTGTTATATTAAAGGAAATTTTTGT  5145

Query  361   TCGAGTTTCATACACTCTAATTTGTATAGGAAAGGGCTTGACTGGAATGGAGACAAATTA  420
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Sbjct  5146  TCGAGTTTCATACACTCTAATTTGTATAGGAAAGGGCTTGACTGGAATGGAGACAAATTA  5205

Query  421   GAAGTATACTTCTATAATAAATCTTCTAGCTACACTCTTAAAAATAATGAAAATTACTGT  480
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Sbjct  5206  GAAGTATACTTCTATAATAAATCTTCTAGCTACACTCTTAAAAATAATGATAATTACTGT  5265

Query  481   GGACGTAATTCACGTTATGACTGAATGATGCATGATGTAAGTGATGGAACGACACAAAAA  540
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Sbjct  5266  GGACGTAATTCACGTCATGACTGAATGATGCATGATGTAAGTGATGGAACGACACAAAAA  5325

Query  541   CGTGTTCTTGAAGATGTATTGaaaaaaaaaTGTAATTTCACATGATGAAGGACACGAAAA  600
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Sbjct  5326  CGTGTTCTTGAAGATATATTGAACAAAAAATGTAATTTCACATGATAAAGGACACGAAAA  5385

Query  601   CGATGGCACCAGATCGTCATTCCCAGAATCAGACACTAAC  640
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Sbjct  5386  CGATGGCACTAGATCGTCATTCCCAGAATCAGACACTAAC  5425
```

3.3 Male mating competitiveness

Table showing the mating competitiveness of OX4476C and OX4476F males compared to background wild-type strain. Results are shown for each experimental replicate.

Mating competitiveness of OX4476F						
		Paternity			Proportion of OX4476F mating	
Rep	Egg Batches	Wild-type	OX4476F	Both	Excluding multiple matings	Including multiple matings
1	9	2	7	0	0.78	0.78
2	10	5	4	1	0.44	0.50
3	9	5	4	0	0.44	0.44
4	8	2	6	1	0.86	0.88
5	9	6	3	0	0.33	0.33
6	10	4	3	3	0.43	0.60
7	7	2	4	1	0.67	0.71
8	10	6	3	1	0.33	0.40
9	10	3	6	1	0.67	0.70
10	10	4	5	1	0.56	0.60
11	5	2	3	0	0.60	0.60
12	5	2	2	1	0.50	0.60
13	7	4	3	0	0.43	0.43
14	6	2	4	0	0.67	0.67
15	7	2	4	1	0.67	0.71
16	4	1	3	0	0.75	0.75
17	5	2	4	0	0.80	0.80
18	9	5	4	0	0.44	0.44
19	8	2	6	0	0.75	0.75
20	8	2	6	0	0.75	0.75

Mating competitiveness of OX4476C						
		Paternity			Proportion of OX4476C mating	
Rep	Egg Batches	Wild-type	OX4476C	Both	Excluding multiple matings	Including multiple matings
1	10	7	3	0	0.30	0.30
2	9	4	5	0	0.56	0.56
3	10	7	3	0	0.30	0.30
4	9	4	4	1	0.50	0.56
5	10	5	4	1	0.44	0.50
6	9	2	7	0	0.78	0.78
7	8	4	4	0	0.50	0.50
8	9	3	5	1	0.63	0.67
9	10	3	3	4	0.50	0.70
10	9	3	5	1	0.63	0.67
11	10	5	3	2	0.38	0.50
12	10	3	7	0	0.70	0.70
13	10	5	5	0	0.50	0.50
14	9	7	1	1	0.13	0.22
15	9	5	2	2	0.29	0.44
16	10	7	3	0	0.30	0.30
17	10	5	3	2	0.38	0.50
18	9	3	6	0	0.67	0.67
19	9	3	5	1	0.63	0.67
20	9	5	2	2	0.29	0.44