Genome Engineering and Gene Drive in the Mosquito *Aedes aegypti*

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Table of Contents

Abstract

Genetic control strategies are a novel method for reducing populations of pest insects such as the yellow fever mosquito *Aedes aegypti*, a major vector of several important arboviral diseases. This thesis describes efforts to develop new tools to engineer the *Ae. aegypti* genome and to better understand existing tools, and furthermore to use these to engineer a gene drive system in *Ae. aegypti*. The piggyBac transposon was found to be extremely stable in the germline of *Ae. aegypti*, and transposons engineered into the germline could not be remobilized with either an endogenous or exogenous source of piggyBac transposase. Conversely, somatic remobilization of piggyBac transposons was found to be readily detectable in the presence of a source of active transposase, the first report of such remobilization in *Ae. aegypti*. Toward new tools for genome engineering, the site-specific integrase from the phage ϕC31 was successfully used to promote exchange between a transgene cassette inserted into the genome of *Ae. aegypti* and a cassette in a plasmid vector, in the first demonstration of recombinase mediated cassette exchange technology in a pest insect species. The integrases from phages ϕRV1 and Bxb1 were not found to be active in the germline of the mosquito. Finally, development of a gene drive system in *Ae. aegypti* using an RNAi-mediated killer-rescue mechanism was attempted. Tissue-specific expression of tTAV-regulated-toxic effectors genes, using the promoter regions of the blood meal induced genes Carboxypeptidase A-1, 30Kb and Vitellogenin A, was possible, but sex-specificity was not achieved. A blood meal inducible lethal phenotype was not possible using the chosen promoters, with expression of the effectors either leading to death in early development or to a sublethal phenotype. RNAi against tTAV fused to the Mnp fragment of the dengue virus' genome was tissue specific, but was found to be highly effective in the fat body suggesting that the Vitellogenin A was the best candidate for the engineering of killer-rescue systems in the mosquito.

Acknowledgements

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This thesis is dedicated to the memory of my mother

Kerra St John

1953 - 2012

"Your life is defined by those who love you and those who do not."

Chapter 1

Introduction

1.1 Mosquito-Borne Diseases

Mosquitoes are responsible for the spread of diseases such as Dengue fever, Yellow fever, Chikungunya, Lymphatic Filariasis and Malaria. Over half the world's population is at risk from these diseases, which cause millions of deaths every year and impose a severe socio-economic burden on the countries to which they are endemic. Climate change, population growth and the evolution of drug and insecticide resistance is causing a worldwide resurgence of many of these mosquito-borne diseases and concurrent increases in their geographical ranges (Abraham et al. 2007, Riehle et al. 2003, Plowe 2009, Hales et al. 2002). Vaccines against these diseases have proved extremely hard to develop and effective low cost vaccines appear to be a long way off (Breman et al. 2007). Because of this, control strategies to combat these diseases have traditionally focused on elimination of the mosquito vectors; however, existing methods have proved costly and largely ineffective in preventing the resurgence of many of these diseases (Gubler 1998, Trape et al. 2002). Global efforts to combat these serious threats to human health are increasingly multinational in nature, focusing on the development of novel methods to combat the health, social and economic problems caused by these diseases (Alilio et al. 2004, Sachs et al. 2002).

1.1.1 Malaria

Malaria is one of the greatest remaining unsolved global health problems. Around 350 to 550 million incidents of the disease occur annually causing one to three million deaths every year, primarily in the developing world and with most of the victims being infants and young children, who have not yet

developed resistance to the disease, in sub-Saharan Africa (Tolle 2009, Snow et al. 2005). The global distribution of malaria is shown in Figure 1.1.

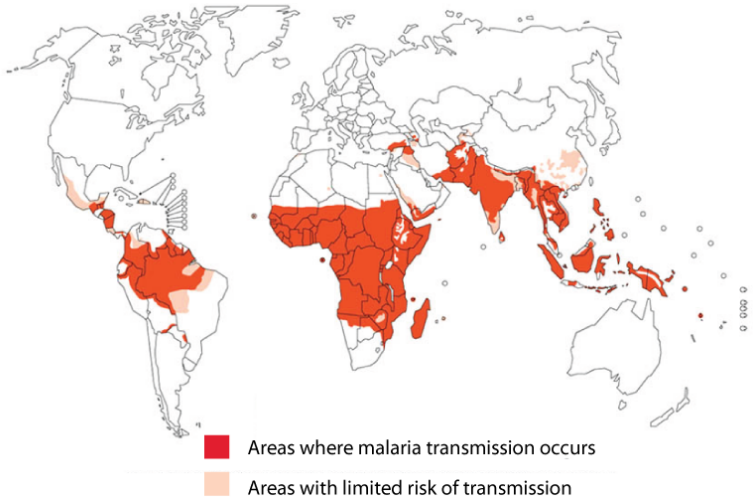


Figure 1.1: Global malaria distribution in 2004. Source WHO, 2004.

Malaria is caused by infection by protozoal parasites of the genus *Plamsmodium* that may infect reptiles, birds or mammals. Human malaria is primarily caused by four *Plamsmodium* species; *P. falciparum, P. vivax, P. ovale* and *P. malariae* with most infections being the result of *P. falciparum* and *P. vivax* (Price et al. 2007). *Plamsmodium* enter the human host and quickly infect hepatocytes within the liver, after an incubation period that ranges from 5 to 14 days, depending on the *Plamsmodium* species, these cells rupture and release plasmodial merozoites into the circulatory system of the host. It is at this stage that the disease becomes symptomatic and when the host can infect the primary vector of human malaria; female mosquitoes of the *Anopheles* genus. Once plasmodial gametocytes are ingested by the mosquito they replicate within the gut eventually developing into a motile form that can colonize the salivary glands and begin the cycle of infection anew once the female takes

another blood meal (Cook et al. 2009). *Anopheles gambiae* is the most important vector of *P. falciparum* and is responsible for the majority of cases in Africa. Controlling the populations of this vector is the primary strategy for combating malaria. The use of pyrethroid-impregnated bednets and indoor residual spraying has greatly decreased the incidence of malaria in some parts of the world by reducing the number of vector competent mosquitoes around human habitation (Chandre et al. 1999). However, control of malaria is becoming increasingly challenging due to the emergence of strains of *Plasmodium* resistant to anti-malaria drugs such as chloroquine, one of the most effective anti-malarials of the last fifty years. The evolution of insecticide resistance in *Anopheles* mosquitoes, in particular pyrethroid resistance, has also become a hindrance to control efforts (Myint et al. 2004, Martinez-Torres et al. 1998). Many attempts have been made to develop an effective vaccine against malaria but the Plasmodium's life cycle and genetic diversity makes this a challenging approach. Within a few years a moderately effective vaccine may exist but vector control will continue to play an important part in combating malaria incidence (Plowe et al. 2011).

1.1.2 Viral diseases

Mosquitoes are the primary vectors of several viral diseases that affect humans. The most important of these are Dengue Fever, Yellow fever, Chikungunya, Japanese Encephalitis and West Nile Virus. These diseases are not as great a public health concern as Malaria but they can still impose a substantial health, social and economic burden in areas to which they are endemic. In most cases the urban cycles of the disease are maintained by sylvatic cycles involving lower

primates and canopy dwelling mosquitoes that act as reservoirs for the viruses (Gubler 2004).

1.1.3 Dengue Fever

Dengue Fever (DENV) is caused by the four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) of an arbovirus from the *Flavivirus* genus in the family *Flaviviridae*. These have positive sense single-stranded RNA genomes approximately 10,500bp in size. Dengue fever is the most important of the mosquito borne viral diseases, with 2-3 billion people worldwide at risk of infection, making it the most wide spread of all arboviral diseases (see Figure 1.2)(Schmidt 2010). Annually 50-100 million people worldwide are infected by Dengue fever with 0.5-1% of cases progressing to the life-threatening hemorrhagic form of the disease (Gubler 2002, Guzman et al. 2003, Watts et al. 1999). Dengue is unique among the arboviruses in that it has adapted entirely to humans and does not require flow of the virus from enzootic cycles to maintain itself in a human population (Gubler 1998).

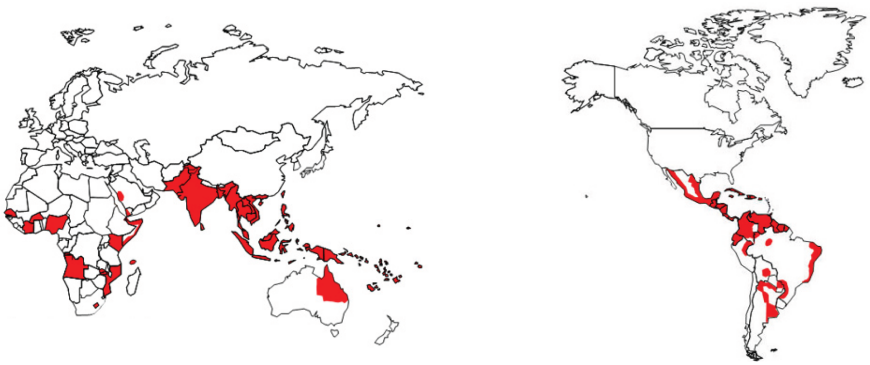


Figure 1.2: Global distribution of Dengue Fever. Image source: CDC, 2005.

The primary vectors of Dengue Fever are the mosquitoes *Aedes aegypti* and *Aedes albopictus*, which are also capable of transmitting other arboviruses such

as Yellow fever and Chikungunya. After feeding on a viremic human the epithelial cells of the mosquito's midgut are infected by the virus. Replication occurs in these cells followed by migration of the virus through the hemocoel resulting in eventual infection of the salivary glands 7 to 10 days after the initial blood meal. After this period the mosquito suffers a lifelong persistent infection that can be spread to humans upon further blood feeding (Salazar et al. 2007, Whitehead et al. 1971).

In recent years the incidence of Dengue fever has been increasing, especially in South America and southeast Asia, and the geographical range of the disease appears to be increasing (Gould et al. 2010). This increase has largely been attributed to the failure of long-term control of the *Ae. aegypti* mosquito (Gubler 1998). This problem is likely to become greater if the worst predictions of climate change occur and global temperature increases result in a wider inhabitable zone for the vector mosquitoes (Hales et al. 2002, Hopp et al. 2001). Development of a vaccine for Dengue Fever has proved challenging due to difficultly of simultaneously immunizing against all four serotypes of the virus for long periods of time. Development of a low cost vaccine to address the dengue burden in the developing world is still some way off, so vector control remains and important tool to prevent outbreaks (Guirakhoo et al. 2001, Guirakhoo et al. 2004, Whitehead et al. 2007).

1.1.4 Yellow Fever

Yellow Fever (YFV) is another *flavivirus* transmitted by mosquitoes that is endemic to the rainforests of Africa and South America (see Figure 1.3). It has a positive sense single-stranded RNA genome approximately 11,000bp in size.

Infection with the virus can lead to symptoms varying from a light fever to a serious illness that develops in around 15% of cases. (Robertson et al. 1996) The virus has two major cycles: sylvatic and urban. In the sylvatic cycle the virus is transmitted by a variety of mosquito species between lower primates; however, this cycle can result in incidental human infection, which can lead to development of an urban cycle where the virus is transmitted between humans by anthropophilic mosquito species, such as *Ae. aegypti*, resulting in an outbreak of Yellow Fever (Barrett et al. 2007).

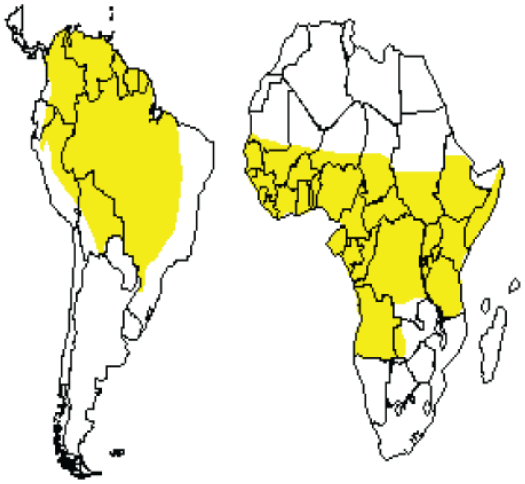


Figure 1.3: Global distribution of Yellow Fever. Image source: CDC 1996.

The vector responsible for the urban cycle is *Ae. aegypti* with the mechanism of transmission being identical to that of Dengue Fever and other *flaviviruses*. A combination of an effective vaccine – the 17D vaccine – and vector control using DDT led to near elimination by the 1970s of Yellow Fever in many countries where it was once endemic. However recent years have seen a resurgence of the disease; with significant outbreaks in Bolivia, Peru, Ecuador, Brazil and Sudan; as vector control programmes have declined and vaccination rates have

decreased (Gubler 2004, Robertson et al. 1996, Gould et al. 2008). As the urbanization of populations in endemic countries increases it is feared that Yellow Fever may once again become a major health concern, returning to areas where it was once eradicated (Barrett et al. 2007).

1.1.5. Chikungunya

Chikungunya (CHIKV) is an arboviral disease with many of the same symptoms as Dengue Fever. Unlike Dengue Fever it can cause severe arthritis that may persist for several years post-infection. The virus that causes it belongs to the *Alphavirus* genus of arboviruses and has a 12,000bp single-stranded RNA genome. Its biology is less well characterized than Dengue's, with its reservoirs thought to consist of a variety of vertebrates. Compared to dengue it is relatively uncommon but like many of the other viral diseases transmitted by *Aedes* mosquitoes it is undergoing a resurgence, with recent outbreaks in India and Reunion island infecting more than a million people (Pialoux et al. 2007, Borgherini et al. 2007). It is endemic to parts of Africa and Asia and recently has become established in Southern Europe (Rezza et al. 2007). No commercial vaccines are currently available to protect against Chikungunya, meaning that it remains a serious health threat in many areas. Recently, effective vaccines have been developed in nonhuman primates but these have yet to be shown to be efficacious in humans (Akahata et al. 2010). *A. albopictus* is native to many temperate developed countries, including some in Southern Europe, fueling fears that Chikungunya may be imported by travelers into these regions resulting in outbreaks of the virsus (Krastinova et al. 2006).

1.1.6. Japanese encephalitis and West Nile virus

Japanese encephalitis (JEV) and West Nile virus (WNV) are diseases also caused by arboviruses of the *Flavivirus* genus. They both have positive-sense single strand RNA genomes 11-12,000bp in size. Japenese encephalitis is endemic to southeastern Asia, Papua New Guinea and northern Australia, while West Nile virus is found in Africa, southern Europe, India, the Middle East and was recently introduced into North America (Mackenzie et al. 2004). Both viruses have avian reservoirs with transmission to humans mediated by *Culex*. spp. mosquitoes. Infection with either virus is often asymptomatic but 20-30% of cases may progress onto serious viral encephalitis that can result in death or long term neurological damage. No approved vaccine exists for West Nile virus though recently clinical trials of a low cost Japanese encephalitis vaccine have been effective (Nakano 2011, Monath et al. 2006). Recent emergence of these diseases are thought to be due to changes in land usage and bird migratory patterns, both of which are making Japanese encephalitis (JEV) and West Nile virus (WNV) an increasing health concern (Mackenzie et al. 2004).

1.1.7 Lymphatic filariasis

Lymphatic filariasis is caused by infection with the filarial nematode worms *Wuchereria bancrofti, Brugia malayi* and *Brugia timori*. The worms are spread by mosquitoes of several genera and are endemic to most of Africa and Southern Asia as well as parts of Latin America. The disease can produce elephantitis resulting in life long disfigurement if not treated. The use of antifilarial drugs has been largely successful in reducing incidents of Lymphatic filariasis (Molyneux et al. 2003). Eradication of filarial diseases may prove

possible in the coming years aided largely by the fact there are no reservoir species for some of the worms, meaning that elimination from humans with effective drug interventions will result in their extinction (Ottesen et al. 2008).

1.2 The Yellow Fever Mosquito, *Aedes aegypti*

1.2.1 Systematics and morphology

Aedes aegypti (Linnaeus), commonly called the yellow fever mosquito, is a medium sized mosquito with a body approximately 0.4 to 0.7cm in length. It is distinctively marked with a lyre shaped pattern of white scales upon the dorsal surface of the thorax, with white scales continuing onto the abdominal tergites, as shown in Figure 1.4.

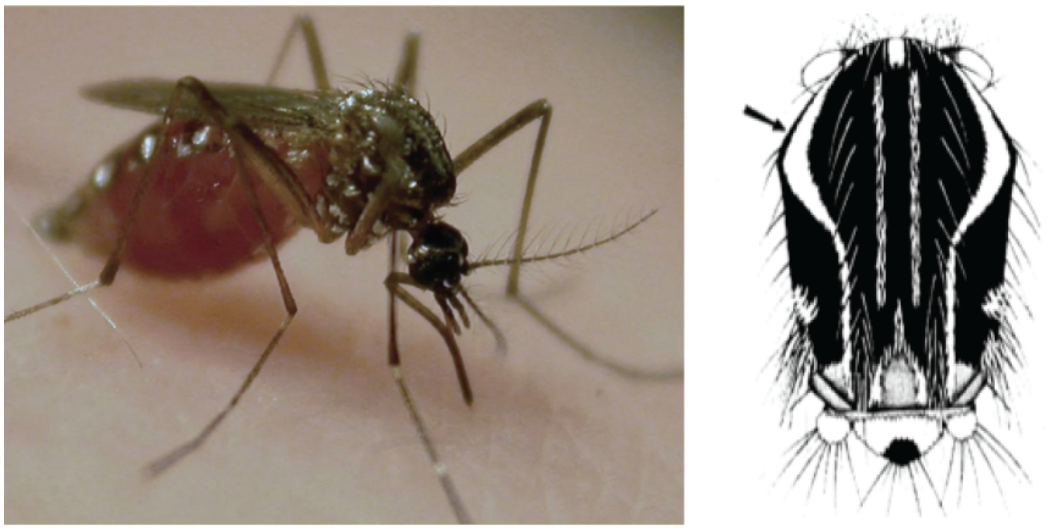


Figure 1.4: The yellow fever mosquito, *Aedes aegypti*, taking a blood meal and the characteristic lyre pattern of white scales on the dorsal surface of the thorax. Image sources: Oxitec Ltd. and Huang (1981).

Of African origin this species has been spread around the world and is now present in virtually all of the tropics and other regions between latitudes 40°N and 40°S. It has also proved adaptable to more arid environments and populations exist in the drier regions of Madagascar (Fontenille et al. 1989).

Mattingly (1957) proposed the existence of two subspecies of *Ae. aegypti*; *Ae. aegypti aegypti*, the globally distributed form, and *Ae. aegypti formosus*, a darkly colored form, that lacks pale scales on its first abdominal tergite, which is confined to sub-Saharan Africa and is associated with sylvan rather than urban environments (Tabachnick et al. 1979, Mattingly 1957). Mattingly (1957) supposed that the domestic *Ae. aegypti aegypti* subspecies arose from a sylvan *Ae. aegypti* formosus population in West African forests, and recent population genetic analyses of gene flow have confirmed this theory (Sylla et al. 2009).

1.2.2 Genome and Genetics

The draft genome sequence of *Ae. aegypti* was published in 2007 and is around 1.38 Gb in size, with approximately 16,000 protein-coding genes (Nene et al. 2007). Of the protein coding genes identified by Nene et al. (2007) 67% had an ortholog with genes identified in the mosquito *An. gambiae* compared to 58% with an ortholog in the fruit fly *D. melanogaster*. Analysis of the protein domains transcribed by the genes indicated an increase in the number of genes associated, among others, with olfactory systems, the insect cuticle and detoxification pathways, linked to increased resistance to insecticides, as compared to *An. gambiae* (Nene et al. 2007, Strode et al. 2008). The genome of *Ae. aegypti* is approximately 5 times larger than the published genome of *An. gambiae*, the two species having diverged around 150 million years ago (Holt et al. 2002). This increase in size is due in large part to the high number of transposable elements found in the intergenic regions and introns of the genome. These elements account for approximately 47% of the genome's size, with over one thousand species of transposable elements having been identified

within it, the majority of which have been inactivated through the loss of their open reading frames. Miniature inverted repeat transposable elements (MITEs) are the most common of the elements, comprising 16% of the genome, though a single non-long terminal repeat retrotransposon element accounts for around 3% of genome (Nene et al. 2007). This high number of duplicated transposable elements results in the genome's short interspersed repeat pattern, where repetitive regions are more closely grouped than in other Dipetra such as *D. melanogaster* (Warren et al. 1991).

Both *An. gambiae* and *Ae. aegypti* have three pairs of chromosomes that show a high level of synteny between the two species, with whole-arm or near-whole-arm conservation being observed (Severson et al. 2003). However, *Ae. aegypti* do not possess heteromorphic sex chromosomes, differentiating them from *Anopheline* mosquitoes. Rather, sex is determined by the dominant male determining allele, M, at an autosomal locus. The exact mechanism of sex-determination in *Ae. aegypti* has yet to be identified and although orthologs of the genes involved in sex-determination in other Diptera, such as the *D. melanogaster* doublesex (dsx) double-switch gene, have been identified, their role in sex-determination in the mosquito remains unclear (Salvemini et al. 2011).

1.2.3 Behavior and Life History

Ae. aegypti is closely associated with humans and human habitation and rarely breeds outside urban environments. While males will attempt to mate with multiple females, females of *Ae. aegypti* are monogamous and are not receptive to further sperm uptake after an initial mating (Craig 1967). Once mated

females undergo several gonotrophic cycles, all of which require blood feeding to obtain the necessary protein for egg production (Briegel et al. 2001).

Ae. aegypti is strongly anthropophilic, although populations of the *Ae. aegypti formosus* subspecies have been found to primarily feed on other mammal species (Reeves 1965). Females rarely feed on sources of sugar in the wild, preferring to take multiple blood meals to maintain their energy reserves (Scott et al. 2000). This preference for human blood may to be due to the blood meal's biochemistry, which provides *Ae. aegypti* females with a source of energy that is not otherwise easily attainable in urban environments. This also promotes a continued close association of the mosquito with humans, which has important ramifications for the mosquito as a disease vector (Harrington et al. 2001).

After a blood meal development of embryos take between 3 and 5 days (Christophers 1960). In natural environments eggs are deposited in places such tree-holes or leaf axils. In urban environments sites such as pots, drainage ditches or tyres are preferred (Crovello et al. 1972). Upon hatching the progeny take at least 8 to 9 days to develop from larvae to adults. This development time is strongly dependant on both temperature and food availability (Tun-Lin et al. 2000). Competition for food resources is particularly important during the larval stage and survival of the early instars is strongly density dependant, with larval competition for limited food resources within the habitat limiting the number of adults that will successfully emerge (Southwood et al. 1972). After emergence males and females will begin mating immediately, although sperm transferral does not occur until the adult female is 1 to 2 days old (Lea 1968).

This is due to females being refractory to mating during this period and the necessity for inversion of the male genitalia that begins after emergence and takes around 24 hours. This refractory period is thought to encourage dispersal from the breeding site, thus limiting inbreeding (Hartberg 1971). Mating of *Ae. aegypti* does not involve swarming but occurs primarily around the host, both before and after blood feeding, with males detecting females through a combination of pheromones and sound signals of a specific frequency. After a female enters or leaves the vicinity of the host, a male will initiate mating by grasping the female and orientating itself venter-to-venter with the female (Jones et al. 1965). Mating takes approximately 10 seconds with the success of the mating being strongly dependent on male size and age. Transferral of accessory proteins during sperm transfer is thought to be important for maintaining monandry in females (Ponlawat et al. 2009).

1.2.4 *Ae. aegypti* as an invasive species

It is assumed that the worldwide spread of *Ae. aegypti* occurred through the accidental transport of eggs of the West African *Ae. aegypti aegypti* subspecies during the slave trade between the New World and Africa that took place in the 15th to 19th centuries. Eggs of *Ae. aegypti* are extremely tolerant of desiccation and may survive several months in relatively dry conditions between the time of laying and hatching (Sota et al. 1992). This has allowed eggs to be readily transported during human movement. Genetic studies have shown that urban colonization by African *Ae. aegypti* has occurred independently at numerous times over the past few centuries, suggesting that the mosquitoes behavioral flexibility makes it adept at invasion (Brown et al. 2011). The mosquito's

adaptation for man-made breeding sites has also aided its invasion by allowing it to avoid competition, filling an empty ecological urban niche. Where competition does occur – for example with another invasive *Aedine* mosquito, *A. albopictus* – *Ae. aegypti* populations can be greatly decreased or even locally driven to extinction due to the effects of interspecific larval competition (Juliano et al. 2004).

1.2.5 *Ae. aegypti* as a vector of disease

As discussed in section 1.1 *Ae. aegypti* is a vector of several medically significant arboviral diseases, most notably Dengue Fever for which it is the primary vector (see Figure 1.5). It is not a vector for human malaria, though it is an important model for malarial studies since it can transmit the avian malaria parasite, *Plasmodium gallinaceum* (Kilama et al. 1969).

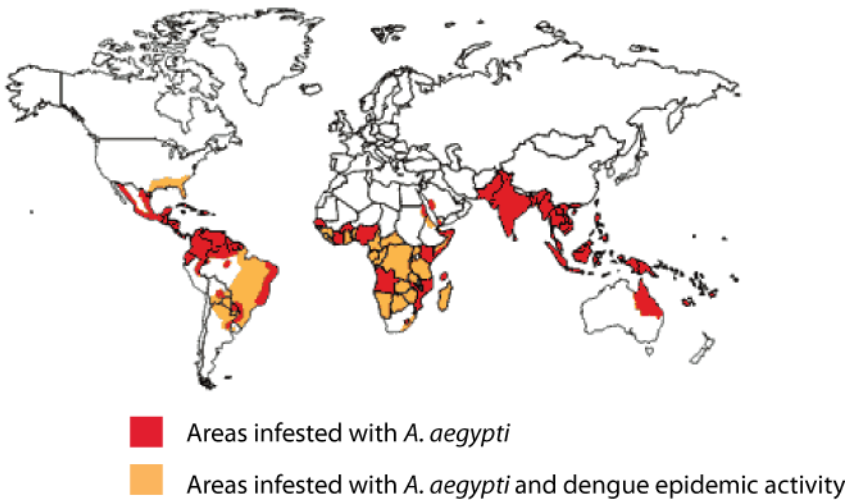


Figure 1.5: Global geographic distribution of *Ae. aegypti* and Dengue Fever. Image source: Gubler et al. (2003)

Three key factors are important when determining if the virus can successfully infect the mosquito and then be transmitted onto a human. These three

barriers to propagation of the virus are the midgut infection barrier (MIB), the midgut escape barrier (MEB) and the salivary gland escape barrier (SEB). To initially infect the mosquito, after feeding on a viremic host, the virus must interact with receptors on midgut epithelial cells and penetrate the cells. Any mechanism that hinders the virus at this early stage is a MIB. After infection of the midgut replicated virons must escape into the hemocele and infect secondary organs, including the salivary glands. Anything that prevents this from occurring, such as the mosquito's immune response, is a MEB. Finally to complete the cycle the virus must overcome the SEB to secrete virons into the lumen for injection into a secondary host (Black et al. 2002).

The vector competence –the intrinsic permissiveness of a vector to infection, replication, and transmission– of *Ae. aegypti* is extremely variable between populations, as it is for many other vector mosquito species (Black et al. 2002). Vector competence of *Ae. aegypti* for the dengue virus was found to vary between 24 and 83% in North American populations because of the variance in the extent of MIB and MEB in the populations (Bennett et al. 2002). Similarly the vector competence of *Ae. aegypti* for yellow fever varies greatly between populations from different regions of the world, with Caribbean populations being more susceptible than Asian populations to infection (Tabachnick et al. 1985). Vector competence is closely correlated with the genetic grouping of *Ae. aegypti* populations, with studies suggesting that susceptibility to arboviruses is regulated by genes at multiple loci acting in concert to determine how effective barriers to propagation are. Barriers to gene flow between sub-populations can

therefore produce extreme variances in vector competence (Bosio et al. 1998). The vector competence of the mosquito is also dependant on the peculiarities of the strain of virus. Different strains of the DENV-2 virus have been shown to infect *Ae. aegypti* with different efficiencies (Armstrong et al. 2001, Lozano-Fuentes et al. 2009). Competing parasitic organisms may also affect the vector competence of *Ae. aegypti*. The maternally inherited symbiotic bacteria *Wolbachia* induces upregulation of the mosquito's immune responses by production of reactive oxygen species resulting in greater resistance, or even refractoriness, to arbovirus or *Plasmodium* infection (Pan et al. 2011, Moreira et al. 2009). In contrast, infection with Filariasis may dramatically increase vector competence by eliminating the MEB in *Ae. aegypti* through physical damage to the midgut (Vaughan et al. 2009).

Environmental factors also affect the vector competence of *Ae. aegypti*, which may result in differences in infectivity even within populations of the mosquito. Temperature and humidity both have an impact on the time interval between ingestion of a viremic blood meal and oral transmission of virus, as well the extent of viral replication, and may play a role in the seasonal variation in outbreaks of some arboviruses (Watts et al. 1987, Thu et al. 1998). The larval diet of developing *Aedine* mosquitoes has also been shown to affect vector competence, and small adults have been reported to be more susceptible to infection due to reduced barriers to infection resulting from poor nutrition at the larval stage (Nasci et al. 1994, Grimstad et al. 1991).

1.2.6 Immune response of *Ae. aegypti*

The immune response of *Ae. aegypti* to infection with an arbovirus is still not well understood. However, high-throughput gene expression technology has increasingly been used to study the effect of infection in the mosquito and elucidate the mechanisms behind the mosquito's immune response. Both RNA interference (RNAi) and immune specific pathways are thought to contribute toward viral defense in *Ae. aegypti*. The Toll pathway was first identified in *D. melanogaster* where it upregulates the expression of several antimicrobial peptides that are released into the haemolymph in response to infection by microorganisms (Hoffmann 2003). This pathway is activated in *Ae. aegypti* upon infection with DENV-2, resulting in expression of anti-microbial peptides Cecropin A and Defensin A. This suggests that the Toll pathway is part of the mosquitoes immune response to viral infection and that the anti-microbial compounds may also play a role in limiting replication of an infecting virus (Pan et al. 2011, Xi et al. 2008, Ramirez et al. 2010). The Janus kinase signal transducer and activator of transcription (JAK-STAT) pathways also seems to be involved in the *Ae. aegypti* viral immune response, being upregulated in the midgut during the early and late stages of infection with the Dengue virus (Souza-Neto et al. 2009).

RNAi is the most important of the *Ae. aegypti* immune responses to infection with an arbovirus (Blair 2011). This immune response relies upon the recognition of viral RNA by the mosquito's RNAi machinery. Viral RNA is then processed into 21-24bp small interfering RNAs (siRNAs) by the ribonuclease Dicer. These siRNAs are then used as templates by the mosquito RNAi

machinery to guide sequence specific silencing of viral mRNAs by endoribonucleic cleavage mediated by an Argonaute protein complex called the RNA-induced silencing complex (RISC) as shown in Figure 1.6 (Ding 2010).

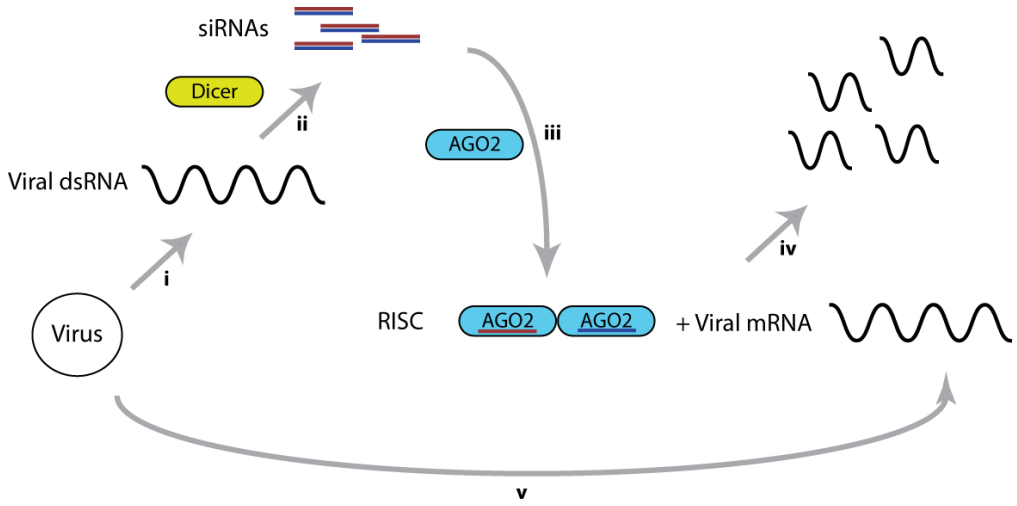


Figure 1.6: The theorized mechanism of the mosquitoes RNAi immune response. Viral replication (i) in mosquito cells produces viral dsRNAs that are processed by Dicer (ii) into 21-14bp siRNA duplexes. These complex (iii) to dimers of the Argonaute-2 protein (AGO2) forming the RIS complex. This mediates (iv) sequence specific degradation of viral mRNAs produced during the viral replication process (v).

Production of virus specific siRNAs appears to occur via a double-stranded RNA (dsRNA) intermediate produced during the replication cycle of positive-strand RNA viruses such as Dengue Fever (Aliyari et al. 2008). This is supported by the observations that replication of alphaviruses and flaviviruses in *Ae. aegypti* can be inhibited by activating the RNAi response with injection of virus derived dsRNAs before inoculation with the virus; and that accumulation of viral dsRNAs and derived siRNAs occurs in cells after infection (Campbell et al. 2008). Shutting down the RNAi response in *Ae. aegypti* by silencing of key genes in the RNAi pathway results in increased replication of the dengue virus confirming that this pathway plays a large role in reducing vector competence

(Sanchez-Vargas et al. 2009). Though, from the mosquito's point-of-view limiting the effects on fitness caused by high levels of viral replication is the primary intent.

1.2.7 Strategies for the control of *Ae. aegypti*

As an important vector of arboviruses, *Ae. aegypti* has been the subject of numerous control programs. During the first half of the 20th century these were largely successful in reducing many populations of *Ae. aegypti* around the globe, with incidents of Yellow Fever being virtually eliminated from Central and South America. However, many social and political factors have resulted in the decline of these control programs and a resurgence in the populations of *Ae. aegypti* and the reemergence of the diseases they transmit (Gubler 1989). Control programs targeting *Ae. aegypti* are often said to rely on three key aspects: sustainability of the program over time, continuous surveillance of mosquito populations, and effective tools for controlling mosquito numbers (Morrison et al. 2008). Maintenance and monitoring of mosquito control programs is complex and expensive and is often challenging to achieve in undeveloped nations where control program are most needed. Tools for controlling *Ae. aegypti* include insecticide treated materials, lethal ovitraps, space repellants and larvicides (Gubler et al. 1994, Gratz 1991) However, these a have proved largely ineffective at preventing the resurgence in *Ae. aegypti* populations and combined with the increase in *Ae. aegypti* resistance to commonly used insecticides it is clear that new methods of control are needed to combat the threat posed by this mosquito (Zaim et al. 2002).

1.2.8 Novel control strategies

A variety of novel control strategies for *Ae. aegypti* have been proposed. The development of novel chemical agents could overcome existing insecticide resistance however, such development is not currently seen as commercially viable, which has resulted in few new candidates being brought to market (Zaim et al. 2002). The sterile insect technique (SIT) –the release of sterile male insects to prevent mating of fertile wild females with wild males– has also been proposed as a method to control *Ae. aegypti*. The use of sterile males would be particularly attractive as it is extremely species specific and the mosquitoes own mate-seeking behavior can target populations that are hard to reach with traditional control techniques. Initial attempts to use SIT to control mosquitoes in India were not successful due a combination of failures in implementation and production of the sterile males (World Health Organization 1976, Pates et al. 2005). In particular, techniques to produce sterile males, such as the creation of strains carrying chromosomal aberrations using classical genetics or radiation sterilization, may severely impact the fitness of the males, limiting their efficacy (Alphey 2002). Most recently, genetic engineering of the mosquito genome has been proposed as a method for controlling *Ae. aegypti* and advances over the last two decades in insect transgenesis has made this approach possible (Collins et al. 1996).

1.3 Genetic Control of Mosquito Vectors

1.3.1 The development of mosquito transgenesis

Mosquitoes are a particularly attractive candidate for genetic control approaches due to the failings of traditional control methods such as insecticides, larval habitat destruction or the sterile insect technique. (Curtis

1968, Knols et al. 2007). This has resulted in a great deal of research into the potential for genetic engineering of important vector mosquitoes, particularly *Ae. aegypti* and *An. gambiae*. The development of exogenous DNA delivery techniques involving the microinjection of engineered DNA into preblastoderm embryos, to transform the developing pole cells, has allowed germline transformation of mosquitoes to become commonplace (Handler 2000). More recently, publication of the genome sequences of *Ae. aegypti* and *An. gambiae* has greatly increased the understanding of the biology of both species and has allowed for increasingly precise engineering of their genomes (Nene et al. 2007, Holt et al. 2002).

Since its initial germline transformation with the mariner transposon the *Ae. aegypti* mosquito has been engineered in a wide variety of ways that include expression of anti-malaria effector molecules, RNAi systems to induce refractoriness to the DENV-2 virus and repressible dominant-lethal systems to sex-specifically kill female mosquitoes (Coates et al. 1998, Kokoza et al. 2000, Franz et al. 2006, Fu et al. 2010). Such engineered mosquitoes are on the cusp of being deployed into the field and recently the first field trial of a genetically modified strain of *Ae. aegypti* occurred in the Cayman islands (Harris et al. 2011).

1.3.2 Strategies for the genetic control of mosquitoes

The control strategies that employ transgenic mosquitoes generally have one of two major goals; either the total replacement of indigenous populations with strains refractory to disease transmission, or the local suppression or elimination of targeted populations around human habitations (Alphey et al.

2002, Scott et al. 2002). With advances in molecular biology, the development of transgenic strains for both approaches has become a reality.

The use of engineered strains for local suppression is the most developed of the strategies. A genetically engineered strain of *Ae. aegypti* has been created that displays repressible late-acting dominant lethality, resulting in the death of individuals not raised in the presence of a tetracycline food additive (Phuc et al. 2007). Recently this has been improved upon by making the dominant lethality sex-specific. This resulted in an engineered strain in which the female mosquitoes are flightless and so functionally sterile (Fu et al. 2010). Such engineered mosquitoes can be used in a control strategy named Release of Insects carrying a Dominant Lethal (RIDL), an improvement of the traditional SIT technique that involves the release of male mosquitoes that are homozygous for a dominant lethal gene that is expressed in the absence of an dietary antidote. These males mate with wild females, passing the lethal gene on to their offspring in whom it is expressed due to the absence of the antidote, resulting in the death of the progeny (Alphey 2002). The use of genetically engineered mosquitoes overcomes many of the problems of traditional SIT, such as sterilisation reliability and male fitness. Engineered males have be found to be sufficiently competitive for use in suppression programs and the first uses of this technique in the field have been recently reported (Harris et al. 2011, Bargielowski et al. 2011).

The development of strains for population replacement has been more technically challenging but is increasing rapidly. Transgenic mosquitoes that

are highly or completely resistant to infection by Dengue Fever or Malaria have already been created (Ito et al. 2002, Sanchez-Vargas et al. 2004). Despite these successes, a key aspect of the technology remains undeveloped; the gene drive systems with which engineered refractoriness may be introgressed into wild populations. These are discussed in detail in section 1.4.

Developing strains of mosquitoes for use in any genetic control programme relies upon several factors, including: the development of gene vectors that can integrate into the mosquito genome efficiently; and the identification of effector genes that can be used in population suppression or population replacement strategies (Alphey et al. 2002).

1.3.3 Transposons for *Ae. aegypti* germline transformation

Insect germ-line transformation using nonautonomous transposons such as *Mos1*, *Hermes*, *Minos* and *piggyBac* has proved to be an efficient and practical method for integrating molecular constructs into the genomes of pest insects (Handler 2000). These transposons belong to the class II inverted terminal repeat-type transposable elements that transpose using a cut-and-paste mechanism via a DNA-mediated intermediate. This is accomplished by recognition of the inverted terminal repeats (ITRs) that flank the transposon by the appropriate transposase enzyme, which then cuts both strands of DNA at the termini of the ITRs (Wicker et al. 2007).

Three of these transposon-based vectors have been successfully used to mediate germline transformation in *Ae. aegypti*. Transformation of *Ae. aegypti* using the *MosI* transposon was first demonstrated by Coates et al. (1998) who

reported a transformation efficiency of about 4%. Similarly, *Hermes* was employed by Jasinskiene et al. (1998), who demonstrated an efficiency of around 8% – although this transposon displays non-canonical integration in *Ae. aegypti*, with some insertions failing to occur through the expected precise cut-and-paste mechanism (Jasinskiene et al. 2000). The most efficient of these transposon systems is *piggyBac*, which was first used in *Ae. aegypti* by Lobo et al. (2002) and has since become the most regularly used transposon for transformation of *Ae. aegypti* and many other insect species (Abraham et al. 2007).

1.3.4 Post-integration stability of transposons in *Ae. aegypti*

The post-integration stability of a transposon has important ramifications for the transposon's use as a gene vector. A transposon can theoretically remobilise in the presence of a suitable source of transposase, which could lead to the loss of an inserted transgene or movement within the genome. The post-integration stability of a variety of transposons has been tested in mosquitoes. The *Minos* transposon's stability in *An. stephensi* was investigated using homozygous 'jumpstarter' strains expressing the *Minos* transposase. These were crossed to lines carrying a X-linked *Minos* transposon and the resulting offspring were analysed for evidence of remobilisation using transposable element display, a DNA fingerprinting method. No remobilisation events were detected in the germline (based on 35,000 G1 progeny), however remobilisation events in somatic cells were consistently recovered in ~40% of screened individuals (Scali et al. 2007). Similar experiments with the *MosI* mariner transposon in *Ae. aegypti* identified a single instance of germline remobilisation

in 14000 screened G1 offspring, with somatic movement found to be more common (Wilson et al. 2003). An autonomous *Hermes* transposon appeared not to remobilize in the germline of *Ae. aegypti* but somatic movement was consistently detected (O'Brochta et al. 2003). More recently, using a combination of non-autonomous *Hermes* transposons and helper lines expressing the transposase, it has been shown that germline remobilisation does occur. This was observed in less than 1% of progeny, suggesting that the rate of remobilisation is very low, and furthermore occurred via an aberrant mechanism (Smith et al. 2011). It is notable that the rate of remobilisation of these transposons is host-specific: *MosI* displays a high frequency of remobilisation in *D. mauritiana*, from which it was first isolated, and *Minos* can be efficiently remobilised in the germline of *D. melanogaster* (Metaxakis et al. 2005).

1.3.5 The *piggyBac* transposable element

The *piggyBac* transposon, first identified in the genome of a baculovirus isolated from cell lines of the cabbage looper moth *Trichoplusia ni*, is a 2472bp class II transposable element that encodes a 594 amino acid transposase (Cary et al. 1989). It demonstrates precise cut-and–paste integration and excision; inserting exclusively into TTAA tetranucleotide sequences and consistently restoring the donor site to its pre-transposon state (Mitra et al. 2008). *PiggyBac* is classified as member of subclass 1 of the class II transposable elements (those that transpose via a DNA intermediate), along with other transposons such as members of the *Tc1–Mariner* superfamily of transposons, which are also able to mediate transposition in insects (Wicker et al. 2007). In its preference for TTAA

sites, *piggyBac* transposase shares little similarity with other class II transposons and the structure of the transposase's catalytic domain has not yet been well categorized; however, the aspartate residues at positions D268, D346, and D447 of the catalytic domain (the so called DDD structure) are thought to be vital for recruiting the metal ions used to promote double stranded DNA breakage (Keith et al. 2008).

Structurally, *piggyBac* transposons are flanked by inverted terminal repeat domains (ITRs) with the 5' and 3' ITRs consisting of 19bp and 13bp inverted repeat sequences separated by 3 and 30bp respectively (Fraser et al. 1996).

PiggyBac transposition is accomplished when the transposase excises the transposon via DNA 3' nicking at the flanking ends of the ITR. The free 3'-hydroxyl groups then attack the complementary strand forming a hairpin intermediate. This intermediate is resolved by the transposase into the mobile transposon with 5'-TTAA overhangs. Finally the transposase promotes nucleophilic attack by the 3' –hydroxyl groups at the transposon ends on the 5' end of the TTAA target site, resulting in reinsertion and complete transposition (See Figure 1.7). This direct nucleophilic attack on the target DNA makes *piggyBac* mechanistically similar to the DDD/DDE recombinases, though it shares little sequence homology with them (Mitra et al. 2008, Keith et al. 2008).

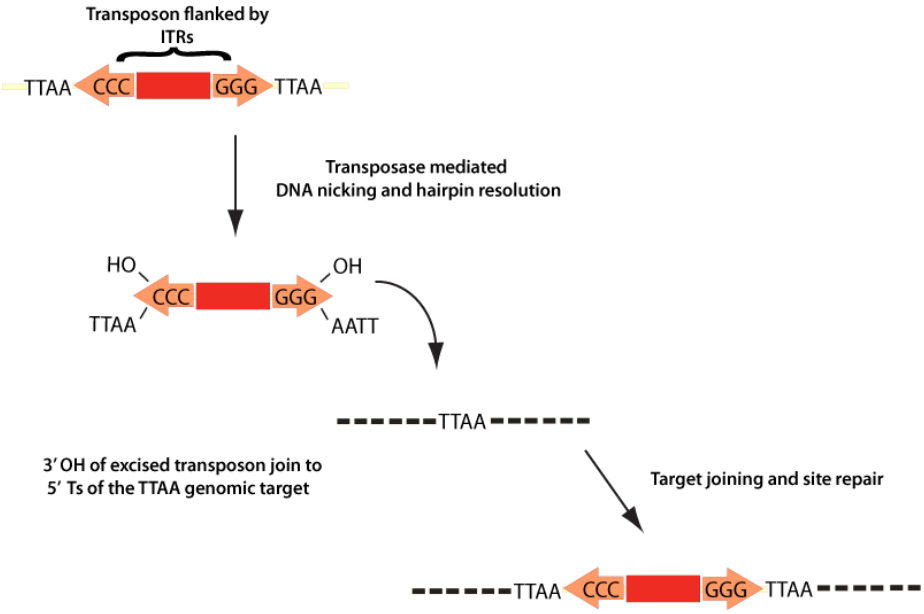


Figure 1.7: The mechanism of *piggyBac* cut-and-paste transposition into a genomic TTAA site. Nicking at the 3' transposon end initiates *piggyBac* transposition liberating the 3'OH. Attack of the 3'OH on the complementary strand of the transposon then creates a hairpin intermediate. The transposase then resolves the hairpin linearising the transposon. The nucleophilic 3'OH then attacks the TTAA target site resulting in integration and duplication of the TTAA site.

Nonautonomous *piggyBac* transposons –those that do not encode the transposase gene– have been regularly used to accomplish germline transformation of *Aedine* and *Anopheline* mosquitoes. These include: *An. gambiae* (Grossman et al. 2001), *Anopheles albimanus* (Perera et al. 2002), *Anopheles stephensi* (Nolan et al. 2002), *Ae. aegypti* (Lobo et al. 2002), *Aedes fluviatilis* (Rodrigues et al. 2006) and *Ae. albopictus* (Labbé et al. 2010). Transformation in all of these species is achieved by microinjection of preblastoderm embryos with a donor plasmid carrying the *piggyBac* transposon and a source of the *piggyBac* transposase (Morris 1997, Adelman et al. 2004).

When used as an artificial gene vector for germline transformation, internal identification sequences (within the ITRs) at each end of the transposon are

required for efficient transposition. (Li et al. 2005). Like other transposons *piggyBac* transposition efficiency is influenced by the amount of internal sequence within the ITRs, decreasing at an approximately logarithmic rate as a function of the transgene size (Handler et al. 1999, Karsi et al. 2001). This limits the size of genetic constructs that may be inserted efficiently using this transposon to around 14Kb (Ding et al. 2005a). *PiggyBac* appears to insert preferentially into certain sites in eukaryotic genomes, with a particular preference for transposing into regions surrounding transcriptional start sites and into long terminal repeat elements (Thibault et al. 2004a, Wilson et al. 2007).

PiggyBac transposons have proved to be particularly effective gene vectors for the engineering of *Ae. aegypti* and are capable of inserting a wide variety of transgenic constructs. From simple marker gene bearing constructs (Lobo et al. 2002), to constructs carrying sites for further integration (Nimmo et al. 2006) as well as constructs with more complex regulated gene expression systems (Phuc et al. 2007). *PiggyBac* transposons could potentially be useful tools for the creation of *Ae. aegypti* strains that are refractory to Dengue Fever and which could be used in population replacement strategies (Olson et al. 2002, Olson et al. 2006). However, the suitability of *piggyBac* for use in a strain intended for release into the wild must be evaluated based its post-integration behaviour in the genome.

1.3.6 Site-specific recombination systems for use in *Ae. aegypti*

Transposons such as *piggyBac* integrate randomly into the host genome at their preferred target sites. The positional effects of these random insertions may

complicate analysis of the insertion or prevent correct function of an inserted transgene due to neighbouring enhancing or silencing elements (Wilson et al. 1990, Bischof et al. 2007). Insertion into specific genomic sites allows the problem of randomness to be overcome. Systems based on the Cre-loxP and FLP-FRT recombinase systems have been successfully used to manipulate transgenes in many organisms including *Ae. aegypti* (Werdien et al. 2001, Jasinskiene et al. 2003). However, the reversible nature of the integration means that efficiencies are low due to the entropically unfavourable nature of the integration reaction, and because secondary recombination events are possible in the presence of any residual recombinase leading to instability of the transgene (Albert et al. 1995, Baer et al. 2001).

1.3.7 The bacteriophage site-specific integrases

Bacteriophages employ a class of enzymes called site-specific integrases to integrate their genomes into that of their host at specific attachment sites. The phage integrases are members of the serine recombinase family of site-specific recombinases. This is a large family of proteins that comprises many resolvases, invertases and transposases characterised by their highly conserved N-terminal domains that contain a catalytic serine residue (Stark et al. 1992, McEwan et al. 2009, Smith et al. 2002, Calendar et al. 2006). The large serine recombinases are characterised by the size of their C-terminal catalytic domains that are around 300-500aa, much larger than those of the rest of the serine recombinases that have domains of only 141-183aa (Rowley et al. 2008). The use of phage integrases as gene vectors has become increasingly popular due to their versatility and

efficiency in several phylogenetically diverse species (Groth et al. 2004a, Nkrumah et al. 2006, Allen et al. 2005, Gregory et al. 2003).

The function of the phage integrases depends upon the structure of their phage and bacterial attachment sites, attP and attB, where recombination occurs at a central non-palindromic dinucleotide crossover site (Smith et al. 2004). The sequence structure of the sites is critical for formation of the reactive synapse required for recombination, and their polarity determines the orientation in which recombination takes place, resulting in integration, excision or inversion of the sequence enclosed by the sites. (Gupta et al. 2007). The minimum size of these sites is generally in the region of 30-50bp though efficiencies of recombination are generally improved with larger attachment sites. Although their catalytic mechanism is not completely understood; the phage integrases are thought to promote recombination by bringing together the two attachment sites in a synaptic tetramer. Cleavage of the DNA strands by the integrase then occurs via a nucleophilic attack on the scissile bonds by the serines of the N-terminal catalytic domains creating staggered 2bp breaks in the strands, with the recessed 5' ends being bound to the catalytic serine residue via a covalent phospho-serine bond (Johnson et al. 1989, Grindley 2002). Rotation of one pair of the integrase sub-units relative to the other pair then brings the cleaved attachments half-sites into proximity with their complementary half-site allowing recombination of the strands and formation of the new hybrid attachment sites; attL and attR (see Figure 1.8) (Rowley et al. 2008, Kamtekar et al. 2006).

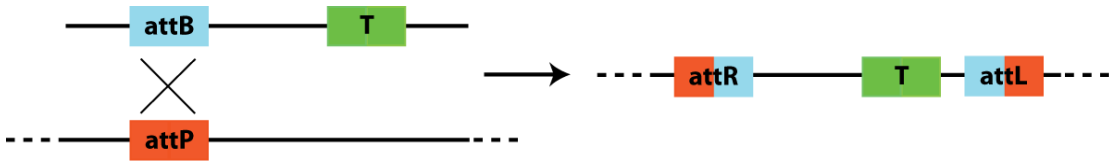


Figure 1.8: Recombination of attB and attP sites mediated by a site-specific integrase. Recombination results in formation of the hybrid sites attR and attL that are inert to further exposure to the integrase. If one site is placed with the genome and the other on a plasmid vector carrying a transgene (T), recombination will result in integration of the transgene into the genome.

When bound to the hybrid sites, the integrase is unable to obtain the correct conformation necessary for synapsis and so further recombination is prevented. A separately encoded phage recombination directionality factor (RDF) may allow the reverse reaction to take place under certain conditions but it is not known if RDFs exist for all integrases (Thorpe et al. 1998, Ghosh et al. 2005). The asymmetrical attachment sites and the specific requirements for the forward and reverse reactions enforces strict control over the directionality of integrase mediated recombination (Groth et al. 2004, Thorpe et al. 2000). This separates the site-specific integrases from the Cre and FLP recombinases whose identical attachment sites allow for reversible recombination reactions (O'Gorman et al. 1991). However, like the Cre and FLP recombinases the phage integrases generally do not require host specific co-factors to promote recombination and hence can be used in a wide variety of species for site-specific genome engineering (Keravala et al. 2005).

The site-specific integrase from the bacteriophage ϕC31 has seen extensive use as a gene vector and has been shown to promote efficient site-specific recombination in a wide range of eukaryotic organisms (Ow 2007). The minimum size of the attP and attB sites that have been shown to function in

vivo are 39bp and 34bp respectively (Groth et al. 2000). Recombination occurs at the central 5' TG dinucleotide of the attachment sites, which in the attP site is flanked by symmetrical imperfect repeats (Gupta et al. 2007). The use of the integrase from phage ϕC31 has already been demonstrated in *D. melanogaster* and was used to efficiently integrate multiple transgenes into a single genomic locus (Groth et al. 2004b). More recently the same system has also been shown to function in *Ae. aegypti* and *A. albopictus* making it a valuable tool for highly efficient site-specific integration into mosquito genomes (Labbé et al. 2010, Nimmo et al. 2006). Site-specific integration into the germline with other phage integrases has yet to be demonstrated in any insect species, but they offer the possibility of a large, efficient toolset for site-specific manipulation of the *Ae. aegypti* genome.

1.4 Gene drive systems

1.4.1 Gene drive systems and population replacement

Gene vectors such as *piggyBac* and the phage integrases provide a powerful toolset for the creation of transgenic mosquitoes and have allowed for the engineering of mosquitoes refractory to some of the diseases for which they are the primary vectors. In order to permanently or temporarily replace wild populations with these refractory strains a genetic drive mechanism will be required that can introgress a cargo of transgenic antipathogen effector genes into the indigenous population (Alphey et al. 2002, Sinkins et al. 2006). Several possible gene drive systems capable of spreading a genetic cargo after release of a practical number of mosquitoes have been proposed, and some of them have been effective in the laboratory setting. These include autonomous

transposable elements, meiotic drives, homing endonuclease genes, *Medea* elements and *Wolbachia* induced cytoplasmic incompatibility.

1.4.2 Criteria for a successful gene drive system

Gene drive systems must meet a stringent set of criteria before they can be considered for use in the field. These considerations are both scientific and political. The ability of gene drives systems to potentially drive transgenes across international borders makes them a complex technology to regulate, and to be effective in population replacement strategies the systems must be extremely well engineered (James 2005, Braig et al. 2001). Braig (2001) and James (2005) have proposed a set of key requirements that gene drives for engineering mosquito populations must meet. The most important of these is that the invasiveness of the drive must be great enough to overcome any fitness effects imposed by the transgene cargo, and great enough to drive the cargo to fixation in a wild population over a reasonable time scale, overcoming factors such as the invasion of vector competent individuals from outside the control area. Fitness effects imposed by transgenes are likely to vary greatly depending on the mode of action of the transgenes and studies have identified either positive or negative impacts of transgenes on engineered mosquito fitness (Bargielowski et al. 2011, Marrelli et al. 2006b, Marrelli et al. 2007). The strength of a gene drive system depends to a large extent on its particular mechanism, and the invasiveness of different systems may vary greatly (Marshall 2009). Another important factor in the design of any gene driver is the linkage between the transgene cargo and the drive mechanism. Ideally, the driver and the cargo must be linked in a way that prevents separation of the components

over time by processes such as meiotic recombination or mutation. Were the gene driver to become separated from the effector the unlinked driver would spread through the population to the exclusion of the complete system, which would likely have a greater fitness penalty associated with it and so would be driven less efficiently (James 2005). An ideal gene driver should also be able to drive multiple effector genes as a contingency against inactivation of one or more of them (Sinkins et al. 2006). This is of particular concern because mutation or recombination of the refractory gene or its promoter may either be selectively neutral or beneficial in some systems, meaning that refractoriness might be lost over time (Hahn et al. 2004, Boete et al. 2003). Multiple refractory mechanisms would also decrease the chance of the targeted pathogens evolving resistance to the refractory mechanism.

There are also several criteria that are important from a social, regulatory and epidemiological perspective. Risk assessment before the release of any gene drive will be critical, and the potential effects of the system on the mosquito population, as well non-target organisms, must be carefully considered to ensure that undesirable side effects do not occur (Benedict et al. 2008). Recallability of a gene drive system must be considered before release. The ability to replace or remove the transgene cargo from the population will be important, should post-release monitoring reveal unintended epidemiological consequences after release of a gene drive (James 2005). Relatively high release thresholds to achieve fixation are also desirable to insure against accidental release of any driver carrying individuals from laboratories or field cages. This

is of particular concern for highly invasive drivers which could theoretically spread through partially isolated subpopulations with the release of only a few individuals (Marshall 2009).

1.4.3 Autonomous transposons

Autonomous transposons that encode their own transposase were among the first gene drives proposed (Kidwell et al. 1992, Crampton et al. 1990). A natural example of introgression of a transposon into a population was the P transposon that spread itself throughout the worldwide *D. melanogaster* population within a few decades due to its ability to replicatively transpose (O'Hare et al. 1983). For transposons to function as gene drives, they must have a high rate of transposition in the germline. This can be difficult to achieve due to the carrying capacity of transposons, which decreases with the length of sequence between the ITRs, and because of endogenous mechanisms that protect the germlines' of organisms from transposon movement (Ding et al. 2005b, Zamudio et al. 2010). The *Ae. aegypti* genome contains a great deal of transposon sequence with these elements now appearing to be immobile, suggesting that such protective mechanisms exists in *Ae. aegypti* (Nene et al. 2007). The mobility of various class II DNA transposons in *Ae. aegypti* has been studied and no good candidates have been found for transposon-based gene drive systems, with post-integration stability appearing to be high (Wilson et al. 2003, Smith et al. 2011, Sethuraman et al. 2007). Even if highly mobile transposons can be identified, they pose a regulatory challenge. Engineering recallability of a highly active transposon would be technically difficult and the risk of horizontal transfer of the transposon, which has happened numerous

times over evolutionary history, could be high (Zimowska et al. 2006). These hurdles have meant that transposons have seen little recent development as gene drive system in mosquitoes.

1.4.4 Meiotic drive mechanisms

Meiotic drive mechanisms are common in nature and rely on a heterozygous allele segregating with a frequency greater than the normal Mendelian ratio of 0.5 through impairment of a sensitive responder allele on the homologous chromosome (Lyttle 1991). This results in favouring of the gametes that contain the drive by preventing development of those that carry the responder allele. The best characterized of these natural meiotic drive mechanisms is the Segregation Distorter (SD) from *D. melanogaster*, which causes failure in chromatin condensation on chromosomes bearing the drive's responder allele (Crow 1999).

An endogenous meiotic drive allele (M^D), closely linked to the sex-determining locus on chromosome 1, has been identified in *Ae. aegypti* in which it distorts meiosis to favour male-determining gametes (Hickley et al. 1966, Mori et al. 2004). The precise mechanism of this meiotic drive is not yet fully understood, although some evidence suggests that the Ras superfamily of regulatory small GTPases may be involved in the chromosomal breaking (Shin et al. 2011). Previous attempts to use this meiotic allele as a gene drive fro *Ae. aegypti* have not always been successful, with resistance to the drive quickly developing in populations due to the strong selective pressure to repress the drive through selection of insensitive responder alleles (Hickey et al. 1966, Wood 1976). However, more recently Cha at al. (2006) showed that simultaneously release of

insensitive allele bearing individuals and drive carrying individuals could rapidly drive the insensitive allele into the population. Linkage of such an insensitive allele to a refractory cargo could be used as the basis for a gene drive system in *Ae. aegypti* (Mori et al. 2004, Cha et al. 2006).

The use of a naturally occurring meiotic drive has the advantage that there is virtually no risk of horizontal transfer of the drive and cargo. However, modelling predicts that such systems may not be able to fix their cargo stably in populations if resistance alleles to the meiotic drive exist, and may be severely hampered by fitness costs associated with the cargo (Huang et al. 2007). Engineering of artificial meiotic drives using endonucleases may result in more robust systems, but such systems have yet to be demonstrated in *Ae. aegypti*.

1.4.5 Homing endonuclease genes

Homing endonuclease genes encode a class of DNA endonucleases that introduce double stranded breaks into DNA at specific 18 to 30bp genomic sites that are not already interrupted by the gene (see Figure 1.9). If one of a pair of homologous chromosomes lacks the homing gene then, in the germline, cutting by the endonuclease may result in the homologous recombination repair mechanism inserting the homing gene into the broken chromosome, leading to an increased proportion of gametes carrying the homing gene (Burt 2003). Linkage of a cargo to the homing endonuclease gene could therefore be used as a gene drive system, however homing endonucleases genes may also be used as a refractory mechanism in of themselves (Sinkins et al. 2006, Deredec et al. 2008).

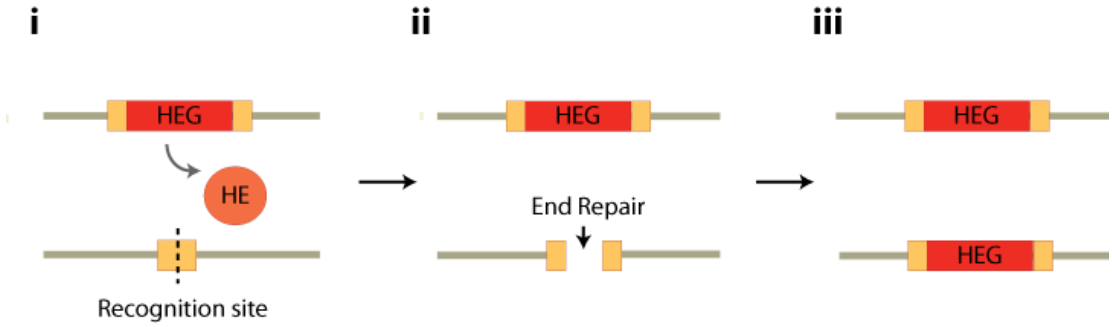


Figure 1.9: Mechanism of homing endonuclease gene (HEG) duplication onto a homologous chromosome. i) the homing endonuclease (HE) cuts the homologous chromosome at its recognition site resulting in a double stranded break. ii) this double stranded break is repaired by the homologous recombination repair mechanism using the HEG as a template resulting. iii) this results in duplication of the HEG.

Homing endonuclease genes have recently been shown to be active in the mosquito *An. gambiae* and the *I-SceI* homing endonuclease gene was recently shown to rapidly introgress itself into populations of the mosquito that had been engineered with a *I-SceI* recognition site (Windbichler et al. 2011, Windbichler et al. 2007). These results suggest that homing endonucleases could be very effective at engineering mosquito populations, and homing endonucleases have been shown to be active in *Ae. aegypti*, suggesting that such systems could be used in this species (Traver et al. 2009). However, homing endonucleases are not known to exist naturally within the nuclear genomes of insects, which means that their recognition sites are also absent. To be used as gene drive systems in *Ae. aegypti*, or other mosquitoes, existing homing endonucleases would have to be engineered to recognise sequences within mosquito genomes. Altering the specificity of homing endonuclease is possible using directed evolution, or even predictive modelling, and by exploiting sequence similarity within the mosquito genome to existing homing endonuclease recognition sites this approach should be possible (Windbichler

et al. 2011, Ashworth et al. 2006, Doyon et al. 2006). Homing endonucleases could be used for both population suppression and replacement. By targeting the cutting site at a vital gene or a sex-determining locus the population could be reduced. Alternatively by targeting a gene involved in vector competence, refractoriness could be driven into the population by removing vector competent individuals (Deredec et al. 2008, Deredec et al. 2011). Modelling predicts that homing endonuclease genes could be successfully used to engineer mosquito populations. However, the evolution of resistance is a major concern, as is the identification of suitable sites in mosquito genomes to serve as targets for the endonucleases (Deredec et al. 2011).

1.4.6 Maternal-effect dominant embryonic arrest elements

Maternal-effect dominant embryonic arrest (*Medea*) elements are selfish elements that induce maternal-effect lethality of all gametes not inheriting the element from the maternal and/or paternal genome (Chen et al. 2007). The first *Medea* element was described in the flour beetle *Tribolium castaneum* and its mechanism is thought to involve a maternal lethal toxin that kills gametes that have not inherited the element, and a zygotic antidote that protects those that have, as shown in Figure 1.10 (Beeman et al. 1999, Beeman et al. 1992).

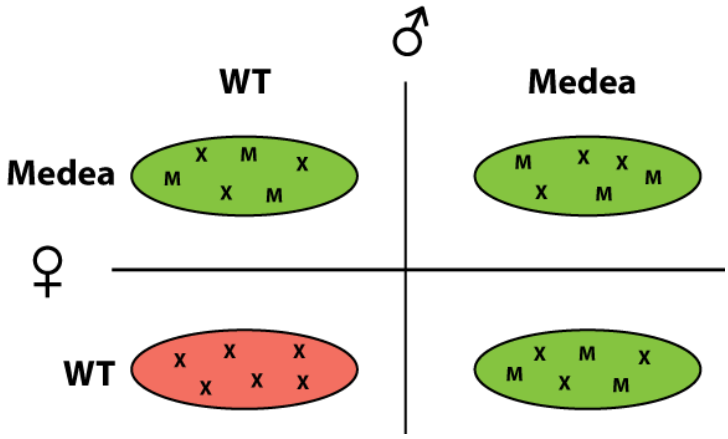


Figure 1.10: Mechanism of *Medea*'s maternal lethal effect. Females hemizygous for the Medea allele deposit a toxin (X) in gametes that results in death of the gamete (red). If gametes inherit a medea element from either the mother (♀) or father (♂) they survive (green), as the *Medea* antidote (M) prevents the toxin from killing the gametes. Based on the figure of Chen et al. (2007).

An artificial *Medea* element engineered into *D. melanogaster*, which used miRNA directed RNAi to silence a vital developmental gene as the maternal lethal, was shown to successfully introgress into small cage populations (Chen et al. 2007). *Medea* elements have yet to be engineered into mosquitoes; however, efforts to that effect are underway and there is no known reason why they should not be functional (Hay et al. 2010). Particular advantages of *Medea* elements are that recall and replacement are achievable by releasing wild-type mosquitoes or second generation constructs at a high enough frequency. For recall, engineering of a *Medea*-specific suicide transgene that suppressed the antidote would also be possible (Hay et al. 2010). Modelling suggests that *Medea* could be used as an effective gene drive for a variety of mosquito populations, resulting in fixation of a refractory cargo within a 1-2 year period after mass release of males engineered with a *Medea* element (Ward et al. 2011).

1.4.7 Killer-rescue type gene drive systems

A variety of gene drivers based on the use of engineered killer-rescue genetic constructs have been proposed. Each of these systems has a core component that consists of a dominant lethal gene that kills the mosquito when endogenously expressed and a rescue gene that suppresses the lethal phenotype. Such gene drive systems include; underdominance, which uses cross-suppressing lethal genes as the driving mechanism; *Semele*, which uses a toxin expressed in the semen of transgenic males and an antidote expressed in inseminated females as the drive (Davis et al. 2001, Magori et al. 2006, Marshall et al. 2011b, Gould et al. 2008). Introgression is achieved by releasing a large number of individuals carrying the killer and rescue constructs into a population. Since individuals lacking the rescue gene will be at a selective disadvantage in a population containing the killer gene; modelling predicts that the rescue gene will be introgressed into the population at a rate dependant on the initial release ratio and the fitness costs associated with the engineered constructs (Magori et al. 2006). By linking a refractoriness inducing cargo to the rescue gene such systems should be able to drive the cargo into mosquito populations (Davis et al. 2001). Particular advantages of killer-rescue type gene drives are that they are easily recallable or replaceable, through release of new constructs or wild mosquitoes at the necessary release ratio, and that they are predicted to be practical even when the engineered constructs have some degree of fitness penalty associated with them. This makes them particularly advantageous when geographic containment of a gene drive systems is desirous (Marshall 2009).

1.4.8 *Wolbachia* and paratransgensis

Paratransgensis is also a potential approach for population replacement strategies. The intracellular bacterium *Wolbachia* is able to spread itself through populations of insects, including *Ae. aegypti*, by use of Cytoplasmic incompatibility (CI). Infected males are unable to fertilize the eggs of uninfected females but due to a rescue function are able to successfully mate with infected females; resulting in selection for the infected state (Xi et al. 2005, Curtis et al. 1998, Sinkins 2004). Some strains of *Wolbachia* can induce refractoriness to a variety of pathogens, which is thought to occur due to upregulation of the mosquito's immune response (Kambris et al. 2009, Pan et al. 2011, Moreira et al. 2009). Alternatively, engineered *Wolbachia* strains could be used to carry and drive transgenes into mosquito populations and could be employed in a wide range mosquito species (Sinkins et al. 2004). Strains of *Wolbachia* have already been successfully transfected into *Ae. aegypti,* but as of yet no *Wolbachia* have been successfully transformed with transgenes that can promote refractoriness (McMeniman et al. 2009, Ruang-Areerate et al. 2006).

1.4.9 Gene drive systems: future development

Many of the gene drive system described above have attractive properties that make them potential candidates for use as gene drive systems in *Ae. aegypti* population replacement strategies. However none of them currently meets all of the desirable criteria for a gene driver that is to be deployed in the field. Of particular concern are the issues of linkage of the refractoriness inducing cargo to the drive mechanism, and recallability of the gene drive after release (Benedict et al. 2008). Additionally, for initial field trials of refractory

mechanisms, methods to confine a gene drive to a local population would be desirable to prevent spread of the cargo beyond the trial area (Marshall et al. 2011a). This may necessitate the design of self-limiting gene drives, such as killer-rescue systems, for the purposes of testing refractory cargos before they are engineered into highly invasive drive mechanisms (Gould et al. 2008). For these reasons the release of mosquitoes carrying a gene drive mechanism is some way off, and there is still a great deal of potential for the development of new systems.

1.5 Summary

Aedes aegypti is the vector of several important diseases that are responsible for social and economic problems worldwide. Efforts to prevent the recent resurgence and reemergence of these diseases have largely failed, primarily due to the lack of effective mosquito control strategies. For this reason the development of genetic control strategies, such as population suppression and replacement, has been a priority for researchers in recent years. The development of such strategies requires gene vectors that can integrate stably into the genome and ensure predictable expression of transgenes. The *piggyBac* transposon is the most widely used and efficient of these vectors, but its post-integration stability is still in question. Site-specific gene manipulation tools will also be of great use for the creation of engineered mosquito strains. Site-specific engineering with recombinases can allow for conservative and irreversible integration into previously characterized sites, ensuring predictable expression of a transgene at a known genomic locus. With these genome-engineering tools available, the insertion of complex engineered genetic constructs into the genome can be achieved. A particular goal of such detailed genome engineering is the creation of gene drive systems that can be used to introgress refractory genes into wild populations of *Ae. aegypti*. Such gene drive systems must meet some key design criteria if they are to be suitable for release into wild populations. Many gene drive systems have been proposed, and some have been successfully engineered into *Ae. aegypti* and other mosquitoes as proofs-of-principle. However, no gene drive system has yet been created that

simultaneously combines all the key design criteria, and some systems remain undeveloped.

1.6 Scope of Work

This thesis describes three main topics of research. The first is the investigation of the post-integration stability of the *piggyBac* transposon in *Ae. aegypti*, with the aim of determining the likelihood of germline and somatic movement of an inserted transposon (See Chapter 3). The second is the development of new site-specific genome engineering tools in *Ae. aegypti*, with the aim of allowing for more complex manipulations of transgenes in the mosquito in a sequence conservative and site-specific manner (See Chapter 4). Finally, this thesis details attempts to create an RNAi mediated killer-rescue system that could be used as the basis for construction of killer-rescue or underdominance gene drives (See Chapter 5).

Chapter 2

General Methods and Materials

2.1 *Ae. aegypti* strain and rearing procedures

The strain of *Ae. aegypti* used for all transformations and crossing experiments (hereafter referred to as wild-type) was the Rockefeller strain of the mosquito obtained from an existing colony at the University of Manchester, UK. Mosquitoes were raised using previously described procedures that are standard for the strain (Crampton et al. 1997). Briefly, all colonies and crosses were maintained at approximately 28°C and 60% relative humidity, with a 12 hours day-night cycle. Immature stages were raised in trays containing approximately 2.5 l of deionised water. Larval stages were fed with Tetramin fish food (Tetra GmbH, Germany). Pupae were then transferred to 100 ml weigh boats (VWR, UK), if necessary after screening (described in section 2.3). Adults were maintained on a 10% sucrose solution containing 14 μg ml^{-1} of the antibiotics penicillin and streptomycin, included to prevent bacterial growth. Mated females were fed on defibrinated horse blood (TCS Biosciences Ltd., UK) for 45 minutes three days after mating and then fed again two days later. Eggs were collected on wet 90mm diameter filter papers (Whatman, UK) 8 days after the initial mating of the female mosquitoes. Eggs were either stored for up to three months before hatching or dried for at least three days before hatching in 200ml of deionised water. Hatching was induced using a vacuum pump to deoxygenate the water.

2.2 Microinjection of *Ae. aegypti* and establishment of transgenic lines

2.2.1 Preparation of capped mRNA

mRNA of *piggyBac* transposase or ϕC31 site specific integrase was transcribed from the OX3081 and OX3869 plasmids of Oxitec Ltd. as describes in Fu (2010)

and Nimmo (2006) respectively. Briefly, these plasmid contained the transposase or integrase coding sequence under control of the T7 promoter and the 3'UTR of the *D. melanogaster vasa* gene. The *vasa* gene is expressed during germline development in the pole cells of developing embryos and it is thought that the 3' UTR can aid in directing protein expression to these cells (Sano et al. 2002). These plasmids were linearized by digestion with the *HindIII* restriction endonuclease and 2 µg used to transcribed capped mRNA with the mMESSAGE mMACHINE T7 kit (Ambion, USA) using the manufacture's protocol. Capped mRNA was then purified using the MEGAclear kit (Ambion, USA), precipitated with ammonium acetate and resuspended in 20 µl of nuclease-free water.

2.2.2 Preparation of Injection solutions

Injection solutions were prepared by purification of the plasmid vector, containing the genetic construct to be transformed, with the EndoFree Plasmid Maxi kit (Qiagen, Germany). The purified plasmid and capped mRNA were diluted to final concentrations of 300 ng μl^{-1} and 900 ng μl^{-1} respectively in endonuclease-free water (Ambion, USA) and injected along with injection buffer (final concentration, 5mM KCl and 0.1 mM NaH_2PO_4, pH 6.8) into freshly laid embryos.

2.2.3 Microinjection

Microinjection of 1-2 hour old embryos was performed using a FemtoJet microinjector system (Eppendorf, UK) using the procedure of Morris et al. (1989). The only modifications to this procedure being that the injection oil was removed from the injected embryos immediately after injection. Injected embryos were then left in an atmosphere of 100% relative humidity for 3 days.

2.2.4 Establishment of transgenic lines

Injection survivors were sexed and separated into pools, with 30 females or 10 males per pool. These were then crossed to virgin wild-type mosquitoes at a ratio of 1 male to 3 females. These pooled individuals were blood fed and G1 eggs collected. G1 progeny were then screened for presence of the expected fluorescent transformation marker(s) (as described in section 2.3). Transgenic lines were established from G1 individuals positive for the marker(s).

2.3 Screening of transgenic strains

Transgenic lines were screened on ice as 4th instar larvae or early pupae for fluorescence using a Leica MZ95 microscope with filter sets from Chroma Technology (Rockingham, USA). The filters used for each fluorophore were: ECFP (exciter D436/20x; emitter D480/40m), DsRed2 (exciter HQ545/30x; emitter HQ620/60m) and AmCyan (exciter ET436/20x; emitter ET480/40m). Pictures of larvae and pupae were taken with a Canon PowerShot S5IS with an MM99 adaptor (Martin microscopes, UK).

2.4 Genome walking for the retrieval of flanking sequences of *piggyBac* insertions

All primer and oligonucleotide sequences are described in Appendix 1.

2.4.1 Genomic DNA extraction

Genomic DNA was extracted from three pooled pupae of the line whose insertion site was to be determined using the GeneJET Genomic DNA Purification kit (Fermentas, Lithuania) according to the manufacturers protocol.

2.4.2 Genome walking with degenerate primers

Genomic DNA was used as the initial template for a series of sequential PCR amplifications employing nested primers. Primers specific to the *piggyBac* 5'and 3' ITRs were designed, as well as a set of four degenerate primers whose first seven nucleotides consisted of the 5'-ACGCTCC-3' motif followed by 10 degenerate nucleotides, followed by the 5'-SCATGS-3', the 5'-SGATCS-3', the 5'-SACGTS-3' or the 5'-SAGCTS-3' motif. These degenerate primers were designed to anneal randomly to DNA approximately every 1000bp. Three rounds of PCR were conducted with successive use of the nested primers specific to the ITRs. The nested primers used are described in Table 2.1.

Table 2.1: Nested primers specific to the *piggyBac* 5' and 3' ITR regions, used for the three rounds of PCR for genome walking with degenerate primers. Sequences of the primers are described in Appendix 1.

***PiggyBac* ITR**	**PCR Round**	**Name**
5'	1	PB5'-nested-01
	2	PB5'-nested-02
	3	PB5'-nested-03
3'	1	PB3'-nested-01
	2	PB3'-nested-02
	3	PB3'-nested-03

Amplification reactions were preformed using DreamTaq DNA polymerase (Fermentas, Lithuania) with the nested primers at a final concentration of 0.8µM μl^{-1} and each of the four degenerate primers at a final concentration of 2µM μl^{-1}. The thermal cycling conditions used for each PCR round were: an initial activation step of 94°C for one minute, followed by 3 cycles of 97°C for 7 seconds, 55°C for 40 seconds and 72°C for 2 minutes. The remaining 33 cycles

were carried out at 97°C for 7 seconds, 55°C for 20 seconds and 72°C for 2 minutes. PCR products were visualized on an Agarose gel after the third PCR round and extracted using the Minelute Gel Extraction kit (Qiagen, Germany). These were cloned into pJet plasmid vectors using the GeneJET PCR cloning kit (Fermentas, Lithuania) and transformed into XL-10 competent cells (Stratagene, USA) using the manufacture's standard protocol. Positive clones were purified with the GeneJET Plasmid Miniprep Kit (Fermentas, Lithuania) and sent for sequencing (GATC Biotech, Germany).

2.4.3 Genome walking with cassette PCR

Adapters specific to the 5' overhangs generated by the *MspI* (5'-CG) and *DpnII* (5'-GATC) restriction endonucleases were created by annealing of the oligonucleotides Msp-adapter-01 or Dpn-adapter-01 with the adapter-long oligonucleotide. These adaptors both contained sequence specific to the primer adapter-diag-01 and could be ligated to DNA cut with the *MspI* and *DpnII* enzymes.

Approximately 1µg of genomic DNA of the line whose flanking sequence was to be determined was digested with one unit of the *MspI* or *DpnII* restriction endonucleases (NEB, UK). Digested DNA was ligated to the appropriate adapter (final concentration: 0.5µM μl^{-1}) with T4 DNA ligase (NEB, UK) at 18°C for 12 hours. This ligation mix was then used as the template for sequential nested PCR reactions to recover the 3' and 5' flanking sequence. Nested primers specific to the *piggyBac* ITRs and the adapater specific adapter-diag-01 primer were used for these amplifications. The nested primers were: PB2 & PB1 for the 5' *piggyBac* ITR, and PB4 & PB3 for the 3' *piggyBac* ITR. PCR reactions were

performed using DreamTaq DNA polymerase (Fermentas, Lithuania) with the primers at a final concentration of 0.5µM μl^{-1}. The thermal cycling conditions used for each PCR round were: an initial denaturation step of 1 minute at 94°C, 35 cycles of 94°C for 10 seconds, 55°C for 45 seconds and 68°C for 90 seconds with a final elongation step of 68°C for 9 minutes. PCR products were visualized on an Agarose gel after the second PCR round and extracted. These were then cloned into pJet plasmid vectors and sent for sequencing as described in section 2.4.2.

2.5 Analysis of sequence data

Electronic sequence files from GATC Biotech, who performed all sequencing reactions, were analyzed using VectorNTI Advanced 11.0 (Invitrogen, USA). Recovered flanking sequences were compared to the published *Ae. aegypti* Liverpool LVP strain genome sequence using the BLAST algorithm available at the webpage of the VectorBase bioinformatics resource centre (Lawson et al. 2009).

Chapter 3

Stability of *piggyBac* Transposons in *Aedes aegypti*

3.1 Introduction

3.1.1 Importance and ramifications of *piggyBac* remobilization

The post-integration stability of *piggyBac* transposons has important ramifications for their use in mosquito transgenesis and disease control programmes. Any field release of a genetically engineered mosquito will have to meet a stringent set of regulatory criteria before a release can proceed (Benedict et al. 2008, Benedict et al. 2003). Of particular concern is the stability of any insertion and its potential for movement within the genome and between genomes (Beech et al. 2009). An insertion of a nonautonomous transposon – one that does not encode the transposase gene – can theoretically be remobilised in the presence of a suitable transposase source. The absence of such a source cannot be absolutely guaranteed in populations released into the wild where viral or bacterial vectors or hybridisation could hypothetically result in the transposase gene being inserted into the genome. Given that *piggyBac* elements have been identified in a phylogenetically diverse range of *Lepidoptera* and *Diptera*, as well as other organisms, such horizontal transfer of transposases has clearly happened repeatedly, albeit on evolutionary (Myr) timescales (Zimowska et al. 2006, Handler 2002, Sarkar et al. 2003). However, movement of a transposon from an infectious agent into an insect genome has never been observed experimentally and as such the rate of such events cannot be easily estimated (Handler 2002).

Remobilization or excision of a transposon after exposure to a source of the transposase could result in elimination of an engineered transgene from individuals in the wild after a release or influence the strain's fitness by

transposon mediated mutagenesis (Horn et al. 2003). Both these could have a severe impact on population replacement strategies where stable insertion of a refractory element is required (Sinkins et al. 2006, Handler 2004b). Also of potential environmental concern is horizontal transfer of the transgene to non-target organisms which could occur in the wild or in the process of mass rearing a transgenic strain for release, though this is predicted to be much rarer still (Wimmer 2003, Dafa'alla et al. 2006). Nuclear polyhedral viruses have been shown to be capable of acquiring genomic nucleotide sequences during replication in insect cells, and transposons have almost certainly been horizontally transferred from insects to infecting viruses (Fraser et al. 1983, Jehle et al. 1998). The intracellular parasitic bacterium *Wolbachia*, that infects many mosquito species, may be another possible route for horizontal gene transfer, and transfer of DNA from the bacterium to its host appears to be common (Hotopp et al. 2007, Schaack et al. 2010). The precautionary principle, which governs much of the regulation covering the release of modified organisms, suggests that a cautious approach be taken and if at all possible transposons should either be inactivated or alternatives identified (Spielman et al. 2002).

3.1.2 Stability of *piggyBac* transposons in *Ae. aegypti*

Germline remobilisation of *piggyBac* transposons has been observed at high rates in the fruit fly *D. melanogaster* (Horn et al. 2003), the beetle *Tribolium castaneum* (Lorenzen et al. 2003), the silkworm *Bombyx mori* (Uchino et al. 2008) and the medfly *Ceratitis capitata* (Dafa'alla et al. 2006). Studies on the remobilisation of *piggyBac* in *Ae. aegypti* have failed to detect any evidence of

germline or somatic remobilisation. Strains carrying nonautonomous *piggyBac* elements in five independent genomic locations have been crossed to jumpstarter strains – expressing *piggyBac* transposase under control of the Hsp70 promoter – and have also been injected with helper plasmids expressing the transposase (Sethuraman et al. 2007, O'Brochta et al. 2003). Exposing the nonautonomous transposon to the transposase by either method did not result in remobilisation in either the somatic or germline cells. The authors indicated that this result was unexpected given the high rate of remobilisation events detected in other insect species. In *D. melanogaster* germline remobilisation is readily induced in the presence of an endogenous transposase (Häcker et al. 2003). Equally, in the beetle *Tribolium castaneum* it can be efficiently remobilised for use in mutagenesis studies (Lorenzen et al. 2007). More recently it has been shown that *piggyBac* transposons can be remobilised in the germline of *Anopheles stephensi* in the presence of a jumpstarter construct integrated at a second genomic locus. This was shown to happen at a high rate with ~6% of the progeny of such crosses carrying the transposon at a new genomic location (O'Brochta et al. 2011).

Previous studies on the post-integration mobility of *piggyBac* transposons in *Ae. aegypti* have examined <200 post-injection Go individuals, which were exposed to a *piggyBac* transposase source, for evidence of somatic remobilisation. However, such numbers may not detect remobilisation if its rate is low (Sethuraman et al. 2007). Additionally, *piggyBac* transposase was provided in the form of helper plasmids which may be less effective at

delivering functional transposase than other available methods; such as the use of capped transposase mRNA (Kapetanaki et al. 2002). Such studies have also used the *D. melanogaster Hsp70* heat shock gene to drive expression of *piggyBac* transposase and this promoter may not induce strong expression in *Ae. aegypti*. For these reasons the 20,000 progeny of the crosses that were screened may not represent a large sample (Sethuraman et al. 2007).

3.1.3 Endogenous suppression of *piggyBac* transposition

It is clear that the rate at which *piggyBac* and other transposons remobilise after insertion into a genome is highly host-specific, and the rate of remobilisation may vary by orders of magnitude between even closely related species. Therefore it is not yet possible to say if *piggyBac* is stable once inserted into the genome of *Ae. aegypti* or whether its rate of remobilisation is just low. Some variation in *piggyBac* transposons' post-integration behaviour is to be expected based on the genomic context of transposons. Chromatin structure and the transcriptional activity of different genomic regions each affect the ability of transposases to mediate transposition (Horn et al. 2003, Lorenzen et al. 2007). The size and structure of the *Ae. aegypti* genome, with its short interspersed repeat pattern, may also exacerbate positional suppression of transposition relative to other insect species (Sethuraman et al. 2007). Additionally qualitative differences in the biochemistry of *Ae. aegypti* may make remobilisation less frequent or impossible. Other class II transposable elements display unusual activity in *Ae. aegypti*, with the Hermes transposon not displaying its canonical cut-and-paste transposition (Jasinskiene et al. 2000). It has been suggested that *Ae. aegypti* may have developed an

endogenous system to regulate transposition within its genome, possibly based on the use of an RNAi suppression system, however knockdown of the dicer genes –integral to the RNAi pathway– do not appear to result in increased rates of transposition (Sethuraman et al. 2007). The transpositional activity of retrotransposons, which transpose via RNA intermediates, is suppressed in the germline of *Drosophila* by the PIWI group of proteins that appear to act in concert with repeat-associated small interfering RNAs to limit retrotransposon activity (Siomi et al. 2008, Kalmykova et al. 2005, Vagin et al. 2006). It is possible that a similar system could be active in *Ae. aegypti* but how it would function to suppress non-autonomous DNA intermediate dependent class II transposons, such as *piggyBac*, is unclear. CpG methylation has also been suggested as a mechanism by which *piggyBac* could be stabilised in a host genome (Handler 2004a). Methylation can play an important role in gene silencing and the extent of germline methylation mechanisms varies greatly between insect species (Finokiet et al. 2007, Kunze et al. 2002). However Sethuraman et. al. (2007) investigated the extent of methylation of inserted *piggyBac* transposons and found no difference between the methylation states of the inverted terminal repeats (ITRs) and the genomic DNA surrounding the integration site.

3.1.4 Stabilising *piggyBac* transposon post-insertion

To overcome the possibility of a *piggyBac* transposon remobilising methods have been developed to prevent transposase-mediated transposition after integration of the element by partial or total removal of the ITRs that bracket the transposon (Handler 2004b, Dafa'alla et al. 2006). The ITRs of a *piggyBac*

transposon play a vital role in its transpositional activity and without them transposition is impossible, as the transposase is unable to bind correctly and form the necessary transposition complex (Li et al. 2001). The use of recombinase systems such as FRT or Cre-loxP has been suggested as a mechanism to excise portions of the ITRs of transposons. However these methods necessitate the flanking of the sequence to be removed with the appropriate recombination sites, which would require these sites to lie within the transposon's ITRs. Considering that the fine structure of *piggyBac's* ITRs appear to be critical to its transpositional activity, and hence its use as a transgene vector, this does not seem to be a practical solution (Li et al. 2005, Handler 2004b) .

Handler *et al.* (2004) developed a method for the partial stabilisation of *piggyBac* insertions in *Drosophila* by creation of transposons containing a head-to-tail tandem duplication of one of the transposon ITRs essentially creating a shorter, and hence more readily mobilised, *piggyBac* transposon internal to the transposon used to insert the transgene. Crossing lines of carrying this construct to a 'jumpstarter' expressing *piggyBac* transposase resulted in excision of the shorter transposon and hence stabilisation of the initial insertion due to deletion of one of the flanking ITRs. Schetelig et al. (2009) demonstrated a variant of this method using a *piggyBac* transposon containing an attachment site for the ϕC31 site-specific integrase (see chapter 4). This site was used to create a shorter transposon for remobilisation through site-specific insertion of a *piggyBac* ITR into the attachment site (Schetelig et al. 2009). A

drawback of both these methods is that a second transposon could integrate near the excised end; remobilising the transgene by providing the necessary ITR (Handler et al. 2004).

An extension of Handler et al.'s system allows for excision of both the ITRs of a *piggyBac* transposon, rendering the insertion completely stable in the presence of the transposase. (Dafa'alla et al. 2006). This method relies on two internal ITRs, engineered to create two shorter flanking transposons around the transgene to be inserted. Remobilisation and excision of these flanking transposons results in a stabilized insertion with no *piggyBac* ITRs surrounding it, as shown in Figure 3.1.

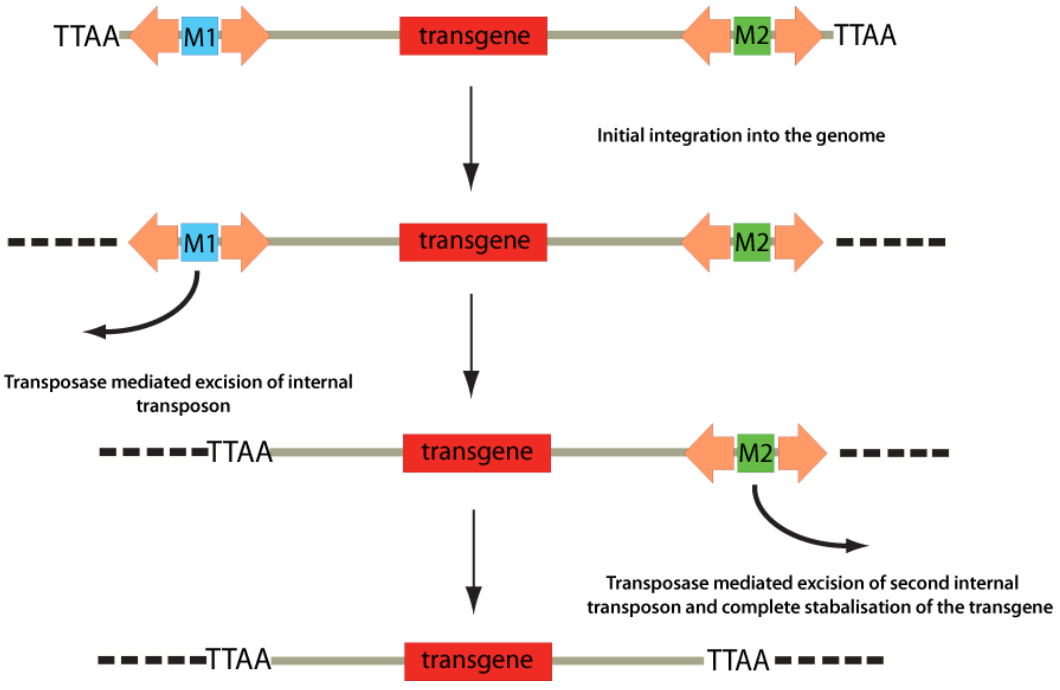


Figure 3.1: Post-integration stabilization of a *piggyBac* insertion, by excision of flanking transposons. Selecting for the stabilized insertion is easily achieved by screening for the loss of markers M1 and M2 over successive generations

Excision of the flanking transposons can be readily achieved by crossing to a jumpstarter construct or by injection with transposase mRNA. Stabilisation can be easily monitored by screening for the loss of the fluorescent markers that have been engineered into the flanking transposons (M1 and M2 in Figure 3.1). With the excision of all of the *piggyBac* ITRs flanking the transgene the insertion is stable to any further exposure to a source of transposase, with only the duplicated TTAA sites remaining as artefacts of the initial transposase mediated insertion. So far the only published applications of this method of transposon stabilisation have been in the Mediterranean fruit fly and the Mexican fruit fly (Dafa'alla et al. 2006, Condon et al. 2007).

Recently Tkachuk et al. (2011) published a new method for the post-integration stabilisation of transposons based on the use of chromosomal double-strand break (DSB) repair, which they demonstrated in *D. melanogaster* using a mariner transposon (Tkachuk et al. 2011). This involved a multi-stage process of first generating an acceptor line with a ϕC31-integrase attachment site and then inserting a secondary construct with segments of DNA homologous to the flanking sequence of the acceptor construct. The rare-cutting I-SceI and I-CreI homing nucleases were then used to introduce double stranded breaks with the integrated construct. The fly's natural single-strand annealing pathway then resulted in the excision of the transposon ITRs and stabilisation of the transgene (see Figure 3.2).

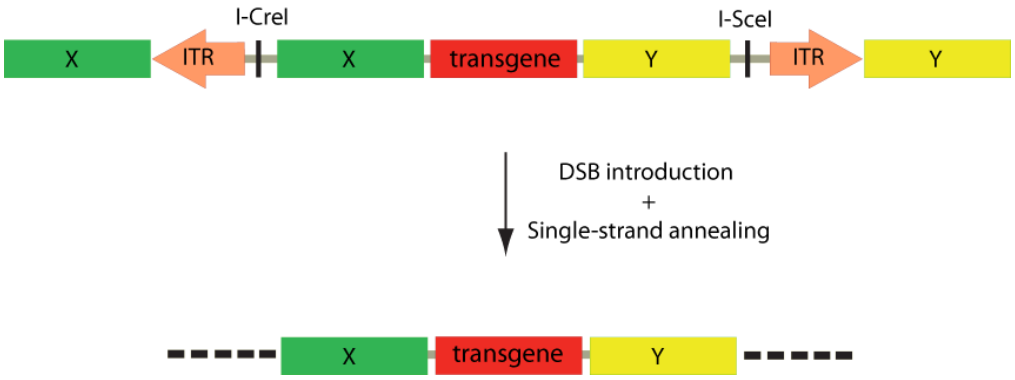


Figure 3.2: Stabilization of a transposon using chromosomal double-strand break repair. Exogenous sequences homologous to the sequence flanking the transposon ITRs (X and Y) are inserted into the transposon using site-specific recombination. The I-SceI and I-CreI homing nucleases are then used to introduce double strand breaks and the single-strand annealing pathway results in elimination of the transposon ITRs.

This method of transposon stabilization has yet to be attempted in *Ae. aegypti*. Both the I-SceI and I-CreI homing nucleases have been shown to be active in *Ae. aegypti* but the efficiency of DSB repair in the mosquito is unknown (Traver et al. 2009).

3.2 Experimental aims

The experiments described in this chapter were conducted with the aim of assessing the rate of remobilization of piggyBac transposons after insertion into the genome of *Ae. aegypti*, in the presence of *piggyBac* transposases. If remobilization could be achieved the further aim was to stabilize genetic constructs after insertion, using the method of Dafa'alla et al. (2006).

3.3 Materials and Methods

3.3.1 Synthesis of plasmid constructs

The OX3885 plasmid was designed by Tarig Dafa'alla of Oxitec Ltd. Briefly, a 3303bp fragment of the *Aedes vasa* germline promoter, complete with the 5' UTR, was amplified from *Ae. aegypti* genomic DNA with the primers *vasa*-up-01

and *vasa*-down-01 containing the *FseI* and *BsaI* restriction sites. Design of the primers was based on the published and annotated *Ae. aegypti* genome sequence with the BLAST algorithm being used to search for ORFs with homology to the published sequence of the *D. melanogaster vasa* gene. Similarly, the *vasa* 3'UTR (537bp) was then amplified from gDNA with the primers *vasa*-3'-F and *vasa*-3'-R and digested with *BsaI* restriction endonuclease. The *piggyBac* transposase gene (1808bp) was amplified from existing plasmid OX3985 with primers PB-transp-F and PB-transp-R and then digested with *BsaI*. Another plasmid, OX3653, was digested with *FseI* and *NotI* giving a 11240bp fragment containing the four-ended transposon system with the flanking transposons containing the AmCyan and DsRed florescent reporter genes under control of the promoter 3xP3. This is an artificial eye specific regulatory element, constructed from three tandem repeats from the Pax-6/Eyeless transcriptional transactivator (Berghammer et al. 1999). The three amplification products were then cloned into this plasmid to create OX3885. Plasmid OX3861 was created by digestion of plasmid OX3586 with *KpnI* and treatment with Klenow DNA polymerase (New England BioLabs) followed by digestion with *BglII*, giving a 2101bp fragment containing the HR5-IE1-AmCyan-Sv40 3'UTR marker. This fragment was then cloned into the *SwaI-BglII* sites of existing plasmid OX3767 giving the final OX3861 construct.

3.3.2 Reverse transcription and Real-time reverse transcription-PCR

RNA was extracted as described in section 2.2.1 from three pooled samples of each life-stage. cDNA was generated from 0.5µg of this RNA using RevertAid reverse transcription kit (Fermentas, Lithuania), using random hexamer

primers. RT-PCR to analyze expression of *piggyBac* transposase in the OX3885 lines was conducted using primers PB-Transposase-F and PB-Transposase-R that were specific to a 412bp fragment of the transposase coding sequence. Real-time RT-PCR to determine the relative expression of *piggyBac* transposase at different life stages of the OX3885-D line was conducted with 0.5 µg of cDNA generated from extracted RNA as above. This cDNA was also used to conduct Taqman real time PCR using primers and probes specific to the *piggyBac* transposase coding sequence and a normalizing gene; the 18S ribosomal protein gene that has previously been shown to have approximately stable expression over the mosquito life cycle (Niu et al. 2000). The primers, probes and product lengths are described in Table 3.1.

Table 3.1: Primers and probes used for real-time PCR of the *piggyBac* transposase transgene and the 18S-normalizing gene. The transposase specific probe was labeled at the 5' end with the HEX minor grove binding reporter dye. The 18S specific probe was similarly labeled with the FAM dye.

Probe	Primer	Sequence	Product (bp)
Transtaq		5'- Hex-CGGGATTCACCCCGCACGATAGC-3'	
	TranstaqF	5'- CAAACGCGAGATACCGGAAGTACTG-3'	91bp
	TranstaqR	5'- GACGAGAGTAAGGGGTCCGTCAAA-3'	-
18Sprobe2		5'-Fam- TTCGTAGACCGTCGTAAGACTAACTAAAGCG -3'	
	18StaqF2	5'-GTATTACGGCGCGAGAGGTG-3'	141bp
	18StaqR	5'- GAAAACATCTTTGGCAAATGCTT-3'	-

The reaction mix used 12.5µl TaqMan Gene Expression Master Mix (Applied Biosystems, UK) combined with 2.5µl of each primer (9µM dilution) and 0.06µl of each probe (100µM dilution), with the final volume brought to 25µl with water. Real time PCR reactions were performed in triplicate on a Mx3005P

thermal cycler (Stratagene, USA) with an initial activation step of 50°C for 2 minutes, followed by denaturation at 95°C for 10 minutes and forty cycles of 95°C for 15 seconds and 60°C for 30 seconds. Data from the reactions were analysed using the MxPro-QPCR software (Stratagene) and R statistical package (R Foundation for Statistical Computing).

3.3.3 Multiplex PCR for analysis of *piggyBac* remobilization

Multiplex PCR to determine if the flanking transposons of the OX3885 construct had remobilized was carried out using Phusion high-fidelity DNA polymerase (NEB, UK). Primers specific to the flanking region of each insertion of the construct and two nested internal primers for each transposon were used to amplify the region lying between the flanking transposons and the core of the OX3885 construct. The internal primers used were, 5'-unresolved-01 & 5'resolved-01, for the 5' transposon (relative to the transposase gene), and 3'-unresolved-01 & 3'-resolved-01 for the 3' transposon. The flanking primers and the expected product sizes from the multiplex PCRs are shown in Table 3.2.

Table 3.2: The line and transposon specific primers used in the multiplex PCRs to test for remobilization of the 5' and 3' flanking transposons of the OX3885-C, D and E lines, and the product sizes expected from the stable transposons and the sequence generated by remobilization. Primers sequences are described in Appendix 1.

Line	Transposon	Flanking Primer	Product-no movement (bp)	Product – remobilization (bp)
C	5'	3885C-5'flank-01	1079	367
	3'	3885C-3'flank-01	1101	574
D	5'	3885D-5'flank-01	1248	536
	3'	3885D-3'flank-01	1088	561
E	5'	3885E-5'flank-01	1116	404
	3'	3885E-3'flank-02	1077	550

Touch-down PCR reactions were carried out with an initial activation step of 98°C for one minute, followed by 3 cycles of 98°C for 10sec, 61°C for 20sec and 72°C for 1 minute. The remaining 30 cycles were carried out at 98°C for 10sec, 58°C for 20sec and 72°C for 35sec. For the OX3885-C line a longer primer was used for the 3' transposon (3'-unresolved-C01) as well as higher annealing temperatures of 65°C and 61°C.

3.4 Results

3.4.1 Creation of a four-ended *piggyBac* construct

To test the remobilization potential of *piggyBac* transposons in *Ae. aegypti* a construct, OX3885, containing a four-ended *piggyBac* system similar to that reported in Dafa'alla et al. (2006) was constructed (see Figure 3.3).

Figure 3.3: Schematic of the OX3885 construct. A *piggyBac* transposase gene, under control of the germline specific *vasa* promoter, and an Hr5*ie*1-DsRed marker (M1) was flanked by two *piggyBac* transposons. These contained the 3xP3-AmCyan (M2) and 3xP3-DsRed (M3) marker genes.

The construct consisted of a 4948bp *piggyBac* transposon containing the *piggyBac* transposase coding region under control of the *Aedes vasa* promoter, with the *vasa* gene's 3'UTR after the stop codon of the transposase gene. The endogenous *vasa* gene is expressed during oogenesis and the VAS protein is localized in the posterior end of developing oocytes, where it is present in germline cells throughout development (Liang et al. 1994, Sano et al. 2002). A DsRed fluorescent reporter gene, under control of the Hr5*ie*1 baculovirus

promoter, was placed downstream of the *piggyBac* transposase gene to act as a transformation marker. The *piggyBac* ITRs used were 560bp and 864bp for the 3' and 5' ends (relative to the transposase gene) respectively; these were identical in length to those used by Dafa'alla et al. (2006). Within this transposon two smaller, flanking transposons were engineered by insertion of inverted ITRs identical to those previously described (Dafa'alla et al. 2006). These flanking transposons contained fluorescent marker genes under control of the artificial eye specific promoter 3xP3 (Berghammer et al. 1999). The marker gene in the 5' transposon (relative to the transposase gene) was DsRed and in the 3' transposon AmCyan. These markers were intended to be easily distinguishable to allow for convenient screening for their presence or absence during analysis of remobilization.

3.4.2 Generation of OX3885 lines

The OX3885 construct, containing the four-ended system, was injected into 1334 embryos of *Ae. aegypti* in the presence of *piggyBac* transposase capped-mRNA. A total of 193 G0 individuals survived to adulthood and were crossed to wild-type virgin mosquitoes. The OX3885 construct was successfully inserted into the genome with an efficiency of 41%. Of the G1 transformants 34% carried all three fluorescent markers indicating that the entire construct had been successfully inserted. 66% of transformants carried one of the 3xP3 promoted markers but neither the other 3xP3 marker nor the Hr5*ie*1-DsRed marker, indicative of one the flanking transposons being inserted into the genome. A single G1 individual carried the central Hr5*ie*1-Dsred marker and the 3xP3-DsRed marker but not the 3xP3-AmCyan marker. This suggested that insertion

of the entire transposon had occurred followed by transposase-mediated excision of the AmCyan containing flanking transposon, or that the marker in one of the flanking transposons had been silenced. Unfortunately, this individual died before a line could be established from it so the nature of the insertion could not be determined. Five single males carrying all three markers were outcrossed to virgin females and three independent, single insertion lines established from these crosses. These were OX3885-C, OX3885-D and OX3885-E, the expression profiles of the three markers in each line is shown in Figure 3.4.

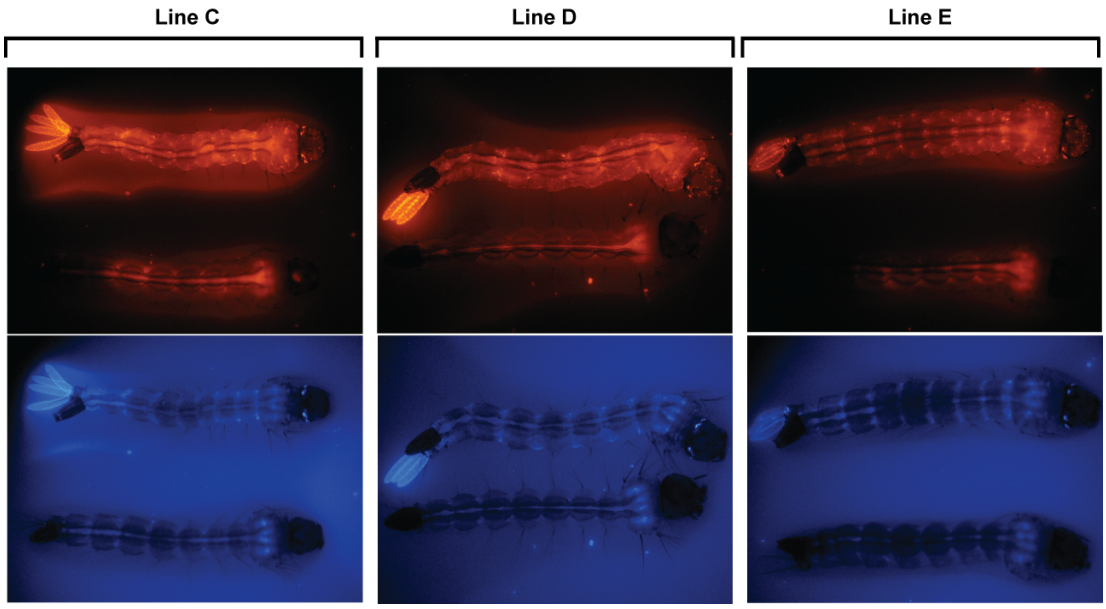


Figure 3.4: Expression of the DsRed and AmCyan reporter genes in 4th instar larvae of the three lines (C, D and E) of OX3885 (top of each image) compared to wild type (bottom of each image). Expression of the Hr5*ie*1-Dsred reporter is clearly visible in the cuticle. Expression of the 3xP3-DsRed and AmCyan reporters is visible in the optic nerves and anal papillae.

For each line the flanking sequence of the insertion was obtained. Lines C and E had inserted via canonical *piggyBac* mediated transposition into TTAA sites in the genome. The BLAST algorithm was used to locate the insertions within

the published *Ae. aegypti* Liverpool LVP strain genome sequence (Lawson et al. 2009). The OX3885-C insertion was located in an intergenic region of supercontig 1.304. The OX3885-E line had inserted into the putative AAEL012755 gene on supercontig 1.735, but the flanking sequence differed slightly from the published sequence with a 50bp deletion upstream of the TTAA site. The OX3885-D line did not insert via canonical *piggyBac* transposase mediated transposition. Progressive PCR of the construct regions revealed that insertion had occurred at an FRT site (the attachment site of FLP recombinase) present in the plasmid, an artifact from one of the plasmid precursors of the OX3885 construct, at the 3' end of the Hr5*ie*1-Dsred marker (see Figure 3.5).

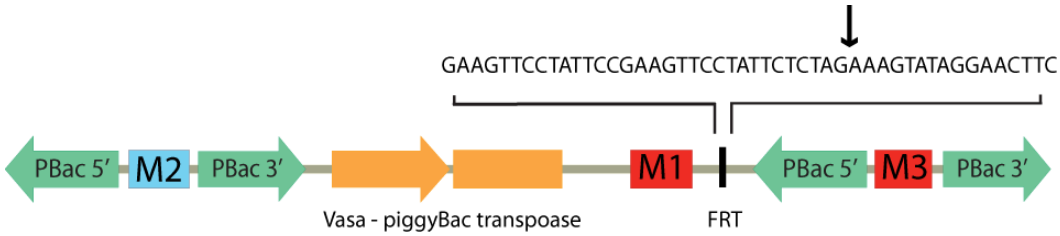


Figure 3.5: Insertion site of the OX3885-D line. Insertion of the OX3885-D line was non-canonical, with recombination (indicated by the arrow) occurring at an FRT site, integrating the entire plasmid into the genome.

This insertion was not mediated by FLP recombinase and the FRT site was not reconstituted after insertion and resulted in the backbone of the plasmid vector being inserted in its entirety. This suggests that the insertion was due to an anomalous recombination event, rather than *piggyBac* transposase-mediated insertion that occurs at TTAA sites. *PiggyBac* transposons have previously been reported to undergo non-canonical integration events in *Ae. aegypti*. The mechanism these integrations is unclear but may be related to endogenous

factors that interact with the transposase (O'Brochta et al. 2003). The insertion was located on supercontig 1.688 of the published *Ae. aegypti* genome in an intergenic region with no obvious similarity to the FRT site. The flanking sequences and insertion sites for each line are summarized in Table 3.3.

Table 3.3: Insertion sites and the first 20bp of the 5' and 3' flanking sequences of the OX3885 C, D and E lines. Lines C and E inserted at TTAA sites via canonical *piggyBac* mediated transposition. Line D inserted at a CACA site, without duplication of the site, via an unknown recombination mechanism.

Line	5' flanking sequence	Insertion Site	3' flanking sequence
C	GATTCCTAATCAAGCGATCG	**TTAA**	CTGAAAAATGAGCATTGTTC
D	CAATGTCTTTCTCAGCTGGT	**CACA**	ATCTGCTCGTTGGGTCAGC
E	GGCTTCGTTTCACCTTCACT	**TTAA**	AGCAATCAAATTTCGATGAC

3.4.3 Germline expression of *piggyBac* transposase

In the OX3885 construct, the *piggyBac* transposase gene was under control of the *Ae. aegypti vasa* promoter fragment. To determine if this artificial promoter was driving endogenous expression of *piggyBac* transposase in the OX3885 lines, RT-PCR was performed using *piggyBac* transposase coding sequence specific primers. The template for the reaction was DNAse treated RNA extracted from adult female abdomens 48 hours after a blood meal and pooled embryos 3 hours after deposition. The results of the RT-PCR are shown in Figure 3.6.

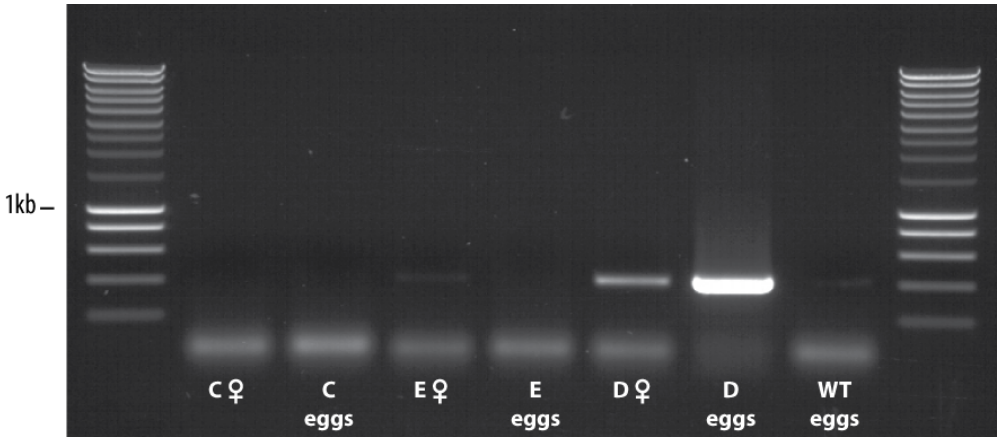


Figure 3.6: RT-PCR of *piggyBac* transposase transcripts in female abdomens (♀) 48 hours after a blood meal and embryos (eggs) 3 hours after deposition from the three lines of OX3885. Primers specific to the transposase coding sequence were used with an expected amplicon of 412bp. Samples were treated with DNAse to prevent false positives and no-transcriptase samples were run (data not shown) to ensure observed bands were not due to contamination. RT-PCR was also performed on RNA extracted from wild type (WT) embryos as a control.

Strong amplification of transposase transcripts was observed only in female and embryo samples of the OX3885-D line, with the RNA from embryos producing the strongest band. Although this assay is only semi-quantitative the stronger band seen produced from embryo RNA may be indicative of higher expression. In general lines C and E did not show any evidence of transposase expression in females or developing embryos. A faint band was observed for females of the E line but this was likely due to contamination as no evidence of expression was seen in embryos.

From these results it was unclear if the *vasa* promoter fragment was simply not functional, and if the expression in the D line was due to serendipitous insertion of the construct near genomic enhancer elements that lead to expression in the germline; or if the *vasa* promoter was highly sensitive to genomic context, rendering it inactive in the C and E lines. The 3085bp *Ae.*

aegypti vasa promoter region engineered into OX3885 was homologous to the *D. melanogaster vasa* promoter region that has previously been used to drive expression of transgenes in the fruit fly (Bischof et al. 2007). However it was possible that the fragment used lacked the necessary elements for complete activity since the size of the 5' flanking region of the VAS gene has previously been shown to have a great impact on its expression profile (Sano et al. 2002).

To determine if the *vasa* promoter fragment was regulating the transgene as expected, the expression of the transposase at different life stages of the hemizygous OX3885-D line was examined using quantitative RT-PCR with primers and probes specific to the coding sequence of the *piggyBac* gene (see Figure 3.7). The expression levels (dR) of *piggyBac* transposase, relative to the expression in male pupae, of extracted mRNA were examined in triplicate and normalized against the expression of the 18S ribosomal protein gene.

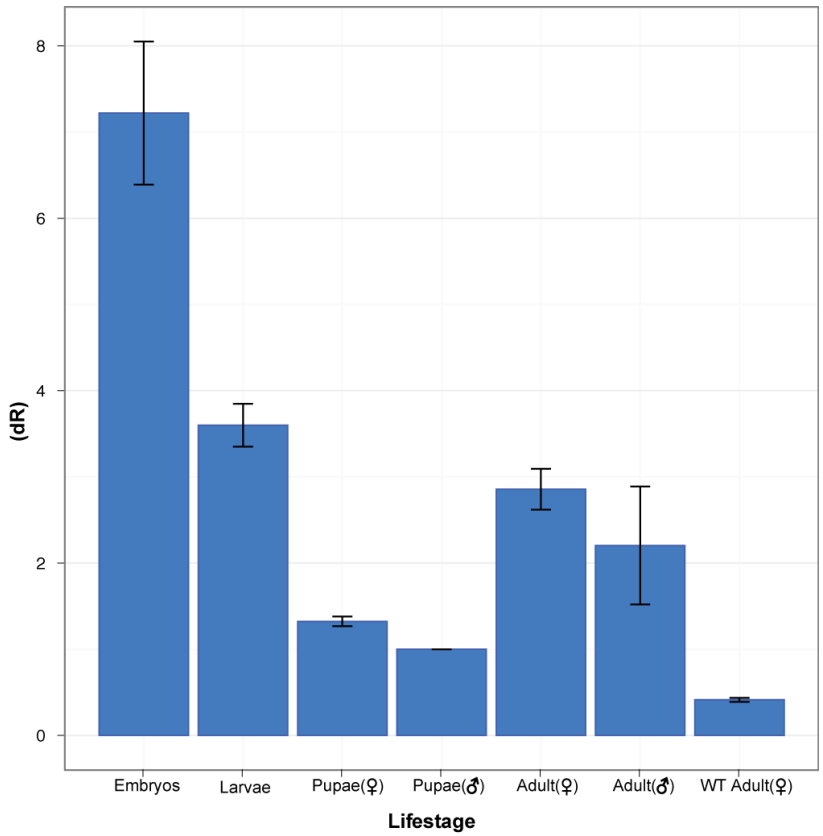


Figure 3.7: Mean relative expression levels of the *piggyBac* transposase transgene in the OX3885-D line at different life stages in hemizygous males and females. Expression levels were determined using Taqman based duplex real time RT-PCR and are relative to the expression of the transgene in male pupae. The results were normalized against the expression of the 18S gene. Error bars show the standard error of the mean of the three experimental replicates of three pooled individuals or approximately two hundred pooled embryos. Amplification in wild-type adult females (resulting from contamination or non-specific binding) is included for comparison.

In OX3885-D transposase transcript levels were greatest during early development declining to their lowest levels in males and females at the late pupal stage. Expression began to increase again in adults after mating (but before blood feeding), with the highest expression observed in embryos three hours after deposition by a female. This expression profile is very similar to that reported for the *Drosophila vasa* gene (Sano et al. 2002, Raz 2000). This suggests that, in the context of line D, the *vasa* regulatory region was functioning as expected but had been inactivated in lines C and E.

3.4.4 Mobilization of an exogenous transposon

It was considered that RT-PCR was not incontrovertible proof of expression, or lack thereof, of the *piggyBac* transposase protein. The absolute quantity of transposase mRNA may have been extremely low making detection through RT-PCR difficult and giving false negatives for the C and E lines. Furthermore the presence of transposase transcripts is not necessarily proof of the presence of active transposase protein, which would be required for any remobilization in the germline. To test for the presence of an active source of *piggyBac* transposase, the OX3885 lines were injected with a second plasmid construct, OX3861. This construct consisted of a single *piggyBac* transposon containing the AmCyan fluorescent reporter gene under control of the baculovirus promoter Hr5*ie*1. OX3861 was injected into hemizygous embryos of the OX3885 lines as above but without the addition of capped *piggyBac* mRNA to the injection solution. The Go survivors were outcrossed to virgin wild type mosquitoes and the G1 progeny screened for transformation with the OX3861 construct. The number of injected embryos, the survival and transformation efficiency of these injections are summarized in Table 3.4.

Table 3.4: Transformation efficiency of the OX3861 construct injected into the three lines of OX3885, without an exogenous source of *piggyBac* transposase. Fertile G0s were assumed to comprise 50% of the G0 individuals surviving the microinjection process.

Injection Line	Eggs injected	Fertile Gos	Transformation Eff.(%)
C	1040	191	0
D	1249	166	3
E	1063	213	0

Injection into lines C and E resulted in no transformants. Injection into line D, with an identical injection mix, resulted in two separate integration events; OX3861-A and OX3861-B. These lines carried the Hr5*ie*1-AmCyan marker, which independently segregated from the markers of the OX3885 insertion; suggesting insertion into unlinked genomic loci. The two lines also had different profiles of fluorescence suggesting they had inserted into different genomic contexts (see Figure 3.8).

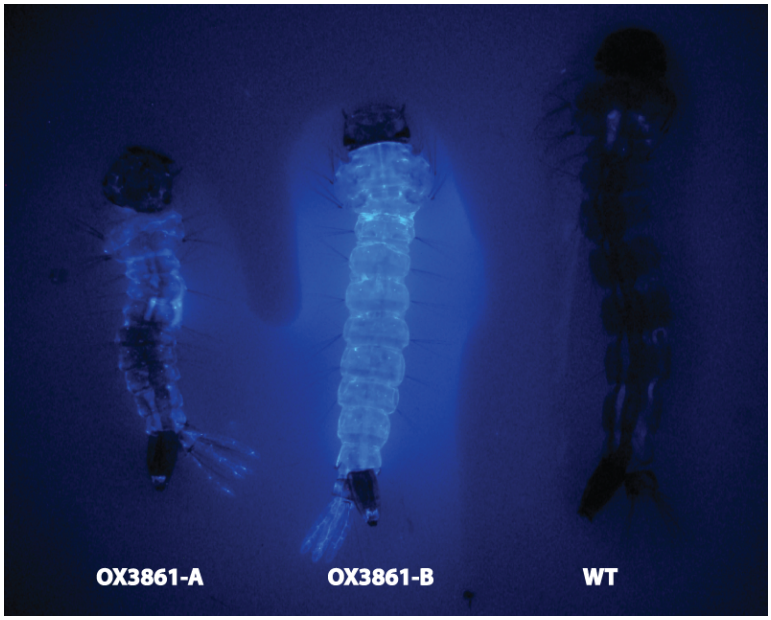


Figure 3.8: Expression of the AmCyan fluorescent protein in 3rd instar larvae of the two lines of OX3861 and wild type (WT). Lines A and B showed distinctly different profiles of expression, with line B being much brighter than the patchy expression in line A.

The flanking sequences of the two OX3861 lines were determined using genome walking with degenerate primers. This revealed that in both lines the construct had inserted via *piggyBac* mediated transposition with characteristic duplication of the genomic TTAA site (see Table 3.5).

Table 3.5: Insertion sites and flanking sequences of the OX3861 A and B lines.

Line	5' flanking sequence	Insertion Site	3' flanking sequence
A	TGAATATCAAAAGATTGTTC	**TTAA**	GACAAAGTTTTGATTTTCTA
B	GTACTTCATTTGAATATTAT	**TTAA**	CTGGGTCAAATCAATTGATT

The results of these injections supported the hypothesis that functional *piggyBac* transposase was being endogenously expressed in the germline of OX3885-D allowing it to act as a 'jumpstarter' for other transposons and confirmed the results of the RT-PCR. In lines C and E there was no evidence for active transposase being present. Unfortunately this precluded using them in germline remobilization assays that required an endogenous source of the transposase. However, the flanking transposons of these insertions were still potentially mobile, so it was decided to use an exogenous source of *piggyBac* transposase to attempt remobilization.

3.4.5 Germline remobilization of *piggyBac* transposons

Males of the OX3885-D line, which expressed *piggyBac* transposase in the germline, were outcrossed to wild type females over the course of ten generations (see Table 3.6). At each generation the progeny were screened at the larval stage for loss of either the 3xP3-DsRed or 3xP3-AmCyan markers contained with the flanking transposons. Progeny were also screened for individuals carrying only the 3xP3-regulated markers, which could arise from remobilization followed by reintegration (though this is expected to have a relatively low probability), and for unusual expression patterns of any of the markers, which could indicate movement of the transposons within the

genome. In total over 30,000 individuals were screened for evidence of germline remobilization in their parents. No such evidence for germline movement of the *piggyBac* transposons was observed and the expression profile of each of the markers was extremely stable over the screening generations.

Table 3.6: The number of 4th instar larvae of the OX3885-D line screened over ten generations.

Screening Generation	Number of Individuals Screened	Individuals lacking M1	Individuals lacking M2
1	973	0	0
2	576	0	0
3	1037	0	0
4	2671	0	0
5	3924	0	0
6	4601	0	0
7	4688	0	0
8	3913	0	0
9	5104	0	0
10	3222	0	0
Total	**30709**	**0**	**0**

Occasionally individuals with atypical fluorescent profiles were observed, and these were collected and crossed to wild type mosquitoes. Progeny of these crosses expressed the typical fluorescent profile without independent segregation of the markers suggesting that individual variation rather than transposon movement was responsible.

The remaining lines of the OX3885 construct did not express *piggyBac* transposase endogenously in the germline, and so capped *piggyBac* transposase mRNA was injected into embryos of these lines with the aim of remobilizing the flanking transposons. Injections into each of the lines yielded

approximately 1000 Go survivors from each set of injections, these were then outcrossed to virgin wild type mosquitoes (see Table 3.7).

Table 3.7: Results of injections of *piggyBac* transposase into embryos of the OX3885-C and OX3885-E lines. The number of G1s screened was estimated from taking a 1ml sample of the 500ml of water that each pool of G1 progeny was hatched in and counting the number of 1st instar larvae present, assuming a constant distribution of larvae.

Injection Line	Eggs injected	Go survivors	Approximate numbers of G1s screened (2 s.f.)
C	3665	995	21,000
E	4153	1051	18,000

The G1 progeny were screened for the loss of either of the flanking markers as was done for the OX3885-D line. Once again no evidence for germline remobilization was observed and all the progeny examined were positive for the markers lying within the flanking transposons and the central region of the OX3885 construct.

Visual screening for the loss of markers will have a failure rate associated with experimenter error meaning that, if the rate of remobilization is very low, then events may be overlooked with visual screening. For this reason PCR screening of progeny was also used to detect putative remobilization events. Primers specific to the flanking genomic sequence of each end of the insertion lines were designed and used in a multiplex-PCR with primers binding either within the flanking transposon or beyond its internal ITR (see Figure 3.9). The amplicon formed in the case of remobilization was, by design, the shorter of the two to ensure it was favored if present.

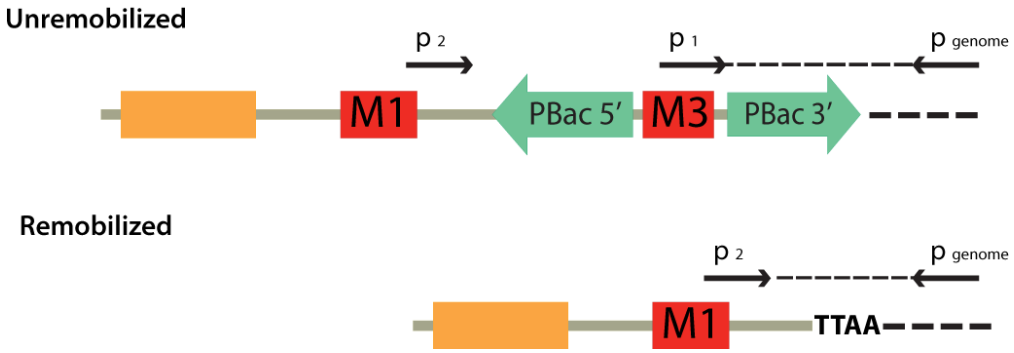


Figure 3.9: Schematic of the multiplex PCR reaction designed to test for remobilization of the flanking *piggyBac* transposons in lines of OX3885. Primer P_{genome} is specific for the flanking sequence of the transposon; primers P_1 and P_2 are specific to sequences within the flanking transposon and beyond its internal ITR respectively. If the transposon has remained in place the amplicon between P_{genome} and P_1 is favored if remobilization has occurred then the amplicon between P_{genome} and P_2 is favored.

DNA was extracted from ten pools of ten hemizygous individuals from the 8th generation of the OX3885-D line and from the G1 generation of lines OX3885-C and E injected with *piggyBac* transposase. This DNA was used as the template for the multiplex PCR experiment. The results of these PCRs are shown in Figure 3.10.

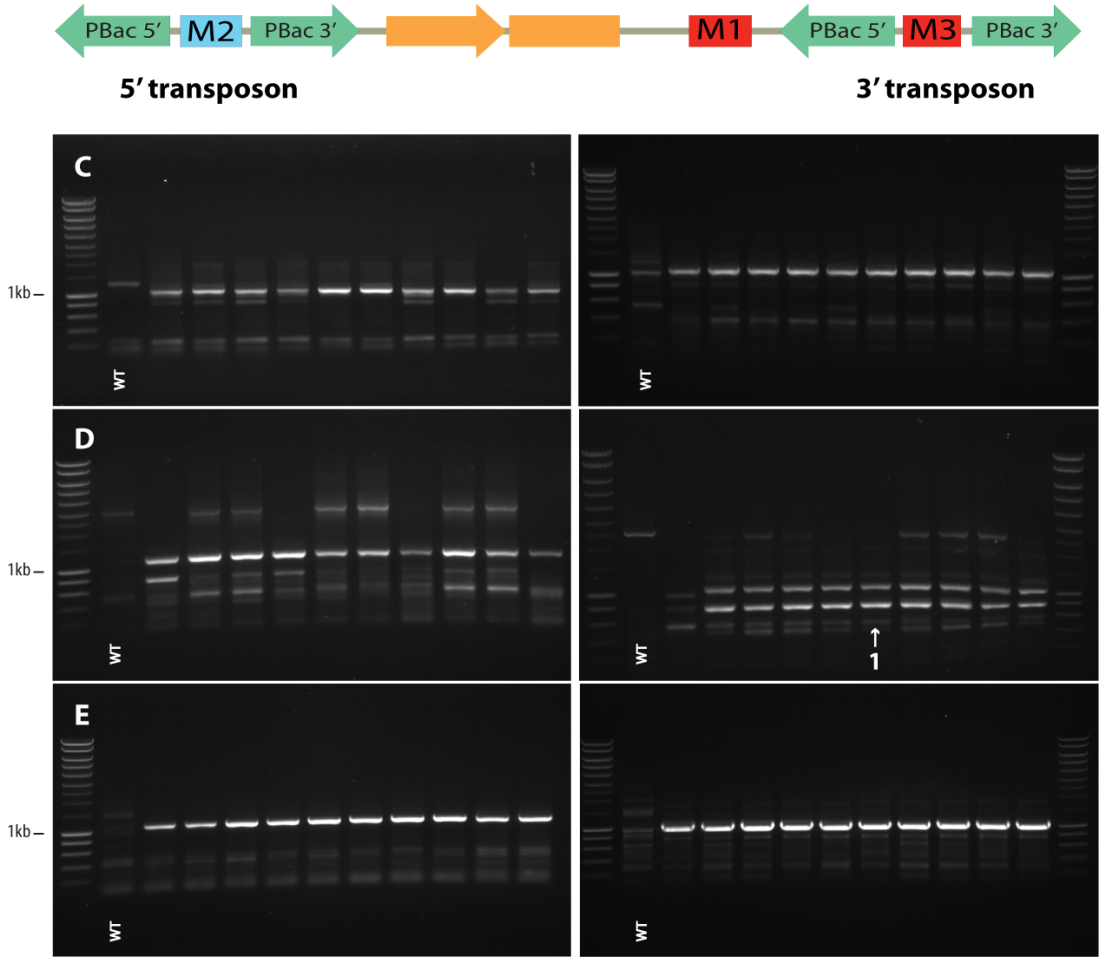


Figure 3.10: Multiplex PCR to detect germline remobilization of the 5'and 3' transposons of three OX3885 lines; C, D and E. DNA from 10 pools of 10 individual 3rd instar larvae from each line was used as the template for the multiplex reaction. The flanking and internal primers were designed such that in the case of remobilization a product of 300-500bp would be amplified. If no remobilization had occurred a product of 1000-1200bp was expected. Wild samples (WT) are included to show non-specific products of amplification. The arrow indicates the band resulting from potential movement of the 3' transposon in line D.

In general the multiplex PCR assay revealed no evidence of germline movement of the flanking transposons in the parents of the screened individuals. The primary amplicon identified in every reaction was the 1000-1200bp (depending on where the flanking primers for each junction of each line lay, see Table 3.2 for the exact product sizes) product expected from amplification of the stable ends. The OX3885-D line in particular produced many non-specific products

due to the highly repetitive region of DNA that it had inserted into, which was duplicated perfectly at least thirty times in the *Ae. aegypti* genome according to BLAST searches of the published genome (Lawson et al. 2009). Although most of the bands were not of the size expected for the amplicon produced by remobilization they were extracted and sequenced revealing that they were indeed due to non-specific binding of the primers rather than remobilization of the flanking transposons. One of the bands (band 1 in Figure 3.10) in the screen of the 3' transposon of the D line was of the 599bp size expected from remobilization. Extraction and sequencing of this band revealed that a TTAA site, indicative of canonical *piggyBac* transposition, formed the junction between the internal construct and genomic sequences. All of the pooled hemizygous individuals used in the screening of the OX3885-D line had been carefully examined for presence of all three markers. For this reason it was assumed that this band was not due to germline movement of the 3' transposon but rather due to somatic movement caused by non-specific expression of the *piggyBac* transposase in somatic tissues, which was previously observed in the RT-PCR analysis of *piggyBac* transposase expression the OX3885-D line. An alternative explanation is that remobilization had occurred followed by reinsertion into the genome at a different locus, resulting in the continued presence of all three markers. This was considered the less likely of the scenarios however, as the 599bp remobilization band was seen in all but two of the screened pools. Reintegration, if it occurs, is expected to be less common than simple excision as the probability of an excised transposon successfully searching for and inserting into a secondary TTAA site is likely to be low.

Furthermore separate segregation of the markers was never observed during the screening of progeny over ten generations, as would be expected from a even a moderate rate of reinsertion of one of the markers at a new and unlinked locus.

Previous studies of the germline stability of *piggyBac* transposons in *Ae. aegypti* have examined at most 20,000 individual progeny resulting from crosses of *piggyBac* transposon containing lines to 'jumpstarter' lines expressing the *piggyBac* transposase; or have examined the progeny of only 141 Go individuals injected with a source of transposase (O'Brochta et al. 2003, Sethuraman et al. 2007). The results presented here represent a much more extensive investigation of *piggyBac* transposons' potential for germline movement, though regrettably only the OX3885-D line expressed the transposase endogenously. The genomic context of transposable elements may have a great impact upon their mobility, and their potential for transposition can be greatly reduced by a closed chromatin structure of local DNA (Berg et al. 1991, Bancroft et al. 1993). It could be that the OX3885-D insertion was in a genomic location that inhibited remobilization, however the unusual insertion of the construct in this line should mitigate any such effect, for the 3' flanking transposon at least, since sequence from the plasmid vector surrounds the flanking transposons. This plasmid DNA should mask the transposons from local intrinsic factors that might suppress transposition, maintaining their activity (Esnault et al. 2011). Observation of somatic movement of the 3' transposon supports this conclusion.

3.4.6 Somatic remobilization of *piggyBac* transposons

The potential for somatic remobilization of the flanking transposons of the OX3885-C and E lines was also investigated. The Go individuals resulting from the transposase injections into the OX3885-C and E lines were screened for somatic movement after they had been mated to virgin wild type mosquitoes. DNA was extracted individually from twenty of these Go injection survivors, from each line, and the same multiplex PCR described in Section 3.4.5 was used to assay remobilization of the flanking transposons in somatic cells exposed to the injected *piggyBac* transposase. The results of these multiplex screens are shown in Figure 3.11.

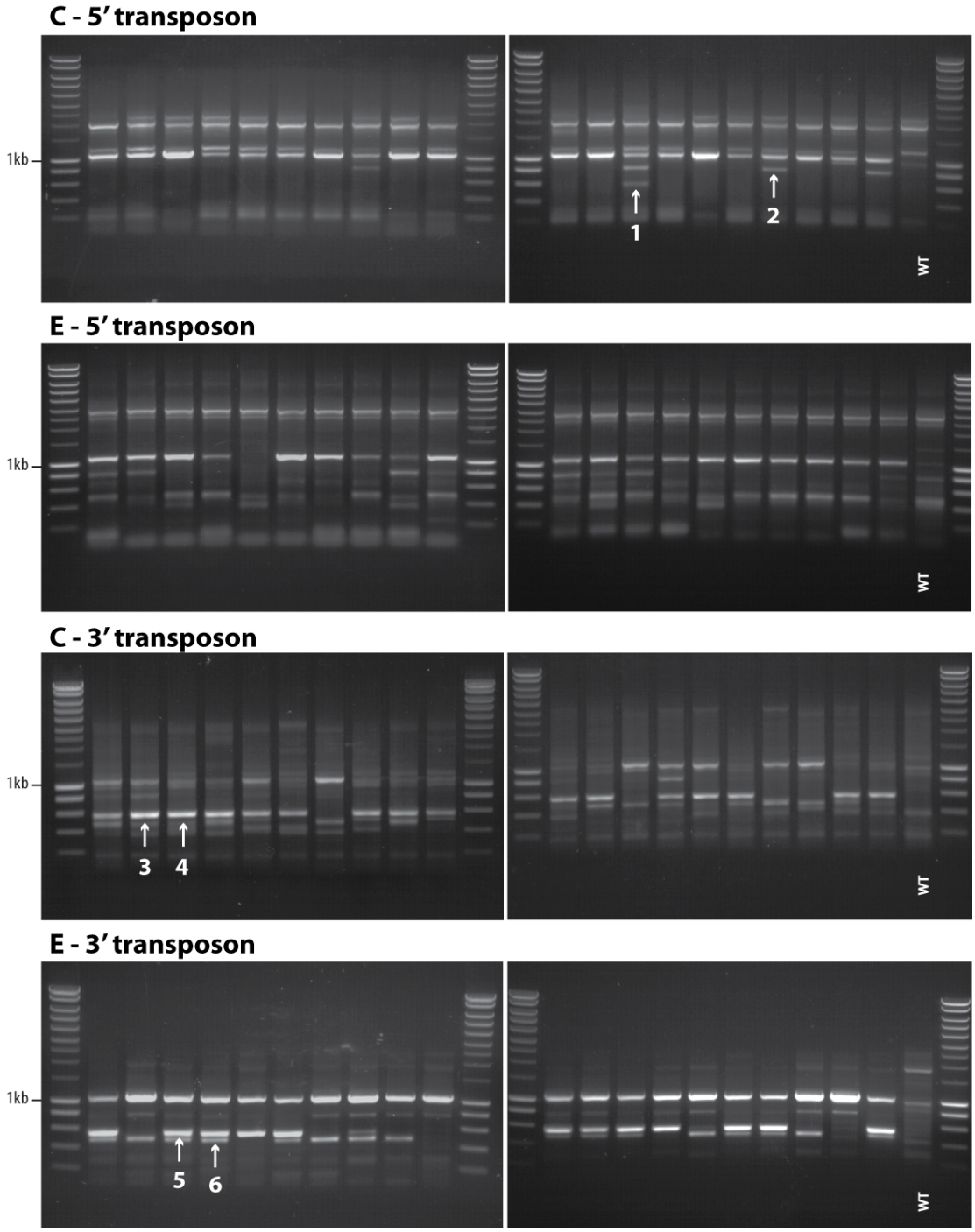


Figure 3.11: Multiplex PCR to detect somatic remobilization of the 5' and 3' transposons of the OX3885-C and E lines injected with *piggyBac* transposase mRNA. DNA from twenty individual G0 transposase injection survivors was assayed for remobilization. The flanking and internal primers were designed such that in the case of remobilization a product of 300-500bp would be amplified. If no remobilization had occurred a product of 1000-1200bp was expected depending of the particular transposon. Wild samples (WT) are included to show non-specific products of amplification. Arrows indicate bands that were extracted and sequenced to determine if they resulted from remobilization.

High salt concentration in the extracted DNA, due to the DNA extraction protocol, meant that unspecific amplification products were present in many of the multiplex reactions. These bands may also have been due to plasmid contamination (used as a template for the mRNA) in the injected *piggyBac* mRNA. In the screen of the 5' transposons no evidence for somatic remobilization was observed, with the ≈1000bp band, specific to the stable transposon, being the primary product. For the OX3885-C line two smaller bands were observed (bands 1 and 2 in Figure 3.11), these were larger than the 367bp amplicon expected from amplification of remobilization of the transposon but were extracted and sequenced. This revealed that they were due to non-specific binding of the primers rather than remobilization.

Screening of the 3' transposon showed an amplicon of the expected size (571bp for line C and 550bp for line E) for remobilization in 80% of the individuals from line C and 65% of the individuals from line E (bands 3, 4, 5 and 6 in Figure 3.11). Extraction and sequencing of these bands revealed that they were indeed due to remobilization of the 3' transposon. In each case this remobilization had formed a TTAA motif at the junction of the construct and genomic sequence indicating that it had occurred via canonical *piggyBac* mediated transposition (Mitra et al. 2008). The junction sequences recovered through sequencing of the 3' remobilization events are shown in Figure 3.12.

Figure 3.12: Junction sequences formed by remobilization of the 3' transposon in the three lines (C, Dand E) of the OX3885 construct. A central TTAA tetranucleotide is formed between the internal construct sequence and the flanking genomic sequence as expected from *piggyBac*-mediated transposition. Due to its unusual insertion the 'genomic sequence' for the D line originates from the plasmid vector.

These results demonstrate that *piggyBac* transposons can remobilize in somatic cells of *Ae. aegypti* when provided with either an exogenous or endogenous source of transposase. The 3' transposon was consistently observed to remobilize in somatic cells in all three lines of the OX3885 construct suggesting that this ready post-integration mobility was not an artifact of position effect but rather a general property of the transposon. Interestingly the 5' transposon was never observed to remobilize. This is unlikely to be due to position dependent inactivation of the transposon considering that the 3' transposon was capable of movement and that such inactivation would require all three independent insertions to have transposed into regions of the genome inimicable to remobilization. It is possible that the sequence structure of the OX3885 plasmid itself was responsible for inactivation of the 5' transposon. However, this seems unlikely considering that an identical construct design was previously employed by Dafa'alla et al. (2006) who demonstrated efficient germline remobilisation in the Medfly, *Ceratitis capitata*, in multiple genomic contexts.

3.5 Discussion

Using transposons as the vectors for engineered genetic constructs allows for the creation of engineered mosquitoes that have great potential for the control or replacement of vector populations. *Ae. aegypti* is a particularly attractive target species for these approaches since it is readily transformed with gene vectors such as *piggyBac* transposons (Kokoza et al. 2001). Despite its potential, regulation of this novel technology is still undeveloped in many parts of the world, often where such releases are likely to occur (Lavery et al. 2008, Marshall 2010). While there are many criteria for a potential release strain to meet before an open field release can occur one of the most important is that the engineered construct is stable within the genome of the mosquito (Beech et al. 2009, Touré et al. 2004). Germline stability is important to ensure that the construct remains at the characterized genomic locus within the engineered mosquitoes during mass rearing and, if population replacement is intended, within the field population (Sinkins et al. 2006). This ensures that the properties conferred by the construct, such as refractoriness or lethality, are predictable and reliable. Movement of a transposon could result in inactivation of a refractory gene, rendering population replacement futile. Similarly, inactivation of a lethal gene could result in releases of vector competent females, increasing the size of a disease transmitting population rather then suppressing it. Somatic stability is important to allay fears about the horizontal movement of transgenes from released engineered mosquitoes to other organisms (Macer 2005). Horizontal transfer to viruses or intracellular bacteria is of particular concern and transgenes could theoretically be transferred to

these organisms if somatic movement of the transposon is possible. The probability of horizontal transfer of elements engineered into an insect genome has not been investigated. Studies on horizontal transfer from transgenic plants to bacteria have identified such events but the probability of such movement is thought to be extremely low (Droge et al. 1998, Kay et al. 2002). For horizontal transfer to occur a sequences of events, each with low probability, would have to occur. The first of these is that excision and then transposition of a transposon would need to occur, not into another locus in the insect genome but into the much smaller bacterial or viral genome. This would necessitate a source of the relevant transposase, which itself is only likely to be present due to a rare horizontal transfer event (since no strain would ever be intentionally released into the wild carrying an active transposase source, except for population replacement purposes). Finally, if transposition were to occur it would need to happen in such a way as to prevent a severe fitness penalty to the virus or bacterium. This is thought to be very unlikely and if transfer were to occur it has been supposed that the majority of transformants would simply die, with little chance of passing the transgenes onto any further generations (de Vries et al. 2005, Silva et al. 2004).

This chapter represents the most comprehensive study of the stability of *piggyBac* transposons in *Ae. aegypti* yet undertaken (O'Brochta et al. 2003, Sethuraman et al. 2007). *PiggyBac* transposons were found to be extremely stable in the germline when exposed to *piggyBac*. Screening of the OX3885-D line, which expressed transposase in the germline, revealed no evidence of

movement in over 30,000 individuals examined over 10 generations. Injection of an exogenous source of transposase into lines OX3885-C and E similarly revealed no evidence of movement in the germline, despite the fact that injected transposase mRNA is known to be extremely active in the germline of *Ae. aegypti* where it readily mediates transposition into the genome (Lobo et al. 2002). Somatic movement was found to readily detectable, the first time such remobilization has been observed in *Ae. aegypti* and contrary to the findings of Sethuraman et. al. (2007) who saw no evidence of *piggyBac* remobilization in the mosquito. Somatic remobilization of the 3' transposon was detected through multiplex PCR analysis in all three lines of the OX3885 construct, whether the transposase was provided endogenously or exogenously, though the 5' transposon remained stable.

It is impossible to prove that *piggyBac* is completely stable in the germline of *Ae. aegypti* using the methods described in this chapter, since its rate of remobilisation may be orders of magnitude less than that which can easily be screened for. However, the results presented here demonstrate that its post-integration stability in the germline is substantially greater than that observed in other species. In *D. melanogaster* germline remobilisation occurs at a high rate with around 70% of progeny (from adults expressing a transposase source) carrying a transposon at a new locus (Thibault et al. 2004b). In the mosquito *An. stephensi* germline remobilisation is also readily achieved with 10% of progeny having transposed elements (O'Brochta et al. 2011). This demonstrated stability of *piggyBac* transposons in the germline of *Ae. aegypti* makes them an

ideal choice for the creation of engineered mosquitoes for use in control programmes where stable insertion of a transgene is desirous. The only drawback is that this same stability prevents the use of methods that employ post-integration remobilisation to excise transposon ITRs around transgenes (Handler et al. 2004).

The readily detectable remobilisation in somatic cells suggests that the germline must have some specific protective mechanism against the remobilisation of transposons that is not active in somatic cells. Such protection against transposon movement in the germline has already been demonstrated in *D. melanogaster* and would clearly be advantageous for *Ae. aegypti*. Transposons may carry elements that can alter the expression of nearby genes, disrupt genes, or dramatically alter the chromatin structure of DNA (Feschotte 2008, Khurana et al. 2010). This can severely impact development of progeny and so systems that protect the hereditable genetic information in the germline are likely to be strongly selected for. What mechanism *Ae. aegypti* may use to suppress remobilisation of a class II DNA transposon (such as *piggyBac*) is unknown, but it is likely to involve complex, multi-component epigenetic silencing of the transposase (Zamudio et al. 2010, Feschotte 2008). The observed somatic remobilisation of *piggyBac* transposons by the transposase does potentially make such insertions vulnerable to horizontal gene transfer to other organisms, which may be of regulatory concern. Such transfer necessitates the presence of an active source of the transposase and in the work presented here this source was artificially

provided, but in nature it is likely to be extremely uncommon. However, *piggyBac* like elements have been identified in the mosquito *Anopheles gambiae* and horizontal transfer of class II DNA transposons has occurred in recent evolutionary history of mosquitoes so such concerns cannot be dismissed (Holt et al. 2002, Diao et al. 2011).

Chapter 4

Aedes aegypti Genome Engineering with Site-Specific Recombinases

4.1 Introduction

4.1.1 The site-specific integrase from phage ϕC31

Random or pseudo-random insertion of transgenes into the genome, for example by *piggyBac* transposase, has the drawback that expression of the transgene may be greatly influenced by local enhancer or suppressor elements. This may be a problem when tight control of regulation is required, such as for tissue or time specific expression of effector proteins. Site-specific engineering of the genome can minimize the impact of chromosomal position effects by inserting transgenes into previously characterized sites (Metzger et al. 1999, Thyagarajan et al. 2001b). The large serine recombinase, also called an site-specific integrase, from the *Streptomyces* phage ϕC31 has become a widely tool for such site-specific integration in numerous species (Groth et al. 2004a, Thyagarajan et al. 2001a, Belteki et al. 2003). It promotes recombination between two attachment sites, *attP* and *attB*, which are different in sequence, sharing only a common TG nucleotide core about which recombination takes place (see Figure 4.1).

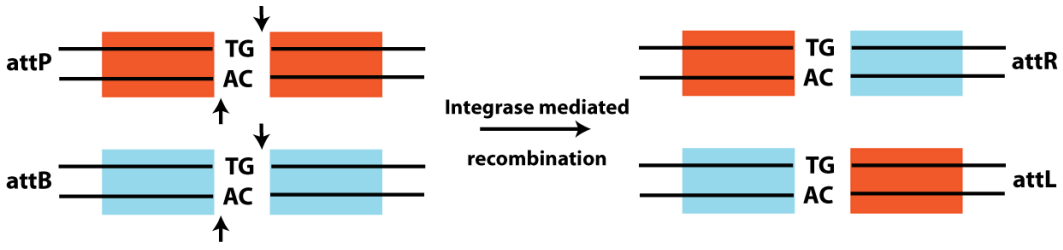


Figure 4.1: ϕC31 mediated recombination between *attP* and *attB* sites. Recombination occurs around the central nonpalindromic dinucleotide resulting in the crossover of the *attB* and *attP* half-sites forming the hybrid sites *attR* and *attL*.

This recombination is unidirectional rendering insertions inert to further exposure to a source of the recombinase except in the presence of accessory

factors found only in the bacteriophage, though these have yet to be identified for the ϕC31 integration system. The recombinase from ϕC31 has already been used to engineer *Aedes* mosquitoes because of its efficiency, site-specificity and post-integration stability (Labbé et al. 2010, Nimmo et al. 2006). However, a drawback of its use for the insertion of plasmid constructs is that recombination between the two attachment sites results in the insertion of unwanted sequence into the genome. Such extraneous sequence; including the donor-plasmid backbone, antibiotic resistance genes and transformation markers; may perturb the expression of the transgene, or lead to additional fitness costs of transgenesis (Marrelli et al. 2006b). Additionally, incorporation of any functional sequence that is not directly involved in obtaining the desired transgenic phenotype may be undesirable from a regulatory perspective, where the minimum amount of exogenous sequence possible is likely to be preferred (Benedict et al. 2008). This is particularly important in the case of antibiotic resistance genes whose introduction into the environment is considered undesirable due to the risk of horizontal gene transfer (Daniell et al. 2001).

4.1.2 Bxb1 and ϕRV1 site specific integrases

The site-specific integrase from the phage ϕC31 has proved to be functional in a wide variety of model organisms and so has been widely employed. There are many other potential bacteriophage recombinases that could prove to be useful tools for genome engineering. Two such large serine recombinases are the site-specific integrases from the mycobacteriophages Bxb1 and ϕRV1 that integrate their genomes into those of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* respectively (Ghosh et al. 2003, Bibb et al. 2005). The 500-residue

Bxb1 integrase is capable of site-specific integration into plant, mammalian and plasmodium genomes (Yamaguchi et al. 2011a, Crabb et al. 2007, Yau et al. 2010). Recently it was shown to be functional in *D. melanogaster*, promoting integration with high efficiency (Huang et al. 2011). The 469-residue site-specific integrase from ϕRV1 has been used successfully in mammalian cells to mediate efficient site-specific integration but has not yet been tested in any insect model (Keravala et al. 2005). As members of the large serine recombinases family of enzymes, the Bxb1 and ϕRV1 integrases promote integration in a mechanistically identical fashion to ϕC31 integrase, with a conserved 150-residue N-terminal domain vital for catalytic activity. Bxb1 integrase promotes recombination between *attP* and *attB* sites at a central 5'GT dinucleotide site. The minimum size of these sites is 48 and 38bp respectively and they posses only a single recombinase binding site (Ghosh et al. 2003). The common core of the ϕRV1 recombination sites is a 5'-TG dinucleotide and the minimal sizes for the *attP* and *attB* sites are 40 and 52bp respectively. As is the case for ϕC31 integrase, recombination is unidirectional except in the presence of a phage-encoded 'recombination directionality factor'. In the case of the ϕRV1 integrase this factor has been indentified as a 28 kDa protein called gp47 (Ghosh et al. 2006).

A characteristic of using phage recombinases in genome engineering is that in general the target site is destroyed after integration, being converted to *attR*/*attL* sites in the case of the site-specific integrases. Hence, after insertion of a plasmid construct, further rounds of integration are not possible. Addition

of multiple attachment sites recognized by a single recombinase onto a plasmid greatly favors intra-plasmid recombination rather then integration (Bischof et al. 2007). For this reason the identification of novel site-specific integrases active in *Ae. aegypti* will be of great use; for there is little homology between the sequences of the attachment sites of different integrases, preventing the possibility of undesired recombination events. A combination of the integrases from Bxb1 and ϕC31 has recently been used for successive integration into *D. melanogaster* for the purpose of creating knock-in alleles (Huang et al. 2011). The R4 serine recombinase has also been shown to be active in *Aedes* cell lines for the purpose of successive integration techniques, though its germline activity has not yet been demonstrated (Chompoosri et al. 2009).

4.1.3 Recombinase mediated cassette exchange

Recombinase mediated cassette exchange (RMCE) is a sequence conservative, site-specific method for genome engineering. This can be achieved by using inverted pairs of recombinase attachment sites flanking a cassette containing the sequence to be inserted (see Figure 4.2). This cassette may be interchanged with another, flanked by the complementary attachment sites, that has been pre-inserted into the genome, in the presence of the recombinase (Baer et al. 2001).

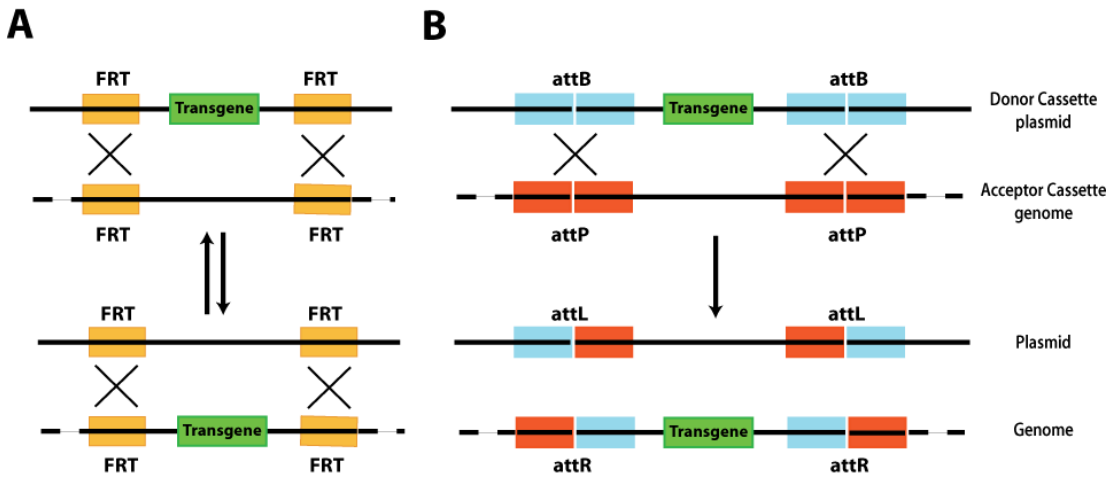


Figure 4.2: Recombinase mediated cassette exchange (RMCE). A: Recombination between FLP recombinase FRT sites, results in exchange of the cassettes and reformation of the FRT sites; making the recombination reversible. B: Recombination between the ϕC31 recombinase attachment sites *attP* and *attB* results in irreversible exchange of the cassettes flanked by the sites, insertion of the transgene of interest, and the formation of the *attR* and *attL* sites. Adapted from Bateman et al. (2006).

Because only the cassette of interest is inserted; no extraneous sequence need be included in the final insertion. This sequence conservative integration makes RMCE an attractive strategy for site-specific genome engineering of mosquitoes intended for field release.

The recombinases that are widely used for RMCE are the Cre and FLP recombinases from Bacteriophage *P1* and the yeast *Saccharomyces cerevisiae*. These promote recombination between the short nucleotide sequences loxP (34bp) and FRT (48bp) respectively. Unfortunately, since the recombination reaction is reversible in the presence of these recombinases, the rate of RMCE is extremely low. Furthermore, recombination is hampered by the thermodynamically disfavored insertion of any donor cassette larger than the acceptor cassette, caused by the decrease in entropy this reaction necessitates (Baer et al. 2001). As such, Cre and FLP recombinases are poor choices for the

creation of efficient and stable RMCE systems. Their reversibility may also make them undesirable from a regulatory perspective.

ϕC31 site-specific integrase can overcome the problem of poor integration efficiency encountered by reversible recombinases because the *attL* and *attR* sites formed after recombination are inert to further recombinase exposure. An efficient RMCE system using ϕC31 integrase was developed by Bateman et al. (2006) to allow for negative-marker selection of transgenic *D. melanogaster*. However RMCE has yet to be demonstrated in mosquitoes, or indeed any pest insect.

4.1.4 Recombinase mediated integration into pseudo-sites

A disadvantage of the use of site-specific integrases is that at least one of the attachment sites must have been previously inserted into the genome. This is normally accomplished by using a transposon vector to insert an *attP* integrase attachment sites into the genome (Labbé et al. 2010, Nimmo et al. 2006, Bischof et al. 2007). This has the advantage that transposition is pseudo-random, allowing attachment sites to be inserted into a diversity of genomic locations which may be useful in enhancer trapping studies or for insertions on particular chromosomes (Ryder et al. 2003). The disadvantage is that a transposon vector must be used, which increases the requirement for extraneous sequence and may mean that any post-integration movement of the transposon will affect the stability of the attachment site. This issue can be overcome with the use of so called pseudo-integration. This relies on the imperfect recognition of endogenous genomic sequences by the integrase enzyme, promoting recombination between these so-called pseudo-attachment

sites and the wild-type attachment site. Such pseudo-integration is rare, occurring in *D. melanogaster* and *Ae. aegypti* at a rate an order of magnitude smaller than normal integration in the case of ϕC31 integrase (Nimmo et al. 2006, Bischof et al. 2007, Huang et al. 2011). Pseudo-*attP* sites are the most common sites for ϕC31 integrase mediated pseudo-integrations to occur, and have been identified in the genomes of a diverse range of model organisms (Bischof et al. 2007, Allen et al. 2005, Chalberg et al. 2006, Ma et al. 2006). This is likely due to the higher sequence specificity requirement of *attB* sites whose structure guides formation of the reactive tetramer that promotes recombination (Gupta et al. 2007). In the case of ϕC31 integrase pseudo-*attP* sites, the core motif can share as little as 24% similarity to the wild-type *attP* site, however there is a strong correlation between the efficiency of integration in pseudo-sites and the presence of a palindromic sequence motif similar to that found in the wild-type *attP* site (Thyagarajan et al. 2001b, Chalberg et al. 2006). ϕC31 integrase-mediated pseudo-integration has been proposed as a method for delivery of gene therapies and it is estimated that around 370 pseudo-*attP* sites exist within the human genome (Chalberg et al. 2006, Ginsburg et al. 2005). ϕC31 integrase pseudo-sites have also been used to integrate into the *D. melanogaster* genome. Constructs bearing wild-type *attP* sites were successfully integrated into these pseudo-sites, making them targets for successive rounds of recombination; however, the efficiency of integration was ~27 times lower than that observed for wild-type sites (Bischof et al. 2007).

The *Ae. aegypti* genome is approximately 40% of the size of the human genome which should mean fewer potential pseudo-*attP* sites; however, there should be >100 potential sites available for pseudo insertion by ϕC31 integrase, assuming linear scaling with genome size. Potentially, integrase mediated pseudo-integration into the mosquito genome could be an effective method of genetic transformation, reducing the need for transposons and concern about their stability. Multiple site-specific integrases could be used to pseudo-randomly insert the attachment sites of other integrases into the genome in a semi-random fashion; allowing for successive, site-specific and irreversible transposon-free transformation.

4.2 Experimental aims

The experiments described in this chapter were conducted with the aim of designing new systems for the site-specific and sequence conservative engineering of the *Ae. aegypti* genome. The three main objectives were:

- To establish whether ϕC31 integrase could be used to mediate RMCE in *Ae. aegypti* efficiently.
- To determine if the integrases from mycobacteriophages Bxb1 and ϕRV1 were able to promote recombination between their specific attachment sites in *Ae. aegypti*.
- To examine whether it was feasible to use pseudo-integration into the *Ae. aegypti* genome as a method for engineering the species, and if the use of multiple integrases would allow for the development of a transposon-free transformation methodology.

4.3 Methods and Materials

4.3.1 Capped mRNA transcription

The T7 expression plasmids for mRNA generation were synthesized by creation of a master expression construct with *NotI* and *FseI* restriction enzyme sites downstream of the T7 polymerase promoter. These cloning sites were flanked by the *Ae. aegypti nanos* gene 5' and 3' UTRs. The *nanos* gene expresses its product in the developing germline cells and it was hoped that its UTRs would localize the expression of the integrase protein to this tissue (Adelman et al. 2007). This master construct was created by digestion of the mRNA expression plasmid OX3947, previously synthesized at Oxitec Ltd., with restriction enzymes *BglII* and *EcoRI*. Into this 5211bp fragment were ligated two PCR products amplified from the OX3947 plasmid with primer sets; 3947-entire-NheI-F with 3947-outer-1 (product: 1240bp), and 3947-entire-SpeI-f with 3947-outer-2 (product: 418bp). The individual integrase expression constructs (OX4460 and OX4504) were synthesised by amplification of the Bxb1 or ϕRV1 integrase genes from plasmids; pMA1 and pLB45, obtained from the Hatful lab (Pittsburgh university). These are both pET21a derivatives used for expression of the integrases, as described in Ghosh(2006) and Bibb(2005). The primers used for amplification were PhiRV1-Int-Diag1 with PhiRV1-Int-Diag2 and Bxb1-int-F with Bxb1-int-R. These constructs were then purified using the EndoFree Plasmid Maxi kit (Qiagen, Germany) and then used to transcribe Bxb1 and Rv1 integrase capped mRNA as described in section 2.2.1.

The Bxb1 transcription construct, OX4596, that had the *D. Melanogaster* germline specific *vasa* gene 3' UTR appended to the Bxb1 integrase coding

region, was synthesized by amplification of the Bxb1 integrase gene with primers bxb1-5'-kpn1 and bxb1-3'-avrII. This PCR amplicon was then cloned into the *KpnI* and *AvrII* sites of the OX3081 construct described in section 2.2.1.

4.3.2 Construct synthesis

The acceptor construct for ϕC31 RMCE, OX4372, was synthesized based on the design of Bateman et. al. (2006). The 221bp ϕC31 *attP* site from the pBac[3xP3-ECFPaf]-*attP* plasmid described by Nimmo et. al. (2006) was amplified with primers *attP*-ApaI and *attP*-XhoI, mutating restriction sites onto the ends of the fragment. This fragment was digested with *ApaI* and *XhoI* restriction endonucleases and cloned into the *ApaI*/*XholI* sites, at the 5' end of the 3x3P-DsRed cassette, of the pBac[3xP3-DsRed] plasmid (obtained from Oxitec Ltd.). A second inverted *attP* site was then cloned into the *SwaI*/*EagI* sites of this intermediate, at the 3' end of the 3x3P-DsRed cassette, using the primers *attP*02-EagI and *attP*02-blunt, followed by digestion with *EagI* and *DpnI*. The donor plasmid, OX4312, was synthesized by amplification of a 310bp *attB* site from the pB*attB*[3xP3-DsRed2nls-SV40]lox66 of Nimmo at al. (2006) with the primers *attB*-PacI and *attB*-HpaI. An Hr5*ie*1-AmCyan marker cassette was amplified from the plasmid OX3861 (Oxitec Ltd.), using primers 3861-markerF-PacI and 3861-markerR-ApaI. The *attB* and marker fragments were then ligated into the *HpaI* and *ApaI* sites of a 3635bp fragment of plasmid OX3636, which contained a second *attB* site, inverted relative to the first.

The Bxb1 and ϕRV1 *attP*-containing construct, OX4619, was created by synthesis of the two integrase *attP* sites by Geneart (Germany). These recombination sites were cloned into the 6000bp fragment created by digestion of the OX4372

fragment with the *XhoI* and *FseI* restriction endonucleases. Into the *NotI* and *AscI* sites of this intermediate was cloned the 2403bp Hr5*ie*1-DsRed-Sv40 3'UTR marker cassette from the existing construct OX4513. This resulted in the marker cassette flanked by the ϕC31 *attP* site on one side and the Bxb1 and ϕRV1 *attP* sites on the other, the sites orientated for RMCE.

The transposon free insertion construct, OX4592, was synthesized by insertion of the marker cassette from OX4372 into a vector containing the *attB* sites specific to Bxb1 and ϕRV1 integrase. Briefly, Ox4372 was digested with the *SalI*, *PacI* and *BglII* restriction enzymes and the 1501bp fragment blunt ended with Klenow Polymerase (New England Biolabs, UK). This fragment was ligated into the similarly blunted *KpnI* site of the *attB* vector synthesised by Geneart (Germany). Into the *BssHII* site of this construct, at the opposite end of the marker cassette, was cloned a 387bp fragment containing a ϕC31 integrase specific *attP* site from the OX4338 construct.

4.3.4 PCR analysis of RMCE in the lines of OX4372

To test for exchange of the AmCyan and DsRed cassettes in the OX4372 line injected with the OX4312 construct, primers DsRed-5' and DsRed-3', internal to the two cassettes, were designed to anneal to the 5' and 3' ends of the DsRed cassette (relative to the ORF of the DsRed gene) and AmCyan-5' and AmCyan-3' similarly for the AmCyan cassette. Primers specific to the attachment sites: *attP* (*attP*-Diag1) and *attR* (*attR*-Diag1) were then designed to bind over the central TG motif of the sites. PCR amplification between the attachment site-specific primers and the cassette specific primers was then preformed using DreamTaq polymerase (Fermentas, UK) to determine whether recombination

had occurred in transformed individuals. PCR reactions were carried out with the primers at a final concentration of 0.2µM μl^{-1} and betaine at a final concentration 1µM μl^{-1}. Thermo cycling was performed with an initial activation step of 95°C for two minutes, followed by 3 cycles of 95°C for 30 seconds, 45°C for 30 seconds and 72°C for 90 seconds. This was followed by 3 cycles of 95°C for 30 seconds, 49°C for 30 seconds and 72°C for 90 seconds. The remaining 29 cycles were carried out at 95°C for 30sec, 52°C for 30 seconds and 72°C for 90 seconds.

The nucleotide sequence of the *attR* sites formed after RMCE were determined by amplification with primer pairs *attR*-Diag2/ *attR*-Diag3 or *attR*-Diag4/ *attR*-Diag5, depending on their orientation. Amplification products were then cloned in the Pjet2.1 vector and sent for sequencing with a Pacific Biosciences PacBio RS platform.

4.3.5 PCR analysis of Homologous recombination

The potential rate of inversion due to homologous recombination between the *attR* sites in the final recombined line was investigated. For each line, genomic DNA was extracted from 100 3rd instar larvae, in pools of ten individuals using the GeneJET Genomic DNA Purification kit (Fermentas, Lithuania) according to the manufacturers protocol. Primers specific to the *piggyBac* 3' ITR (*attR*-Diag6), the Sv40 3' UTR of the marker cassette (*attR*-Diag7) and the Hr5*ie*1 promoter region (*attR*-Diag7) were designed so that upon inversion a 525b product would be amplified as opposed to a 888bp amplicon for the stable sequence. Multiplex PCR was conducted on the pooled mosquito gDNA using DreamTaq polymerase (Fermentas, UK) with an identical reaction mix and

thermocycling conditions to those described in section 4.3.4. gDNA from wild-type mosquitoes was also included in the analysis as a control for non-specific amplification products.

4.3.6 PCR analysis of RMCE in the lines of OX4619

Testing for exchange of the DsRed and AmCyan cassettes after injection of the OX4678 construct into the OX4619 construct was performed in a similar manner to that described in section 4.3.4. Primers, C31-sitediag01 and Bxb1-sitediag01, specific to the outer half-sites (those that would form the *attR* sites upon recombination) of the ϕC31 and Bxb1 sites were designed. Primers specific to the DsRed and AmCyan cassettes were also designed. These were C31-DsRed-01 and C31-AmCyan-01 for the 5' end of the cassettes (relative to the ORF of the marker gene) and Bxb1-DsRed-01 and Bxb1-AmCyan-01 for the 3' end of the cassettes. These sets of primers were then used to amplify the *attP* or *attR* sites of the OX4619 lines before or after recombination with OX4678 construct. DreamTaq (Fermentas, UK) was used for the amplification reactions and the thermocycling conditions are described in section 4.3.4.

4.3.7 Plasmid rescue of OX4592 insertions

5µg of DNA extracted from pooled larvae of each OX4592 insertion line was digested with 5 units of either; *BglII*, *FseI* or *RsrII* (NEB, UK) restriction endonucleases in a 100µl volume for 4 hours at 37°C. Cut DNA was circularized with one unit of T4 DNA ligase (NEB, UK), after dilution to 0.0081µg μl^{-1}, at 18°C for 12 hours. Circularized DNA was precipitated with an equal volume of propan-2-ol and resuspended in 10µl of water. This circularized DNA was then transformed into XL-10 competent cells (Stratagene, USA), using the

manufacturers standard transformation protocol, and the resulting ampicillin resistant colonies were purified using the GeneJET Plasmid Miniprep Kit (Fermentas, Lithuania) following the manufacturer's protocol. The purified DNA was progressively sequenced (GATC BioTech, Germany) using primers binding within the known sequence.

4.4 Results

4.4.1 Construction of Donor & Acceptor Constructs

To study the viability of RMCE in *Ae. aegypti* two simple genetic constructs were synthesized: an acceptor construct containing the *attP* attachment sites and a donor construct containing the *attB* sites (see Figure 4.3). To create the acceptor construct, OX4372, a marker cassette flanked by a pair of inverted *attP* sites from the *Streptomyces* phage ϕC31 was constructed. These *attP* sites were 221bp in length, consisting of the central TG crossover site required for recombination and approximately 100bp of upstream and downstream flanking sequence from the phage genome, identical to those used in Nimmo et al. (2006). The DsRed2 fluorescent marker gene was placed within this cassette, under control of the artificial eye-specific 3xP3 promoter (Berghammer et al. 1999). This marker cassette was then placed within a *piggyBac* vector to allow it to be inserted into the *Ae. aegypti* genome via *piggyBac* transposase-mediated transgenesis.

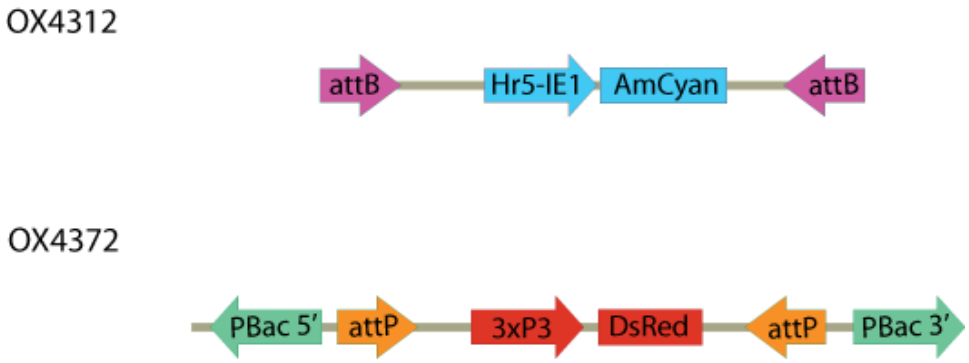


Figure 4.3: Schematic diagram of the acceptor (OX4372) and donor (OX4312) constructs for RMCE.

The donor construct, OX4312, was synthesized with two inverted 310bp *attB* sites, from the bacteria *Streptomyces lividans*. These recombination sites flanked a cassette containing the AmCyan fluorescent marker gene, driven by the Hr5*ie*1 promoter from the *Autographa californica* nuclear polyhedrosis virus. The enhancer-transactivator properties of this regulatory region have previously been shown to drive strong expression of fluorescent marker genes in the cuticle of *Ae. aegypti* (Fu et al. 2010).

The different fluorescent markers and promoters used in the donor and acceptor constructs, with their distinct spectral and expression profiles and tissue specificities were intended to allow for easy discrimination of cassette exchange events.

4.4.2 Generation of OX4372 acceptor lines

The acceptor construct, OX4372, was co-injected with *piggyBac* transposase capped-mRNA into embryos of *Ae. aegypti* (Go survival: 25%, transformation efficiency: >70%). Outcrossing of male G1 transformants to wild-type females resulted in three acceptor lines containing a single insertion of the OX4372

construct: OX4372A, OX4372F and OX4372I. Each of these lines showed strong expression of the 3xP3-DsRed marker in the optic nerves and anal papillae. Sequencing of the regions flanking the insertions was attempted using PCR amplification between nested primers within the known sequence of the 5' and 3' *piggyBac* recognition sequences and degenerate primers designed to bind randomly to the genome. For lines A and I the flanking sequences were recovered and indicated canonical *piggyBac* insertions at genomic TTAA tetranucleotide sites (see Table 4.1).

Table 4.1: First 20bp of the flanking sequences of the OX4372 lines recovered by genome walking. Highlighted TTAA motifs are the duplicated sites characteristic of *piggyBac* transposase mediated insertions. Flanking sequence of OX4372F was not recovered despite multiple attempts.

Line	5' flanking sequence	3' flanking sequence
A	TTTTGTGTAAGTTTTGTATT**TTAA**	**TTAA**GGAGTTCAACCTGCAAACCT
F	Not determined	Not determined
I	TCTATCAGTATACATTGGCC**TTAA**	**TTAA**ATCTAAAGAACTGCTACGAT

The BLAST algorithm was used to compare these flanking sequences to the *Ae. aegypti* Liverpool LVP strain genome sequence (Lawson et al. 2009). This showed 100% identity between the flanking sequences of the A and I insertions and intergenic regions of supercontigs 1.49 and 1.17 respectively. The genomic flanking sequence of line OX4372F could not be obtained using this method, with no amplification bands produced, or with the use of cassette PCR using double-stranded oligo adaptors (Tonooka et al. 2009). This suggested that in this line the transposon had inserted into a region that was extremely difficult

to amplify, such as one high in repetitive sequence structure (O'Hare et al. 2002).

4.4.3 ϕC31 Mediated Cassette Exchange in Lines of OX4372

The donor construct, OX4312, was injected into embryos of the three OX4372 acceptor lines along with ϕC31 recombinase capped mRNA. The average survival of the injected Go embryos to adulthood was 16%, compared to 25% and 23% obtained by Labbe et al. (2010) and Nimmo et al. (2006) for site-specific integration into *Aedes* mosquitoes, using an equivalent micro-injection protocol. G1 progeny of these injections were then screened for the presence or absence of the DsRed and AmCyan markers using fluorescence microscopy. Injections into the A and I lines resulted in potential germline recombination events, producing G1 offspring carrying the AmCyan marker (see Table 4.2).

Table 4.2: Efficiencies of ϕC31 recombinase mediated cassette exchange, between construct OX4312 and lines OX4372A, F and I. Fertile G0 individuals were assumed to comprise 50% of the individuals surviving the microinjection process.

Line	Eggs injected	Fertile Gos	Recombination Eff.(%)
A	1594	85	8.0
F	1450	121	0
I	2158	231	9.5

Microinjection into OX4372F did not result in any detectable recombination events despite a similar number of Go injection survivors to the other lines. Previous work in *D. melanogaster* has suggested that the efficiency of ϕC31 recombinase mediated recombination is dependent on the chromosomal

location of the attachment sites within the genome (Bischof et al. 2007). In *Ae. aegypti* attachment sites also display variable recombination efficiency (Nimmo et al. 2006). To catalyze recombination between its attachment sites, ϕC31 recombinase must form a tetrameric complex, each monomer composed of a 67kDa protein, around the *attP* and *attB* attachment sites (Thorpe et al. 2000, Grindley et al. 2006). The size and associated steric requirements of this tetrameric complex may mean that the secondary, tertiary and quaternary structure of the genomic sequence surrounding the attachment sites can have a profound effect on the ability of the recombinase to promote recombination. The inability to resolve the flanking sequence of the F lines using two different PCR-mediated genome walking techniques suggests that the F insertion was in a region of the genome rich in structure, which may have prevented both annealing of primers and recombination. Though, without further microinjection experiments it is impossible to say whether the F insertion is completely inert to recombination.

G1 transformants from the injections into line I showed cuticular expression of the AmCyan marker and no evidence of red fluorescence in the optic nerves (see Figure 4.4). These were named I + 4312. This suggested recombination between both pairs of attachment sites and complete inter-molecular cassette exchange. G1 transformants from the injections into line A showed both patchy cuticular expression of AmCyan and expression of DsRed in the optic nerves. These were named A + 4312 (intermediate). This combination of markers was indicative of incomplete cassette exchange, due to only a single pair of

attachment sites recombining. Embryos of this partially recombined line were injected with additional ϕC31 recombinase mRNA. This resulted in a second recombination step and G1 progeny (transformation efficiency: 37%) that showed no expression of the DsRed marker and much brighter expression of the AmCyan marker in the cuticle. These were named A + 4312 (complete). The loss of the DsRed marker suggested that intra-molecular recombination had occurred between the remaining set of *attB* and *attP* sites, resulting in complete cassette exchange, through an intermediate step. The much higher transformation efficiency of the second round of injections was also indicative of recombination between two attachment sites in close proximity, where the recombinase has a far smaller search volume of potential sites to contend with.

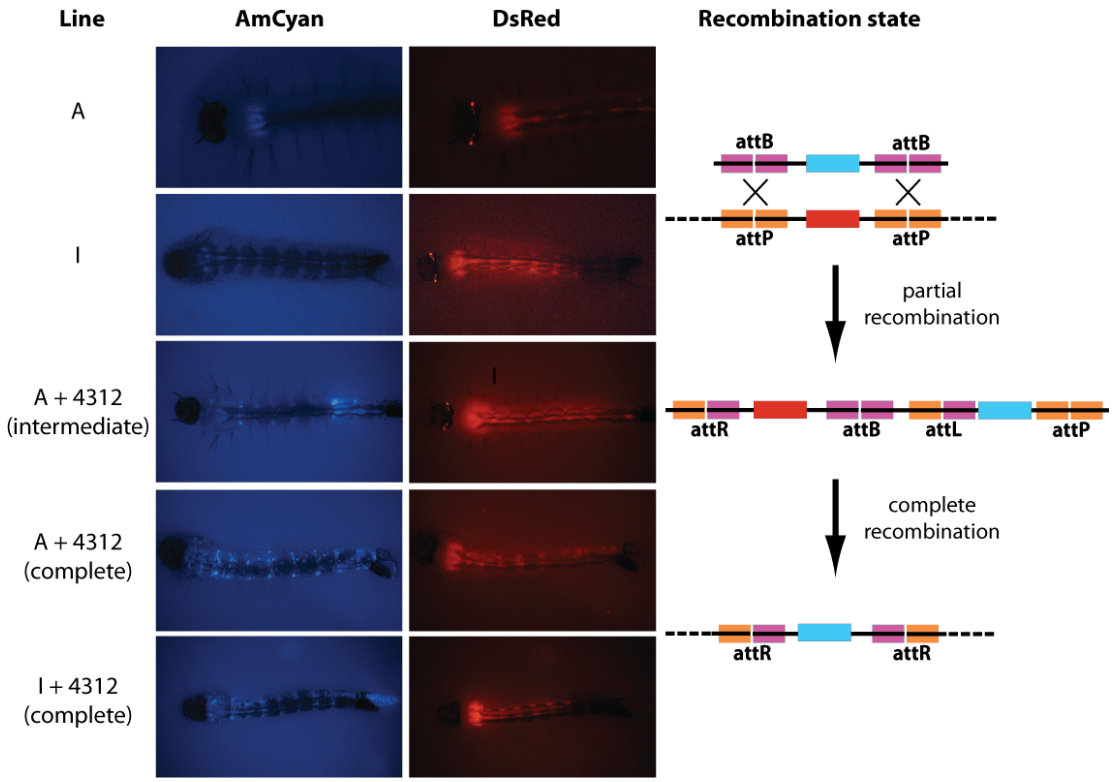


Figure 4.4: 4th instar larval expression of the fluorescent reporter genes AmCyan and DsRed and schematic diagram of recombination between constructs OX4372 and OX4312. In lines of OX4372 before and after ϕC31 mediated recombination with construct OX4312. Lines A and I show only DsRed expression in the optic nerves. After recombination of one set of attachment sites A + 4312 (intermediate) shows expression of both reporter genes. After recombination of both sets of sites in lines A and I, and complete RMCE, only the AmCyan reporter was observed.

The recombination states of the 5' and 3' (relative to the AmCyan gene) attachment sites in the OX4372 lines before and after RMCE were confirmed using PCR. Primers specific to either the initial *attP* attachment site or the post-recombination *attR* sites were designed to anneal over the central TG motif. Amplification of the sequence between these primers and primers internal to the AmCyan or DsRed cassettes confirmed the progress of recombination suggested by analysis of the fluorescent markers (see Figure 4.5). The initial OX4372 lines showed no evidence of *attP*/*attB* recombination

at the 5' or 3' attachment sites, with only the expected bands for the *attP* sites being observed.

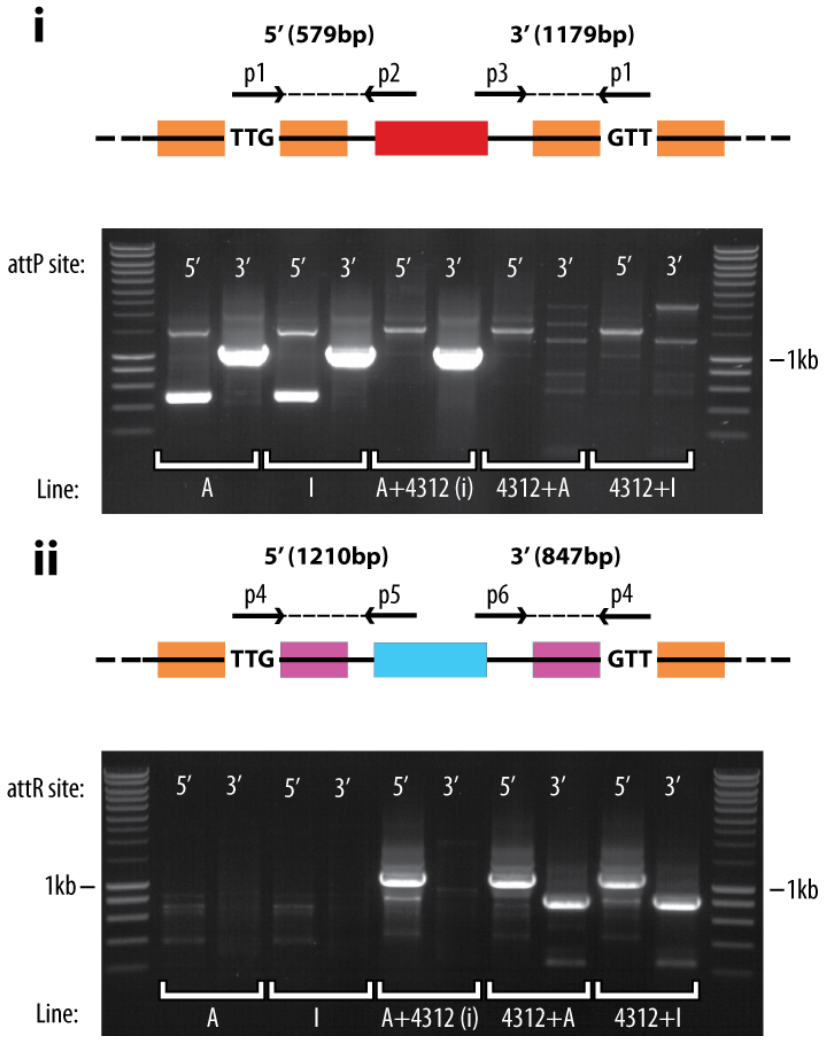


Figure 4.5: PCR amplification between, i: the 5' and 3' *attP* sites and the DsRed cassette in lines OX4372A and I; and ii, the 5' and 3' *attR* sites and the AmCyan cassette in the lines post-cassette exchange. Lines OX4372 A and I, the A + 4312 intermediate and the final A and I + 4312 lines were tested for the presence of the *attP*-DsRed junction, expected in the acceptor lines, or the *attR*-AmCyan junction expected in the recombined lines. The A + 4312 and I lines showed the formation of the *attR* junctions with loss of the *attP* junctions, expected from complete RMCE. The I + 4312 intermediate was positive for the 5' *attR* and 3' *attP*, indicative of recombination between one pair of attachment sites.

The A + 4312 (intermediate) line only produced a band for the 5' *attR* site, proving that incomplete recombination had occurred in this line. This was

confirmed by the presence of a band for the intact *attP* site at the 3' end of the insertion. The final A and I + 4312 (complete) lines showed bands for both *attR* sites; showing that complete RMCE has taken place in these lines, either via an intermolecular/intramolecular 2-step recombination or a single-step intermolecular recombination.

Amplification of the *attR* sites of the A + 4312 and I + 4312 lines with flanking primers, followed by sequencing of the product, revealed that the *attB*/*attP* attachment sites had recombined around the central TG dinucleotide core to form *attR* sites typical of ϕC31 recombinase-mediated recombination (Groth et al. 2000).

4.4.4 Stability of the inserted cassettes

The *attR* attachment sites resulting from complete RMCE are potential sites for homologous recombination, particularly during meiosis in the germline (Shiroishi et al. 1995). The sites are inverted so any recombination should result in inversion of the intervening sequence with no loss of the cassette or the transgene it contains (Bollag et al. 1989). Homologous recombination should not therefore affect the stability of a transgene inserted using RMCE. However inversion could alter the influence of nearby enhancer elements on the transgene by changing the relative orientation of the cassette. As such an understanding of the rate of inversion was considered useful. Multiplex PCR primers, internal and external to the AmCyan cassette, were designed such that were inversion to occur, a smaller amplicon would be produced by the reaction

(see Figure 4.6). One hundred 3rd instar larvae of lines A and I + 4312 were tested for inversion events in pools of 10 individuals.

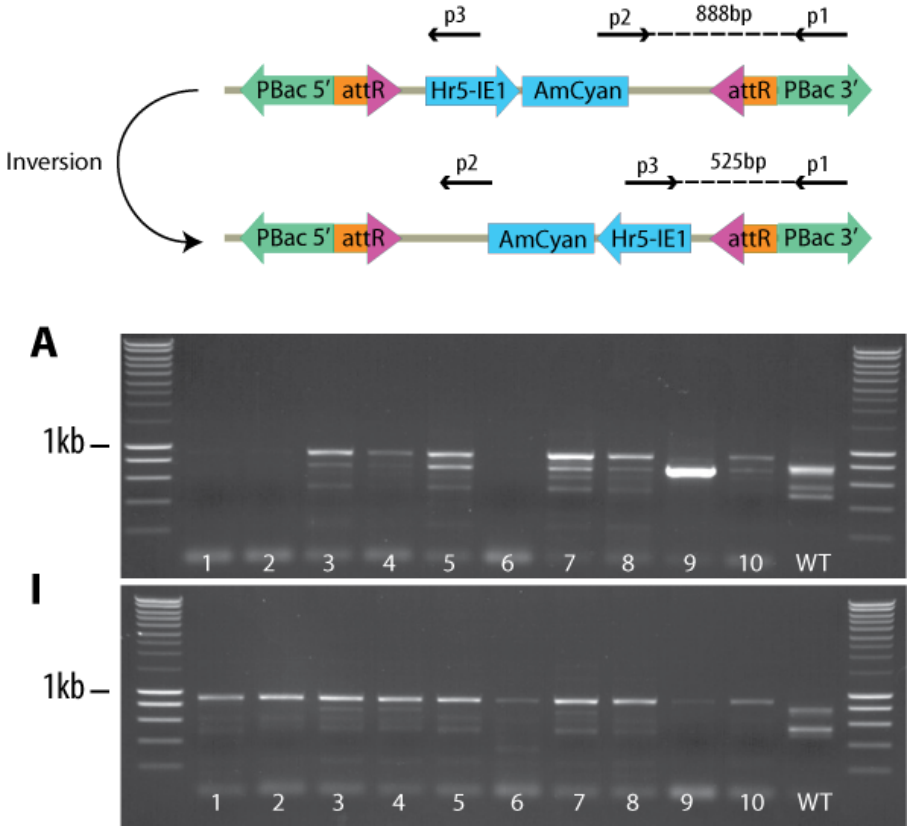


Figure 4.6: Multiplex PCR amplification of potential inversion events in lines A and I + 4312. 10 pools of 10 individuals from each of the lines with an inserted AmCyan cassette were tested for inversion events. Wild-type (WT) DNA was included for elucidation of non-specific binding of the primers. A 525bp amplification product was expected from any inversion events. Only the expected 888bp from the stable sequence was observed.

No bands of the expected size were observed in any of the pools suggesting that germline inversion due to homologous recombination was not occurring at a detectable rate in the germline of the insertion lines. Recombination rates may vary widely across the genome of an organism so with only three lines it is not possible to estimate the risk of inversion events between attachment sites in general (Nachman 2002). However, in the case of this limited screening of 100 individuals, inversion does not appear to be common.

4.4.5 Generation of Bxb1 and ϕRV1 *attP* acceptor lines

To determine if Bxb1 and ϕRV1 integrases could mediate recombination between their attachment sites in *Ae. aegypti*, genomic acceptor (OX4619) and plasmid donor (OX4678) constructs were synthesized (see Figure 4.7).

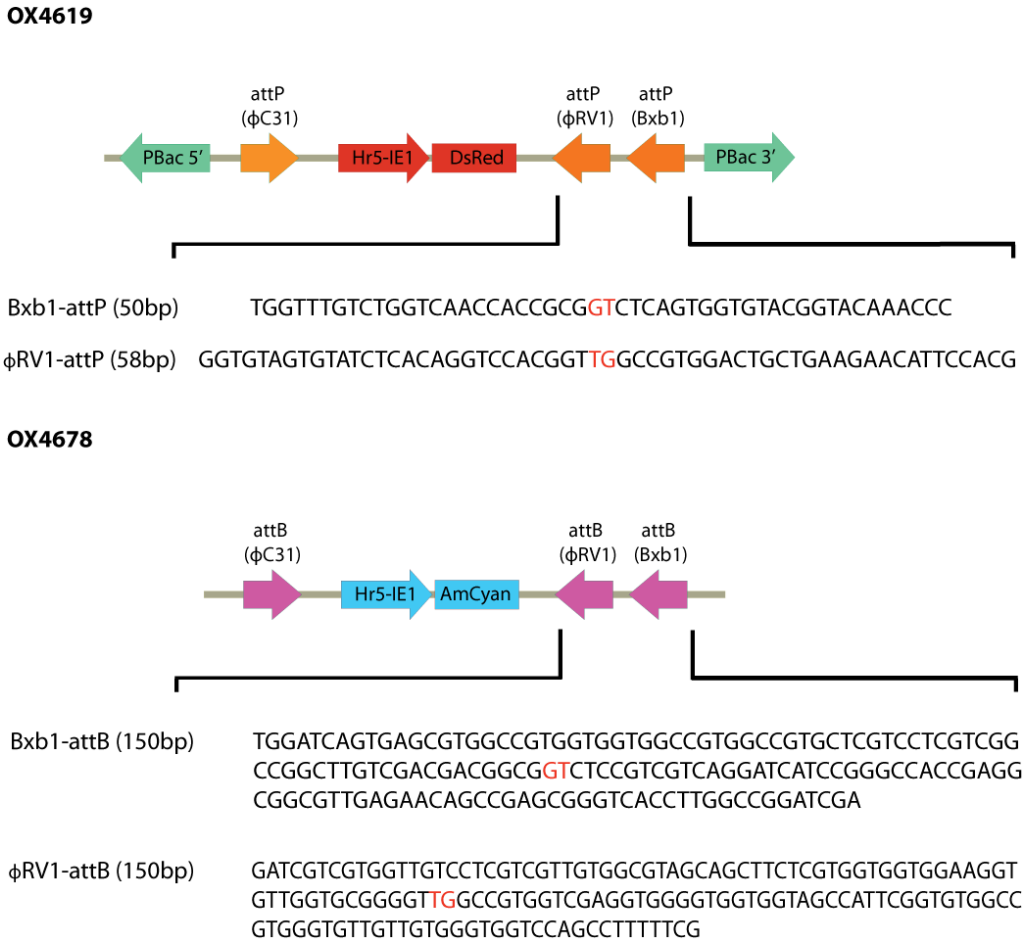


Figure 4.7: Schematic diagram of the acceptor (OX4619) and donor (OX4678) constructs used to test the recombination ability of Bxb1 and ϕRV1 integrases. The lengths and sequences of the attachment sites used are shown beneath each construct with the central dinucleotide highlighted in red.

These had a similar design to the OX4372 and OX4312 constructs (see section 4.4.1), with a ϕC31 att site at one end of a marker cassette and the Bxb1 and ϕRV1 att sites at the other. The other major change was the substitution of the 3xP3-DsRed marker for the Hr5*ie*1-DsRed in the OX4619 construct. The attachment sites were in such an orientation as to allow for RMCE. The *attP* fragments

used were 50 and 58bp respectively in length, symmetrical about the central dinucleotide. This is larger than the minimal sizes that have previously been shown to be active for both integrases (Ghosh et al. 2005, Bibb et al. 2005). The structure of the *attB* sites is thought to be essential for formation of the synaptic tetramer that promotes recombination, so a conservative 150bp of bacteriophage sequence was used for the BxB1 and ϕRV1 *attB* sites, 75bp upstream and downstream of the central dinucleotide. This is approximately 100bp larger than the minimally active sites for both integrases.

The OX4619 construct was successfully transformed into *Ae. aegypti*. Of the 1611 embryos injected, 28% survived to adulthood. These Go individuals were outcrossed to virgin wild-type and produced transformants with an efficiency >90%. This high transformation rate was likely due to the small size of the construct. Individual males from separate Go pools were used to establish five independent lines. Outcrossing G2 males to wild-type once more resulted in three lines carrying single insertions of the acceptor construct; OX4619-A, OX4619-B and OX4619-D. Each of these lines displayed typical expression of the Hr5*ie*1-DsRed florescent marker, shown in Figure 4.8.

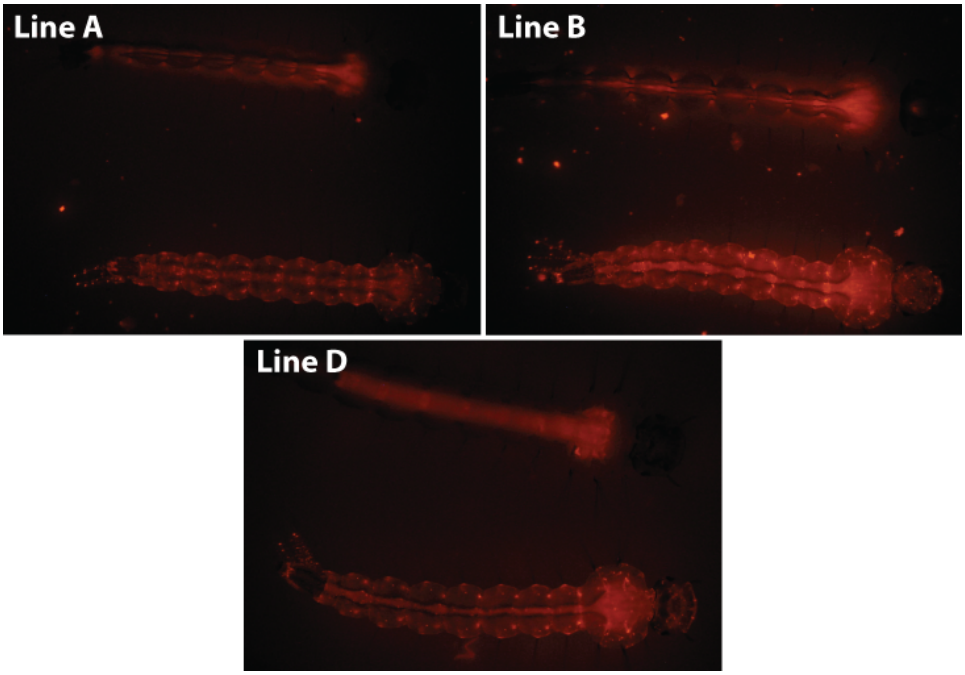


Figure 4.8: Expression of the DsRed reporter gene in 4th instar larvae of the three lines (A, B and D) of OX4619 (bottom of each image) compared to wild-type (top of each image). Expression of the Hr5*ie*1-Dsred reporter is clearly visible in the cuticle of transgenic individuals. Expression in line B was brighter than either line A or D.

The flanking sequences of these lines were obtained by genome walking with degenerate primers and confirmed that the lines resulted from canonical *piggyBac* mediated insertions at genomic TTAA sites. The flanking sequences are shown in Table 4.3.

Table 4.3: First 20bp of the flanking sequences of the OX4619 single insertion lines recovered by genome walking. Highlighted TTAA motifs are the duplicated sites characteristic of *piggyBac* transposase mediated insertions. The p value shown is the p statistic of the Chi-squared test with Yates correction to determine if segregation of the transformation marker (from N number of screened G3 progeny) differed significantly from the expected Mendelian inheritance of 50% for a single insertion.

Line	5' flanking sequence	3' flanking sequence	p
A	Not Determined	**TTAA**GCAAAACTGAATGACT	0.77 (N=144)
B	AGAGAGGAGCTGTGAC**TTAA**	**TTAA**GACACTAGCCGGACAG	0.41(N=69)
D	GTATCTTTCAGACCTC**TTAA**	**TTAA**AGAACACACTTAGAAG	0.51(N=112)

Comparison to the *Ae. aegypti* genome using the BLAST algorithm revealed that the B and D insertions had occurred in intergenic regions of the genome, and were located on supercontigs 1.38 and 1.412 respectively (Lawson et al. 2009, McGinnis et al. 2004). For the A line the retrieval of the 3' flanking sequence revealed only partial similarity to the putative AAEL000540 gene. The 5' sequence could not be recovered using either genome walking with degenerate primers or cassette PCR.

4.4.6 Activity of Bxb1 and ϕRV1 integrases in *Ae. aegypti*

The three OX4619 insertion lines –inbred so that >95% of progeny carried at least a single insertion – were injected with the donor plasmid OX4678 in the presence of either Bxb1 or ϕRv1 integrase capped mRNA. This mRNA was generated with the *Ae. aegypti* germline specific *nanos* gene 3' and 5'UTRs flanking the coding region of the integrase gene. Mariner transposase mRNA generated with these UTRs has previously resulted in greatly increased transformation rates in *Ae. aegypti* in the labs of Oxitec Ltd. (data not shown).

Injections with these sources of integrase failed to produce any transformants expressing the Hr5*ie*1-AmCyan marker of the OX4678 construct, despite a large number of injections as shown in Table 4.4. Injection of a similar number of embryos from each line was not always possible due to the low fecundity of lines B and D, which may have been associated with the insertions.

Table 4.4: The number of embryos injected, surviving G0s and the transformation efficiency of injections of the OX4678 construct into the three lines of OX4619 with either RV1 or Bxb1 integrase mRNA with the *nanos* gene UTRs.

Line	Integrase mRNA	Eggs injected	Gos	Recombination Eff.(%)
A	RV1	1464	366	0
B	RV1	914	94	0
D	RV1	685	60	0
A	Bxb1	1767	226	0
B	Bxb1	1283	89	0
D	Bxb1	1421	144	0

It was theorized that the absence of recombination was due to the presence of the *nanos* gene UTRs, either preventing correct transcription or misdirecting the expression of the protein. Therefore, a second mRNA transcription construct was created with the 123bp *D. melanogaster vasa* gene 3' UTR appended after the stop codon of the Bxb1 integrase (an equivalent ϕRV1 integrase was not made due to time constraints). This UTR appended to *piggyBac* transposase has previously been shown to be capable of producing high transformation rates in *Ae. aegypti* (Fu et al. 2010, Labbé et al. 2010). The three acceptor lines were injected with this new source of capped Bxb1 mRNA simultaneously with ϕC31 integrase capped-mRNA in an attempt to promote RMCE with the OX4678 construct. It was thought that simultaneous expression of the two integrases would improve the recombination efficiency of Bxb1 by initial recombination of the ϕC31 attachment sites resulting in the attachment sites of Bxb1 being brought into close proximity. It was hoped that this would reduce the need for sequence searching by the Bxb1 integrase after binding to

the *attB* site and increase its recombination efficiency, as was seen for two ϕC31 attachment sites in close genomic proximity (see section 4.4.3).

The injections into the OX4619-A and B lines resulted in transformants carrying the Hr5*ie*1-AmCyan marker of the OX4678 construct; the recombination efficiencies of these injections are shown in Table 4.5. Injections into the OX4619-D line did not result in any transformants, which may have been due to the low number of embryos injected, which was once again a result of the line's low fecundity.

Table 4.5: Efficiencies of ϕC31 and Bxb1 recombinase mediated cassette exchange, between construct OX4678 and lines OX4619A, B and D. Fertile G0s were assumed to comprise 50% of the G0 individuals surviving the microinjection process.

Line	Eggs injected	Fertile Gos	Recombination Eff.(%)
A	1084	76	2
B	1139	131	16
D	953	62	0

Complete RMCE should result in the loss of the DsRed marker and insertion of the AmCyan marker. However, the transformants from lines A and B carried the Hr5*ie*1-DsRed marker of the original OX4619 lines suggesting that recombination had occurred between a single pair of the integrase attachment sites, rather than both pairs as would be expected for complete RMCE (see Figure 4.9).

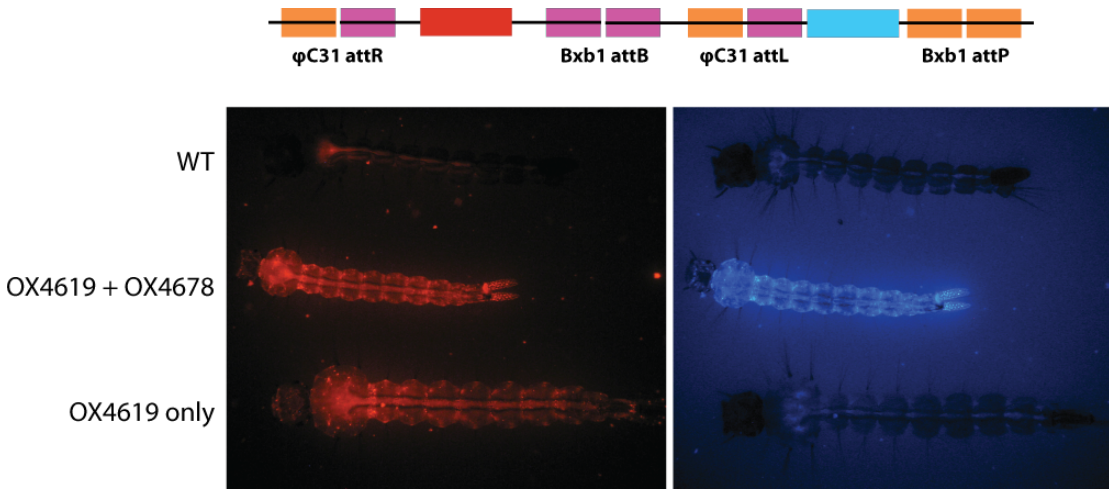


Figure 4.9: Expression of the DsRed and AmCyan fluorescent proteins in 4th instar larvae of the G1 progeny of injections of the OX4678 construct into the OX4619B line compared to the OX4619B line alone and wild-type (WT). The schematic of the supposed recombined structure of the OX4619 and OX4678 constructs is also shown.

PCR analysis, using primers specific to the stationary half-site of each *attP* site (the half-site that recombines to form the genomic *attR* site) and primers internal to either the DsRed or AmCyan cassettes, was used to confirm the recombination events that had taken place, as shown in Figure 4.10. This revealed that in the OX4619 + OX4678 lines the Bxb1 *attP* site remained unchanged, having not recombined with the Bxb1 *attB* site. However, a product of the expected size was seen as a result of recombination of the ϕC31 *attP* and *attB* sites. This confirmed the evidence from visual screening of the fluorescent markers that only incomplete RMCE had taken place. An unexpected larger amplicon was seen in the Bxb1 *attP* amplifications in the OX4619 + OX4678 lines. Sequencing revealed that this was due to unspecific binding of the cassette specific primer in the plasmid backbone that had also inserted as a result of incomplete RMCE.

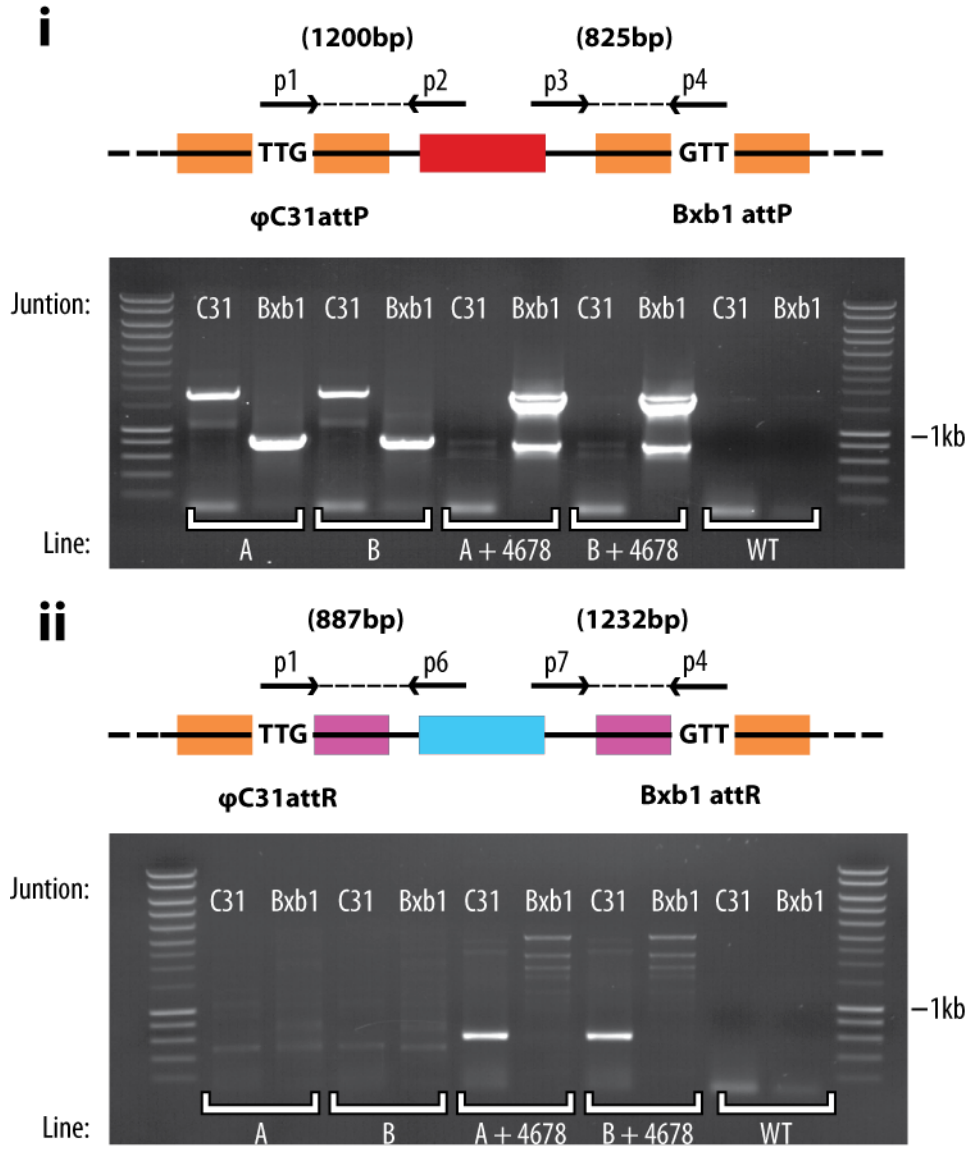


Figure 4.10: PCR amplification between, i: the ϕC31 and Bxb1 *attP* sites and the DsRed cassette in lines OX4619-A and B; and ii, the ϕC31 and Bxb1 *attR* sites and the AmCyan cassette in the lines after recombination with OX4678, A + 4678 and B + 4678. To determine which of the *attP* sites had recombined diagnostic PCRs were conducted with primers specific to the *attP* sites before recombination (p1 to p2 for the φC31 site and p3 to p4 for the Bxb1 site) and to the *attR* sites that would be formed after recombination (p1 to p6 for the φC31 site and p4 to p7 for the Bxb1 site). The A + 4678 and B + 4678 lines showed bands diagnostic for the unrecombined Bxb1 *attP* site and the recombined φC31 *attR* site, indicative of only incomplete RMCE. Wild-type (WT) DNA was also used as a template to elucidate any non-specific products.

Position effects can alter the efficiency of Bxb1 recombination between *attB* and *attP* sites at different genomic loci, but considering that the ϕC31 recombination was very efficient in these lines (in the case of the B line at least)

it seems unlikely that the properties of the insertion site would completely inactivate Bxb1 recombination in all three lines (Nkrumah et al. 2006, Huang et al. 2011).

4.4.7 Bxb1 mediated integration into pseudo-*attP* sites in the *Ae. aegypti* genome

Simultaneously with the work on the OX4619 construct, the OX4592 construct was injected into embryos of *Ae. aegypti* in the presence of the capped Bxb1 integrase with the attached *D. melanogaster vasa* gene 3' UTR. This construct contained an 3xP3-AmCyan reporter gene flanked by a Bxb1 integrase *attB* site and a ϕC31 *attP* site (see Figure 4.11).

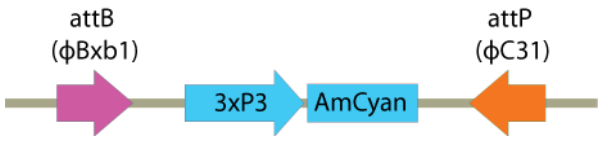


Figure 4.11: Schematic diagram of the pseudo-integration construct, OX4592.

This design was intended to allow Bxb1-mediated insertion into the genome via recombination of the Bxb1 *attB* site on the plasmid construct with pseudo-*attP* sites in the *Ae. aegypti* genome. It was hoped that this would result in irreversible transformation without the need for a transposon vector. The ϕC31 *attP* site would then allow for successive rounds of recombination with ϕC31 integrase, allowing functional transgenes to be site-specifically inserted. The efficiency of integration achieved by these injections was low. In total 2875 wild-type embryos were injected, resulting in 503 G0 survivors. These were outcrossed to pools of virgin wild-type and the G1 progeny screened for expression of the AmCyan marker in the eyes. The estimated transformation

efficiency was approximately 1%, with three transformants recovered from three separate pools. These were lines OX4592-A, OX4592-B and OX4592-C. Injection of a ϕC31 *attB* containing plasmid construct carrying a 3xP3-DsRed marker, OX4105 (previously described by Labbe et al. 2010), into the A and C lines, in the presence of ϕC31 integrase, resulted in transformation with an efficiency of 10 and 12% respectively (see Figure 4.12). This confirmed that the ϕC31 *attP* site had been successfully inserted into the genome and was capable of successive rounds of recombination in the presence of the ϕC31 integrase.

Figure 4.12: Expression of the AmCyan and DsRed reporter genes in the optic nerves of a G1 transformant (4th instar larvae) resulting from the injection of the OX4105 construct into line OX4592-A.

Since the entire plasmid construct had inserted, along with its ampicillin antibiotic resistance gene, plasmid rescue was used to determine the genomic insertion sites of the OX4592 lines by digestion of the flanking genomic DNA with restriction endonucleases *BglII*, *FseI* or *RsrII*, which did not lie within the original plasmid. Bacterial colonies were only obtained from the digestion of the A and C lines with the *BglII* endonuclease, suggesting that the B line had no nearby recognition sites for the selected endonucleases. Sequencing of the recircularized plasmids revealed that the initial insertions of the OX4592-A and

C lines had not occurred as a result of Bxb1 mediated recombination between the integrase's *attB* sites and pseudo-*attP* sites in the genome. Instead, recombination had occurred between the plasmid backbone, in the region lying between the ampicillin antibiotic resistance gene and the Bxb1 *attB* site. This recombination did not appear to be Bxb1 mediated, and no obvious similarity was found between the genomic insertion sites or the plasmid recombination sites and the attachment sites of Bxb1 integrase.

It is extremely unlikely that no potential Bxb1 pseudo-sites exist within the *Ae. aegypti* genome given that they are plentiful in the genomes of other eukaryotes (Chalberg et al. 2006). Hence the lack of observed recombination suggests that Bxb1 integrase may not be active in *Ae. aegypti*, confirming the results of the assays using OX4619 and OX4678 that carried the wild-type attachment sites. Recently, Bxb1 integrase has been shown to be active in the germline of *D. melanogaster*, successfully promoting recombination between its wild-type attachment sites inserted on plasmids and within transposons inserted into the fly genome (Huang et al. 2011). That Bxb1 should be active in another Dipteran and not *Ae. aegypti* is disappointing but not surprising, considering that FLP recombinase does not appear to be active in *Ae. aegypti* but is highly efficient in *D. melanogaster* (Nimmo et al. 2006). The biochemistry of *Ae. aegypti* may prevent the Bxb1 integrase protein from folding correctly, thus preventing its activity. Alternatively, cofactors necessary for recombination activity may be lacking in the mosquito, preventing formation of the tetrameric complex that promotes recombination.

4.5 Discussion

This chapter has demonstrated the first example of RMCE in a pest-insect. Two of the three OX4372 acceptor lines proved viable for cassette exchange with a donor construct carrying the *attB* attachment sites. The efficiency, 8-9.5%, of the RMCE in lines A and I was less than those previously reported for recombination between single pairs of attachment sites in *Aedes* mosquitoes, which range from 17-32% (Labbé et al. 2010, Nimmo et al. 2006). However they were comparable to the efficiencies of RMCE reported in *D. melanogaster* (9-24%) using similarly designed constructs (Bateman et al. 2006).

The results presented here suggest that ϕC31 recombinase mediated cassette exchange can be a valuable tool for engineering the *Ae. aegypti* genome in a site-specific and sequence conservative manner. Since ϕC31 recombinase has also been shown to be active in *Ae. albopictus* and *An. gambiae,* it is probable that this method can be used in a range of medically significant mosquito species (Chompoosri et al. 2009). Such systems, employing inverted recombinase attachment sites, may be vulnerable to homologous recombination, though this was not detected in small-scale assays. Even though this would result in inversion of the inserted cassette rather than deletion, such recombination could be of regulatory concern (Handler 2004a). This problem could potentially be mitigated by the use of shorter attachment sites that are less likely to be targets for homologous recombination. The minimal functional *attP* and *attB* sites for ϕC31 integrase are 39bp and 34bp respectively; however, such truncated sites may well affect the efficiency of recombination (Groth et al. 2000). An alternative solution would be to use non-homologous attachment

sites from two different recombinases, eliminating the risk of homologous recombination.

In the research presented here it was demonstrated that the site-specific integrases from bacteriophages Bxb1 and ϕRV1 do not appear to be able to mediate canonical integration in the germline of *Ae. aegypti*. Neither was able to promote recombination between their wild-type attachment sites, and Bxb1 was not able to mediate recombination with pseudo-*attP* sites in the *Ae. aegypti* genome using the *attB* attachment site. This was despite Bxb1 having recently been employed successfully in *D. melanogaster* transgenesis (Huang et al. 2011). These results were disappointing given that these two integrases are the most extensively characterized of the large-serine recombinases (aside from ϕC31 integrase), and have been shown to be active in a variety of model organisms (Bibb et al. 2005, Yamaguchi et al. 2011b). Other potential site-specific recombinases have been identified and some have already been used in recombination systems in a limited number of model organisms (Zhang et al. 2008, Rashel et al. 2008, Olivares et al. 2001). They may well prove to be active in the germline of *Ae. aegypti*, and provide the most promising route to making the systems investigated in this chapter viable in the future.

Chapter 5

Development of an Engineered Gene Drive in *Aedes aegypti*

5.1 Introduction

5.1.1 Gene drive systems for *Ae. aegypti*

Gene drivers have the potential to revolutionize the control of mosquito borne disease such as Dengue Fever and Malaria by replacing wild populations with engineered strains refractory to the disease. Despite their great potential concerns exist about the use of gene drives in the field. These include their ability to be 'recalled' should unintended consequences present themselves after release, and their rate and range of invasiveness into areas that may not have approved their use (James 2005, Benedict et al. 2008, Benedict et al. 2003, Knols et al. 2006). Theoretically some gene drive systems, such as those employing transposable elements or homing endonucleases, have a very low release threshold for establishment in wild populations that can allow them to easily invade geographically isolated subpopulations either through intentional or unintentional release (Marshall 2009, Burt 2003, Ribeiro et al. 1994). Self-limiting and easily recallable gene drives are therefore attractive from a regulatory standpoint. Two such artificial gene drives have been proposed based on the use of killer (an autocidal gene) and rescue (a repressor of the killer) elements. These gene drives systems are engineered underdominance and the simpler killer-rescue system.

5.1.2 Engineered Underdominance

In natural diploid populations it is sometimes the case that homozygous individuals are advantaged in reproductive success relative to heterozygous individuals. This heterozygote disadvantage is termed underdominance (Altrock et al. 2011). In nature, single-locus underdominant alleles are bi-stable

and can either be lost from or become fixed in a population depending upon the alleles frequency relative to a certain threshold which is dependant on the fitness of the different genotypes (Li 1955). Naturally occurring chromosomal rearrangements can give rise to underdominance, though unless the population is extremely small the probability of fixation of these underdominant alleles is very low (Altrock et al. 2010).

The use of underdominance as a gene drive system was first proposed by Curtis (1968) who suggested that reciprocal chromosomal translocations created with selective breeding could be used to ingress genes into insect pest populations, since the progeny of the wild-type strain and the pure translocated strain would be at a substantial fitness disadvantage (Curtis 1968). However the use of these underdominant systems has not proved to be viable as a gene drive system due to the difficulty in creating refractory translocated strains, the poor viability of homozygous translocated strains that make mass rearing impossible and the high release ratio required for fixation (Curtis 1968, Robinson 1976).

With recent advances in molecular biology the engineering of artificial systems, based on the use of mutually suppressing dominant killer constructs inserted on non-homologous chromosomes, has become a viable approach to construction of an underdominance gene drive (Magori et al. 2006, Davis et al. 2001). Davis et. al. (2001) were the first to suggest such an engineered underdominance system and demonstrated that, in the absence of fitness costs associated with the constructs, a transgene could be successfully introgressed into populations using this gene driver. The proposed mechanism for such

engineered underdominance systems relies upon paired constructs consisting of a killer transgene and its promoter; a trans-acting suppressor; and the refractoriness gene to be driven under control of a second promoter (see Figure 5.1).

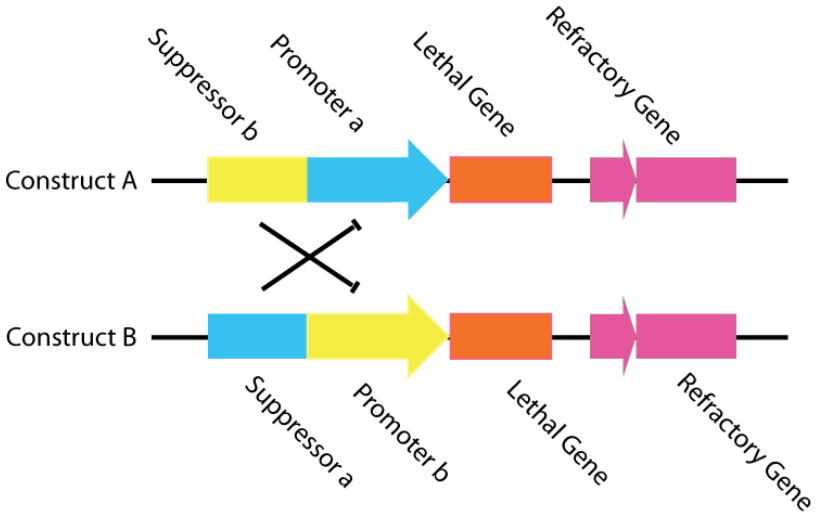


Figure 5.1: Diagrammatic representation of an engineered underdominance system. Two constructs, A and B, are inserted on non-homologous chromosomes. Each construct has a suppressor that suppresses the expression of the lethal gene on the other construct. A refractory gene driven by an independent promoter is also present on each construct (adapted from Davis et al 2001).

The suppressor on one chromosome would then rescue the killer on the other chromosome and vice versa (Davis et al. 2001). Individuals who carry both constructs are viable whereas individuals carrying only one construct, without the cross-suppressor, express the killer gene and die. If individuals homozygous for both constructs are released into a wild population then the offspring (G1) will be heterozygous for both constructs. When mating with wild-type individuals the G1s will produce progeny; 25% of which carry both constructs

and so are viable, 50% that carry only one and so die, and 25% that carry neither (see Figure 5.2).

Figure 5.2: Genotypes produced from an underdominance system with cross-suppressing constructs at two unlinked loci (aabb) and the wild type alleles (AABB), over three generations. Genotypes highlighted in red are not viable due to the absence of the associated cross-suppressor.

For constructs inserted at unlinked chromosomal loci a bi-stable inflection point exists above which the genes become fixed in the population and below which they are eventually removed, as is the case for single-locus underdominance.

Mathematical modelling of engineered underdominance predicts that the release ratio –the number of engineered individuals that must be released for every wild-type individual– required for fixation of the refractory genes is as low as 0.03, though this figure does not take into account fitness effects from insertion of the constructs or leakiness of the lethal genes when suppressed (See Figure 5.3) (Davis et al. 2001). When the constructs have an associated fitness cost the release ratio required for fixation is substantially increased (Gould et al. 2004). This can be offset with multiple insertions of the cross-suppressing constructs allowing release ratios of approximately 0.25 to achieve fixation, a figure that can be further reduced by release over multiple

generations (Magori et al. 2006). The release ratios required for underdominance systems are substantially higher than those of other gene drive systems, such as HEGs or Medea-like drives, making them less economically attractive, all other things being equal, than systems with a lower threshold for invasion for replacement of populations over large geographic areas.

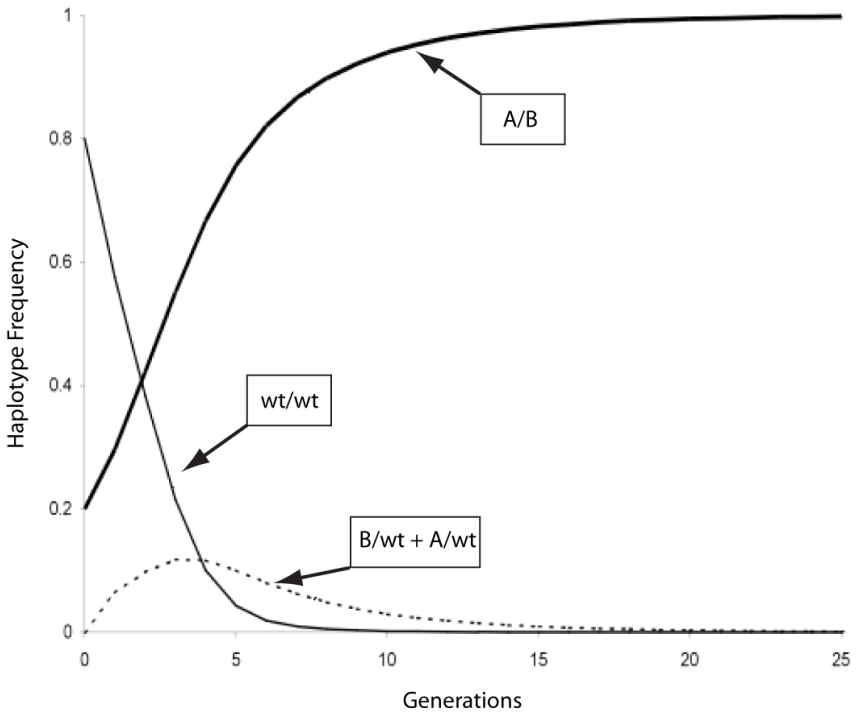


Figure 5.3: The frequency of cross suppressing underdominance constructs, A and B, based on the models of Davis et al. (2001). For a single release with a release ratio of 0.2 males homozygous for A and B for every wild type (wt) male A/B become fixed in the population with all other haplotypes declining to zero. The model assumes no fitness cost for the insertion of A or B.

However, their relatively low invasiveness makes them ideal for modifying local populations such as those around major urban centres, where further spread of the transgenes may not be desired and where the impact of vector-borne

diseases is often highest (Sinkins et al. 2006, Fernandez et al. 2003). The high release threshold also makes underdominance systems particularly attractive from a regulatory point of view since any accidental release, even of considerable magnitude, will quickly result in extinction of the transgenes (Marshall 2009). Furthermore the existence of a bi-stable inflection point allows an underdominance gene drive to be recalled from the environment by releasing enough wild type to disfavour the underdominant alleles.

Underdominance systems have several other traits that make them desirable gene drivers. The use of independently inserted refractory genes means that both genes would have to mutate for refractoriness to be lost, preventing loss of activity due to a single point mutation. Additionally using refractory genes with different activities in each construct would make the evolution of resistance of the pathogen more prohibitive (Sinkins et al. 2006, Magori et al. 2006). For the same reason that an underdominance gene drive would be recallable it would also be replaceable by release of a new pair of constructs, with different trans-suppressors/promoters, at the release ratio required for fixation (Davis et al. 2001). This could allow for rapid response to changes in pathogen or vector competence in a control programme.

The construction of a functional underdominance system is technically challenging, requiring the identification of suitable promoters to drive effective lethal genes and development of suppressors able to prevent expression of these. Using current germline transformation techniques simultaneous integration of both constructs at different chromosomal loci would be

impractical. Therefore the constructs would have to be created separately with some way to prevent expression of the lethal gene in the absence of its trans-suppressor. Even if the necessary components are successfully combined into the engineered two-construct system they must not impose too great a fitness penalty if the final system is to be viable as a gene drive system. Hence a great deal of optimisation will be required for any functional engineered underdominance gene drive.

5.1.3 The killer-rescue system

The killer-rescue system was first proposed by Gould et al. (2008) as a simpler alternative to existing gene drivers that could more easily be constructed in non-model organisms, such as mosquitoes. The system consists of a single killer construct and a single rescue construct linked to a refractoriness gene. This arrangement ensures that the frequency of the refractory gene would be identical to that of the rescue gene in a release population (Gould et al. 2008). The killer gene acts as the driver in this system and while its frequency remains high the frequency of the rescue construct increases rapidly as offspring lacking the repressor gene die. If individuals homozygous for the killer and rescue constructs were to be released modelling predicts that the refractory gene would be introgressed into a population, potentially reducing the number of vector competent individuals to very low levels. However, if the rescue construct has even a negligible associated fitness cost then this initial introgression will eventually be reversed, the constructs becoming extinct from the population (see Figure 5.4). This property of killer-rescue systems renders them inherently reversible which may often be advantageous. In the case of a

2:1 release of engineered mosquitoes into a wild population the killer-rescue system could result in 95% of mosquitoes being refractory for over 40 generations, even assuming a 10% fitness cost for each construct, before the constructs eventually become extinct from the population (Gould et al. 2008).

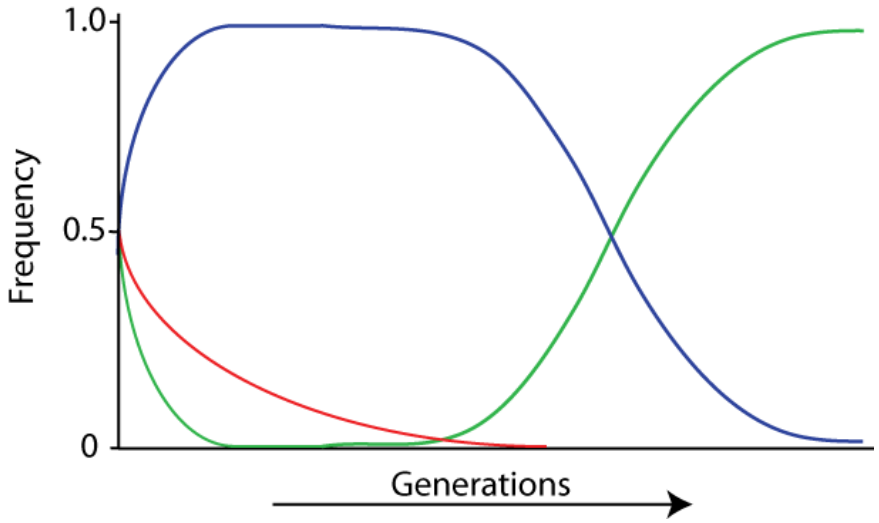


Figure 5.4: Killer (red) and rescue (blue) allele frequencies and wild type genotype (green) frequency over time after a 2:1 ratio release of a killer-rescue strain relative to wild-type. Based on the models of Gould et al. (2008) assuming a fitness cost of 0.1 for both the killer and rescue constructs.

Killer-rescue systems are also vulnerable to migration of wild type individuals into the release area that, in the absence of the killer allele, will quickly dilute out the rescue and refractory allele. This self-limiting behavior and limited invasiveness prevents killer-rescue systems from being useful in large-scale population replacement strategies where the permanent replacement of vector population is desired, because the genetic constructs are likely to have an associated fitness penalty (Bargielowski et al. 2011, Irvin et al. 2004, Marrelli et al. 2006a). Conversely this same self-limiting behavior makes the killer-rescue system attractive for situations where limited spread and eventual removal of the introduced transgenes from the population are required. This would be

particularly useful for the testing of refractoriness genes in wild populations where regulators could be assured of the eventual removal of any modification from the population. As such the development of a killer-rescue system may provide a useful tool for the development of other, more invasive, gene drive systems intended for wider scale use.

5.1.4 Construction of Underdominance and killer rescue systems

Strains of *Ae. aegypti* carrying suppressible, highly penetrant, dominant lethal genes have already been created that use tetracycline responsive elements (tRE) to suppress lethality in the presence of the antibiotic tetracycline (Fu et al. 2010, Phuc et al. 2007). It is possible that artificial tREs could be used in underdominance drives or that other conditional expression systems, such as the Q system, could be adapted for use as the rescue element of the drive (Potter et al. 2010). Another possible basis for construction of an underdominance system is RNA interference (RNAi), which can efficiently silence both endogenous genes, such as AaeIAP1, and exogenous transgenes, such as EGFP, in *Ae. aegypti* (Adelman et al. 2008, Pridgeon et al. 2008).

5.1.5 Engineered RNAi in *Ae. aegypti*

Refractory mosquito strains that express siRNAs targeting the prM region of the dengue type 2 (DENV-2) ssRNA viral genome have been created and been shown to be resistant to the virus through RNA-mediated gene silencing (Franz et al. 2006, Olson et al. 2002, Mathur et al. 2010). This has been achieved through expression of a double stranded RNA hairpin consisting of an inverted-repeat sequence from the prM region of the dengue virus, called Mnp (Adelman et al. 2002). Franz et al. (2006) used the promoter region from the

Ae. aegypti Carboxypeptidase A-1 (CarbA) gene –that is upregulated in the midgut of female mosquitoes 24-28 hours after a blood meal is taken– to drive expression of the Mnp hairpin in the midgut of females. This resulted in significantly reduced DENV-2 viral titer in female saliva, which was attributed to RNAi mediated interruption of viral replication in the midgut after blood feeding. Mathur et al. (2010) used an identical Mnp fragment under control of the *Ae. aegypti* 30Kb promoter –one of the promoter regions of the bidirectional 30K promoter of the anti-platelet protein gene, which is upregulated in the salivary glands of female mosquitoes after a blood meal– to create a strain in which salivary viral titers were significantly reduced (around 6 fold less than the wild type control) (Mathur et al. 2010, Yoshida et al. 2006). A strain with the Mnp hairpin under control of the promoter region of the *Ae. aegypti* Vitellogenin A (VgA1) gene –a gene that is strongly upregulated in the fat body around 24 hours after blood feeding, producing the yolk protein precursor vitellogenin– has also been created, and shows a similar reduction in salivary viral titer (Ken Olson, personal communication)(Romans et al. 1995).

More recently RNAi has been used to construct gene drive systems in *Drosophila*, where miRNA (short RNA strands that are directly transcribed from some genes) was used to silence a gene essential for embryogenesis (Chen et al. 2007). These results indicate that RNAi has the potential to be both the refractory mechanism and the rescue method in underdominance and killer-rescue systems.

5.1.6 Tissue and time-specific expression

The identification of time and tissue-specific promoters is of great importance to the development of underdominance and killer-rescue gene drives. Both systems require that fitness effects be minimised or ideally, in the case of underdominance, eliminated to ensure a practical release ratio (Magori et al. 2006, Gould et al. 2008). Engineered killer and refractory genes are likely to have fitness costs associated with them (Bargielowski et al. 2011, Marrelli et al. 2006a, Moreira et al. 2004). For this reason, reducing the range of tissues in which the genes are expressed, as well as the time over which they are expressed, could substantially reduce the impact of fitness effects associated with the transgenes on the final engineered strain (Sinkins et al. 2006, Gould et al. 2008). Therefore, the identification of time and tissue-specific promoters that can tightly regulate transgene expression will be necessary for the development of underdominance and killer-rescue gene drive systems.

5.2 Experimental aims

The experiments described in this chapter were conducted with the aim of developing and testing components of a killer-rescue system that could be used in an underdominance or killer-rescue gene drive. The key idea behind this work was to use RNAi as both the refractory element in the system and as the repressor of the lethal. This was to be achieved by fusing the Mnp fragment of the dengue virus genome, the target sequence of Mnp RNAi hairpin, to the tTAV protein of the tet-off system (see Figure 5.5). The intention was that RNAi against the Mnp portion of the dengue genome should knockdown both the

tTAV transcripts, thus preventing expression of the toxic effector, and viral transcripts, preventing replication of the virus.

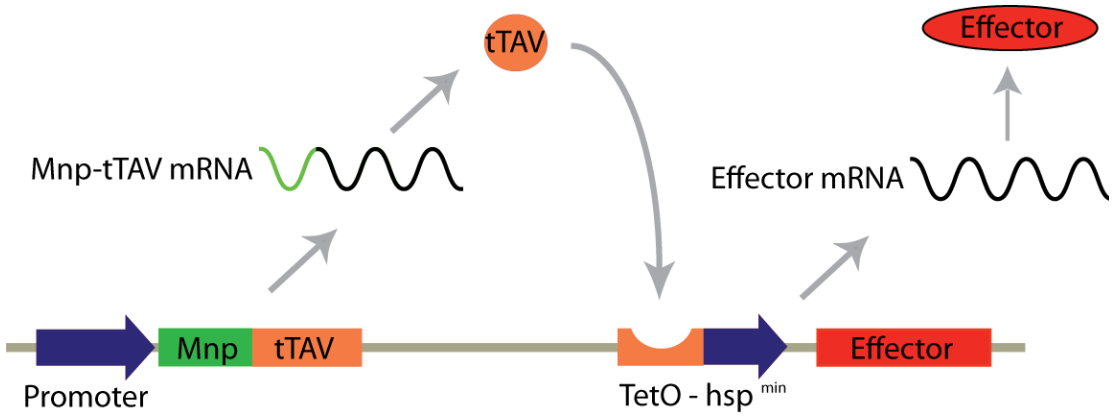


Figure 5.5: Explanatory schematic of the intended killer-rescue system. A tissue-specific promoter drives expression of tTAV mRNA fused to the Mnp fragment from the DEN2 virus. tTAV protein then binds to the tetO transactivator that in turn drives expression of a toxic-effector.

The primary advantage of this design is that separation of the gene drive from its cargo refractory element becomes impossible as any loss of the refractory element prevents rescue of the killer. A practical advantage of this approach was that lines expressing RNAi against the Mnp fragment have already been created, in the laboratories of Ken Olson and Anthony James, and have been shown to be effective against dengue virus replication in *Ae. aegypti*.

This chapter describes the testing of different tissue-specific promoters and toxic effectors for use in such a system and investigates the potential for RNAi-mediated knockdown of these effectors when endogenously expressed in *Ae. aegypti*.

5.3 Materials and Methods

5.3.1. Standard Procedures

General rearing, screening, microinjection and crossing techniques are described in chapter 2. Mosquitoes that had to be reared in the presence of tetracycline were reared during the immature stages in water containing 30µg ml^{-1} of the chlorinated tetracycline salt; chlortetracycline hydrochloride (Sigma-Aldrich, UK). Adult mosquitoes were fed on a concentrated sugar solution containing the same concentration of chlortetracycline hydrochloride.

5.3.2. Synthesis of plasmid constructs

The tissue specific tTAV constructs and tetO-toxic effector constructs were built by Sarah Scaife of Oxitec Ltd. Briefly, the Carboxypeptidase A-1 tTAV construct (CarbA-tTAV) was made by amplifying the Carboxypeptidase promoter region from the construct of Franz et al. (2006) with primers CarbproAscF and CarbproAvBaR. The 1169bp fragment was then digested with *AscI* and *Eag1* and ligated into the *AscI* and *XmaI* sites of the OX4386 construct (previously constructed at Oxitec ltd.) along with the SV40 3'UTR amplified from the construct OX4181 with the primers Sv40NotBaF and Sv40XmaR. Into the *AvrII* and *NotI* sites of the resulting construct was ligated the Mnp fragment of the dengue genome, which was amplified from the plasmid of Franz et al. (2006) with primers Den2AvF and Den2EcoR, fused to the 5' end of tTAV coding sequence.

The 30Kb-tTAV construct is identical to the CarbA-tTAV but with the Carboxypeptidase 1-A promoter replaced with the *Ae. aegypti* 30Kb promoter. It was constructed by the removal of the Carboxypeptidase promoter region from

the CarbA-tTAV construct with restriction enzymes *AscI* and *AvrII* and cloning into these sites of the 30Kb promoter fragment that was amplified from the OX4452 construct with primers 30KXbaR and CarbproAscF.

Using the same method the Vitellogenin A-1 tTAV construct (VgA1-tTAV) was created by amplification of the Vitellogenin A-1 promoter region with primers VitAscF and VitAvR and cloned into the CarbA-tTAV construct.

The tetO-AaHIT and tetO-BmKIT toxic effector constructs were made by insertion of the AaHIT and BmKIT genes from constructs synthesized by Geneart (Germany), using the published sequences of the two genes, into the *AsisI* and *AvrII* sites of the OX4098 construct that contained a Hsp70 minimal promoter under control of the tetO transactivator, as well as an Hr5*ie*1 regulated DsRed marker.

5.3.3. Mosquito dissection

Mosquitoes were dissected under a dissection microscope. Guts and salivary glands were dissected in pH7.5 phosphate buffered saline solution (Sigma Aldrich, UK).

5.3.4. Real time Reverse transcription-PCR

To determine the relative expression of tTAV transcripts before and after blood feeding real time reverse transcription-PCR (RT-PCR) was performed using primers specific to the tTAV coding sequence. RNA was extracted using the using Tri Reagent (Ambion, USA) and treated with the DNase I enzyme (Roche, UK) and quantified on a Pharmacia Biotech GeneQuant II RNA/DNA calculator. cDNA was generated from 0.5µg of RNA with the RevertAid reverse

transcription kit(Fermentas, Lithuania), using random hexamer primers. A 0.5µg sample of this cDNA was then used to conduct Taqman real time PCR using primers and probes specific to the tTAV coding sequence and the normalizer; the 18S ribosomal protein gene. The primers, probes and product lengths are described in Table 5.1.

Table 5.1: Primers and probes used for real-time PCR of the tTAV transgene and the 18S-normalizing gene.

Probe	Primer	Sequence	Product (bp)
TaqVp16probe2		5'- Hex-CGGGATTCACCCCGCACGATAGC-3'	
	TaqVp16F2	5'-CATGCCGACGCGCTAGA-3'	139
	TaqVp16R2	5'- GGTGAACATCTGCTCGAACTCGAAATC-3'	-
18Sprobe2		5'-Fam- TTCGTAGACCGTCGTAAGACTAACTAAAGCG -3'	
	18StaqF2	5'-GTATTACGGCGCGAGAGGTG-3'	141
	18StaqR	5'- GAAAACATCTTTGGCAAATGCTT-3'	-

The reaction mix used 12.5µl TaqMan Gene Expression Master Mix (Applied Biosystems, UK) combined with the two sets of primers to a final concentration of 0.9µM each and the two probes to a final concentration of 24µM each, with the final volume brought to 25µl with water.

Real time PCR reactions were performed on a Mx3005P thermal cycler (Stratagene, USA) with an initial activation step of 50C for 2 minutes, followed by denaturation at 95C for 10 minutes and forty cycles of 95C for 15 seconds and 60C for 30 seconds. Data from the reactions were analysed using the MxPro-QPCR software (Stratagene) and R statistical package (R Foundation for Statistical Computing).

5.3.5 Determining toxic-effector expression with RT-PCR

To determine if the tetO activated toxic-effectors were being upregulated by tTAV expression RNA was extracted from three pooled females from crosses of the tTAV lines to the tetO-effectors and cDNA generated as described in section 3.3.2. PCR was carried out on 1µl of template cDNA using DreamTaq DNA Polymerase (Fermentas, Lithuania) and the primers described in Table 5.2.

Table 5.2: Primers specific to the toxic effector cDNAs and their product sizes. Primer sequences can be found in Appendix 1.

Effector	Forward Primer	Reverse Primer	Amplicon (bp)
AaHIT	AaHIT-diag-F	AaHIT-diag-R	315
BmKIT	BmKIT-diag-F	BmKIT-diag-R	304
Michelob-x	Micx-5'-01-F	Micx-5'-01-R	332
NIPP1	NIPP1-01-F	NIPP1-01-R	360

5.3.6 Survival analysis of tTAV lines crossed to tetO effector lines

To study the survival of crosses of the tissue-specific tTAV lines to the tetO-toxic effectors; 30 hemizygous males of each tTAV line were crossed to 100 hemizygous females of each tetO-effector line. Eggs were collected over three gonotrophic cycles and reared in 2l of deionised water at density not exceeding 0.2 larvae/ml. Larvae were screened at the 3rd instar for the presence/absence of the transformation markers of the two constructs. Three separate pools of 100-200 individuals (depending on fecundity of the crosses) carrying both constructs were then transferred to 2L of deionised water. The number of pupae was recorded and pupae sexed. The sexes were separated and reared to adulthood, the number being recorded again. Unmated wild type individuals of

the opposite sex were added to the cages at a ratio of 1:3 for males and 3:1 for females. The number of surviving males and females was then monitored at the point of blood feeding, 24 hours after a blood meal and again 48 hours after a blood meal over three gonotrophic cycles. Blood feeding was performed over the course of 135 minutes with a new blood feeder placed on the cage every 45 minutes. Blood feeders were wiped with human sweat to increase feeding rate. Females that did not blood feed were counted as dead for the purposes of survival analysis and were removed from the experiment. After each gonotrophic cycle females were allowed to deposit eggs over a period of 48 hours. Survival data was analyzed using the R statistical package (R Foundation for Statistical Computing).

5.3.7 Fecundity analysis of tTAV lines crossed to tetO effector lines

Eggs from the three gonotrophic cycles of the crosses described in section 5.3.6 were used for fecundity analysis of the crosses. Eggs were hatched in deionised water and raised to 2nd instar. The number of 2nd instar larvae was counted and, with the data collected during the crossing experiments, was used to calculate the number of viable eggs per female.

5.4 Results

5.4.1 Construction of tissue-specific TTAV constructs

Three constructs were designed and constructed with the tTAV fusion protein under control of the promoter regions from the three tissue-specific genes; Carboxypeptidase A-1 (CarbA), 30kb and Vitellogenin A (VgA1). Fused to the ubiquitin-tTAV fusion protein was the 558bp Mnp fragment of the dengue virus genome, identical to that described by Franz et al. (2006), for use as a target for

RNAi (see Figure 5.6). The 5' UTRs of the tissue-specific genes were also included before the start codon of the tTAV-Mnp coding region, since it was considered likely that these played an important role in the specificity of the promoters.

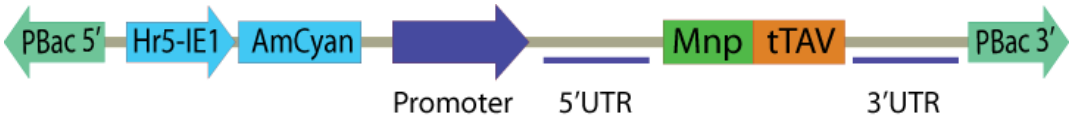


Figure 5.6: Generalized schematic of the tissue specific TTAV expression constructs. In the tTAV constructs three tissue-specific, blood meal inducible promoters were engineered to drive expression of the Mnp-tTAV fusion protein.

The constructs also contained an AmCyan fluorescent marker gene under control of the baculovirus promoter Hr5*ie*1, as a transformation marker. These components were placed within a piggyBac vector to allow insertion of the constructs into the mosquito genome.

5.4.2 Generation of lines with tissue-specific tTAV expression

The three tissue-specific tTAV expression constructs were microinjected into embryos of *Ae. aegypti* in the presence of capped piggyBac transposase mRNA. This resulted in successful transformation of all three lines (see Table 5.3). From the Go injection survivors three single-insertion lines of the CarbA construct were established (CarbA-tTAV-A, CarbA-tTAV-D and CarbA-tTAV-F), as well as three lines of the 30Kb construct (30kb-tTAV-A, 30kb-tTAV-B and 30kb-tTAV-C). Only a single insertion line of the Vg construct (the microinjection of this construct was performed by Pam Gay of Oxitec Ltd.) was successfully established (VgA1-tTAV-A). To confirm that each line carried only a single insertion, single males were outcrossed to virgin females for several

generations until a 50:50 segregation of the transformation marker was seen in the progeny.

Table 5.3: Transformation efficiencies of the three tissue-specific tTAV constructs. Fertile G0s were assumed to comprise 50% of the G0 individuals surviving the microinjection process. Very high transformation rates were observed for the CarbA and 30Kb lines so exact transformation rates were not calculated due to the large number of transgenic G1 progeny. The microinjections of the VgA1 construct were preformed by Pam Gray of Oxitec Ltd. and are included for comparison.

Construct	Eggs injected	Fertile Gos	Transformation Eff.(%)
CarbA	1664	378	>75
30Kb	863	103	>75
VgA1	1654	149	8

5.4.3 Tissue-specific tTAV expression in the CarbA, 30kb and Vg1 lines

To confirm that tTAV was being expressed in a tissue-specific manner in each of the lines of the three constructs; hemizygous males were crossed to females of the OX3576 reporter line (generated previously at Oxitec Ltd.). The OX3576 construct carries a DsRed reporter gene under control of the tetO transactivator that is activated by binding of the tTAV protein. The construct also carried a DsRed transformation marker under control of the artificial eye specific 3xP3 promoter (Berghammer et al. 1999). Male and female G1 progeny of the crosses were screened for expression of the DsRed fluorescent protein before and 48 hours after a blood meal to examine the sex and tissue-specificity as well as blood meal inducibility of tTAV expression in the lines.

When crossed to the reporter line male and females of all three lines of CarbA showed strong DsRed florescence in the midgut with associated, but weaker, expression in the foregut; indicating expression of the tTAV protein in these

tissues (see Figure 5.7). The Malpighian tubules and hindgut showed no evidence of tTAV expression nor did any other tissues, though females displayed dim expression around the rectum. The observed tissue specificity was similar to that previously reported for the Carboxypeptidase A gene, where expression of the gene was restricted to the posterior midgut of blood-fed *Ae. aegypti* (Edwards et al. 2000).

Previous studies using real-time RT-PCR have shown tightly controlled up-regulation of the endogenous Carboxypeptidase A gene around 24h after a blood meal (Edwards et al. 2000, Isoe et al. 2009). Expression of the fluorescent reporter gene was seen in the midguts of females before and after blood feeding in all three of the CarbA lines. This suggested that the CarbA promoter was leaky, with at least some basal expression pre-blood meal in both females and males.

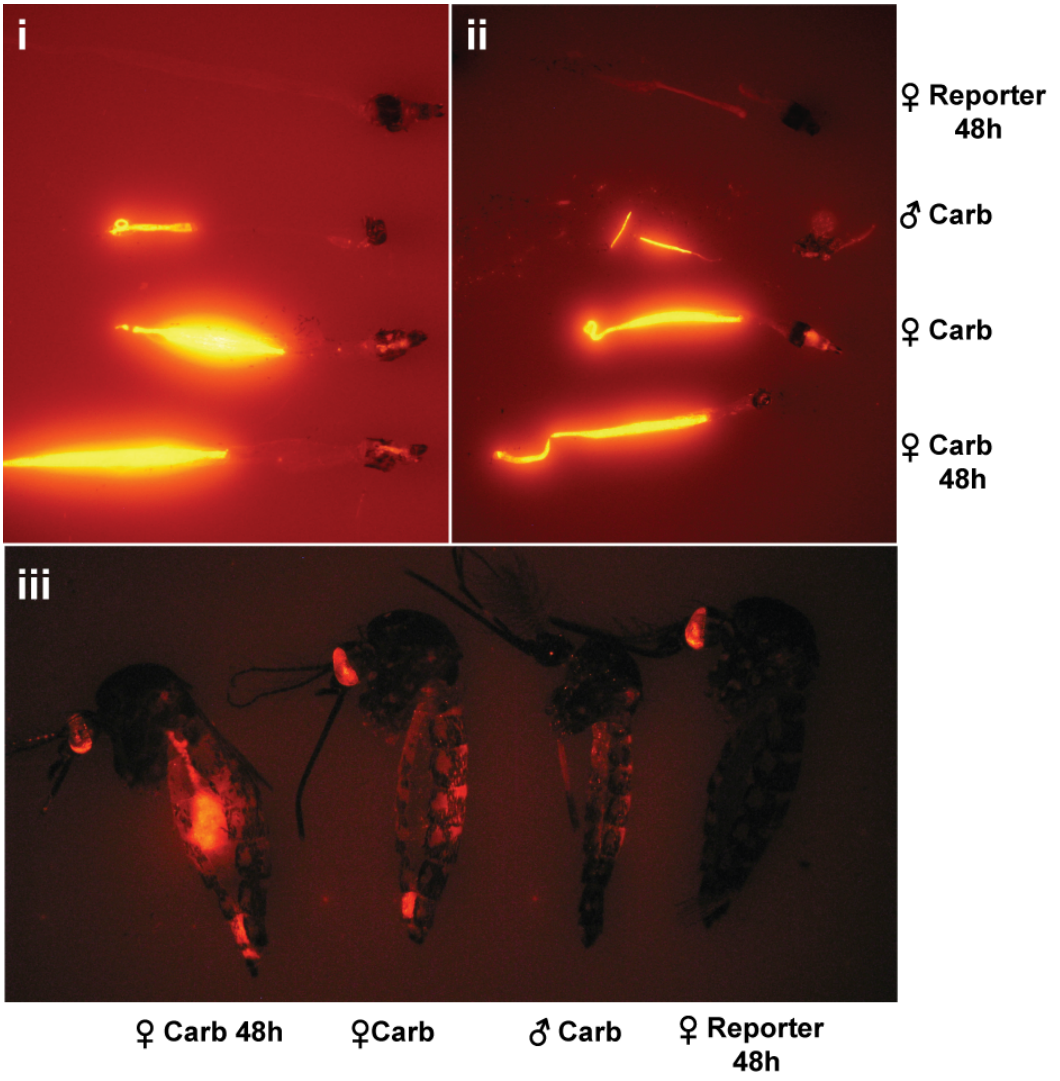


Figure 5.7: Expression of the DsRed fluorescent protein in the dissected guts of the CarbA-tTAV-A (i) and CarbA-tTAV-F (ii) lines, and the whole bodies of the CarbA-tTAV-D (iii) line, crossed to the OX3576 reporter line. i and ii: dissected guts of males (♂Carb), females(♀Carb) and females 48 hours (♀ Carb 48h) after a blood meal of the lines all showed strong expression of DsRed in the midgut. Females of the reporter line alone (♀ Reporter 48h) showed no such expression 48 hours after a blood meal. iii: whole body expression of DsRed in line D, the engorged midgut is clearly visible in the female 48 hours after a blood meal.

Crosses of the three 30Kb lines to the OX3576 reporter line revealed expression of tTAV in dissected salivary glands (see Figure 5.8).

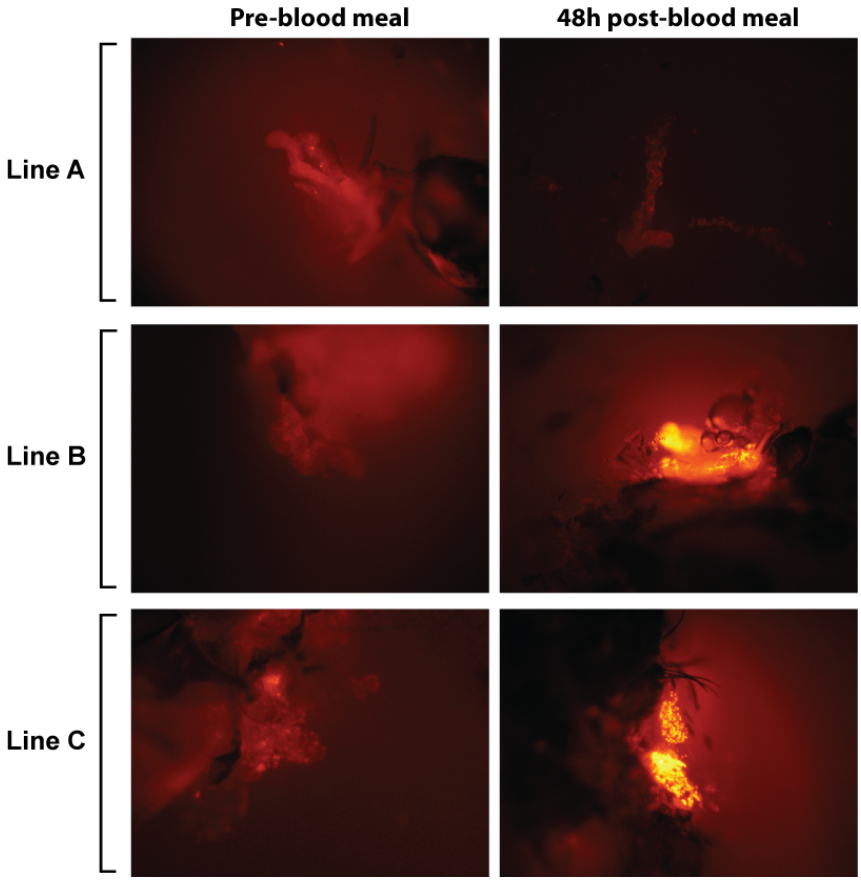


Figure 5.8: Expression of the DsRed fluorescent protein reporter in the; dissected salivary glands of females of the 30Kb-tTAV lines; A, B and C pre-blood meal and 48 hours after a blood meal.

All three lines crossed to the reporter showed dim fluorescence in the salivary glands of females before blood feeding. Lines B and C showed strong expression of the DsRed reporter in the salivary glands 48h after a blood meal, whereas Line A showed only very weak expression. Faint expression in the foregut was seen in all three lines (data not shown), however this did not appear to increase after blood feeding and may have been due to migration of the tTAV protein from the salivary glands into the alimentary canal. Females carrying only the reporter construct showed no visible florescence. The variation in the observed expression levels between lines is likely due to the position effect of nearby genomic elements (Wilson et al. 1990). The expression of tTAV in males was

not examined due to the difficulty of dissecting the smaller male salivary glands.

The single Vg1 line, VgA1-A, was also crossed to the reporter line and females and males screened for DsRed expression in the fat body (see Figure 5.9).

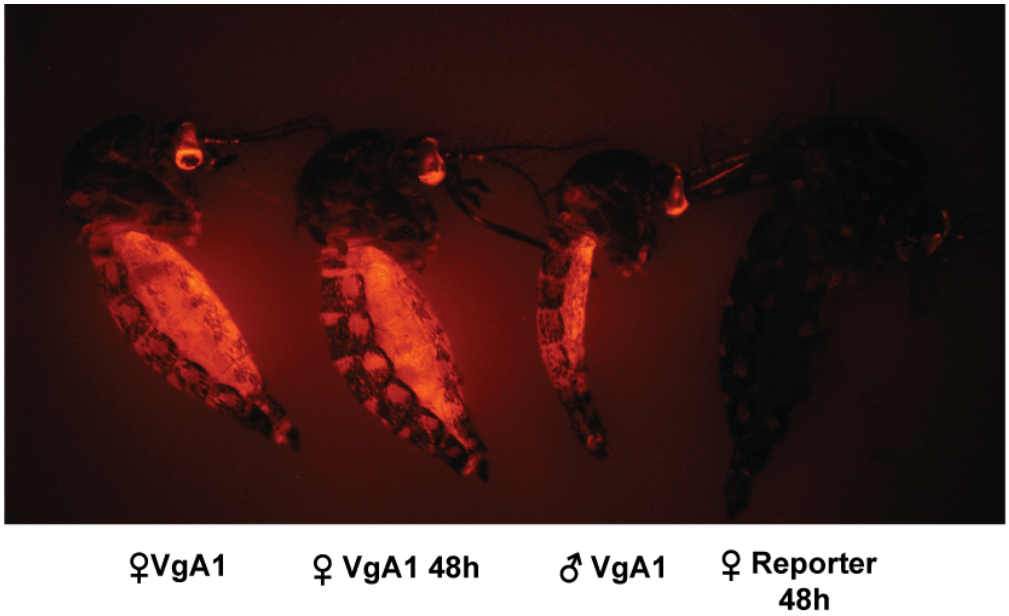


Figure 5.9: Expression of the DsRed fluorescent protein in the fat body of the VgA1-A line crossed to the OX3576 reporter line. Expression of the reporter is strong in males (♂VgA1), females(♀VgA1) and females 48 hours (♀ VgA1 48h) after a blood meal. Females of the reporter line alone (♀ Reporter 48h) showed no such expression 48 hours after a blood meal.

Both males and females showed strong expression in the fat body. There was no obvious upregulation of the reporter 48 hours after a blood meal in females. Dissection of fluorescent individuals revealed that fluorescence was restricted to the fat body and was absent in other structures such as the cuticle and alimentary canal.

All the lines of all three constructs showed expression of the reporter in the expected tissue, suggesting that the promoters were driving the expression of

tTAV with the same tissue-specificity as the endogenous genes. However, in all of the lines of all of the constructs expression of the reporter protein in females was observed before a blood meal, with only the 30Kb-tTAV lines showing a distinct increase in expression after a blood meal. This observation could be due to a poor correlation between the magnitude of DsRed expression and tTAV expression. If the tetracycline operator of the OX3576 line is very sensitive to low levels of tTAV expression, then even basal expression of the tTAV gene will result in saturated fluorescence, with increased expression of tTAV having little effect on the phenotype.

5.4.4 Blood meal inducible upregulation of tTAV in the CarbA, 30kb and Vg1 lines

To determine if tTAV expression was being upregulated after blood feeding quantitative real-time RT-PCR was used to determine the relative abundance of tTAV transcripts at various life stages. One exemplar line of each construct was chosen to examine the expression of the tTAV transgene. Crosses of the CarbA-tTAV lines to the reporter showed identical expression so line F was selected at random. Line C of the 30Kb-tTAV construct was chosen since it avoided the depressed expression of line A. Line A of the VgA1-tTAV-A construct was the only line available. Primers specific to the VP16 domain of the tTAV coding sequence were used to amplify transcripts of the transgene. The expression levels of tTAV mRNA was examined in triplicate and normalized relative to the expression of the 18S ribosomal protein gene that has previously been shown to have approximately stable expression over the pre-vitellogenic and vitellogenic periods (Niu et al. 2000). The relative expression of the tTAV transgene,

determined by real-time RT-PCR, in each of the three exemplar lines is shown in Figure 5.10.

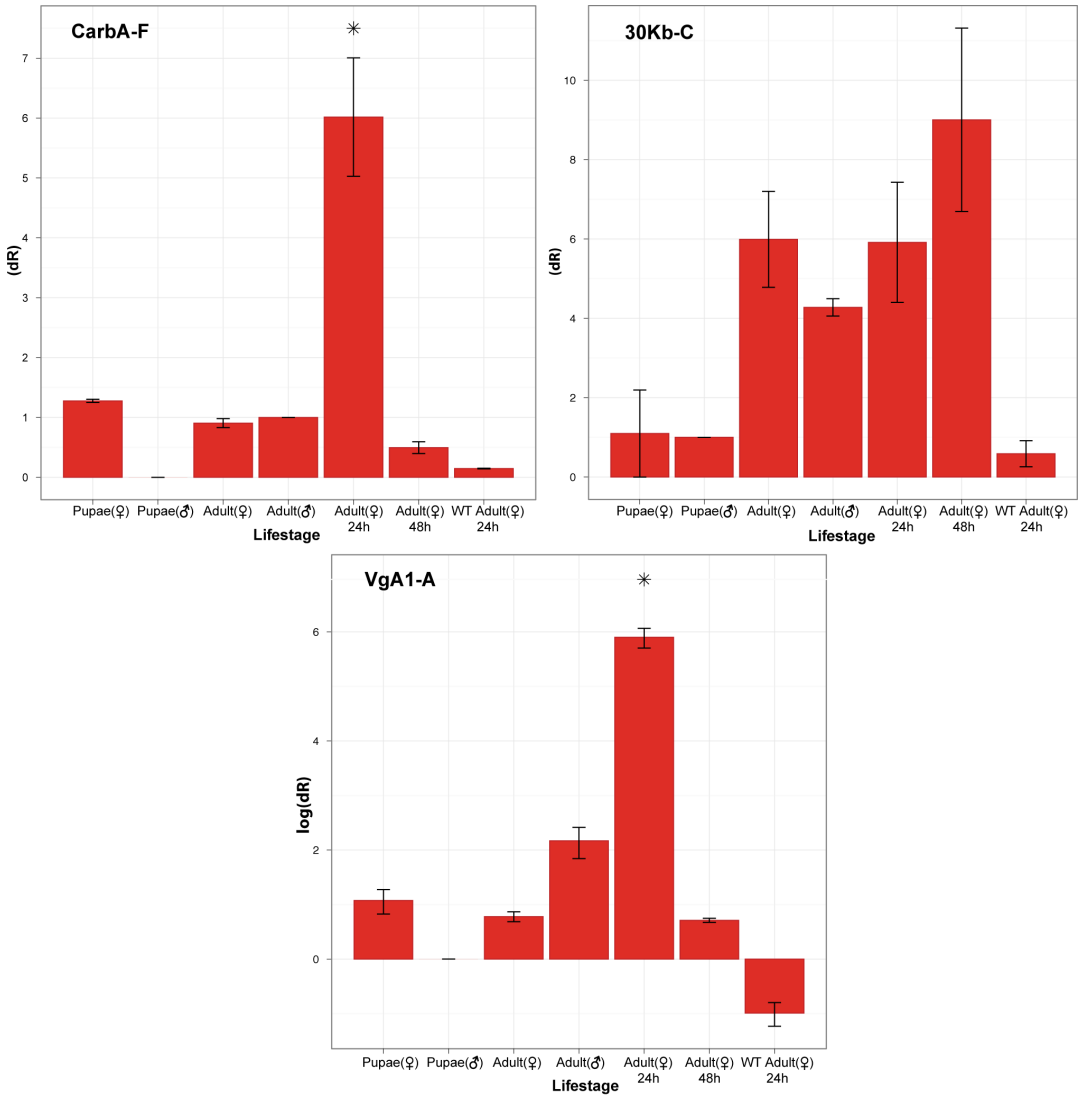


Figure 5.10: Mean relative expression (dR or log(dR) for VgA1-A) of the tTAV transgene in lines of the three tissue-specific constructs at different life stages. Expression levels were determined using Taqman based duplex real time RT-PCR and are relative to the expression in male pupae (for the CarbA-F line expression is reported relative to adult males). The results were normalized against the expression of the 18S gene. Error bars show the standard error of the mean of the three experimental replicates, performed on RNA extracted from three pooled individuals. Significantly difference ($p < 0.05$) from expression from pre-blood meal adult females was analyzed using one way ANOVA and is indicated by a *. A sample from female wild-type (WT) mosquitoes 24 hours after a blood meal is included for comparison.

The CarbA-tTAV-F line showed significant upregulation of the tTAV gene in females 24 hours after a blood meal according to one way analysis of variance.

tTAV transcripts were 6.6-times(p=0.03, t=-5.16, df=2.02) more abundant in females 24 hours after a blood meal. A 25 fold upregulation of the endogenous CarbA-1 gene after blood feeding has previously been reported (Isoe et al. 2009). The lower observed induction of the transgene may be due to regulatory elements that are missing in the artificial promoter region chosen, or positional effects associated with the insertion. Lower induction may also be due to the RNA extraction protocol which used whole abdomens rather than dissected midguts, this may have lowered the sensitivity of the assay by reducing the proportion of transcripts originating from the midgut.

The VgA1-tTAV-A line showed a 182-fold (p=0.03,t=-5.56,df=2.00) upregulation of the tTAV gene in females after a blood meal. Previous studies that have used the Vg1 promoter region in mosquitoes have seen a similarly strong upregulation in the vitellogenic period 1-24 hours after a blood meal (Cho et al. 2006, Nirmala et al. 2006, Racioppi et al. 1986). The real-time RT-PCR also confirmed that expression of the tTAV gene does occur in both males and females before a blood meal.

The 30Kb-C line showed no significant upregulation of the tTAV gene (p = 0.97 and 0.33 respectively) in females after a blood meal at either the 24 or 48 hour periods, though there was a significant, upregulation during the adult life period compared to early pupae; 4.3-fold for males (p=0.004, t=-15.03, df=2) and 5.9 fold for females (p=0.041, t=3.00, df=3.96). These results conflict with those seen in the crosses to the fluorescent reporter, where increased expression of the DsRed reporter was seen 48 hours after a blood meal. This may be

explained by the use of whole thoraces for the RNA extractions, because of the difficulty in dissecting multiple salivary glands. This likely resulted in the tTAV transcripts making up only a very small proportion of the total RNA extracted, greatly reducing the sensitivity of the real-time analysis and potentially obscuring any upregulation of the transgene after blood feeding.

5.4.5 Tetracycline repressibility of tTAV mediated tetO transactivation

The ability to repress expression of the toxic effector transactivated by tetO is of vital importance to the process of developing an underdominance system. Preventing an engineered lethal phenotype from manifesting is required to ensure that lines carrying both a tetO-effector and tTAV source can be reared in the laboratory before crossing to lines carrying an endogenous engineered suppressor. Hence the tetracycline repressibility of the tTAV-tetO system in the three tTAV lines was examined.

Crosses of the three tissue-specific tTAV exemplar lines to the tetO-DsRed reporter line were performed and the G1 offspring reared in the presence or absence of tetracycline. All three exemplar lines showed suppression of the fluorescent reporter in the presence of tetracycline. Both males and females of the CarbA-tTAV-F and VgA1-tTAV-A lines showed complete suppression of the DsRed reporter when raised on 30μg μl^{-1} of tetracycline (see Figure 5.11). Dissection of females from the 30Kb-tTAV-C crosses showed that even raised on tetracycline some cells in the salivary gland still displayed dim DsRed expression, though at a much lower level than that observed in the absence of tetracycline (see Figure 5.11, 30Kb-C). This may be because ingested tetracycline

is not readily transported to the salivary glands or because the 30Kb promoter drives extremely strong expression in this tissue.

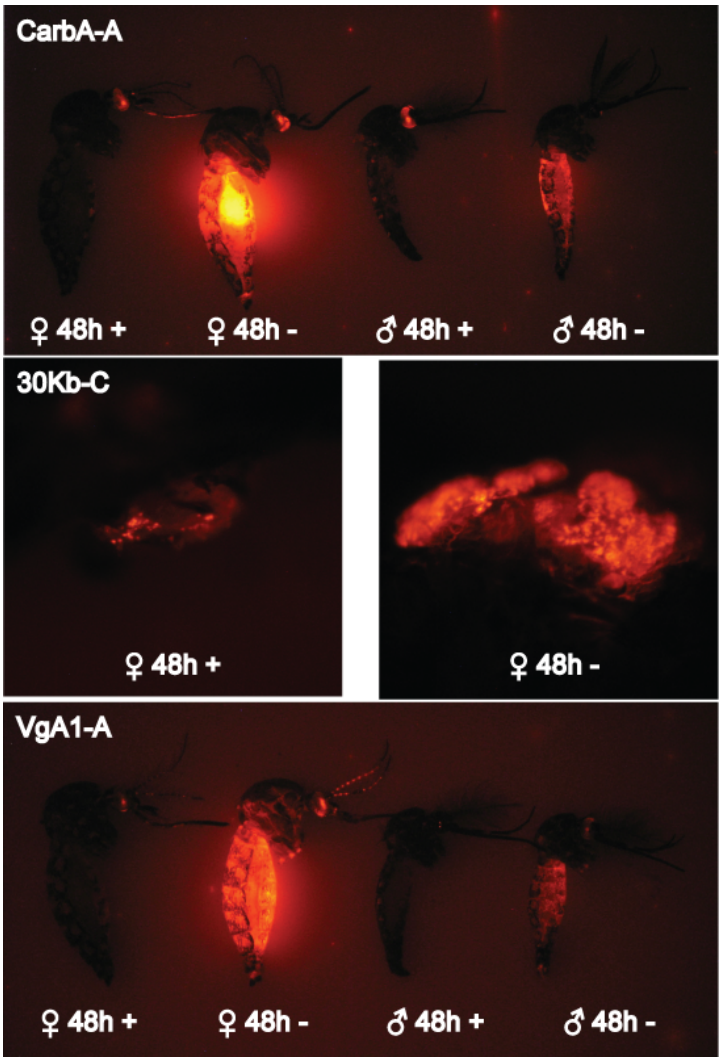


Figure 5.11: Males and females (48 hours after a blood meal) of the tissue-specific tTAV lines crossed to the tetO-DsRed reporter line raised in the presence (+) or absence (-) of tetracycline. Males and females of the CarbA-tTAV-A and VgA1-tTAV-A lines were examined with fluorescence microscopy for expression of the DsRed marker in the midgut and fat-body respectively. Females only of the 30Kb-tTAV-C line were examined for expression in the salivary glands.

The suppression of the marker in the presence of tetracycline confirmed that the fluorescence was being activated by the tetracycline repressible tTAV-tetO system. The results also indicated that inactivation of the tTAV protein in these

lines, in this case by binding of tetracycline, could prevent transactivation of a tetO linked effector. This suggested that an RNAi system capable of degrading tTAV transcripts efficiently could be used as the repressor element in an engineered underdominance system.

5.4.6 Synthesis of constructs for expression of toxic effectors

To determine if expression of tTAV in tissues vital for the propagation of dengue fever could be used to express a lethal phenotype in females post-blood meal, four constructs containing four different putative effector genes were synthesized. Since it was unknown what sort of effector would be most potent in each tissue, four different effectors were chosen to represent a range of possible modes of action. The four effectors chosen were: michelob-x, an inhibitor of apoptosis protein (IAP) antagonist that regulates cell death in mosquitoes (Zhou et al. 2005); NIPP1Dm, a nuclear inhibitor of protein phosphatases from *D. melanogaster* (Parker et al. 2002); AaHIT, an insect-specific excitatory neurotoxin from the scorpion *Androctonus australis* (Higgs et al. 1995, De Dianous et al. 1987); and BmKIT, a second excitatory neurotoxin from the scorpion *Buthus martensi* (Zuo et al. 2004). These effectors were cloned into piggyBac vectors under control of the Hsp70 minimal promoter adjacent to the tetO element. The constructs also contained a DsRed marker under control of the baculovirus promoter Hr5*ie*1 as a transformation marker (see Figure 5.12). This tetO-Hsp70-Effector motif was designed to be activated upon binding of the tTAV protein from the tissue-specific tTAV constructs. It was hoped that this would result in expression of the effector in the tissue of interest and produce a lethal phenotype.

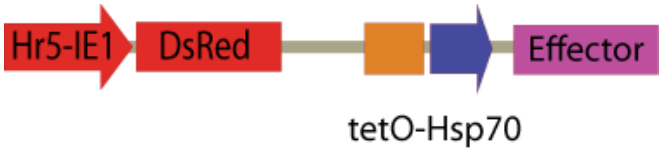


Figure 5.12: Generalized Schematic of the tetO-Effector constructs. The constructs each contained one of the toxic effectors under control of the tetO-Hsp70 tetracycline responsive minimal promoter. The constructs also contained a DsRed transformation marker.

In addition, the two scorpion toxin genes in these constructs were fused to the 14 amino acid secretory signal peptide of the *Ae. aegypti* trypsin gene (Graf et al. 1989). The addition of this signal peptide was intended to allow diffusion of the neurotoxins to the nerve-muscle synapses where they are active. The four effector constructs were named: tetO-michx, tetO-NIPP1, tetO-AaHIT and tetO-BmKIT.

5.4.7 Generation of lines with tissue-specific tTAV expression

Single insertion lines of tetO-michx and tetO-NIPP1 had been previously generated at the labs of Oxitec Ltd. so these were used for further experiments. The tetO-AaHIT and tetO-BmKIT constructs were used to transform *Ae. aegypti* using the standard microinjection procedure. Survival of Gos and the transformation efficiency were both unusually low, as shown in Table 5.4. A possible explanation for this was transient expression of the scorpion toxins in Gos, resulting in lethality. G1 transformants were outcrossed to produce three single insertion lines of each construct. These were lines tetO-AaHIT-D, tetO-AaHIT-F and tetO-AaHIT-G and lines tetO-BmKIT-B, tetO-BmKIT-D, tetO-BmKIT-E.

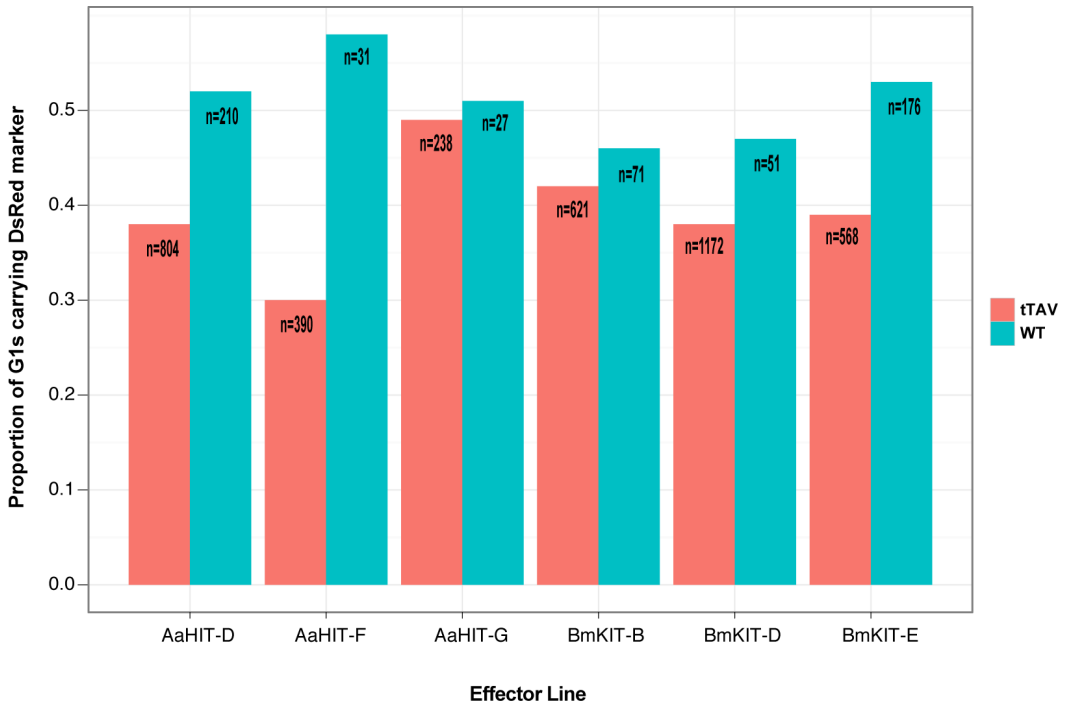
Table 5.4: Transformation efficiencies of the two tetO-scorpion toxin constructs. Fertile G0s were assumed to comprise 50% of the G0 individuals surviving the microinjection process.

Construct	Eggs injected	Fertile Gos	Transformation Eff.(%)
tetO-AaHIT	1397	48	7%
tetO-BmKIT	1509	97	5%

5.4.8 Determining the lethal potential of the scorpion toxins

The tetO-michx and tetO-NIPP1 lines have previously been shown to induce lethality when expressed in the fat body of *Ae. aegypti* (Genevieve Labbe, personal communication). Scorpion toxins have been shown to be active against *Ae. aegypti* when engineered into a Sindbis virus vector and injected intrathoracically but have not previously been endogenously expressed in the mosquito (Higgs et al. 1995). To determine the lethality of the scorpion neurotoxins when expressed in *Ae. aegypti* the lines of the tetO-AaHIT and tetO-BmKIT constructs were crossed to the line OX4351-C2. This line expresses tTAV under control of a 1426bp promoter region of the Ae. aegypti Hexamerin 1-γ (AeHex1-γ) gene, that is strongly expressed during development at the larval/pupal boundary (Gordadze et al. 1999, Krebs et al. 2002). 30 hemizygous females of each tetO-effector lines were crossed to 15 hemizygous males of the OX4351-C2 line. Survival of the G1 progeny was then examined relative to the number of eggs laid. Since the OX4351-C2 and tetO-effector lines shared an identical Hr5*ie*1-DsRed transformation marker it was not possible to tell which progeny carried which construct. Therefore the proportion of G1 pupae carrying the DsRed marker was examined and compared to the 0.75 ratio expected through Mendelian inheritance of the constructs (see Figure 5.13). None of the crosses showed a 0.75 proportion of G1 progeny carrying the DsRed marker.

When compared to crosses of the effector lines to wild-type males no significantly higher proportion of DsRed individuals ($p>0.05$) was observed. Three of the effector lines (AaHIT-D, AaHIT-F and BmKIT-E) examined showed a significantly lower proportion of DsRed marked pupae when crossed to the Hexamerin-tTAV line compared to the effector lines alone. This was likely due to pre-pupal lethality caused by basal expression of the scorpion toxins due to enhancer elements near the insertion sites of the constructs.



Effector Line	Proportion DsRed	Proportion WT	P	X2
AaHIT-G	0.49	0.51	0.885	0.021
AaHIT-F	0.29	0.58	0.002	9.57
AaHIT-D	0.38	0.48	0.013	6.111
BmKIT-B	0.43	0.46	0.700	0.149
BmKIT-D	0.38	0.47	0.257	1.287
BmKIT-E	0.39	0.53	0.001	10.987

Figure 5.13: Proportion of G1 pupae carrying the DsRed Marker resulting from crosses of the tetO-AaHIT and tetO-BmKIT lines to either the Hexamerin 1-tTAV line (tTAV) or wild-type(WT). The total number of G1 progeny (n) from each cross was determined by counting embryos before hatching. The table shows significance of the difference between the tTAV and WT proportions calculated using the binominal proportions test.

The results of these crosses indicated that the 25% of G1 progeny expected to carry both the tTAV and tetO constructs had died by the pupal development stage. This suggested that the tetO driven scorpion neurotoxins were potentially good candidates for achieving a lethal phenotype when expressed endogenously. Lines tetO-AaHIT-D and tetO-BmKIT-E were chosen for use in crossing experiments since they had the most intense expression of the

transformation marker, which was considered useful for screening large numbers of downstream crosses.

5.4.9 Tissue specific expression of toxic effectors

The four toxic effector lines were each crossed to each of the three tissue-specific tTAV lines; CarbA-tTAV-F 30Kb-tTAV-C and VgA1-tTAV-A. Total RNA was extracted from blood fed female G1 progeny of these crosses (thoraxes of the 30Kb line and abdomens of the CarbA and VgA1 crosses) and reverse transcription PCR (RT-PCR) was performed with primers specific to the effector transcripts (see Figure 5.14).

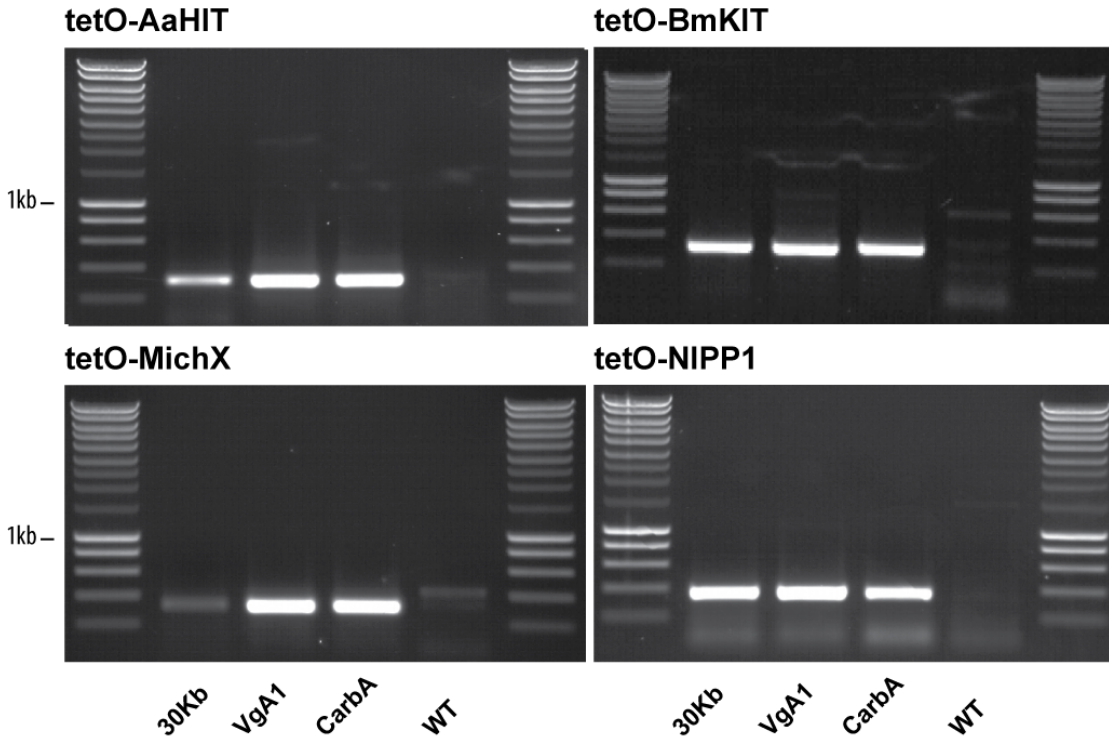


Figure 5.14: RT-PCR on blood fed adult females from crosses of the three tissue-specific tTAV lines (CarbA-F, 30Kb-C and VgA1-A) to the four lethal effector lines (tetO-AaHIT-D, tetO-BmKit-E, tetO-MichX and tetO-NIPP1). Primers specific to the effector transcripts were designed to amplify bands of 315bp for AaHIT, 304bp for BmKIT, 332bp for Michelob-x and 360bp for NIPP1. Additionally a wild-type sample (WT) was included for each primer set. No-transcriptase controls (not shown) were also run to test for DNA contamination and showed no amplification.

The RT-PCRs amplified a product of the expected size from all of the crosses confirming tTAV-mediated expression of each of the toxic effector genes. In light of the Real-time RT-PCR performed on the tTAV expressing lines and the observed fluorescence profiles of those lines crossed to the reporter, it was concluded that tissue specific expression, upregulated by blood feeding, of the toxic effectors was occurring.

5.4.10 Effect of tissue-specific expression of toxic effectors on female survival

To determine if expression of any of the toxic effectors in the salivary glands, midgut or fat body could be used as lethal mechanism in an engineered killer-rescue system, survival analysis of the crosses of each tissue-specific tTAV source to the four toxic effectors was conducted.

G1 progeny of the crosses were raised at a density of <0.2 larvae/ml with an excess of food provided each day. Rearing density can impact survival by increasing larval competition and the rate at which toxic metabolic byproducts accumulate. The low density used was chosen to prevent larval competition and is an order of magnitude less than those used in previous studies that have shown negative consequences for longevity (Bargielowski et al. 2011). Rearing the G1 mosquitoes in pools was chosen over individual rearing to mitigate confounding variables caused by variation in rearing conditions. Deionized water, to prevent tetracycline contamination, was used for rearing and replaced each day to prevent the accumulation of toxins from excretion, dead larvae and the metabolism of surplus food by microorganisms; all of which may have an detrimental effect on larval development and survival (Bédhomme et al. 2005).

The G1 progeny were screened at the 3rd larval instar using fluorescence microscopy and individuals carrying both tTAV and tetO-effector constructs were collected. The sex and survival of these individuals was then recorded from the 3rd larval instar, through to adulthood and then over three gonotrophic cycles. The survival of these individuals was then compared to those carrying the tTAV construct alone.

Kaplan-Meier analysis of the cumulative survival of females revealed that in general there was no significant ($p < 0.05$) decrease in the survival of females carrying the tTAV and tetO constructs as determined by log-rank analysis of the survival curves (see Table 5.5) (Peto et al. 1977). The survival curves for each cross are shown in Figures 5.15 to 5.17.

Table 5.5: Log-rank analyses of the tissue specific-tTAV lines crosses to the four tetO-toxic effector lines. Relative survival (+ better, - worse) of crosses to the effectors compared to crosses to wild type was estimated with Kaplan–Meier survival analysis. The log-rank test was used to determine if any positive or negative difference in survival was significant. Median survival is reported in days post screening of 3rd instar larvae.

Promoter line	Effector line	Relative survival	Median survival (days)	n	X2	p-value
CarbA-tTAV-F	tetO-AaHIT	+	15	372	5.2	0.023
	tetO-BmKIT	+	15	100	4.4	0.036
	tetO-MichX	-	3	324	206.0	0
	tetO-NIPP1	-	3	288	78.4	0
30Kb-tTAV-C	tetO-AaHIT	+	>18	238	17.2	0.000033
	tetO-BmKIT	+	15	150	0.2	0.62
	tetO-MichX	+	16	336	5.1	0.023
	tetO-NIPP1	+	15	264	0.1	0.74
VgA1-tTAV-A	tetO-AaHIT	+	10	193	13.2	0.00024
	tetO-BmKIT	+	9	150	0.6	0.43
	tetO-MichX	+	9	318	3.3	0.069
	tetO-NIPP1	+	10	272	19.2	0.000012

In general females carrying both a tTAV and tetO insertion survived significantly longer or equally long as females carrying only a tTAV construct. Only females of the CarbA-tTAV-F line crossed to the tetO-MichX and tetO-NIPP1 showed significantly lower survival than CarbA-tTAV-F females alone. No increase in mortality was observed in females after blood feeding, as had been anticipated from the observed upregulation in the number of tTAV transcripts (see Figure 5.15). In the case of the CarbA-tTAV-F, tetO-MichX and tetO-NIPP1 crosses, effector induced mortality was observed at the late larval to pupal life stages. This is not surprising given that real-time RT-PCR revealed that the CarbA promoter fragment promoted expression of tTAV before blood

feeding; suggesting that early expression of the toxic-effector gave rise to this effect. Michelob-x is an IAP-antagonist whose over-expression should result in apoptosis and cell death. This may be particularly detrimental during developmental stages, where cell death could prevent correct development of the midgut. This may explain why virtually no females carrying the tetO-MichX and CarbA-tTAV constructs survived to adulthood. Upon dissection dead 4th instar larvae showed no obvious large scale cell death in the midgut; however, cell nuclei were not stained and examined for evidence of fragmentation associated with apoptosis (Vaidyanathan et al. 2006). Similar over-expression of NIPP1 has previously been shown to lead to cell death in *D. melanogaster* through inhibition of the protein phosphatases (Parker et al. 2002).

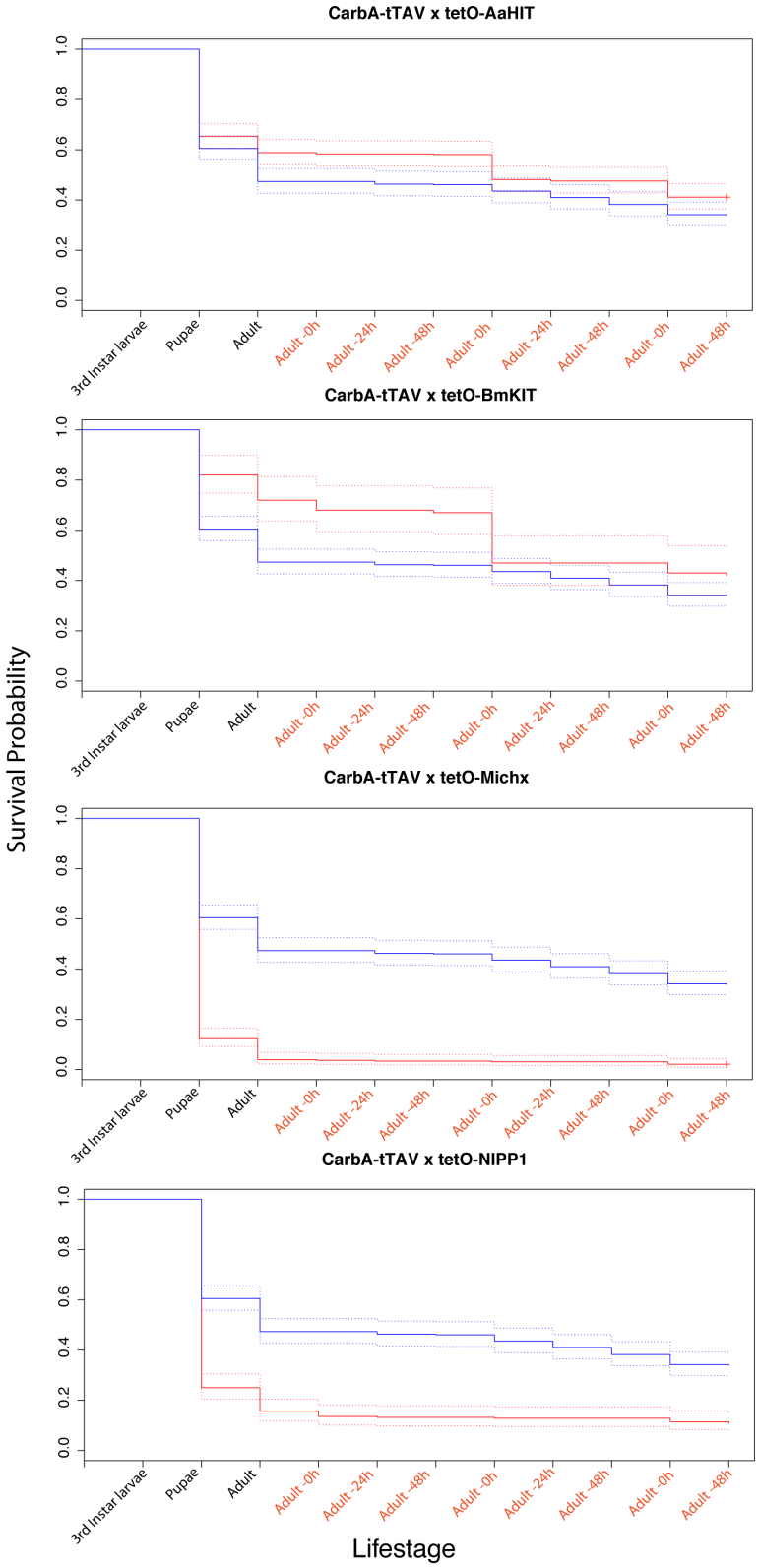


Figure 5.15: Survival curves of females, from 3rd instar larvae to the 3rd gonotrophic cycle, of the CarbA-tTAV-F line crossed to the four lethal effectors (red line) estimated using Kaplan–Meier survival analysis. The cross of the CarbA-tTAV-F line to wild type (blue) is included for comparison in each plot. 95% confidence intervals of the survival curve are drawn as dotted lines. Time points in red represent the three gonotrophic cycles with survival recorded immediately after a blood meal (0h) and then 24 and 48 hours (24h & 48h) subsequently. Sample sizes (n) for each cross are shown in Table 5.5.

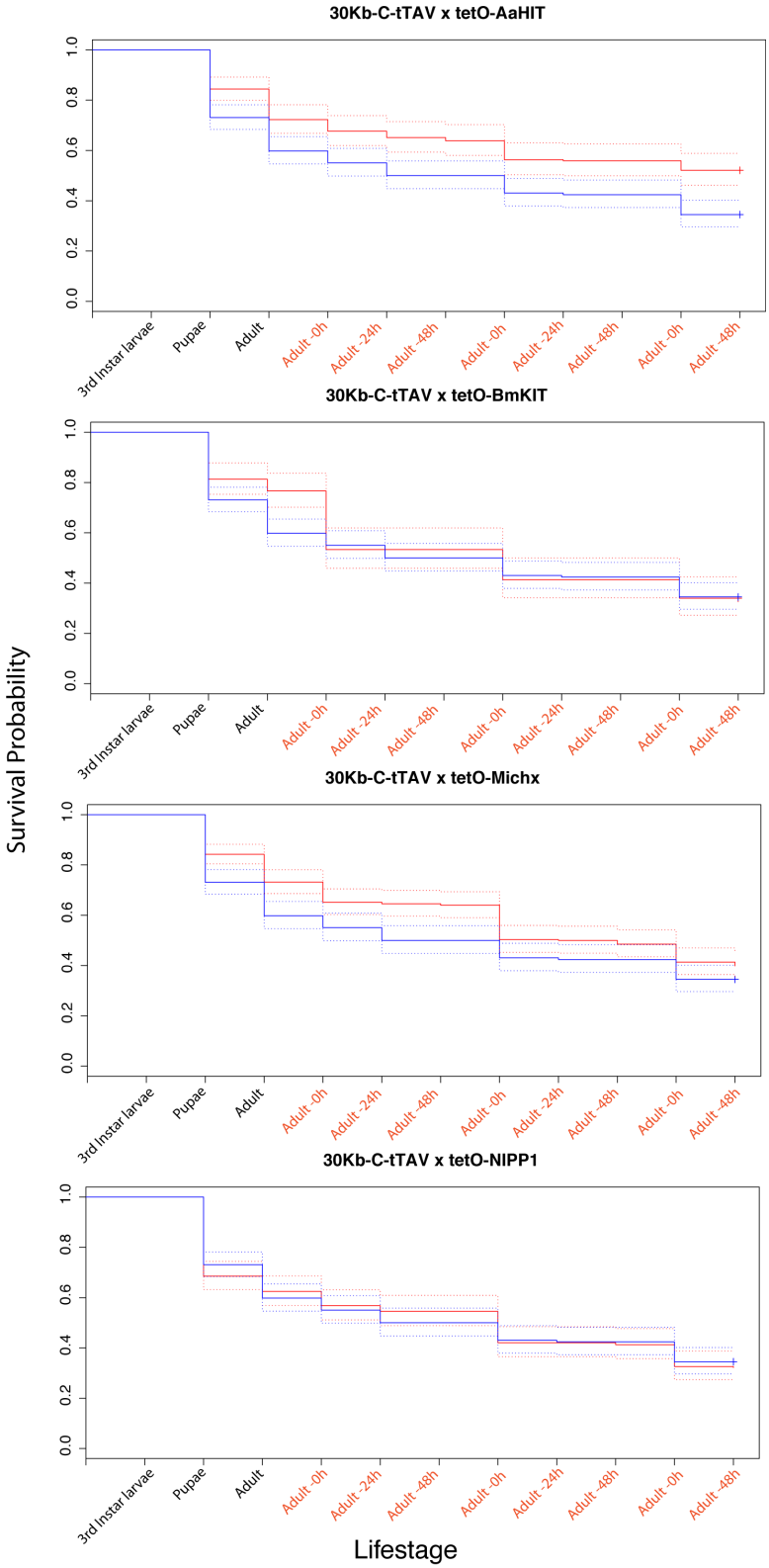


Figure 5.16: Survival curves of females, from 3rd instar larvae to the 3rd gonotrophic cycle, of the 30Kb-tTAV-C line crossed to the four lethal effectors (red line) estimated using Kaplan–Meier survival analysis. The cross of the 30Kb-tTAV-C line to wild type (blue line) is included for comparison in each plot. 95% confidence intervals of the survival curve are drawn as dotted lines. Time points in red represent the three gonotrophic cycles with survival recorded immediately after a blood meal (0h) and then 24 and 48 hours (24h & 48h) subsequently. Sample sizes (n) for each cross are shown in Table 5.5.

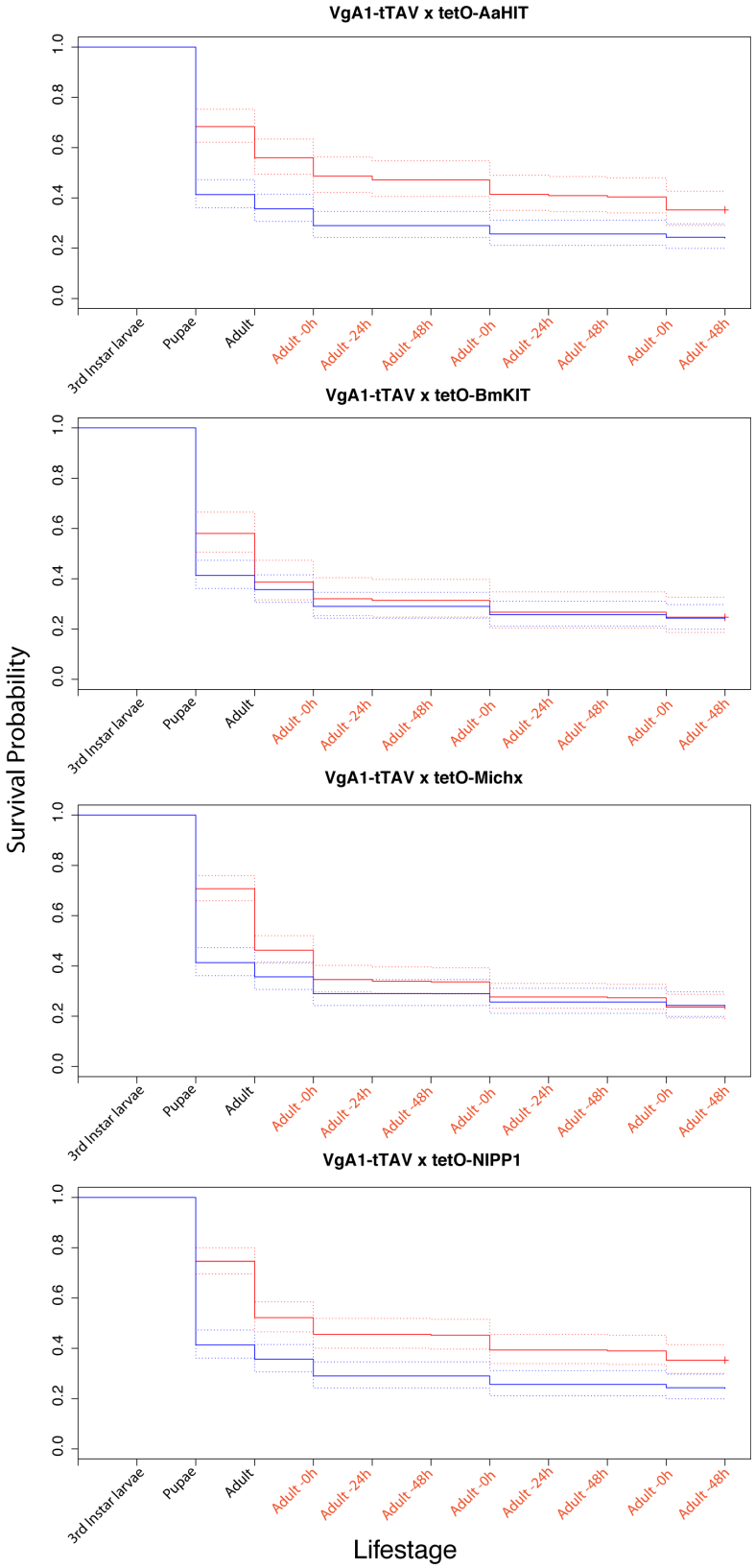


Figure 5.17: Survival curves of females, from 3rd instar larvae to the 3rd gonotrophic cycle, of the VgA1-tTAV-A line crossed to the four lethal effectors (red line) estimated using Kaplan–Meier survival analysis. The cross of the VgA1-tTAV-A line to wild type (blue line) is included for comparison in each plot. 95% confidence intervals of the survival curve are drawn as dotted lines. Time points in red represent the three gonotrophic cycles with survival recorded immediately after a blood meal (0h) and then 24 and 48 hours (24h & 48h) subsequently. Sample sizes (n) for each cross are shown in Table 5.5.

These observations suggest that expression of the four toxic effectors in the salivary glands and the fat body was not sufficient to induce a lethal phenotype or significantly reduce survival.

Visual observation of the behavior of females after blood feeding did reveal the existence of a sub-lethal phenotype in one of the VgA1-tTAV-A crosses. Females of this line carrying the tetO-AaHIT-D construct displayed muscle paralysis approximately 20-24 hours after a blood meal. Females were observed falling to the bottom of the cage apparently paralyzed, their wings beating asynchronously (see Figure 5.18).

Figure 5.18: Female carrying both the VgA1-tTAV-A and tetO-AaHIT-D insertions displaying scorpion toxin induced paralysis 22 hours after a blood meal.

After around four hours of this behavior, which it was assumed corresponded with the peak in the VgA1 promoter's activity, affected females recovered and resumed normal resting behavior. They did not appear to suffer any long-term consequences that could be detected through survival analysis. Fast excitatory

muscle contraction paralysis is associated with the typical mode of action of Na^+-channel blocking excitatory scorpion neurotoxins such as AaHIT (Possani et al. 1999, de Lima et al. 1989). The recovery of affected females suggests that only at the peak of the VgA1 promoter region expression is enough toxin present to induce paralysis in females. AaHIT does not bind irreversibly to receptors of the Na^+-channel, so as the systemic concentration of the toxin decreases it will begin to disassociate, restoring the channel's normal activity (de Lima et al. 1989).

5.4.11 Effect of tissue-specific tTAV expression on survival

Transposon insertions into the genome can have positive or negative consequences for survival depending on their genomic context (Irvin et al. 2004, Woodruff 1992). However, it was surprising that all of the crosses of the 30Kb-tTAV-C and VgA1-tTAV-A lines to the tetO-effector lines showed an improvement in survival compared to the cross to wild type —though the effect was small and not always significant — despite the presence of two insertions one of which expressed a toxic effector. This was likely due to the toxicity of the tTAV protein, whose VP16 domain has previously been reported to display toxic activity in mosquitoes (Fu et al. 2010, Lynd et al. 2011). This toxicity may be mitigated in crosses to lines carrying a tetO element, which binds to tTAV and so may lessen the amount of free protein available for biotoxicity, with any associated upregulation of the tetO activated effector too small to result in its own toxicity.

To determine if expression of the tTAV protein in the different tissues was having a significant effect on female survival, survival curves of the CarbA-A,

30Kb-C and VgA1-A tTAV lines were compared to those of wild type females. Log-rank analysis of the Kaplan–Meier curves, as shown in Figure 5.19, indicated a significant ($p<0.05$) decrease in survival for females carrying the tissue-specific tTAV constructs (see Table 5.6). The median survival of wild type females was greater than 18 days (more than the length of the experiment) post-3rd larval instar. The VgA1-tTAV-A line showed the greatest decrease in survivorship with most death occurring at the larval-pupal boundary. This was similar to the results seen in the crosses of the NIPP1 and michelob-x effectors to the CarbA-tTAV-F line.

Table 5.6: Statistical analyses of survivorship of females of the tissue specific-tTAV lines relative to wild type females. The relative survival of individuals of all three lines was worse (-) than wild type individuals. Log-rank analysis was used to compare the survival curves of the tTAV lines to wild type. Median survival is reported in days post screening of 3rd instar larvae.

Promoter line	Relative survival	Median survival (days)	n	X2	p-value
CarbA-tTAV-A	-	6	395	78.3	0
30Kb-tTAV-C	-	14	316	63.8	$1.3x10^{-15}$
VgA1-tTAV-A	-	3	300	135	0

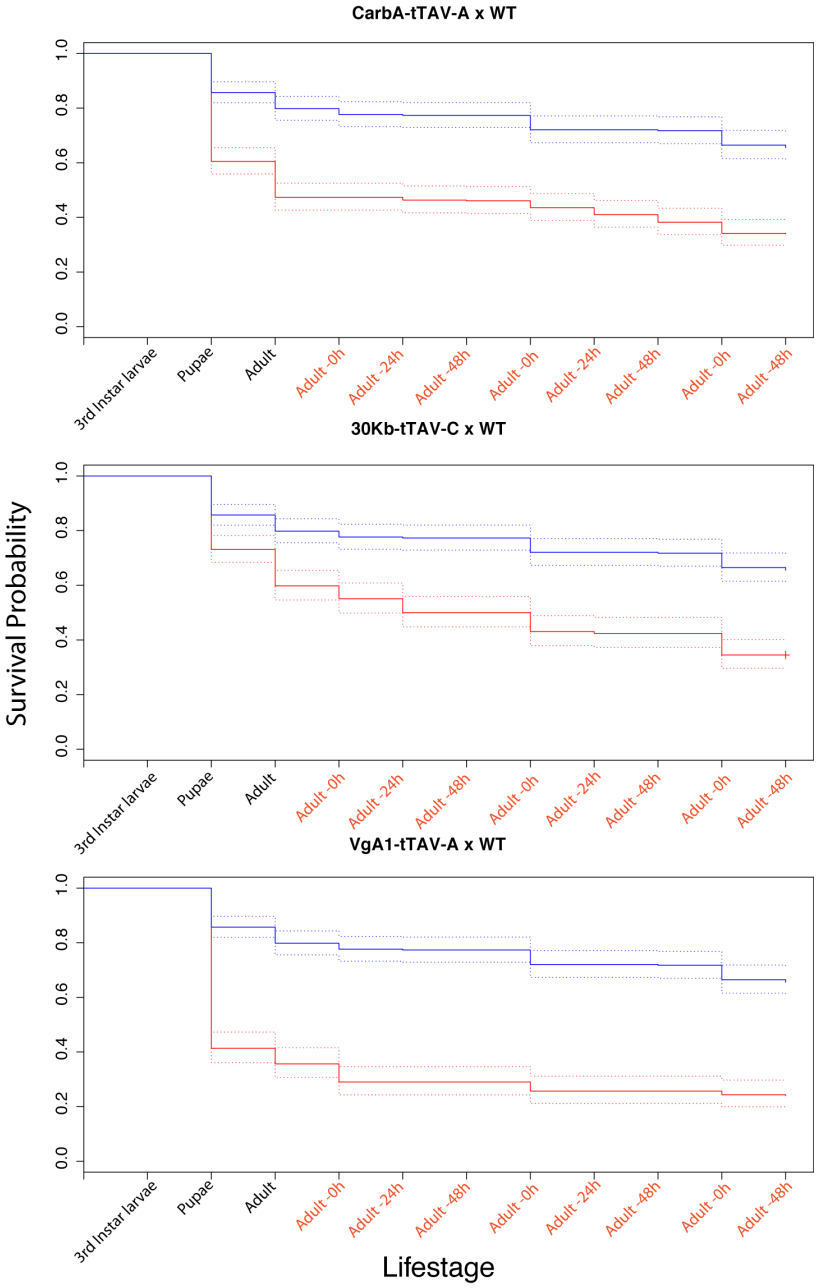


Figure 5.19: Survival curves of females of the tissue-specific tTAV lines (red) compared to wild type females (blue estimated using Kaplan–Meier survival analysis; from 3rd instar larvae to the 3rd gonotrophic cycle. 95% confidence intervals of the survival curves are drawn as dotted lines. Red labels on the x-axis represent the course of blood feeding from 0 hours to 48 hours, over three gonotrophic cycles. Sample sizes (n) for each cross are shown in Table 5.6.

As with the crosses to the toxic effectors blood feeding did not appear to induce an increase in mortality despite the real-time RT-PCR results showing upregulation of the tTAV transgene after blood feeding. This suggests that

females are most vulnerable to toxin-induced damage during their pre-adult development with the transition from larvae to pupae being particularly sensitive. Adult females appear to be far more resilient and increased expression of toxic effectors after blood feeding does not appear to affect survivorship.

5.4.12 Effect of tissue-specific expression of tTAV and toxic effectors on male survival

The effect of tissue specific tTAV expression on male survival was similar to that on females. Males hemizygous for the tTAV insertions survived significantly less than wild type males according to log-rank analysis of the survival curves (see Table 5.7).

Table 5.7: Statistical analyses of survivorship of males of the tissue specific-tTAV lines relative to wild type males (n=322, median survival >18 days).
Log-rank analysis was used to compare the survival curves of the tTAV lines to wild type. Median survival is reported in days post screening of 3rd instar larvae.

Promoter line	Relative survival	Median survival (days)	n	X2	p-value
CarbA-tTAV-A	-	14	395	52.0	$5.45x10^{-13}$
30Kb-tTAV-C	-	15	316	16.5	$4.76x10^{-5}$
VgA1-tTAV-A	-	3	300	38.1	$6.71x10^{-10}$

This was probably due to basal expression of the tTAV protein, as was the case with females. Whereas in females most death occurred at the larval-pupal boundary, the lessened survival of males was due to a higher rate of death in older males in the CarbA-tTAV-F and 30Kb-tTAV-C lines. For the VgA1-tTAV-A line most death did occur at the larval-pupal boundary (see Figure 5.20). The median survival of males for all three tissue-specific lines was greater than females. This could be indicative of elevated basal expression of the tissue-

specific promoters in females, which are in their natural context female-specific, or greater female sensitivity to tTAV or the toxic effectors.

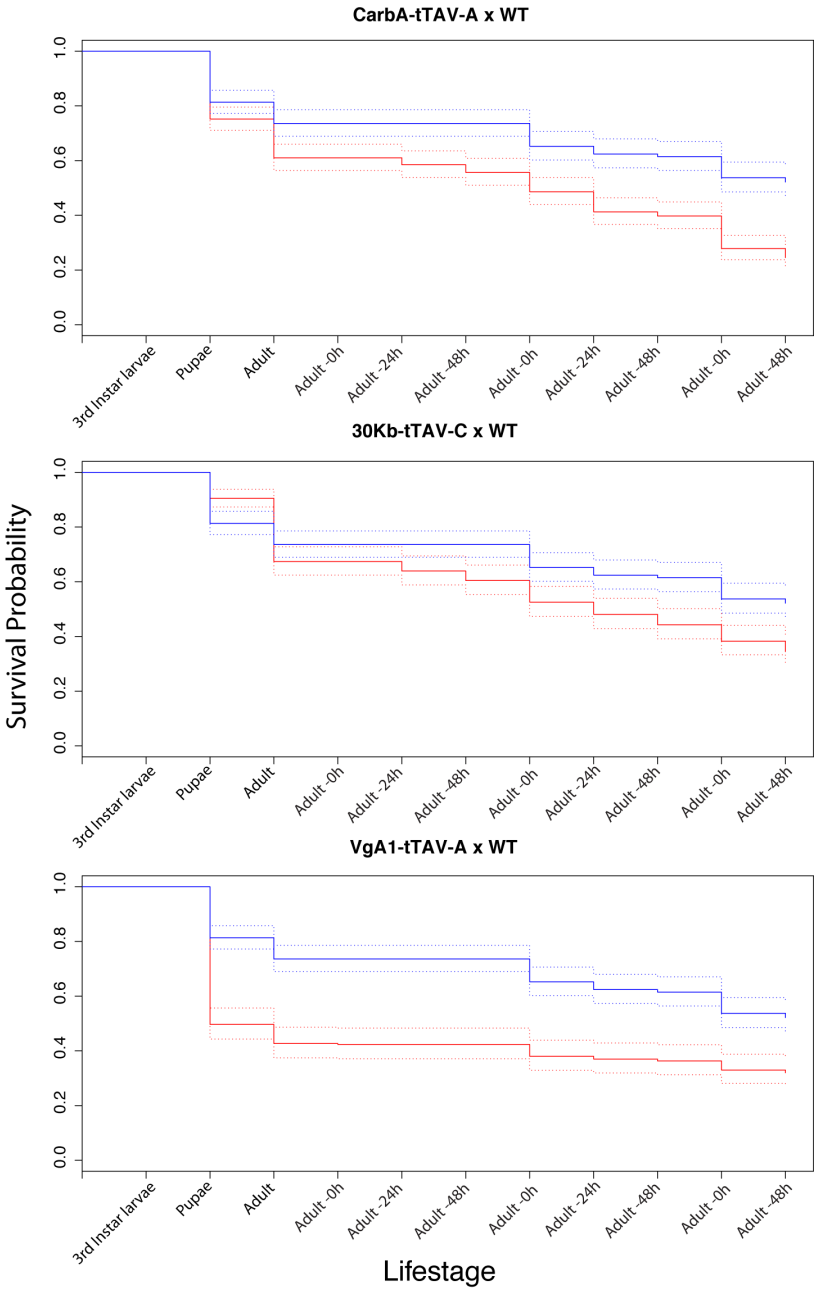


Figure 5.20: Survival curves of males estimated using Kaplan–Meier survival analysis; from 3rd instar larvae to the 3rd gonotrophic cycle (relative to females from the same crosses), of the tissue-specific tTAV lines (red) compared to wild type (blue). 95% confidence intervals of the survival curves are drawn as dotted lines. Sample sizes (n) for each cross are shown in Table 5.7.

In general males of the tTAV line crossed to the tetO-toxic effectors survived significantly longer than those crossed to wild type, though the effect was small (see Appendix 2 for Kaplan–Meier plots). This was similar to the results of survival analysis for females. As was the case with females, males of the Carb-tTAV-F lines crossed to the tetO-Michx and tetO-NIPP1 had significantly shortened lifespans compared to the wild type crosses and only 10-20% survived to adulthood (X^2=295, p=0 & X^2=118, p=0). It is clear from these results that the lethal effect observed in crosses of the tTAV lines to tetO-Michx and tetO-NIPP1 is not sex specific and that the basal expression of the Carboxypeptidase promoter region is occurring in all late instar larvae.

5.4.13 Effect of expression of toxic effectors on female fecundity

Endogenous expression of the four toxic effectors may induce a fitness cost by decreasing female fecundity; here defined as the number of viable eggs laid by a female after blood feeding. To determine if this was the case, the fecundity of females carrying the tissue-specific tTAV and the tetO-effector constructs, as well as those carrying only the tTAV constructs, was examined over three gonotrophic cycles. This data is shown in Figure 5.21.

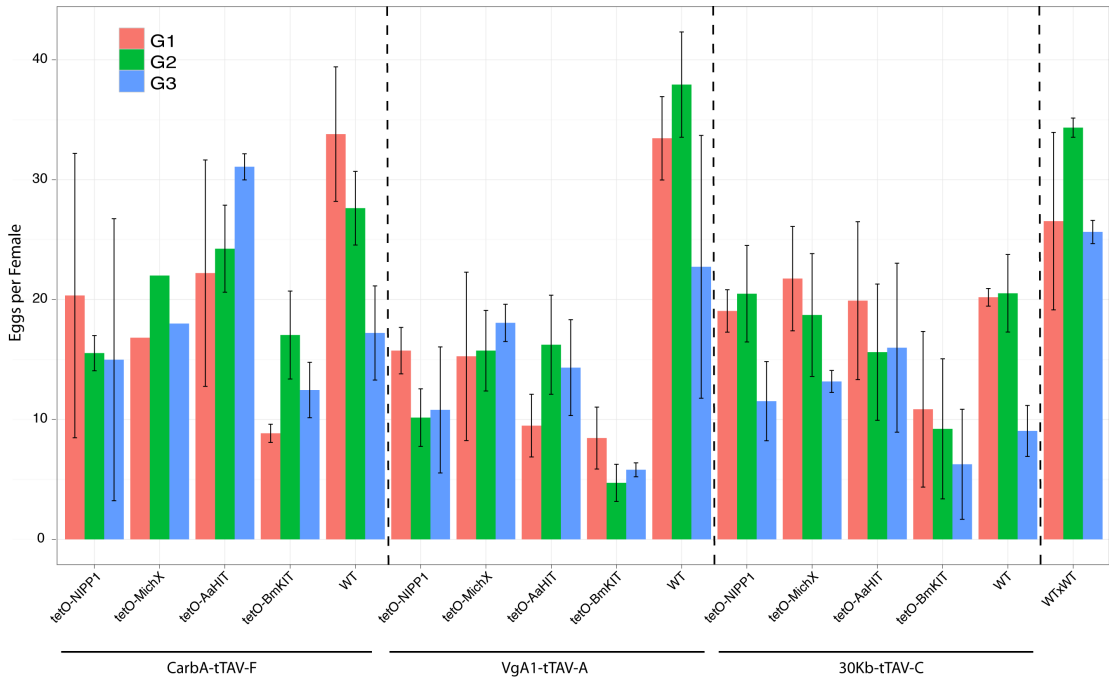


Figure 5.21: Mean number of viable eggs produced over three gonotrophic cycles (G1-G3) for crosses of the tissue-specific tTAV line to the tetO-toxic effectors or wild type (WT). Viable eggs were deduced by counting the number of 2nd instar larvae in hatching pots. Error bars are standard errors of the mean of three replicates of the pooled crosses. The fecundity of wild type females (WTxWT) over three gonotrophic cycles is included for comparison. Crosses of the CarbA-tTAV-F line to the tetO-MichX line resulted in eggs from only one of the replicates due to low female survival.

The number of viable eggs produced per female was extremely variable over all three gonotrophic cycles, with wild type females showing the smallest variance in egg production. For the CarbA-tTAV-F crosses to the tetO-NIPP1 and

Michelob-x effectors this was due to the very low number of individuals reaching adulthood. For other crosses this may be because females were fed in pools, which could have prevented females from taking blood meals of equal volume. The fecundity of females was also low compared to some previous studies of *Ae. aegypti*, where around 80-110 eggs per female per gonotrophic cycle were reported (Briegel et al. 2002, Liles et al. 1960). This reduced fecundity, even amongst the wild type control, might be caused by the pooling of females leading to increased competition for resources such as food and resting space, alternatively it may be an artifact of other rearing conditions. Female size is strongly positively correlated to fecundity and although the rearing density of larvae used was low it may have resulted in the production of smaller females that were less fecund (Briegel 1990).

In most cases the presence of both the tTAV source and tetO-toxic effector did not significantly ($p<0.05$) affect fecundity over the three gonotrophic cycles according to one-way analyses of variance (ANOVA) (see Table 5.8).

Table 5.8: Results of one-way analyses of variance (ANOVA) on the mean egg production per female, for each gonotrophic cycle and for each tissue-specific tTAV line.

tTAV line	Gonotrophic cycle	Degrees of Freedom	Mean squares	F	p-value
CarbA-tTAV-F	1	4	388.14	2.64	0.098
	2	4	226.83	3.22	0.061
	3	4	152.13	2.61	0.127
30Kb-tTAV-C	1	4	40.52	0.76	0.577
	2	4	51.66	0.80	0.554
	3	4	35.42	0.73	0.595
VgA1-tTAV-A	1	4	285.52	5.77	0.013
	2	4	438.58	12.12	0.001
	3	4	107.77	0.97	0.469

Only the VgA1-tTAV-A crosses showed a significant difference in gonotrophic cycles 1 and 2. In these cases the tetO-effector crosses showed a significant reduction, 2-5 fold, in the number of eggs laid per female compared to the cross to wild type, according to the student's t-test with Yate's correction (see Table 5.9).

Table 5.9: Results of pair-wise student's t-test on the mean fecundity of each cross of VgA1-tTAV to the four tetO-effectors compared to the cross of VgA1-tTAV to wild type.

Effector Cross	Gonotrophic Cycle	Degrees of freedom	t	p-value
tetO-AaHIT	1	3.71	5.51	0.007
tetO-BmKIT		2.07	4.41	0.045
tetO-MichX		2.99	5.77	0.010
tetO-NIPP1		3.13	4.45	0.019
tetO-AaHIT	2	3.26	5.56	0.009
tetO-BmKIT		2.24	5.94	0.021
tetO-MichX		2.92	5.78	0.011
tetO-NIPP1		2.75	4.62	0.023

It was possible that the VgA1 promoter fragment was causing very early expression of the toxic-effector in embryos, resulting in death before screening of the 2nd instar larvae and so artificially reducing the observed fecundity. To determine if this was case the number of larvae carrying the two transformation markers of the tTAV and tetO constructs was checked to determine if it deviated significantly from its expected Mendelian inheritance ratio of 0.25. The results of this screening are shown in Table 5.10 and reveal that there was no significant ($p<0.05$) deviation from the expected Mendelian inheritance ratio except in the VgA1-tTAV-A/tetO-NIPP1 cross, where a lower than expected proportion of individuals carrying both constructs was observed.

Table 5.10: Mendelian inheritance of the VgA1-tTAV construct and tetO-toxic effectors in crosses of line VgA1-tTAV-A. L2 larvae were screened with fluorescence microscopy for the presence of absence of the two transformation markers and the ratio of those carrying both markers calculated. Deviation from the expected inheritance ratio of 0.25 was tested with the Chi-squared test of significance.

tetO effector	Ratio	X2	p
AaHIT	0.27	1.2539	0.263
BmKIT	0.24	0.3226	0.5701
MichX	0.3	0.0085	0.927
NIPP1	0.17	5.8357	0.0157

These results suggested that early expression of the NIPP1 effector might be killing embryos before development to the 2nd instar. For the remaining crosses they suggested that differences in viable egg number were due to the action of the toxic effectors on the mother.

The Vitellogenin A-1 gene plays an important part in oogenesis in *Ae. aegypti*, producing the yolk protein precursor vitellogenin, a major component of developing eggs, in the fat body (Hagedorn 1974). Damage to the fat body caused by expression of cell lethal effectors, such as Michelob-x and NIPP1, may well prevent normal synthesis and transport of vitellogenin to developing eggs and so inhibit egg production. Expression of the insect-specific scorpion neurotoxins may also prevent normal egg development via transport of the toxic peptides into the developing embryos. Such transport of small molecules into eggs during the vitellogenic stage has previously been demonstrated for immunoglobulins and the scorpion neurotoxins are only 6-8kDa in size (Ramasay et al. 1988, Taniai et al. 2002). Alternatively they may alter the

behavior of gravid females, preventing them from depositing eggs normally or triggering resorption of developing eggs through toxic stress (Canyon et al. 1999).

5.4.14 Suppression of tTAV expression using small interfering RNAs

Although a toxic effector capable of inducing post-blood meal lethality was not identified it was considered important to determine if RNAi could be used as the suppressor in an engineered underdominance system. Generation of lines capable of efficient RNAi is challenging and labor intensive and constructs generating hairpin RNA are vulnerable to position effects which may dramatically alter the effectiveness of RNAi (Franz et al. 2006, Sandy et al. 2005, Roignant et al. 2003). This can necessitate a great deal of screening to find lines capable of producing a strong RNAi effect. To avoid the time associated with this process it was decided to obtain tissue-specific RNAi lines from the laboratories' of Oxitec Ltd.'s collaborators. These lines have previously been shown to induce RNAi-based resistance to DENV-2 through the endogenous expression of a DENV-2-derived hairpin that is processed into siRNAs. These siRNAs prevent replication of the virus via RISC-mediated degradation of viral transcripts and so produce a dengue resistant phenotype in the lines. Carb109 is a midgut specific RNAi line identical in structure to the Carb77 line of Franz et al. (2006). Its functional component is the Mnp hairpin: a 578-bp fragment of the DENV-2 prM protein-encoding region arranged in sense and antisense orientation around the minor intron of *Ae. aegypti* sialokinin I gene. Expression of this hairpin is controlled by the Carboxypeptidase 1-A promoter fragment which is identical to that used in the CarbA-tTAV construct (Franz et al. 2006).

The 30K-GM-15 line is similar in structure to Carb109 but with the 30Kb promoter, identical to that used in the 30Kb-tTAV line, driving expression of the Mnp hairpin. Finally the Vg40 line has the Mnp hairpin driven by Vitellogenin A-1 promoter fragment, again identical to that used in the tTAV construct.

Female mosquitoes hemizygous for the tissue-specific tTAV constructs and the OX3576 DsRed reporter construct were themselves crossed to hemizygous males of the RNAi lines expressing Mnp hairpins in the corresponding tissue. The progeny of these crosses were screened for the presence of the three constructs' transformation markers and the expression of the DsRed reporter protein examined before and after blood feeding. This proved to be difficult as the tTAV and effector constructs shared transformation markers with the RNAi constructs. By screening at the larval stage most of the lines could be identified through their unique expression profiles caused by the position effects of each constructs' insertion; however, it proved impossible to distinguish those individuals that carried the 30K-GM-15 from those that did not.

Before blood-feeding, expression of the DsRed reporter in CarbA-tTAV-A females crossed to the OX3576 line was not obviously different in those that also carried the Carb109 insertion from those that did not (see Figure 5.22).

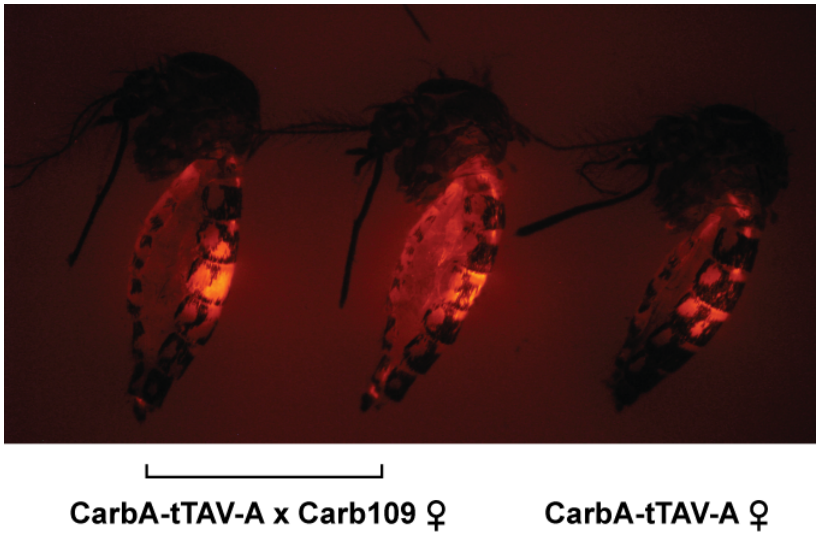


Figure 5.22: Pre-blood meal expression of the tetO-DsRed reporter in undissected females of the CarbA-tTAV-A line that either carried the Carb109 RNAi insertion or did not. Expression of the DsRed marker in the midgut and alimentary canal can be clearly seen through the abdominal wall.

24 hours after blood feeding expression of the DsRed reporter was observed in the presence or absence of the Carb109 insertion. However, in those individuals carrying the Carb109 insertion expression of the DsRed reporter was distinctly dimmer in the engorged midgut, as shown in Figure 5.23.

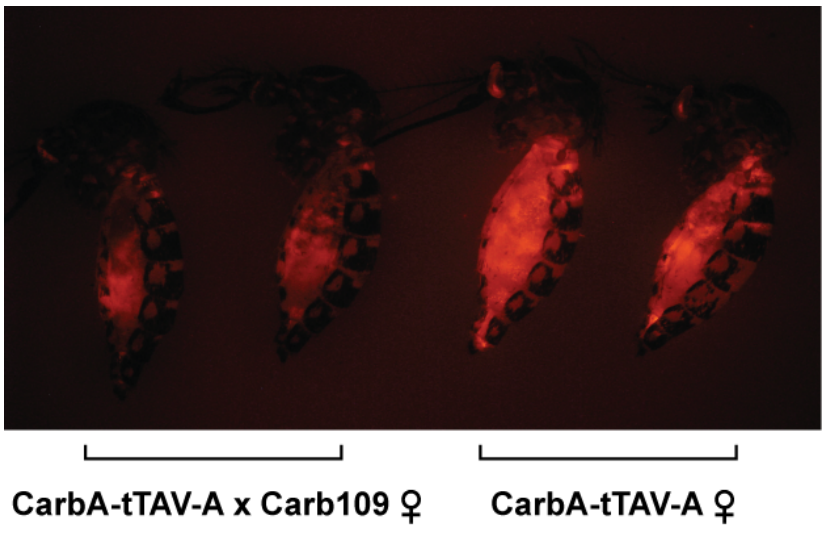


Figure 5.23: Expression of the tetO-DsRed reporter in undissected females of the CarbA-tTAV-A line, that either carried the Carb109 RNAi insertion or did not, 24 hours after a blood meal. Expression of the DsRed marker in the engorged midgut and alimentary canal can be clearly seen through the abdominal wall. Individuals carrying the Carb109 insertion showed patchy expression in the midgut.

Dissection of the midguts revealed patchier expression of DsRed in those carrying the Carb109 insertion. This suggests that RNAi may have been partially degrading mRNA transcripts of the tTAV gene fused to the Mnp fragment from the DENV-2 virus thus limiting transactivation of the tetO element. This interference was apparently not strong enough to prevent some synthesis of the tTAV protein resulting in patchy expression of the reporter.

Crosses of the Vg40 RNAi line to the VgA1-tTAV-A line showed similar results. Before blood feeding reporter fluorescence was visible in females irrespective of whether they carried the Vg40 insertion (not shown). Twenty-four hours after a blood meal fluorescence was still visible in all individuals but those carrying the Vg40 insertion generally displayed dimmer DsRed expression in the fat body as shown in Figure 5.24. Once again this suggested that RNAi might be suppressing expression of the tTAV protein, though not completely.

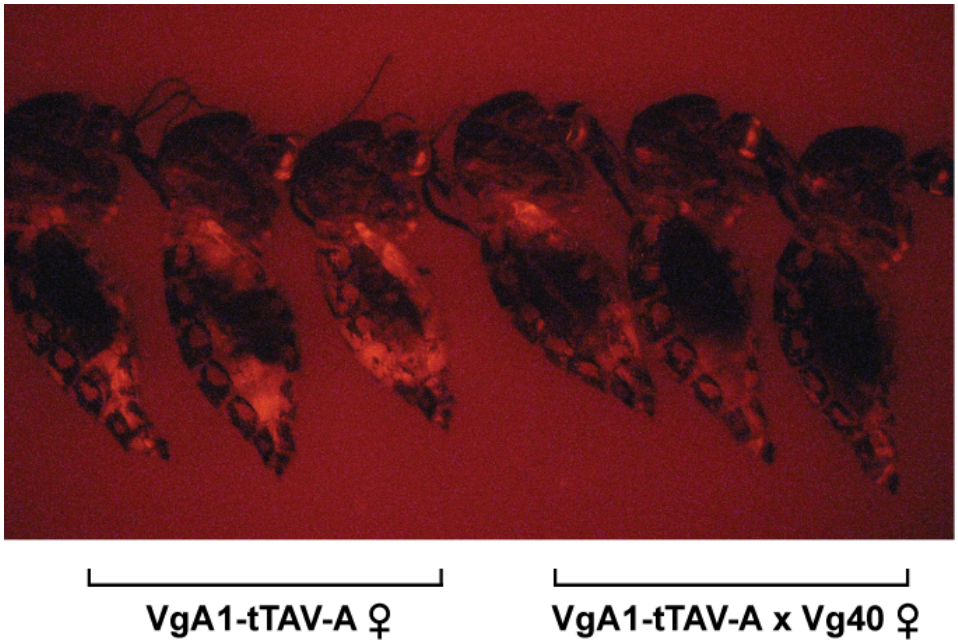


Figure 5.24: Expression of the tetO-DsRed reporter in undissected females of the VgA1-tTAV-A line, that either carried the Vg40 RNAi insertion or did not, 24 hours after a blood meal. Expression of the DsRed reporter was generally dimmer in the fat body of females carrying the Vg40 RNAi insertion, although this was variable between individuals.

It was not possible to determine which individuals carried the 30K-GM-15 insertion in three-way crosses of the 30Kb-tTAV-C line to the reporter and RNAi lines through visual inspection due to overlap of the transformation markers. Hence 100 G1 individuals from the crosses were screened for dimmer expression in the salivary glands after a blood meal. Mendelian inheritance predicts that 12.5% of the individuals of such a hemizygous cross should carry all three constructs. Some variation in expression in the salivary glands was observed however this was only in a very small proportion of G1 offspring (4%) and was more likely the result of individual variation than RNAi.

5.4.15 RNAi mediated knockdown of tTAV transcripts

To check if the reduced expression of the florescent reporter protein in crosses to the RNAi lines was correlated to a lower abundance of tTAV transcripts; real-

time RT-PCR was performed on RNA extracted from the abdomens of screened G1 progeny before and 24 hours after blood feeding. tTAV-specific primers were used to amplify tTAV transcripts and the results normalized against the expression of the 18S gene as described in section 5.4.4.

The results of the real-time PCR for the crosses of CarbA-tTAV-F to Carb109 are shown in Figure 5.25.

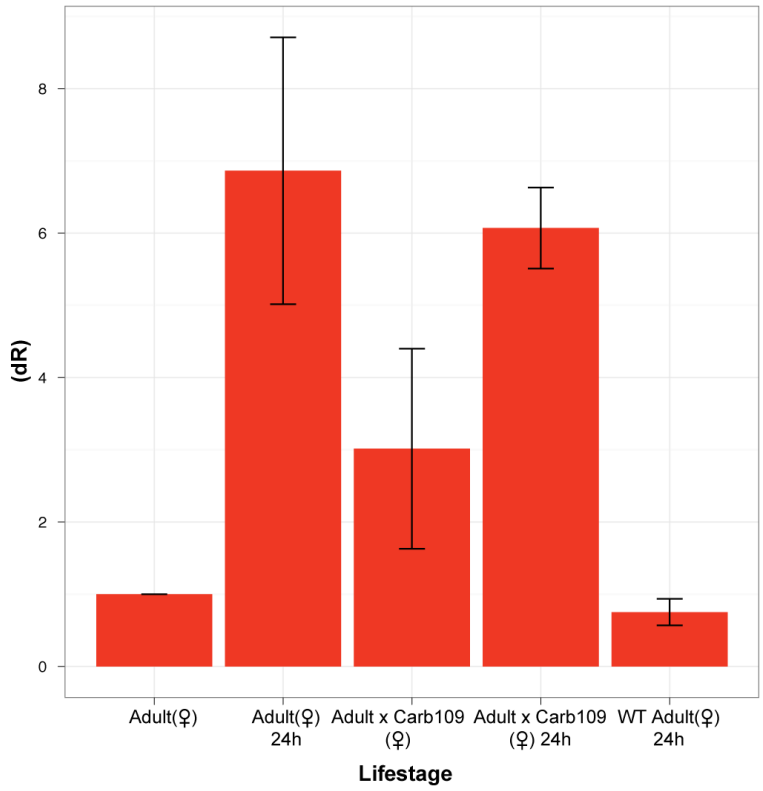


Figure 5.25: Mean relative expression levels (dR) of tTAV in CarbA-tTAV-F females crossed to Carb109 compared to females carrying only the CarbA-tTAV-F insertion, before and 24 hours after a blood meal, determined by real-time RT-PCR. Expression is reported relative to the expression in females of the CarbA-tTAV-F line alone before blood feeding and is normalized against expression of the 18S gene. Error bars show the standard error of the mean of five experimental replicates on RNA extracted from 3-pooled individuals. Expression in wild-type (WT) adult females 24 hours after blood feeding is included for comparison.

There was no significant change in the relative expression of tTAV transcripts when CarbA-tTAV-A lines were crossed to the Carb109 line either before a

blood meal or 24 hours after a blood meal. This was in contrast to the results from the crosses of VgA1-tTAV-A to Vg4o shown in Figure 5.26. In this case tTAV transcript levels in the fat body appeared to decline dramatically when co-expressed with the Mnp +/- hairpin of the Vg4o construct. Before blood feeding, relative expression levels of tTAV in females, crossed to wild type or Vg4o, were not significantly different according to the Student's t-test. However, 24 hours after a blood meal there was a significant reduction in tTAV transcript expression in those females carrying the Vg4o construct of around 12o fold, to approximately the same level seen in wild type (t= 6.366, p=o.oo3).

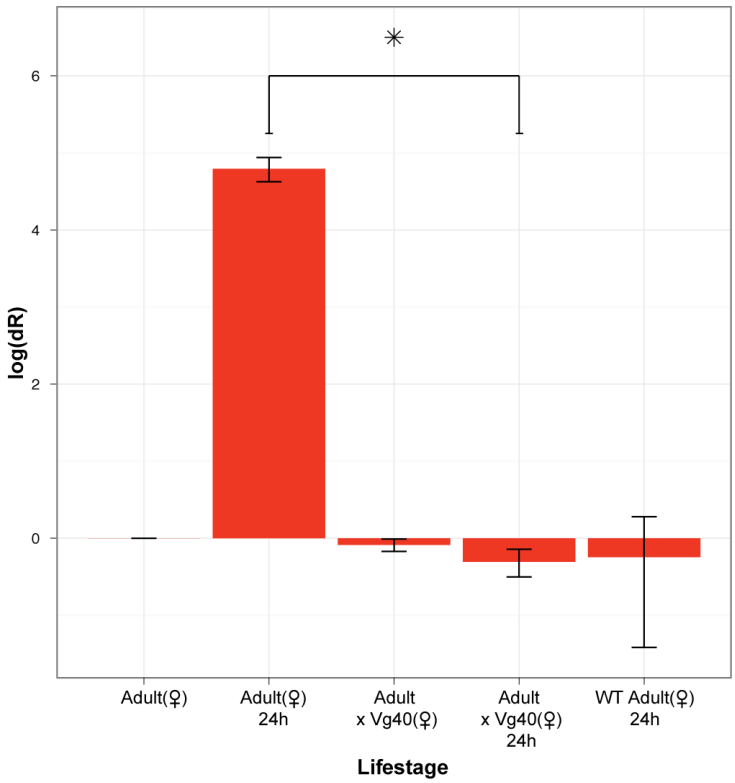


Figure 5.26: Mean relative expression levels (dR) of tTAV in VgA1-tTAV-A females crossed to Vg40 compared to the relative expression in females carrying only the VgA1-tTAV-A insertion, before and 24 hours after a blood meal, determined by real-time RT-PCR. The logarithm of dR is reported relative to the expression in females of the VgA1-tTAV-A line before blood feeding and is normalized against expression of the 18S gene. Error bars show the standard error of the mean of five experimental replicates on RNA extracted from 3-pooled individuals. Significant difference ($p<0.05$) between samples from the same time period are indicated with a *. Expression in wild-type (WT) adult females 24 hours after blood feeding is included for comparison.

The lack of apparent transcript knockdown in the Carb-tTAV-F crosses, despite the observation that fluorescence of the DsRed reporter decreased in females carrying the Carb109 insertion may have a number of explanations. It may be the case that no RNAi was occurring and the reduction in fluorescence was due to titration of Carboxypeptidase-specific transcription factors caused by competitive binding between the endogenous promoter sequence and those in the CarbA-tTAV and Carb109 constructs. The effect that fusing the Mnp sequence to the tTAV coding region may have on its characteristics as a target

for RNAi is unknown, but both the local sequence of the construct and its genomic insertion site could reduce its ability to be suppressed (Yoshinari et al. 2004). It may also be the case that the peak expressions of the Carb109 and CarbA-tTAV-F lines occur at different times post-blood feeding, despite the fact that they share identical promoter regions. This could result in early suppression of tTAV transcripts in the first few hours after blood feeding; and so downstream reduction in DsRed expression. By the 24-hour mark production of siRNAs could have decreased and high levels of transcript expression been reestablished.

The results from the VgA1-tTAV-A crosses to Vg40 suggest that RNAi is a potent suppressor of tTAV transcripts in this context, reducing post-blood meal expression to levels not significantly different from females before blood feeding. The magnitude of the effect may be due to the many fold upregulation of the VgA1 promoter fragment induced by blood feeding, the genomic context of the constructs or the activity of the RNAi pathway in fat body cells. Explaining why such a strong effect is seen in the VgA1-tTAV as opposed to the CarbA-tTAV line is unlikely to be possible without a much more detailed understanding of the RNAi system in mosquitoes. Despite the observed reduction in the number of transcripts DsRed expression was observed in the fat body of females carrying the RNAi line 24 hours after a blood meal (see section 5.4.14). This may be due to early expression of tTAV combined with the long half-life of DsRed, resulting in fluorescence even when protein translation has ceased (Verkhusha et al. 2003).

5.5 Discussion

Both engineered underdominance and killer-rescue gene drive systems require a killer system that is able to produce a lethal phenotype in mosquitoes, and a rescue system able to repress the killer that is in turn linked to the refractory gene desirous of spread. A refinement of this system is to combine the refractoriness and rescue systems into a single component; an RNAi system capable of silencing transcripts of the killer and the Dengue virus. This chapter demonstrates that the construction of such a system is technically challenging, suggesting that functional underdominance or killer-rescue systems may be difficult to achieve in *Ae. aegypti*.

Generating a lethal phenotype through expression of a toxic effector in tissues involved in the dengue virus life cycle was the first hurdle encountered. Achieving the ideal combination of tissue and toxic-effector is not trivial; the primary problem encountered being the identification of suitable promoters. Lines expressing tTAV tissue-specifically were successfully created; demonstrating that targeting of dengue relevant tissues is possible. However, producing lethality through expression of toxic effectors in these tissues proved more challenging. Most of the toxic-effectors tested were inactive or too weak to generate a lethal phenotype when expressed in the midgut, fat body or salivary glands of mosquitoes. Some of those that were active, such as Michelob-x and NIPP1 expressed in the midgut, proved too toxic due to early basal expression of the toxic transgene by the promoter used.

The three tissue-specific promoter elements employed in this chapter have in previous studies been identified as female and time specific regulatory elements in the context of the endogenous genes they control (Edwards et al. 2000, Martin et al. 2001). This chapter demonstrates the difficulty in replicating this specificity when the promoters are used to drive transgenes. While tissue-specificity was obtained in all cases, expression was not limited to females and expression of the transgene occurred from the late larval stage onwards. Previous studies on the promoter regions of CarbA-1 and Vg1A have reported using them to drive transgenes sex and time specifically however, these studies have either failed to monitor males for expression or have used relatively insensitive techniques such as immunoblot analyses or northern analysis to detect expression of the transgene (Kokoza et al. 2000, Adelman et al. 2008, Moreira et al. 2000). The use of Real-Time RT-PCR reported here is a much more sensitive assay for expression and combined with the results from the crosses to the DsRed reporter showed that all of the tissue specific promoters were driving basal expression in both sexes from the late larval life stage onwards. This is the probable cause of the lethality seen in the CarbA-tTAV-F crosses to the Michelob-x and NIPP1 effectors. This early expression makes it unlikely that the RNAi lines, which have been shown to only strongly express siRNAs after a blood meal, could suppress the lethal phenotype (Franz et al. 2006). Early expression of toxic-effectors without simultaneous expression of the rescue system would result in a large associated fitness cost that would make the system undesirable for an underdominance or killer-rescue gene drive; where fitness effects must be minimized to achieve a realistic release

ratio (Magori et al. 2006). The negative impact of early expression on fitness was particularly apparent from the survival analysis of mosquitoes carrying the tTAV constructs only. Longevity of these mosquitoes was greatly reduced compared to wild type individuals no doubt as a result of tTAV expression.

Expression of the insect-specific excitatory neurotoxin AaHIT in the fat body was the most promising of the tissue/effector combinations; resulting in a paralysis phenotype. Unfortunately, females rapidly recovered from this effect and did not appear to suffer any long-term negative consequences. In the field temporary paralysis is likely have much more severe consequences for survival where predation has a severe impact on survival of adult mosquitoes (Strickman 2006). Recovery of affected females suggests that: either the neurotoxin activity was greatly reduced in *Ae. aegypti*, for example due to misfolding of a proportion of the protein molecules whose four disulphide bonds are critical for activity, or that the fat body is a poor choice of tissue for expression of neurotoxins that act on the nervous system (Taniai et al. 2002, Gordon et al. 2007). It is possible that the activity of this effector could be improved by coexpression with a depressant neurotoxin (Possani et al. 1999). Such simultaneous expression of neurotoxins with opposing modes of action on sodium channel receptors and non-competitive binding sites has previously been suggested as a way of greatly increasing neurotoxin lethality in insects (Gordon 1997).

An inability to generate a lethal phenotype after a blood meal was not the only barrier to construction of a functional underdominance/killer-rescue system

using RNAi. It was hoped that the use of the dengue virus Mnp fragment fused to the tTAV protein would allow tTAV transcripts to be knocked down by RNAi against the dengue virus, combing the repressor and refractoriness elements of the system in one. Unfortunately Mnp specific siRNAs proved unable to prevent expression of the tTAV protein via RNAi. In the presence of the RNAi lines there was only slight reduction in the activation of the tetO-DsRed reporter, despite real time RT-PCR showing significant degradation of tTAV transcripts in some of the tissue-specific lines. This suggests that RNAi is not an efficient suppressor for use with tTAV constructs described in this chapter. If a toxic effector were identified that could induce death in adults it would likely require extremely efficient suppression to be effectively rescued. Fusing the coding sequence of the toxic-effector to the Mnp fragment, therefore making the toxic-effector transcripts vulnerable to RISC degradation as well as the tTAV transcripts, could potentially increase the efficiency of RNAi.

Great advances have been made in rendering mosquitoes refractory to dengue fever. The use of Mnp derived siRNAs for RNAi has resulted in the creation of lines incapable of transmitting dengue fever. The research in this chapter was driven by the goal of creating a killer-rescue system that could use these already established lines as the repressor and refractory element. For the construction of such a system each component of the system must meet a specific set of criteria. The killer must be toxic enough to induce a lethal phenotype when upregulated but not so toxic as to cause severe off-target fitness effects. The repressor must be strong enough to completely suppress

the killer at the peak of its expression; and, if it is also the refractory mechanism, must be expressed in a tissue where it can induce refractoriness. Underdominance and killer-rescue systems are advantageous in that fitness effects caused by the systems need not be eliminated just minimized, and even moderate fitness penalties can be overcome by increasing the release ratio (Magori et al. 2006, Gould et al. 2008). Despite this, the work in this chapter has demonstrated that the most obvious candidates for promoters for use in such systems; from the Carboxypeptidase A-1, 30Kb and Vitellogenin 1-A genes are unlikely to be suitable for construction of such engineered gene drives.

Bioinformatical analysis of the recently published *Ae. aegypti* genome as well as gene expression studies have already identified many other candidates for blood meal inducible regulatory elements which may provide more suitable promoters for killer-rescue systems (Sanders et al. 2003). Further engineering of the existing promoters may also produce better results, for example by the introduction of artificial sex or time dependent splicing to more precisely control expression. Many more potential toxic effectors exist that may prove more efficacious when expressed in adult mosquitoes. These include many other insect specific neurotoxins, other IAP antagonists and a host of other insect-specific toxins with a huge variety of modes of action (Zlotkin et al. 1994, Bryant et al. 2008, Kramer et al. 1998). Using these components the constructs described in this chapter could potentially be used to create a functional gene drive using underdominance or killer-rescue to replace naturally occurring populations of *Ae. aegypti* with refractory populations.

Chapter 6

Summary and Conclusions

There is a continued need for new control methods to combat the threat to human health caused by mosquito borne diseases. Billions of people worldwide are at risk of infection and millions die annually as a direct result of mosquito mediated transmission. The worldwide resurgence and reemergence of many of these diseases is ongoing, and multinational efforts, such as the Roll Back Malaria partnership and Grand Challenges in Global Health initiative, are underway to combat them (World Health Organization. Global Malaria Programme 2010). Effective vaccines for many of these diseases remain elusive and it is likely to be years before they can be deployed to the populations that need them most.

Vector control therefore remains an important tool to combat these diseases. However, traditional control methods are often costly and inefficient at controlling mosquito populations and have been particularly ineffective at reducing populations of *Ae. aegypti*, the primary vector of Dengue Fever. An increase in resistance to insecticides is of particular concern to ongoing control programs. This is unfortunate because insecticides, such as pyrethroid-impregnated bednets, have been effective in reducing the mosquito-borne disease burden in many of the world's worst affected areas (Enayati et al. 2010). Unfortunately, such interventions may become increasingly ineffective as evolved resistance spreads through mosquito populations. The need for new technologies to aid in vector control has lead to a great deal of research into the genetic engineering of vector mosquitoes. It is hoped that engineered strains can be used to either suppress wild populations, or replace them with varieties

with reduced, or eliminated, vector competence. With the first field releases of engineered mosquitoes designed to effect population suppression having recently occurred the potential of engineered strains is beginning to be realized (Harris et al. 2011).

As field releases become more common the issue of regulation of transgenic mosquitoes is coming to the fore. Development of engineered mosquitoes that are intended for release into the field, particularly those lacking engineered sterility, that are designed to persist in the wild will require new tools and an improved understanding of the ones already available to ensure that strains intended for release will be deemed acceptable by regulatory bodies (Benedict et al. 2008). Of particular importance will be an understanding of the post-integration behavior of the gene vectors that are used to transform mosquitoes. The development of molecular tools to engineer mosquitoes in a sequence conservative and site-specific manner will also be invaluable for creating strains intended for release. Finally, toward the eventual goal of replacing wild populations, gene drive systems must be developed that can effectively introgress refractory genes into wild populations. Such driving mechanisms must meet a variety of scientific and regulatory criteria if they are to be deemed suitable for engineering wild populations, but many of the possible systems remain undeveloped or have not yet been demonstrated in a mosquito model.

The work in this thesis was conducted with the aim of developing and understanding the tools required for the creation of components of a gene drive system for use in *Ae. aegypti*. Firstly the post-integration stability of the

piggyBac transposon was investigated, see Chapter 3. The *piggyBac* system is the most widely used and efficient transposon system for engineering *Ae. aegypti* and the initial engineering of a gene drive system would have to employ a vector such as *piggyBac* to insert the genetic constructs into the genome. The stability of *piggyBac* in the germline of *Ae. aegypti* was found to be extremely high, with no evidence of remobilization of either of the flanking transposons in the OX3885 system being observed when a source of transposase was provided, either endogenous or exogenously. This suggests that the germline stability of *piggyBac* transposons in *Ae. aegypti* is at least an order of magnitude greater than that observed in other mosquito species and even more so than that observed in other insects where germline remobilization may by readily induced. These results indicate that *piggyBac* would be a good choice for use as a gene vector for the creation of systems such as gene drivers, where stable expression of a transgene at a characterized locus is highly desirable. Contrary to previous reports, albeit performed with different constructs, somatic movement of *piggyBac* transposons was found to occur at a rate that was readily detectable and clearly mediated by the canonical *piggyBac* transposition mechanism. This observation supports the theory that germline stability of the transposon is enforced by a specific endogenous mechanism, which would account for the great difference in germline remobilization rates observed between *Ae. aegypti* and other insects.

In Chapter 4, the use of site-specific recombinases as tools for complex engineering of the *Ae. aegypti* genome was investigated. RMCE using the ϕC31

integrase system was demonstrated for the first time in a pest insect. Using a combination of fluorescent marker and PCR assays it was shown that this method of RMCE could efficiently interchange two cassettes engineered between the attachment sites recognized by the integrase. The ability to insert transgene cassettes without the need to simultaneously insert the plasmid backbone and associated antibiotic resistance genes will be particularly valuable for the engineering of strains intended for field release. The inclusion of antibiotic resistance genes in genetically modified food plants has been among the most controversial aspects of that technology, so avoiding their insertion into transgenic mosquitoes is desirable (Bradford et al. 2005). Site-specific tools, such as RMCE, will be particularly useful for the engineering of gene drive systems. For example, it may be that after release of a strain designed for population replacement that the refractory element must be changed or updated to account for evolution of resistance of the pathogen. RMCE could allow this to be done without the need to retransform the entire genetic construct, allowing the updated refractory mechanism to be inserted into a previously characterized site, whose properties are understood by both researchers and regulators.

Attempts to expand the RMCE system to use heterotypic attachment sites from multiple site-specific integrases, to overcome the possibility of unintended homologous recombination between identical sites, were not successful. Neither the Bxb1 and ϕRV1 integrases, provided in the form of capped mRNA, were able to promote recombination between their wild-type attachment sites

in the germline of *Ae. aegypti*. This was despite their previously demonstrated activity in a variety of organisms, including *D. melanogaster*. Why they failed to work is unknown; it could be that protein was not being translated from the microinjected mRNA (though the capped mRNA system is successful with the ϕC31 integrase system), or that necessary host co-factors are not present in *Ae. aegypti*. Equally, attempts to use pseudo-integration to mitigate the need for transposon gene vectors failed due to the lack of activity of the Bxb1 integrase, with only non-canonical integrations being observed. Despite these failures, the use of multiple site-specific integrase-systems remains a potentially powerful tool for mosquito genome engineering. As other site-specific recombinases, active in the *Ae. aegypti* germline, are identified these systems should become regularly employed.

The effectiveness of an RNAi mediated killer-rescue mechanism was investigated in Chapter 5. It was hoped that such a system could form the core of a killer-rescue type self-limiting gene drive or an underdominance drive system capable of fixing refractoriness in a population of *Ae. aegypti*. The key design feature in this approach was the combination of the refractory element and the rescue element, with both using RNAi targeted against the Mnp fragment of the DENV-2 genome. Engineering of lines expressing the Mnp-fused-tTAV protein tissue-specifically was achieved using midgut (Carboxypeptidase A-1), salivary gland(30Kb) and fat body(Vitellogenin A-1) specific promoters. This in turn could be used to express toxic effectors in these tissues. Unfortunately, these promoter regions were not as time-specific as had

been anticipated, or expected from previous reports of their activity, and basal expression occurred throughout the mosquitoes' development. This resulted in a reduced survival of the lines, which was attributed to continuous expression of the toxic tTAV protein, and suggested that these promoters are not suitable for use in gene drive systems, where tightly regulated expression is highly advantageous to maintaining the fitness of the engineered strain (Gould et al. 2008).

Expression of the four toxic effectors tested in conjunction with these tissue-specific promoters rarely resulted in significant changes in the life span of the mosquitoes. In the case of midgut expression of the two cell killing effectors, NIPP1 and Michelob-x, a significant reduction was seen but occurred well before the expected blood meal induced upregulation. Expression of the AaHIT insect-specific neurotoxin in the fat body was the most promising of the promoter/effector combinations investigated, resulting a paralysis phenotype, albeit only temporarily. RNAi against the tTAV transcripts fused to the Mnp fragment was also the most effective in the fat body, with a significant reduction in the number of transcripts being observed. This suggests that the Vitellogenin A-1 promoter, which displays strong upregulation after blood feeding, may be the best candidate yet identified for such induced killer-rescue systems. However, like the other promoters, it displayed high basal expression and the conclusion of the experimental work was that none of the promoters used were suitable candidates for construction of a functional killer-rescue system unless they could be further engineered to increase their specificity.

Underdominance and killer-rescue gene drivers have many properties that make them attractive systems for use in mosquito population replacement strategies. However, such systems are technically challenging to construct and rely upon the identification of suitable specific-promoters and highly penetrant effectors.

6.1 Future work and development

The experiments described in this thesis have demonstrated that the *piggyBac* transposon displays a level of germ-line stability not observed in other species, such as *D. melanogaster*. However, the inability to reliably express the active transposase endogenously in the germline leaves open the possibility that transposase protein was not being expressed in significant enough quantities in germ-line cells to promote transposition. This may have resulted in underestimating the germline stability of the transposon. Therefore the synthesis of constructs similar in structure to the OX3885 construct but with active germline promoters will be an important further experiment to elucidate the stability of the *piggyBac* transposon. This could be achieved by increasing the size of the promoter region taken from the endogenous VAS gene, which may include elements critical for promoting high levels of germline expression. In silico methods, such as BLAST and BLAT comparison of homology between the upstream and downstream regions of the *Ae. aegypti* VAS gene with the *An. gambiae* and *D. melanogaster* regions, may be useful in identifying conserved regulatory elements, such as transcription factor binding sites, for this purpose (Davuluri et al. 2001). Alternatively, different germline promoters entirely could be employed. Both the *nanos* and *oskar* genes express products that are

localized near the pole cells in developing embryos, while the *Aaβ2t* gene product is expressed in the male germ-line (Juhn et al. 2006, Smith et al. 2011). The regulatory regions of these genes could be used to drive expression of *piggyBac* transposase in a modified construct. Indeed, the promoter fragments of the *Ae. aegypti nanos* and *Aaβ2t* genes have already been used to drive expression of the *mariner MosI* and *Hermes* transposases endogenously in the mosquito (Aldeman et al 2007, Smith et al. 2011). The ability of *piggyBac* to remobilize in somatic cells may raise concerns about the release of transgenic mosquitoes engineered with the transposon into the wild. Unfortunately, the inability to remobilize the transposon in germline cells precludes the use of post-integration remobilization techniques to excise the inverted terminal repeats of the transposon (Handler 2004b, Dafa'alla et al. 2006). Therefore it will be extremely important to develop novel methods to stabilize *piggyBac* transposons after integration or to identify novel methods of germline transformation that do not require the use of transposons. Several such methods are already being developed involving the use of endonucleases to excise transposon sequences post-integration, or employing site-specific recombinases to replace transposons entirely, as explored in this thesis (Tkachuk et al. 2011).

The use of Bxb1 and ϕRV1 integrases for RMCE and pseudo-integration in *Ae. aegypti* does not appear to be possible using the methods employed in this thesis. It is possible that the injected mRNA, used to provide a source of integrase for the germline, was not being effectively translated into active

protein. Staining the pole cells of dissected embryos with integrase specific antibodies could be used to determine if translation of active integrase protein was being produced by the injected mRNA, to determine the effectiveness of this method of transformation (Bischof et al. 2006). Alternatively, co-injection of synthesized purified protein could replace the use of mRNA, to guarantee the presence of the integrase protein, though this method is both more technically challenging and more expensive (Kaufman et al 1991). Despite the failure of the experiments attempted herein, the need to develop a suite of site-specific recombinases for manipulation of the mosquito genome remains a priority for researchers. There are many other recombinases that have been shown to be active in a wide variety of model organisms (Nern et al. 2011). However, as the work presented here demonstrated, active recombination in even closely related species does not guarantee activity in the species of interest. As such the development of rapid screening procedures to identify recombinases whose activity is not precluded by the mosquito's biochemistry would be desirable. The use of intra and inter-plasmid integration assays in insect cell lines has already been employed to demonstrate the activity of integrases such as ϕC31 and R4 in *D. melanogaster*, making this an ideal method for rapid screening of potential recombinases (Chompoosri et al. 2009). *Ae. aegypti* cell lines are readily available and have previously been used to determine transpositional activity of DNA transposons. Extending this method to site-specific recombinases should not prove challenging and could provide a rapid mechanism to identify recombinases active in the mosquito without the

need to transform and rear strains, which is an extremely time consuming process (Kilnakis et al. 2000, Lobo et al. 1999).

The underdominance gene drive system designed and tested in this thesis remains undeveloped, however there are several approaches that could be taken to improve the system and make it a viable technology for use in the manipulation of wild mosquito populations. The most important step for future development of this system will be the identification of tissue and time-specific control sequences, to ensure that the tTAV protein is produced exclusively in tissues associated with the Dengue virus' life cycle and to lessen the fitness impact of effector and RNAi expression. The Carboxypeptidase A-1, 30Kb and Vitellogenin A-1 promoter fragments investigated in chapter 5 do not seem to be good candidates for this approach, due to the extent of their basal expression which seems to be higher than the endogenous genes from which they originate. Comparative sequence analysis of the regions upstream and downstream of the endogenous genes in *Ae. aegypti* and their orthologs in closely related species could reveal regulatory sequences that might reduce basal expression and improve specificity (Isoe et al. 2009). Alternatively, the use of microarray techniques, combined with the published transcriptomes available for many *Ae. aegypti* tissues, could be used to investigate gene expression profiles to identify novel genes expressed in a highly tissue and time-specific manner (Dissanayake et al. 2010). A great deal of effort has already gone in to such work and has identified genes upregulated in the salivary glands and midguts of the mosquito after blood-feeding (Thangamani

et al. 2009, Brackeny et al. 2011). Recently efforts to identify cis-regulatory elements in promoter regions associated with rapid induction of transcription levels after blood feeding have been undertaken using ensemble methods, such as the SCOPE method for identifying regulatory motifs by through comparison of coordinately regulated genes (Carlson et al. 2007, Bonizzoni et al. 2011). The elements identified by these techniques may lead to the design of artificial promoters more suitable for construction of killer-rescue type systems, and novel constructs engineered around them would be good candidates for the next step in the development process. Using a fluorescent protein reporter assay as done in chapter 5, RNAi did not seem to be capable of suppressing the fluorescent phenotype, despite a reduction in the expression of the tTAV transcripts. This may be because the tTAV-tetO system was too sensitive to even low levels of tTAV, preventing RNAi suppression, which is not 100% efficient, from altering the phenotype. This problem could potentially be overcome by reducing the sensitivity of the tTAV-tetO system to low levels of tTAV expression. This could be achieved in two ways; either by reduction in the binding efficiency of the tTAV transactivator through mutation of the protein, or by alteration of the tetO responder element, whose sensitivity can be reduced by decreasing the number of multimerized tetO sites that comprise the responder (Zhou et al. 2006, Rossi et al. 2000). The effectiveness of the RNAi suppression could also be improved by the inclusion of a second Mnp target sequence fused to the reporter or effector transgenes. This should result in the transcripts being targeted by the RNAi machinery, reducing expression of the reporter or effector protein even if some tTAV transcripts are not

degraded and produce active protein. An alternative development route would be to abandon the use of the RNAi mechanism entirely and instead design the system around the use of a recently developed repressible binary expression system called the 'Q-system'. This expression system, composed of regulatory genes from the *Neurospora qa* gene cluster, is similar activity to the tTAV-tetO system however the suppressor (QS), which unlike tetracycline is a small protein, may be provided by an endogenous transgene and may in turn be inactivated by the provision of dietary quinic acid (Potter et al. 2010). This system has already been shown to be active in *D. melanogaster* and should be transferable to the mosquito, where QS could be used as the cross-repressor in the design of the killer-rescue system (Potter et al. 2011). Finally, towards development of a functional killer-rescue type system in the mosquito, it will be important to identify toxic effectors that are able to produce a lethal phenotype when expressed under control of the blood feeding inducible promoter chosen, while at the same time not being so toxic that even low-levels of basal expression dramatically impact survival. The insect-specific excitatory neurotoxin from the scorpion *Androctonus australis* was the most promising of the effectors identified when expressed in the fat body of the mosquito, producing a temporary paralysis phenotype. The investigation of other invertebrate-specific peptide neurotoxins for use in killer-rescue systems is likely to be the most promising route for further development. An extensive library of these toxins has been characterized and they are often both highly active and extremely specific, an important factor when considering release of a transgene into the wild (Schwartz et al. 2012). Alternatively, lethality could be

induced by a mechanism not relying on production of a toxic protein but rather on knockdown of a vital gene product using RNAi targeted against one or more endogenous transcripts, a method that has already been shown to be possible in *Ae. aegypti* (Pridgeon et al. 2008). The lethal effector chosen will be highly dependant on the tissue it is expressed in and the extent of its expression, increasing the importance of identifying suitable promoter regions to use in killer-rescue type systems.

The development of transgenic vectors for population replacement remains a long-term goal in the development of engineered mosquitoes. There are many competing technologies and it is not yet possible to say which, if any, will prove the most effective. It is likely that different systems will be better suited to different situations and vector control programs. This thesis has increased the understanding of the post-integration behavior of the *piggyBac* transposon, one of the most important gene vectors used for engineering mosquitoes, and demonstrated the first application of RMCE technology in mosquitoes. The development of killer-rescue systems for use in *Ae. aegypti* remains some way off but the work presented here has shown that the core components of such a system can be successfully engineered.

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Appendix 1: List of primers and oligonucleotides

Chapter 2: Methods and Materials

PB5'-nested-01	5'-CCAATGAAGAACCTGGTTGTTC-3'
PB5'-nested-02	5'-CTACCGCTTGACGTTGGCTGCAC-3'
PB5'-nested-03	5'-AGTCACGTAAAAGATAATCATGCG-3'
PB3'-nested-01	5'-GAAGTGCCTGGTACATCAGATGA-3'
PB3'-nested-02	5'-GTGCCAAAGTTGTTTCTGACTGAC-3'
PB3'-nested-03	5'-CAGACCGATAAAACACATGCGTCA-3'
Msp-adapter-01	5'-CGCACCGCCCTCCG-3'
Dpn-adapter-01	5'-GATCCACCGCCCTCCG-3'
Adapter-long	5'-GTGTAGCGTGAAGACGACAGAAAGGGCGTGGTG CGGAGGGCGGTG-3'
Adapter-diag-01	5'-GTGTAGCGTGAAGACGACAGAA-3'

Chapter 3: Stability of *piggyBac* transposons in *Aedes aegypti*

vasa-up-01	5'-ACATGGCCGGCCTTATAGATATTCTCAATCGTT CTTCACACG-3'
vasa-down-01	5'-AGCCGGTCTCGCATTTTGATTTCACCGTCTTACTT AAATG-3'
vasa-3'-F	5'-AGCCGGTCTCAGTGAGGTTAGGGCCATTTAATG CGC-3'
vasa-3'-R	5'-AGCCGGTCTCACTAGTGTTCAAATTACTCGATATGTT TATGGTAGA-3'
PB-transp-F	5'-AGCCGGTCTCAAATGAGTAGTTCTTTAGACGATGAG CATATC-3'
PB-transp-R	5'-AGCCGGTCTCATCAGAAACAACTTTGGCACATATC-3'
PB-transposase-F	5'-AAAGCGGAAGGTGGAGGACC-3'
PB-transposase-R	5'-GATTTCATCTTCATTCGTGTCACG-3'
3885C-5'flank-01	5'-GAGCATGATTATGATCTTCCTGG-3'
3885C-3'flank-01	5'- CGATAACGGTCGTTTCCGGA-3'
3885D-5'flank-01	5'-CACTCATTAGGCACCCCAGGC-3'
3885D-3'flank-01	5'-CGGTATTCACGACAGCAGGC-3'
3885E-5'flank-01	5'- CCATTAAGTGTTCAGTTTGATTGG-3'
3885E-3'flank-02	5'- CGACACCATCTTAAGTCATCG-3'
5'-unresolved-01	5'-CTTGAAGGGCGACGTGACCGCC-3'
5'resolved-01	5'-CGGTATTCACGACAGCAGGC-3'

3'-unresolved-01	5'-TTTCGCTTAGCGACGTGTTCAC-3'
3'-resolved-01	5'-AAACAGTGTCCTTCCACAGATTCC-3'
3'-unresolved-C01	5'-CGAAGAAGTTCCTATTCCGAAGTTCCTATTCT-3'

Chapter 4: *Ae. aegypti* genome engineering with site-specific recombinases

attP-ApaI	5'-TGTAGGGCCCTAGTATGTATGTAAGTTAAT-3'
attP-XhoI	5'-TGATCTCGAGGACTATTAGAGGTAAGAATA-3'
attP02-EagI	5'-TAGTCGGCCGTAGTATGTATGTAAGTTAAT-3'
attP02-blunt	5'-GACTATTAGAGGTAAGAATA-3'
attB-PacI	5'-ACGCTTAATTAAGAATTAGATCCCCGGGCGA-3'
attB-HpaI	5'-CGTAGTTAACCGTTGTAAAACGACGGCCAG-3'
3861-marker-PacI	5'-ACGCTTAATTAACACTGGTCGGTCCGAGAAACT-3'
3861-marker-ApaI	5'-CTATGGGCCCCGGTTCCCACAATGGTTGC-3'
DsRed-5'	5'-CTCGATCTCGAACTCGTGGC-3'
DsRed-3'	5'-CGGGGTACCGCTAGAGTCG-3'
AmCyan-5'	5'-TGCAGAGCTGCAAGTGTGGC-3'
AmCyan-3'	5'-CTTGAAGGGCGACGTGACCGCC-3'
C31-sitediag01	5'-CGTCGGGAATCTATGACAGGTGC-3'
Bxb1-sitediag01	5'-CCAATGAAGAACCTGGTTGTTC-3'
C31-DsRed-01	5'-TGCAGAGCTGCAAGTGTGGC-3'
C31-AmCyan-01	5'-CTTGAAGGGCGACGTGACCGCC-3'
Bxb1-DsRed-01	5'-ACCATCGTGGAGCAGTACGAG-3'
Bxb1-AmCyan-01	5'-GCGCATCACAAAGACATCG-3'
attP-Diag1	5'-GTAACCTTTGAGTTCTCTCAGTTGG-3'
attR-Diag1	5'-TGGGGTAACCTTTGGGCTCC-3'
attR-Diag2	5'-ATCATCCACTGATCGTGCATTACAAG-3'
attR-Diag3	5'-CACGATGCATTTGCCTTTCGCC-3'
attR-Diag4	5'-CTTGAAGGGCGACGTGACCGCC-3'
attR-Diag5	5'-GGGCCACATAACAGGCAGCGAG-3'
attR-Diag6	5'-TCATCTGATGTACCAGGCACTTC-3'
attR-Diag7	5'- ACACCTCCCCCTGAACCTGA-3'
attR-Diag8	5'- ATCATCCACTGATCGTGCATTACAAG-3'
3947-entirenheI-F	5'-TAGTGCTAGCGACGGCAAATACTT-3'
3947-outer-1	5'-CGAAAAGTGCCACCTGACGT-3'
3947-entire-SpeI-f	5'-ATCGACTAGTTTGTTCGTTGATCTCGATCA-3'
3947-outer-2	5'-TGATGTCGGCGATATAGGCG-3'
PhiRV1-Int-Diag1	5'-TACACCTGTGCGTGCTGCTG-3'
PhiRV1-Int-Diag2	5'-CACCCGTTCGGGATTGAATA-3'

bxb1-5'-kpn1	5’-TGATGGTACCATGAGAGCCCTGGTAGTCATCC-3’
bxb1-3'-avrII	5’-GTATCCTAGGCTACGACATCCCGGTGTGTAG-3’

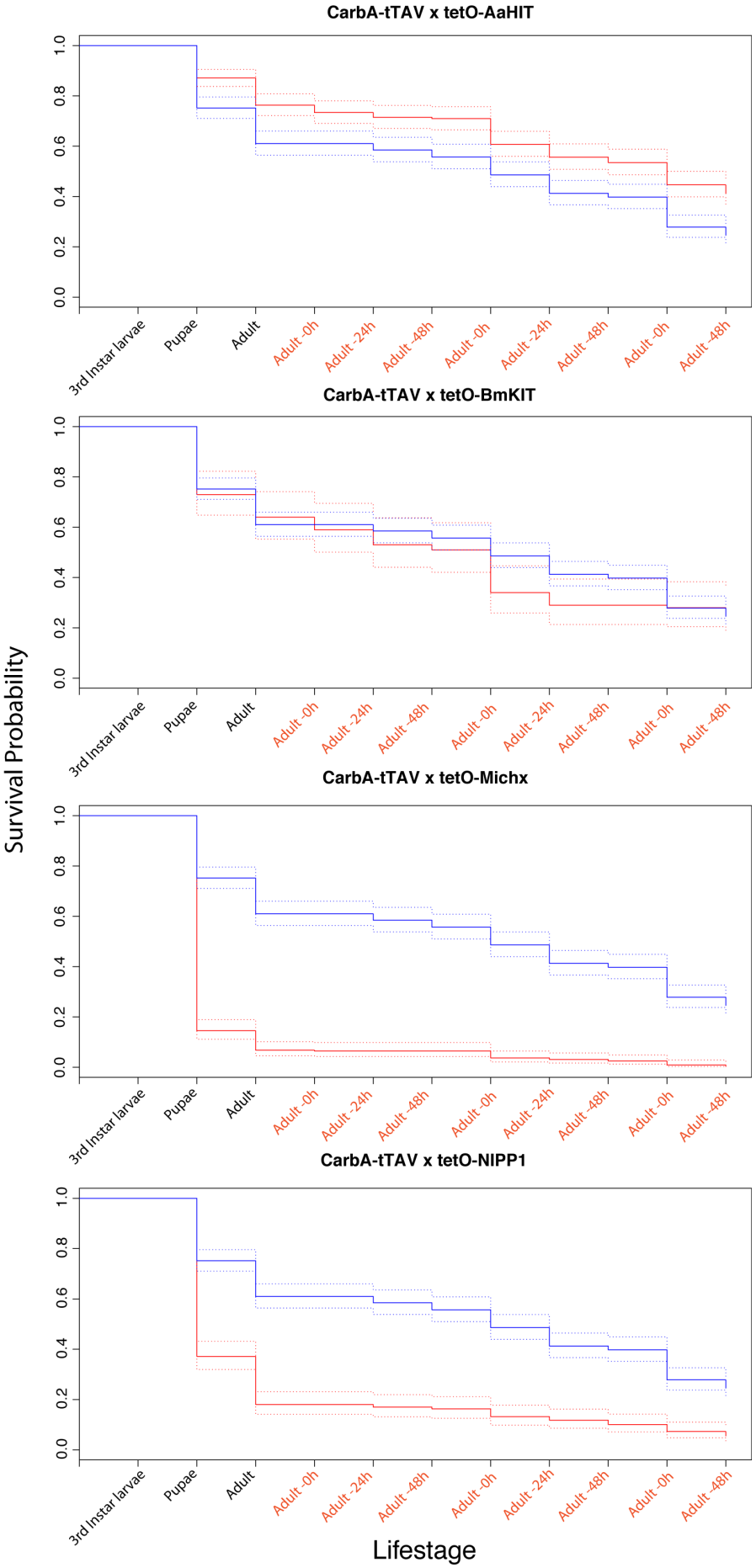
Chapter 5: Creation of an Engineered Gene Drive in *Ae. aegypti*

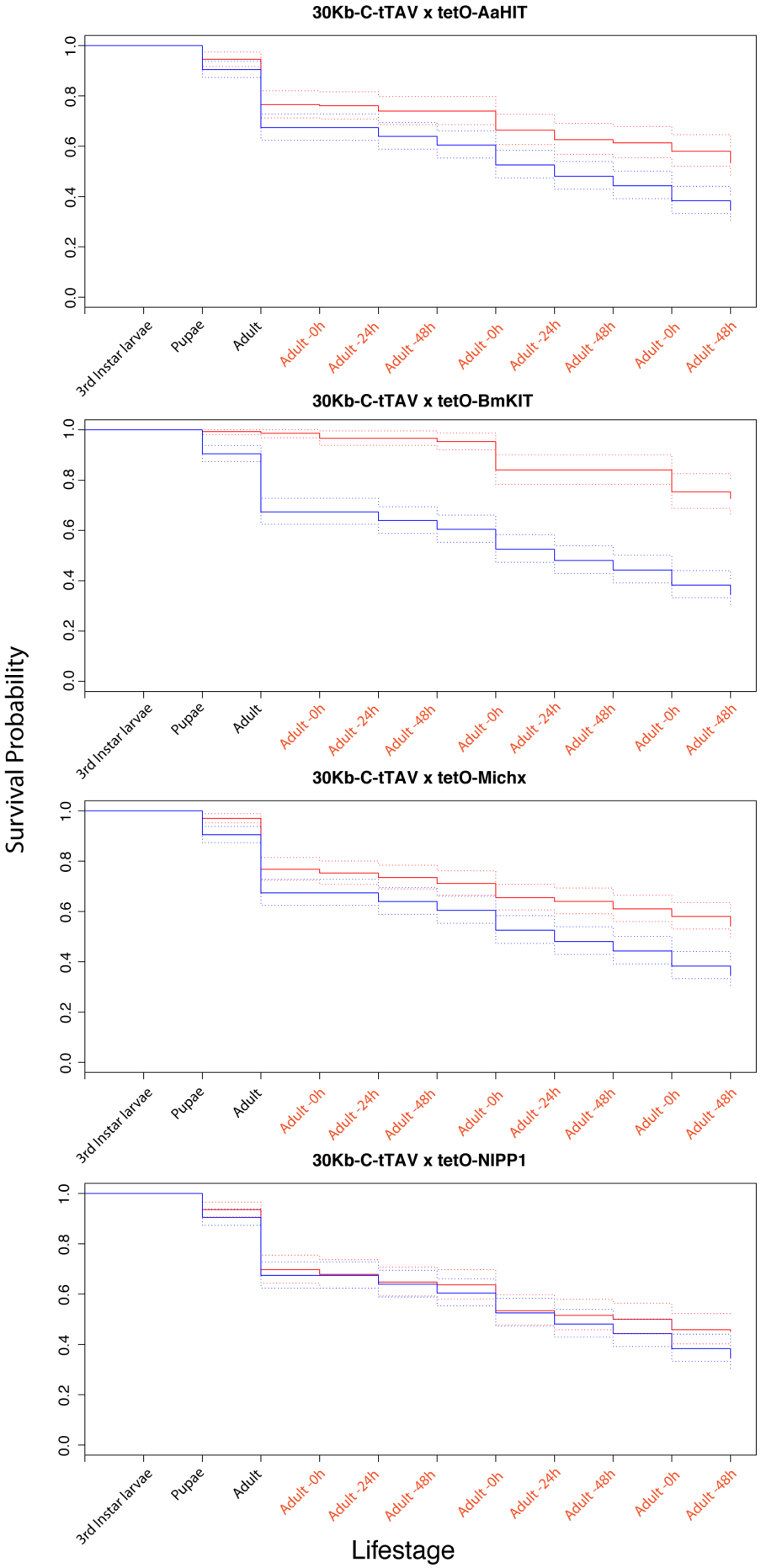
CarbproAscF	5’-GGCCTAGGCGCGCCAAGCTTAAGGTGCACGGCC CAC-3’
CarbproAvBaR	5’-GGTGTGGGATCCATCCTAGGCCGCCTGCAGTTTTC CAACTAAC-3’
Sv40NotBaF	5’-GGTGGGATCCATGCGGCCGCTCGCGTTAAGATACAT TGATG-3’
Sv40XmaR	5’-GGTGTGCCCGGGGATCATAATCAGCCATACCACAT TTG-3’
Den2AvF	5’-GGTGTGCCTAGGCCACCATGGCAGGCGTGATTATT ATGTTGATTC-3’
Den2EcoR	5’-GGTGGTGAATTCCGATATTCCTATGCAACGCAATT-3’
30KXbaR	5’-GGTGTGTCTAGACTTTCTCCTAGGGGCTCGGTATG AC-3’
VitAscF	5’-GGTGTGGGCGCGCCGAATTCCACCACCAGGCAGT GC-3’
VitAvR	5’-GGTGTGCCTAGGCGGGATCCTTCAAGTATCCGGCA GC-3’

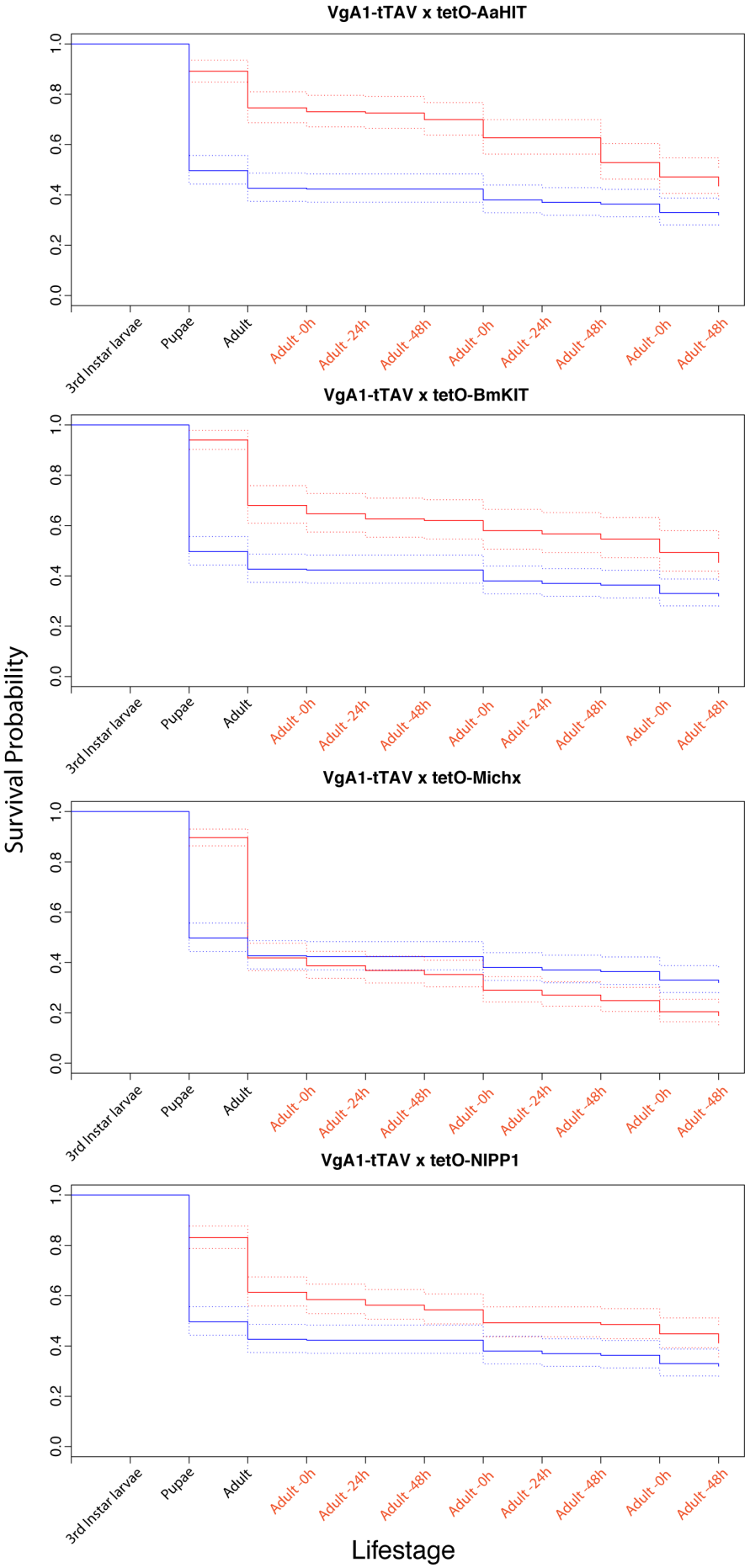
Appendix 2: Survival analysis of males from the tissue-specific-tTAV & tetO-Effector crosses

Survival curves (red line in each plot) of males, from 3rd instar larvae to the 3rd gonotrophic cycle (relative to females from the same cross), of the three tissue-specific tTAV lines (CarbA-tTAV-F, 30Kb-tTAV-C and VgA1-tTAV-A) crossed to the four lethal effectors (red line) estimated using Kaplan–Meier survival analysis. The cross of the tissue-specific tTAV line to wild type (blue line in each plot) is included for comparison in each plot. 95% confidence intervals of the survival curve are drawn as dotted lines. Time points in red represent the three gonotrophic cycles of the females from the same crosses as the males, with survival recorded immediately after a blood meal (0h) and then 24 and 48 hours (24h & 48h) subsequently. The sample size (n) for each cross and the relative survival of males resulting from the crosses (+ better, - worse), compared to the cross of the tTAV line to wild-type, is shown in the table below with the log-rank test used to determine if any difference in the survival curves was significant.

Promoter line	Effector line	Relative survival	Median survival (days)	n	X2	p-value
CarbA-tTAV-F	tetO-AaHIT	+	17	372	26.8	$2.3x10^{-7}$
	tetO-BmKIT	-	14	100	0.4	0.53
	tetO-MichX	-	3	324	295	0
	tetO-NIPP1	-	3	288	118	0
30Kb-tTAV-C	tetO-AaHIT	+	>18	238	19.8	$8.7x10^{-6}$
	tetO-BmKIT	+	>18	150	63.6	$1.4x10^{-15}$
	tetO-MichX	+	>18	336	26.1	$3.2x10^{-7}$
	tetO-NIPP1	+	18	264	4.9	0.028
VgA1-tTAV-A	tetO-AaHIT	+	17	193	16.7	$4.4x10^{-5}$
	tetO-BmKIT	+	17	150	16.6	$4.6x10^{-5}$
	tetO-MichX	-	6	318	0.1	0.82
	tetO-NIPP1	+	14	272	12.9	0.00033


CarbA-tTAV x tetO-AaHIT
CarbA-tTAV x tetO-BmKIT
CarbA-tTAV x tetO-Michx
CarbA-tTAV x tetO-NIPP1
Survival Probability
0.0
0.2
0.4
0.6
0.8
1.0
3rd Instar larvae
Pupae
Adult
Adult-0h
Adult-24h
Adult-48h
Adult-0h
Adult-24h
Adult-48h
Adult-0h
Adult-48h
Lifestage


30Kb-C-tTAV x tetO-AaHIT
30Kb-C-tTAV x tetO-BmKIT
30Kb-C-tTAV x tetO-Michx
30Kb-C-tTAV x tetO-NIPP1
Survival Probability
0.0
0.2
0.4
0.6
0.8
1.0
3rd Instar larvae
Pupae
Adult
Adult -0h
Adult -24h
Adult -48h
Adult -0h
Adult -24h
Adult -48h
Adult -0h
Adult -48h
Lifestage


VgA1-tTAV x tetO-AaHIT
VgA1-tTAV x tetO-BmKIT
VgA1-tTAV x tetO-Michx
VgA1-tTAV x tetO-NIPP1
Survival Probability
0.0
0.2
0.4
0.6
0.8
1.0
3rd Instar larvae
Pupae
Adult
Adult -0h
Adult -24h
Adult -48h
Adult -0h
Adult -24h
Adult -48h
Adult -0h
Adult -48h
Lifestage