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Transgenesis and conditional lethality in *Aedes albopictus*

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Abstract

The Asian tiger mosquito *Aedes albopictus* (Skuse) is a vector of several arboviruses including dengue and chikungunya. This highly invasive species originates from Southeast Asia and has spread across the world in the last 30 years. It is now established in Europe, North and South America, Africa, the Middle East and the Caribbean. In the absence of vaccine or antiviral drugs, efficient mosquito control strategies are crucial. Conventional control methods have so far failed to adequately control *Ae. albopictus*. Using germline transformation technology, a technique known as Release of Insects carrying a Dominant Lethal (RIDL) proposes to enhance the sterile insect technique by replacing irradiation with inherited dominant lethal genes. While this technology has recently shown some success in the field against the yellow fever mosquito, *Aedes aegypti* (L.), it remains to be implemented against *Ae. albopictus*. This thesis presents the development and application of gene transfer and site-specific integration technologies in *Ae. albopictus*, as well as the creation of tetracycline-repressible, female-specific flightless lines for vector control based on the RIDL method. Germline transformation and site-specific integration were performed using the *piggyBac* transposon and the ΦC31 system, respectively. *Ae. albopictus* RIDL strains showing a conditional female-specific flightless phenotype were created using both the *Ae. aegypti* and the *Ae. albopictus Actin-4* regulatory regions. Conditionality was provided by the 'Tet-Off' system, which is suppressed in the presence of tetracycline (and suitable analogues). One of these strains was assessed for attributes relevant to a RIDL control programme. Specific tailoring of the RIDL transgene with alternative transactivator elements was investigated using the ΦC31 system. The work presented in this thesis lays the foundations for the application of the RIDL strategy to *Ae. albopictus*, an innovative vector-control method offering a promising alternative for efficient control of this highly invasive insect.

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Author's declaration

I declare that all the work presented here is my own original research subject to the following acknowledgement: the characterisation of the *Ae. albopictus Actin-4* promoter presented as part of Chapter 3 was done by Sarah Scaife, as indicated in a foot note.

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Chapter 1

Introduction

1. Mosquito-borne diseases

Mosquito-borne diseases, such as dengue fever, chikungunya or malaria, are now, more than ever, major international public health concerns. According to the World Health Organisation (WHO: http://www.who.int/en/) and the Centre for Disease Control and Prevention (CDC: http://www.cdc.gov/), 40% of the world's population are at risk from dengue and malaria, with an estimated 50 to 100 million dengue cases and 350 to 500 million malaria cases worldwide every year.

a/ Dengue

Dengue fever is the most widespread mosquito-borne viral disease of humans. The two main vectors of dengue are *Aedes aegypti* and *Aedes albopictus* (Figure 1-1). Dengue is predominantly found in urban and semi-urban areas of tropical and sub-tropical regions (Figure 1-2). Pandemic infections of dengue started in South-East Asia in the 1950s, and have been spreading to the Americas and Africa since the 1970s (Gubler, 1989), encouraged by the constant urban growth that favours the development of *Aedes* mosquitoes.

The symptoms of dengue fever itself are flu-like and it rarely causes death. However, dengue haemorrhagic fever, a complication of dengue, can cause circulatory failure and death of the patient in only a few days and there is no specific treatment available. Dengue and Dengue haemorrhagic fevers are caused by one of four serotypes of the dengue virus (DEN-1 to -4), of the genus *Flavivirus* and has a single +-strand RNA genome. Infection with one of these serotypes provides life-long immunity against that serotype only and people can suffer more than one dengue infection in their lifetime. Research is under way to develop a vaccine, but it has proved difficult due to the presence of those four closely related but antigenically different viruses (Cardosa, 1998): The vaccine would have to offer protection against all four serotypes.

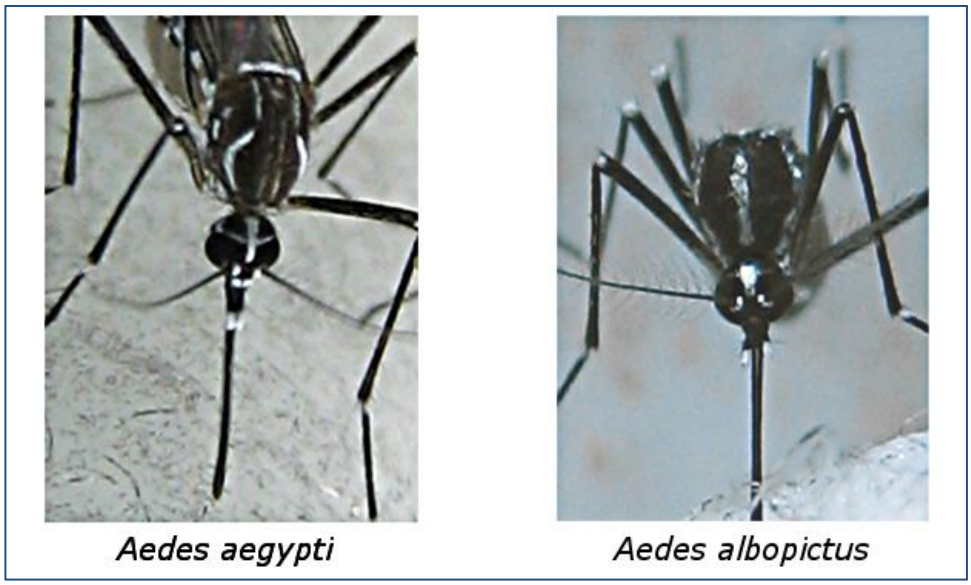

Figure 1-1. The two main vectors of dengue fever and chikungunya, *Aedes aegypti* and *Aedes albopictus*.

Note the white pattern on the thorax: two bands on each side for *Ae. aegypti*, one band in the centre for *Ae. albopictus*. (photogaphs D. Nimmo)

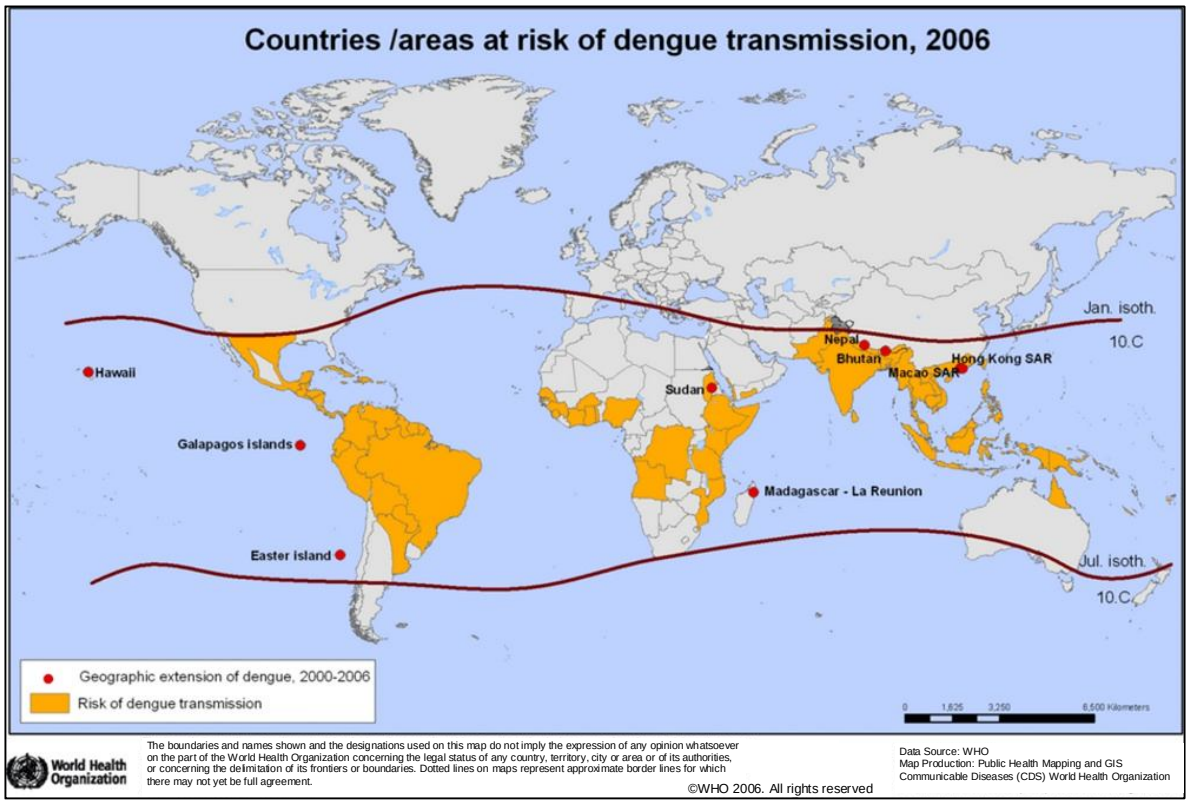

Figure 1-2. Countries / areas at risk of dengue transmission, 2006 (WHO).

b/ Chikungunya

Ae. aegypti and *Ae. albopictus* are also the main vectors of the chikungunya virus. Like dengue, chikungunya is rarely lethal except in old or very young people (Pialoux et al., 2007). It is characterised by flu-like symptoms, extreme joint pain and fatigue (Hochedez et al., 2008). The complete recovery can take up to several months. Chikungunya was first described in Tanzania in the 1950s (Robinson, 1955), and it has since spread through Southeast Asia (Mackenzie et al., 2001), India (Ravi, 2006) and Africa (Jupp and Kemp, 1996), and more recently in the Indian Ocean islands; the La Reunion outbreak in 2005-2006 affected a third of the island's population: about 260,000 cases (Flahault et al., 2007), and Europe; Italy outbreak in 2007 (Rezza et al., 2007) (Figure 1-3). The chikungunya virus is an alphavirus from the family *Togoviridae* and has a single +-strand RNA genome.

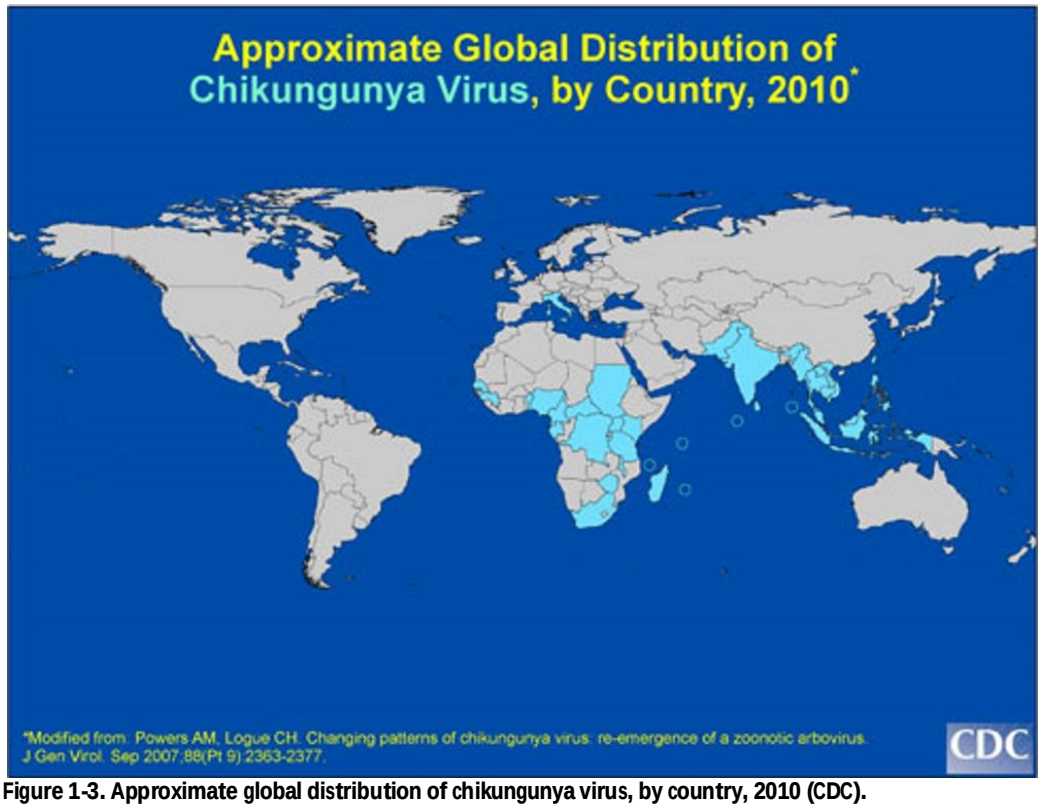

Figure 1-3. Approximate global distribution of chikungunya virus, by country, 2010 (CDC).

c/ Malaria

Malaria is the most deadly mosquito-borne disease affecting humans, with around one million fatalities each year, mainly amongst young children in Africa (www.who.int/mediacentre/factsheets/fs094/en/index.html; www.cdc.gov/malaria/about/facts.html). Malaria is caused by infection with protozoan parasites from the *Plasmodium* genus, the most deadly being *Plasmodium falciparum*. Resistance to anti-malarial drugs has developed in these parasites, and the complexity of their life cycle makes it difficult to develop an efficient vaccine (Rogers and Hoffman, 1999). Unlike dengue and chikungunya, malaria is transmitted by *Anopheles* mosquitoes. More than a third of the global world population lives in a malaria-endemic area (Figure 1-4).

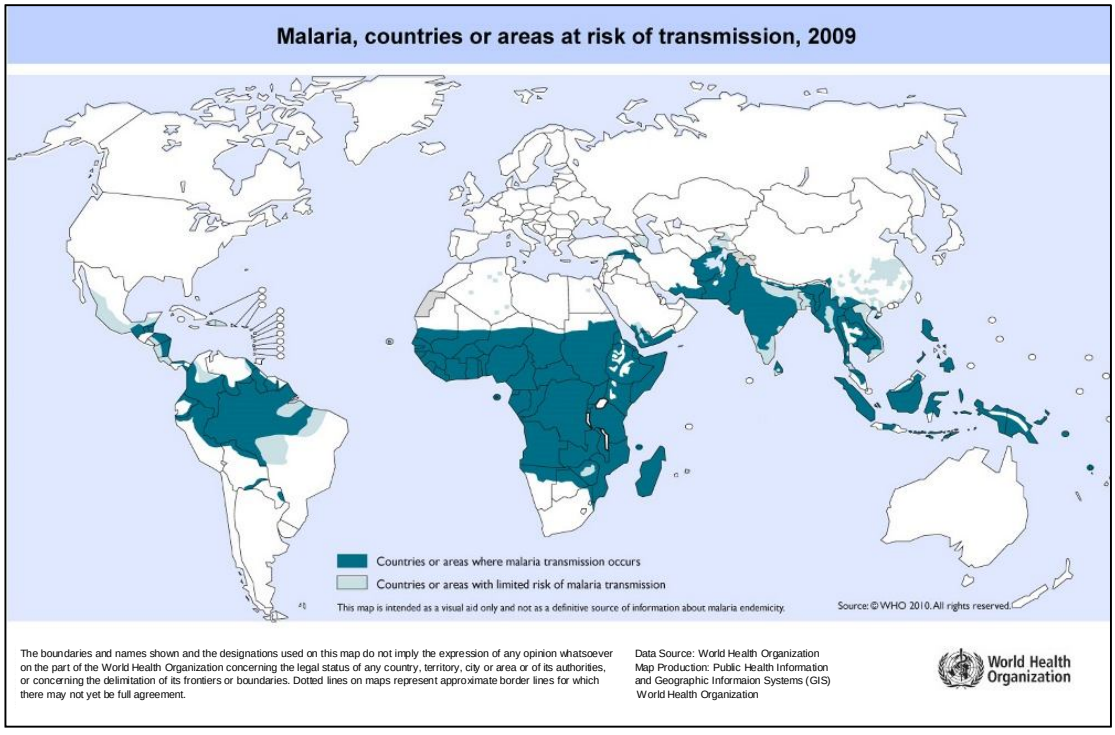

Figure 1-4. Malaria, countries or areas at risk of transmission, 2009 (WHO).

d/ Other mosquito-borne diseases

Yellow fever is caused by another *Flavivirus* transmitted by *Aedes* mosquitoes which can, quite similarly to dengue, cause haemorrhagic illness. Despite the availability of an efficient vaccine

since the 1937 (Monath, 1988), inadequate coverage means there are currently 200,000 cases and 30,000 deaths worldwide each year, 90% of them in Africa. The main vector of yellow fever is *Ae. aegypti*.

West Nile virus, also a *Flavivirus*, is mostly transmitted by Culex mosquitoes. It used to be considered of relatively low importance as infections into humans were usually asymptomatic or mild. Since the 1990s, however, the disease has expanded its geographic range and outbreaks seem to have become more frequent, with more cases involving severe, neurologic syndromes, apparently linked to a new virus variant (Petersen and Roehrig, 2001).

The Venezuelan Equine Encephalitis is a re-emerging disease caused by an alphavirus transmitted mostly by *Ochlerotatus* (formerly *Aedes*) *taeniorhynchus* mosquitoes (Weaver et al., 2004). It is widespread in Central and South America, with mild flu-like to neurological symptoms.

Due to the absence of vaccines and effective drugs against dengue, malaria or chikungunya, disease prevention and control focuses on the vectors of these diseases.

2. The Asian Tiger mosquito *Aedes albopictus*

a/ Biology

Aedes (Stegomyia) albopictus (Skuse) was first described by Skuse (1894) as "the banded mosquito of Bengal" and is now commonly called the Asian Tiger mosquito due to its black and white stripy pattern on the abdomen. It originally comes from Southeast Asia and moved to Madagascar and other Indian Ocean Islands with humans centuries ago (Smith, 1956). It mostly lives at the edge of forests and breeds in tree holes and other natural reservoirs. Adult *Ae. albopictus* are readily distinguished from *Ae. aegypti* by a median longitudinal line of white scales on their thorax (Figure 1-1).

Ae. albopictus is the second main vector of dengue in the world after *Ae. aegypti*. It is also a natural vector of chikungunya virus (Schuffenecker et al., 2006) and a laboratory-competent vector of several other arboviruses such as the Venezuelan Equine Encephalitis (Fernandez et al., 2003), West Nile and La Crosse viruses (Mitchell, 1995). The nematode *Dirofilaria immitis*, a parasite usually found in cats and dogs, can also be transmitted to humans by *Ae. albopictus* (Nayar and Knight, 1999). The lower vectorial capacity of *Ae. albopictus* compared to *Ae. aegypti* is due to its quite different lifestyle (Lambrechts et al., 2010). While *Ae. aegypti* lives in urban areas, tends to bite indoor and feeds almost exclusively on humans, *Ae. albopictus* does not usually live in such close association with humans. It is generally found in more rural areas where the human density and therefore the risk of epidemics are lower, and bites mostly outdoors. It is less anthropophagic, feeding also on other mammals, birds and reptiles (Hawley, 1988). However, the ecology of *Ae. albopictus* is quite flexible and some strains are well adapted to urban conditions where they sometimes even appear to displace *Ae. aegypti* (Lounibos, 2002; Ray and Tandon, 1999; Tandon and Raychoudhury, 1998). In Dakha, Bangladesh, where both vectors are present, the dengue fever outbreak of 2000 seems to have been mostly caused by *Ae. albopictus* (Ali et al., 2003). Dengue outbreaks in places where *Ae. albopictus* is the sole vector tend to be mild (Hawaii 2001-2002, (Gubler, 2003)), but *Ae. albopictus* has nevertheless been responsible for important dengue fever epidemics in Japan (1942-1944), Hawaii (1943) and Seychelles (1976-1977). *Ae. albopictus* also supported a big chikungunya outbreak in the Indian Ocean between 2005 and 2007, infecting a third of the population of La Reunion island (Reiter et al., 2006).

b/ A maintenance and bridge vector

Dengue and chikungunya viruses are maintained in parts of Southeast Asia and Africa within sylvatic cycles involving non-human primates and monkeys (Marchette et al., 1978; McIntosh, 1970; Rudnick, 1965). The distribution of *Ae. albopictus* in both rural and peri-urban areas and its wide range of hosts make it both a vector in sylvatic cycles (Knudsen, 1977) and a bridge vector able to pass viruses on to humans, subsequently starting new urban transmission cycles

and epidemics. *Ae. albopictus* is therefore believed to be a maintenance vector of dengue in endemic countries of Southeast Asia and Pacific Islands (Gratz, 2004).

Vertical transmission of the virus to the vector mosquito offspring is another way by which some viruses can persist in the environment during inter-epidemic periods (Gokhale et al., 2001; Thenmozhi et al., 2007). It has also been reported that *Ae. albopictus* is able to vertically transmit the dengue and chikungunya viruses to their progeny (Angel and Joshi, 2008; Delatte et al., 2008; Rosen et al., 1983).

c/ An invasive species

In the last 30 years, *Ae. albopictus* has travelled the world via the trade of used tires (Reiter, 1998) and "lucky bamboo", a tropical plant (Linthicum et al., 2003), which are common breeding places for this species. It went from South-East Asia and Pacific Islands to Europe, North and South America, Africa, the Middle East and the Caribbean. *Ae. albopictus* shows strong ecological plasticity and is a highly invasive species. Eggs of most *Aedes* species tolerate several months of desiccation and thus can survive long transports. Moreover, some strains of *Ae. albopictus* have developed a photoperiodic egg diapause and freezing tolerance, allowing the establishment of this exotic mosquito in temperate zones, and on every continent except Antarctica (Hawley et al., 1987).

In Europe, it first established in Albania in 1979 (Adhami and Murati, 1987) and has since then spread through most of Italy (Sabatini et al., 1990) and along the Mediterranean coast to Spain (Aranda et al., 2006) and France (Scholte and Schaffner, 2007). It has recently reached Belgium and the Netherlands (Rahamat-Langendoen et al., 2008; Schaffner et al., 2004) although there is no evidence of its establishment in the Netherlands at this time (Scholte et al., 2008). In the USA, it was first found in 1983 (Reiter and Darsie, 1984) and has now established in 26 states, as far north as Illinois (www.cdc.gov/ncidod/dvbid/arbor/albopic_new.htm). In Brazil, it was first reported in 1986 and is now present in 20 of the 27 states (La Corte dos Santos, 2003). *Ae.*

albopictus is in fact present in every Central American country (Benedict et al., 2007). In Africa, it was first established in Nigeria (CDC, 1991) and has recently been found in Cameroon (Fontenille and Toto, 2001), Gabon (Krueger and Hagen, 2007) and Equatorial Guinea (Toto et al., 2003).

d/ A public health threat

The introduction of *Ae. albopictus* in the USA was once perceived as a benefice for colonising the niche occupied by the more competent vector, *Ae. aegypti* (Gubler, 2003; Hobbs et al., 1991; O'Meara et al., 1995). However, since the chikungunya epidemic of La Reunion island in 2006, the public health threat posed by the widespread distribution of *Ae. albopictus* cannot be ignored. The virulence of the chikungunya virus involved in that epidemic was due to a single nucleotide mutation of the virus, which enhanced its ability to infect *Ae. albopictus* (Vazeille et al., 2007). A single amino-acid substitution in the Venezuelan Equine Encephalitis virus was also responsible for enhanced vector infectivity a few years ago (Brault et al., 2004). Viruses are prone to mutations and we cannot rule out that something similar could happen with other viruses including dengue.

The small chikungunya outbreak in Italy in 2007 (Chretien and Linthicum, 2007) showed that *Ae. albopictus* is indeed the key for such viruses to extend their geographic range. Mosquito-borne diseases are not only a burden for developing countries. The establishment of *Ae. albopictus* causes public health concerns regarding the possible emergence of dengue and chikungunya in temperate countries: if introduced by infected travellers, the viruses can now spread in those countries by the incumbent vector, especially during the summer months when the mosquito population is highest (Senior, 2008; Vazeille et al., 2008). It also generates fears of re-emergence and dissemination of other endemic diseases for which *Ae. albopictus* is a competent or bridging vector. This includes West Nile (Farajollahi and Nelder, 2009; Gubler, 2007; Sardelis et al., 2002), La Crosse (Grimstad et al., 1989; Lambert et al., 2010) and Venezuelan Equine Encephalitis (Weaver and Reisen, 2010) viruses in the USA and yellow fever

in Brazil, Argentina and Africa (Maciel-de-Freitas et al., 2006; Savage et al., 1992; Vezzani and Carbajo, 2008).

The spread of *Ae. albopictus* into new territories, its susceptibility to a wide range of arboviruses and other parasites and its ability to bridge with sylvatic transmission cycles also suggests that it could introduce into humans some arboviruses or other parasites only existing in animals so far.

3. Traditional vector control methods

a/ Bed nets and insecticides

Mosquitoes transmit viruses and parasites from one human to another through the bite of the females, which need blood nutrients to develop their eggs. Preventing mosquito bites protects against infection, and insecticide spraying of households and insecticide-impregnated bed nets are the most common vector-control methods (Lengeler, 1998). These methods are efficient (Lengeler, 2004) but the required high coverage is hard to achieve on a large scale. Moreover, the main problem associated with the use of insecticides is the resistance that mosquitoes quickly develop (Brogdon and McAllister, 1998; Curtis et al., 2006; Takken, 2002). These methods are therefore not sustainable and their efficiency is likely to decrease.

b/ Water management

Mosquitoes lay their eggs in water, and after fully aquatic development from eggs to pupae, flying adults emerge. Therefore, any receptacle containing water, or that may collect rainwater, is a potential artificial breeding site for mosquitoes. Close control of these sites helps to reduce the numbers of disease-transmitting adults. Water management is used mainly in big cities, where most breeding sites are artificial. In Singapore, where dengue is a major public health problem, the National Environment Agency (NEA) actively campaigns against dengue (http://www.dengue.gov.sg/) and takes enforcement actions against citizens who allow

mosquitoes to breed on their property. The “Control of Vectors & Pesticides Act” took effect in 1998 but did not prevent a resurgence of dengue since 2002.

c/ Biological control

Biological control agents include predators, parasites and pathogens. The most successful predator of mosquitoes for vector control is the mosquito-eating fish *Gambusia affinis* (Prasad et al., 1993). They should, however, be used with care since they quickly colonise new ecosystems and could become pests themselves (Rupp, 1996). They may also feed on other aquatic insects and zooplankton (Nagdali and Gupta, 2002).

Copepods of the genus *Mesocyclops* are also predators of the early instars of *Aedes* and *Anopheles* mosquitoes. They have recently been used in community programs to control populations of *Ae. aegypti* in Vietnam (Vu et al., 2005). Those results are promising although methods for large-scale deployment of the copepods still need to be developed.

The toxin from the bacterium *Bacillus thuringiensis israelensis* is used as an environment-friendly insecticide since it is specific to the larvae of mosquitoes, black flies and midges, with few effects on other organisms (Lacey and Undeen, 1986). Despite the benefits of lower impact upon the environment, the emergence of resistant mosquitoes occurred in a similar manner to that of chemical insecticides (Boyer et al., 2007).

d/ Control of *Ae. albopictus*

After the trade of used tyres was identified as a source of *Ae. albopictus* dissemination, several governments regulated the import of used tyres from infested countries, although in most cases the economical impact of such decisions was prohibitive (Reiter, 1998). Romi et al. (Romi et al., 1999) report that in Italy, local laws were passed since 1992 to contain the spread of *Ae. albopictus*, but no tyre legislation was passed at national level. However, according to Paul

Reiter, the US efforts in preventing further importations are vain since the number of mosquitoes that could be introduced from overseas is insignificant compared to the vast size and distribution of the existing population. The situation in Italy is probably similar. Nevertheless, even if it seems impossible to prevent the establishment of *Ae. albopictus* within its suitable geographical range, surveillance and control remain critical in order to quickly treat initial invasions and delay its spread, especially its establishment into cities where the risk of epidemics is higher. In France, despite a surveillance network set up by the Ministry of Health in 1999 just after the first infestation (Scholte and Schaffner, 2007), *Ae. albopictus* continues to spread (www.albopictus13.org/cartes_implantation.htm).

Mosquito control largely involves the use of insecticides. In the past, *Ae. aegypti* has been successfully eradicated in some places by use of DDT, but this chemical was prohibited in the 1970s due to its adverse ecological impact (WHO, 1979). Currently, more environmentally acceptable insecticides are employed (mostly pyrethroids against adults and *Bacillus thuringiensis* against immature stages) but their use has to be limited in order to avoid the development of resistance (Curtis et al., 1998). The elimination of artificial breeding sites such as flower-pots is an efficient control method but it requires public awareness and participation, which is hard to sustain. Besides, *Ae. albopictus* also breeds in natural sites such as holes in trees and rocks, which are hard to find and treat. These considerations explain why the 2007 ECDC report on risk assessment of chikungunya in EU states that "once *Aedes albopictus* is known to be established in an area, it is difficult (not to say impossible) to eradicate the mosquito" (Mission Report, Chikungunya in Italy, Joint ECDC/WHO visit for a European risk assessment, 17–21 September 2007).

4. SIT as an area-wide vector control method

a/ Principle of SIT

The Sterile Insect Technique (SIT) (Knipling, 1959) is a vector control method applied to an insect pest population to reduce its numbers (Dyck et al., 2005). It relies on releasing sterile insects in the field to compete with the wild insects, consequently reducing the target population. Unlike conventional control methods, SIT becomes more efficient with time as the density of the target population decreases. It is also area-wide, species-specific and environmentally benign. A traditional SIT programme involves rearing large numbers of insects of the target species, exposing them to ionising radiation to induce sexual sterility, and releasing them into the target population. The released sterile males mate with wild females, preventing them from reproducing. Releasing males alone has proven to be more efficient than both sex releases as only males transfer sterile sperm to wild females (Rendón et al., 2000). If released, sterile females would distract sterile males from inseminating wild females (Rendón et al., 2004). Beyond efficiency, it is highly desirable for mosquito SIT programs to release only males since females, even sterile, would bite and potentially transmit the disease. Releasing large numbers of females would therefore potentially increase the risk of transmission. A stringent sex-separation system ("sexing system") is therefore required for mosquito SIT.

Inundative releases of sterile males result in the decline of the target species population and, potentially, in its eventual elimination (Krafsur, 1998). Since the 1950s, SIT has been used successfully to eradicate the New World Screwworm (NWS) fly *Cochliomyia hominivorax* from the USA, Mexico and other parts of Central America (Wyss, 2000).

Several mosquito releases were performed for purposes related to SIT in the 1960s, 1970s and early 1980s. The largest-scale trials were conducted in El Salvador and India. The highly successful El Salvador field release resulted in elimination of the target population (Lofgren et al., 1974). Unfortunately, political problems in both countries in the mid-1970s interrupted further work. One prominent cause contributing to the failure of several mosquito releases was insufficient production of sterile males due to absence of sexing strains or delays in production (Benedict and Robinson, 2003). The required production was higher than expected due to the

negative impact of insect handling during rearing, sexing and distribution. The efficiency was also reduced due to greater than expected migration of mated females into the release area. Such problems could be overcome in the future by increasing the mass production in order to reach higher release ratios or developing a sexing strain.

Mosquito sexing for those release programs exploited sexual dimorphism based on differential pupal size, using a semi-automated device (Breeland et al., 1974; Lofgren et al., 1974). Male pupae are generally smaller than female pupae although the extent of the dimorphism is species-specific (greater in *Aedes* than in *Anopheles* species) and dependent on the rearing conditions.

Recently, the limits of conventional vector control in the fight against *Ae. albopictus* naturally led Italy to evaluate the feasibility of the Sterile Insect Technique. The Centro Agricoltura Ambiente is currently improving mass rearing and sterilisation of *Ae. albopictus* by irradiation, and conducting experimental field releases of sterile males (Bellini et al., 2007).

b/ Genetic modifications of insects – the early days

The advent of genetic tools in the 1960s provided new solutions for insect sexing: genetic sexing systems were developed by using translocations of an autosome bearing the dominant allele of a selectable marker to the Y chromosome, and crossing male flies carrying the translocation to mutant females (Whitten, 1969). Most of the genetic sexing strains based on translocations used insecticide resistance genes as selectable markers, leaving male progeny resistant and female progeny susceptible at larval and adult stages. Genetic sexing strains of this kind have been developed in many different insects (reviewed in (Robinson, 2002)), including *Anopheles albimanus* (Seawright et al., 1978) for which a field trial was performed in the late 1970s, also in El Salvador (Bailey et al., 1980). The maintenance of such strains as large colonies is not straightforward due to semi-sterility and recombination between the marker and the Y chromosome (Robinson et al., 1999). Moreover, such sexing technology cannot be

easily applied to a wide range of species due the lack of required knowledge of mutations and genetic make-up which would enable to map the chromosomal rearrangements.

Male sterility associated with male-linked chromosomal translocations has also been exploited with the aim of replacing irradiation (Laven and Jost, 1971). However, the development of such strains can be very time consuming and each translocation provides only 50% sterility and comes with a high fitness cost (Weidhaas and Seawright, 1974). These factors, coupled with previously discussed rearing difficulties, mean that irradiation is still a preferred option for sterilisation. For the El Salvador release programs, sterility was induced in pupae of *An. albimanus* by treatment with a chemical mutagen (ENT-61585, (Lofgren et al., 1974)). The fitness cost was lower with this method but the toxicity of the chemical residues makes it unacceptable for current use.

5. Insect transgenesis as a tool for improving SIT

a/ The gene transfer technology

In the 1990s, the development of the gene transfer technology and its potential application to genetic sexing generated strong hopes for SIT improvement (Robinson and Franz, 2000). Indeed, any gene of interest can now be inserted into the genome of many insect species. Such an approach does not require much genetic background information about the species, and once developed, the technology can usually be easily transferred to different species (Benedict and Robinson, 2003; Robinson et al., 2004).

The first transformation system to be successfully used in insects (*Drosophila melanogaster*) derived from the *P* element (Rubin and Spradling, 1982). This element is a class II short inverted terminal repeats transposon of 2.9 kb. The ability of the transposase to act in trans has allowed the development of a binary vector-helper system for transformation (Rubin and Spradling, 1982): the vector is a *P* element incorporating a transgene between the inverted terminal repeats (ITR) in lieu of the transposase; the helper plasmid is a *P* element that provides the

transposase, but rendered non-autonomous by deletion of the 3'ITR (Karess and Rubin, 1984). The co-injection of vector and helper constructs results in the virtually random transposition of the transgene into the host genome. The stability of the integrated transgene is ensured by the dilution and eventual loss of the helper plasmid after several cell divisions.

Despite repeated efforts, the *P* transformation vectors have been unsuccessful for routine transformation of non-drosophilid insect species, including mosquitoes, though a small number of transgenics were generated as low-frequency events that may or may not have been dependent on *P* element functions (Miller et al., 1987; Morris et al., 1989). *P* excision assays confirmed the lack of mobility of the element outside of the Drosophilidae (Handler et al., 1993). Several observations later highlighted that vector function can be dependent on the cellular and genomic environment of each species (Jasinskiene et al., 2000). These results pointed out to the need for new vector systems for gene transfer into insects.

b/ The *piggyBac* transposable element

piggyBac is a class II transposable element from the TTAA-specific family. It was discovered in *Trichoplusia ni* cell lines inserted in the genome of a baculovirus (Fraser et al., 1983). The original *piggyBac* element isolated was 2.4kb in length and its extremities are composed of 13-bp perfect inverted repeats and additional 19-bp inverted repeats located asymmetrically with respect to the ends ((Cary et al., 1989) and Figure 1-5). Elements from the TTAA-specific family insert with the duplication of the TTAA target site. Autonomous *piggyBac* elements have an ORF encoding a functional transposase (Elick et al., 1996). The *piggyBac*-mediated transformation system is similar to the vector-helper system used with the *P* element (Figure 1-6).

piggyBac has been successfully used to transform in a wide range of insect species from several orders, including dipterans, lepidopterans, hymenopterans and coleopterans (reviewed in

(Handler, 2002a), see Table 1-1). Both *Ae. aegypti* and *An. gambiae* have been transformed with *piggyBac* (Grossman et al., 2001; Kokoza et al., 2001).

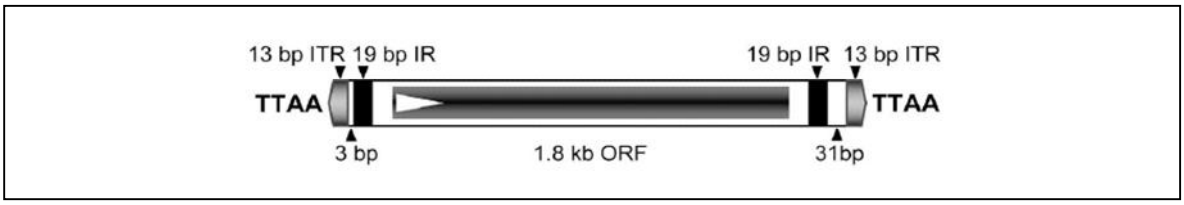

Figure 1-5. Diagram of the 2.47 kb *piggyBac* element (not to scale)

ITR: inverted terminal repeats sequences; IR: sub-terminal inverted repeat sequences; ORF: open reading frame; TTAA: duplicated TTAA insertion site. (*A.M.Handler/Insect Biochemistry and Molecular Biology 32 (2002) 1211-1220).*

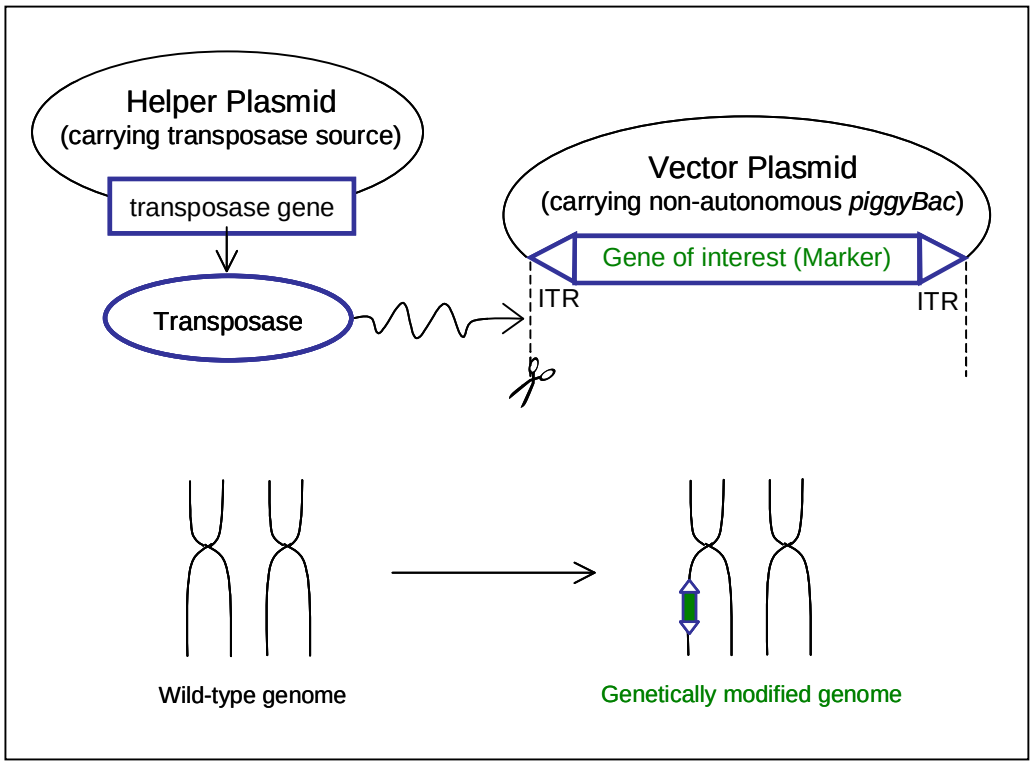

Figure 1-6. Binary vector-helper system based on the *piggyBac* transposable element.

Table 1-1. Species transformed with *piggyBac*, and the markers used for selection (Morrison et al., 2010)

Insect Order	Host species	Marker
Diptera	*Aedes aegypti*	Dm *cinnabar* (Grossman et al., 2001; Kokoza et al., 2001; Lobo et al., 2002)
	Aedes fluviatilis	3xP3-EGFP (Rodrigues et al., 2006)
	Anastrepha ludens	Hr5ie1-DsRed2 (Condon et al., 2007)
	Anastrepha suspensa	PUbnlsEGFP (Handler and Harrell, 2001b)
		PUbnls-DsRed1 (Handler & Harrel, unpub.)
	Anopheles albimanus	PUbnlsEGFP (Perera et al., 2002)
	Anopheles gambiae	hr5-ie1-EGFP (Grossman et al., 2001)
	Anopheles stephensi	actin 5C-DsRed (Nolan et al., 2002)
		3xP3-EGFP (Ito et al., 2002)
	Bactrocera dorsalis	Cc *white* (Handler and McCombs, 2000)
		PUbnlsEGFP
	Bactrocera tryoni	PUb-DsRed1, PUbnlsEGFP (Raphael et al., 2010)
	Ceratitis capitata	Cc *white* (Handler et al., 1998)
		PUbnlsEGFP, PUbnls-DsRed1 (Handler *et al.*, unpub.)
	Cochliomyia hominivorax	PUbnlsEGFP (Allen et al., 2004)
	Drosophila melanogaster	*white*;*white*/PUbnlsEGFP (Handler and Harrell, 1999)
		3xP3-EGFP (Berghammer et al., 1999; Horn et al., 2002)
		3xP3-ECFP, 3xP3-EYFP (Handler and Harrell, 2001a)
		PUb-DsRed1 (Handler and Harrell, 2001a)
		3xP3-DsRed (Horn et al., 2002)
	Lucilia cuprina	PUbnlsEGFP (Heinrich et al., 2002)
	Musca domestica	3xP3-EGFP (Hediger et al., 2001)
Lepidoptera	*Bicyclus anynana*	3xP3-EGFP (Marcus et al., 2004)
	Bombyx mori	Bm *actin* A3-EGFP (Tamura et al., 2000)
		3xP3-EGFP (Thomas et al., 2002)
	Pectinophora gossypiella	Bm *actin* A3-EGFP (Peloquin et al., 2000)
Hymenoptera	*Athalia rosae*	hsp70-GFP-S65T, Bm *actin* A3-EGFP (Sumitani et al., 2003)
Coleoptera	*Harmonia axyridis*	3xP3-EGFP (Kuwayama et al., 2006)
	Tribolium castanum	3xP3-EGFP (Berghammer et al., 1999)
		Tc vermillon (Lorenzen et al., 2003)

c/ The essential role of marker systems

The first successful germline transformations of a non-drosophilid insect utilized transgenes carrying the bacterial neomycin phosphotransferase gene, conferring neomycin resistance to transgenic insects (McGrane et al., 1988; Miller et al., 1987; Morris et al., 1989). However, such selection is not 100% accurate and neomycin is hazardous to researchers.

More recently, fluorescent protein genes have been used as markers. The first fluorescent marker to be developed was the green fluorescent protein (GFP) gene isolated from the jellyfish *Aequorea victoria* (Prasher et al., 1992). GFP has proved functional in an almost universal range of tissues and species, and is easily detectable *in vivo*. However, two properties are not so ideal: the protein is relatively insoluble and its excitation wavelength might damage live organisms (excitation maximum is in near UV). The GFP has been engineered to solve these two problems: the enhanced GFP (EGFP), more soluble and red shifted, can be detected with a longer wavelength of blue light and less intensity, which makes it a very suitable marker for the screening of transgenic insects (Higgs and Lewis, 2000). The successful transformations of Dipterans, Lepidopterans, Hymenopterans and Coleopterans with GFP transgenes testify to the wide applicability of this marker system.

To express fluorescent marker genes in insects several different promoters have been used. The artificial 3xP3 promoter has been designed to express in the eyes of insects: three tandem repeats of the P3 site have been aligned in front of a TATA-box (Berghammer et al., 1999). P3 is the binding site of Pax6, a transcriptional activator that controls the genetic pathway of eye development, and is evolutionarily conserved through all metazoan animals. The 3xP3-EGFP marker has been successfully used in three insect orders (Table 1-1), and the conserved pathway of Pax6 suggests that it should be applicable to all eyed animals. The tissue-specific expression helps distinguish between marker expression and background fluorescence. These features of 3xP3-EGFP indicate that it might be able to serve as a universal marker for insect transgenesis, unless particular eye pigmentation obscures the fluorescence.

Several useful transformation markers were derived from 3xP3-EGFP by replacing the coding sequence for EGFP with the coding sequence for its yellow (EYFP) and cyan (ECFP) spectral variants, and with the red fluorescent protein DsRed (Horn et al., 2002; Horn and Wimmer, 2000). These different markers would allow insect transformations using multi-component genetic systems.

Markers expressed in other tissues have the advantage of providing a distinguishable pattern of expression. Moreover, promoters that express all over the body - such as Hr5IE1 (baculovirus IE1 promoter and Hr5 enhancer, (Rodems and Friesen, 1993)) - are more visible and easier to screen, especially in insects whose eye pigments mask the expression of 3xP3-based markers.

d/ Fitness and SIT

Genetically modified insects may potentially carry a fitness cost due to several factors: i) the cost for cells of producing the exogenous proteins encoded by the transgene and the nature of those proteins; ii) the possible disruption of a gene by integration of the transgene; iii) the limited genetic diversity of transgenic lines: they start from a single transgenic individual and are maintained homozygous by inbreeding. Irradiated insects have an important fitness loss (Helinski et al., 2006) and have nevertheless been successfully used for SIT, but any fitness cost is directly translated into a financial cost, as more insects have to be released in order to reach the same efficiency. It is therefore important to minimise it as much as possible. With transgenic insects, this can be done by creating several lines of the same construct and selecting the fittest one, and out-breeding transgenic lines after their creation. The construct itself can also sometimes be optimised: expressing the marker only in certain tissues to limit the load on the organism; making the RIDL component more adapted to the target species to improve repressibility by tetracycline.

Studies on the fitness of transgenic lines have different conclusions (Catteruccia et al., 2003; Irvin et al., 2004; Moreira et al., 2004) but it seems that inbreeding might be the most influential factor (Marrelli et al., 2006).

For a mosquito SIT (or RIDL) strain, the most relevant fitness parameters are male mating competitiveness and longevity in the wild and the mass rearing potential of the strain.

6. Transgene stability

With the potential release of transgenic sexing strains in the field, ensuring transgene stability is critical to prevent remobilisation by an unintended source of transposase. This could induce the loss of the transgene in mass rearing conditions or, it has been speculated, cause horizontal transfer of the transgene to untargeted wild species. The minimal DNA requirements for the mobility of the different transposable elements are unclear, but at least the terminal (and subterminal) inverted repeat sequences are needed (Li et al., 2005). Consequently, a stabilization system would consist in deletion or rearrangements of these sequences. A. Handler and colleagues developed a system in *Drosophila melanogaster* to delete one of the terminal sequences after germline transformation (Handler et al., 2004): the transgene was formed using three *piggyBac* terminal ends – forming one transposable pair that spanned the entire construct and another pair present at the periphery of the construct. The peripheral construct was remobilized, after insertion of the construct, by crossing this transgenic line to a *piggyBac* transposase-expressing 'jumpstarter' strain. The resulting construct, with excised peripheral pair of *piggyBac* ends, had only one *piggyBac* terminal sequence. In isolation, such a one-ended *piggyBac* insertion is stable and cannot be re-mobilised without provision of the missing end. An improvement to this approach, in which both ends of an integrated construct are rendered *piggyBac*-free, was developed by Dafa'alla *et al.* in the Mediterranean fruit fly, *Ceratitis capitata* (Dafa'alla et al., 2006); Figure 1-7).

This design incorporated two adjacent pairs of *piggyBac* transposable sequence at each end of the construct. After integration of the entire construct into the insect genome, a transposase source, driven by the *Drosophila* hsp70 promoter, in the central domain was used to excise the peripheral pairs of *piggyBac* ends. This rendered it free of *piggyBac* terminal sequence and

therefore incapable of re-mobilisation, even in the presence of transposase. Such a transgene insertion is no more likely than any other part of the genome to be mobilised.

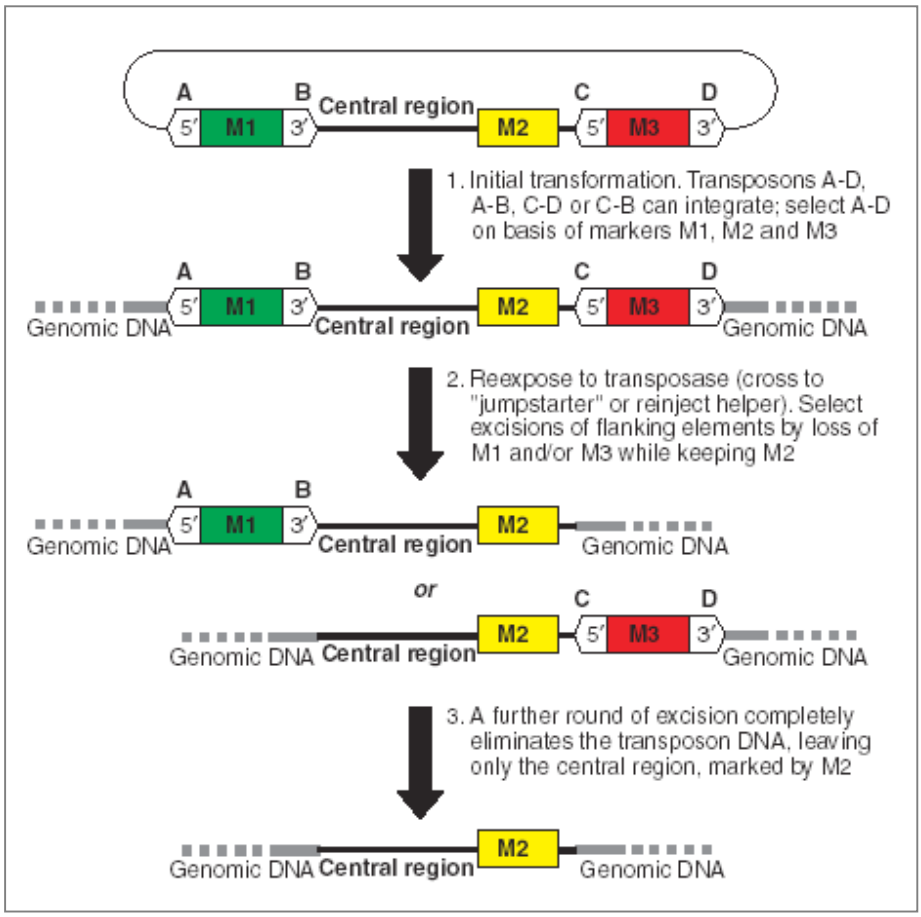

Figure 1-7. Strategy for post-integration removal of transposon ends from transposon-mediated insertions (taken from Dafa'alla *et al*. 2006).

The initial transformation uses a plasmid carrying a composite transposon comprising two pairs of opposed transposon ends. This has four potential transposons (A-D, A-B, C-D and C-B). Insertion of the desired A-D transposon is identified by selecting for marker M2, which marks the central region. The flanking transposons A-B and C-D can then be eliminated by re-exposure to transposase; in principle two rounds of post-integration exposure are required to remove both flanking transposons. The final insertion no longer has any transposon ends and is therefore inert to transposase and subsequent remobilisation.

7. Genetically induced sterility: the RIDL technology

a/ Bisex RIDL technology

Genetic sterility has been engineered in insects using tetracycline-dependent repression of a dominant lethal gene (Heinrich and Scott, 2000; Thomas et al., 2000). This involved a two-component system: the first part contains a promoter controlling the tetracycline-repressed transactivator tTA (Gossen and Bujard, 1992), the second part is a lethal gene placed under the control of the tTA response element. In the absence of tetracycline, the tTA transactivator binds the tTA response element (tetO) and enhances the expression of an hsp70 minimal promoter, thus activating the expression of the dominant lethal gene. The presence of tetracycline represses the system by binding to tTA and preventing it from binding to the tetO sites. This allows the insects to develop normally on a diet supplemented with tetracycline. A variant of this system is a simplified one-component positive feedback system: tTA is placed under the control of a minimal promoter adjacent to the tetO element (Gong et al., 2005). In the absence of tetracycline, the basal amount of tTA produced by the minimal promoter is able to bind tetO, enhancing its own expression in a positive feedback manner (Figure 1-8). The tTA element consists of two parts: the tetR element that binds to tetO, and a VP16 peptide that is a transcription activator (Gossen and Bujard, 1992). The accumulation of large amounts of VP16 in the cell has a toxic effect due to transcriptional squelching (Berger et al., 1990; Gill and Ptashne, 1988). In this system, tTA is both the transactivator and the effector (Gong et al., 2005).

Any insects carrying this system and reared without a tetracycline supplement should die due to the expression of the lethal effector gene (two-component system) or the accumulation of VP16 (positive-feedback system) (Heinrich and Scott, 2000; Thomas et al., 2000). In an SIT-like program, all the released insects would be homozygous for the construct and transmit one copy of the dominant lethal system to their offspring after mating with wild insects. All their offspring would then die in the field due to the absence of tetracycline and subsequent expression of the lethal gene.

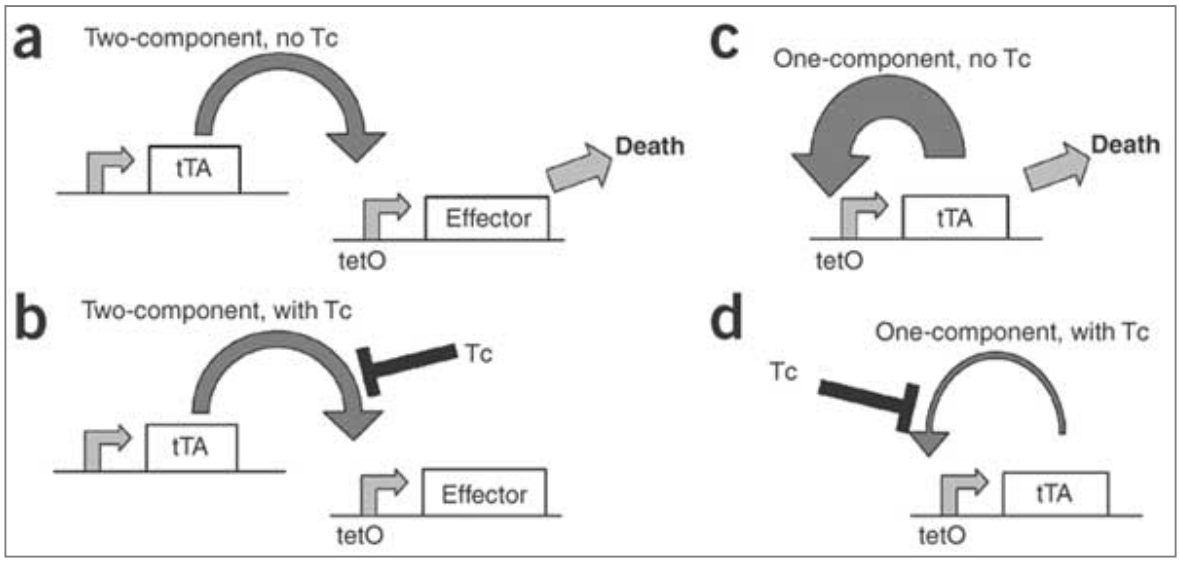

Figure 1-8. Tetracycline-repressible systems (from Gong et al. 2005).

The tTA element consists of the tetR element that binds to the tetO sequence, and a VP16 peptide that is a transcription activator. a, b: Two-component tetracycline repressible lethal system: In the absence of tetracycline, expressed tTA binds to tetO, driving expression of the lethal effector molecule, leading to death. In the presence of tetracycline, tTA preferentially binds to tetracycline, the effector molecule is not expressed and there is no lethal effect. c, d: One-component positive feedback system: Low levels of basally expressed tTA bind to tetO, driving further expression of tTA. When tTA level becomes high, it acts as a lethal effector molecule, leading to death. In the presence of tetracycline, tTA preferentially binds to tetracycline and the level of tTA stays basal. A low level of tTA is not lethal.

Transgenic insects of this kind would not require sterilization before release: the multiple random mutations induced by irradiation, which are the cause of sterility in traditional SIT, are replaced by the lethal transgene. This variant of SIT is therefore called "release of insects carrying a dominant lethal" (RIDL) (Thomas et al., 2000). Considering the high fitness cost associated with irradiation of insects in general and mosquitoes in particular (Helinski et al., 2006), a RIDL program should be more efficient and more economic to run than a traditional SIT program: the RIDL insects should be fitter than irradiated insects so less would have to be released, thus reducing the cost.

Another advantage of RIDL is that the lethality can be engineered to match specific species requirements. The amount of random mutations induced by irradiation causes the embryo to die very quickly, which is the ideal scenario for agricultural pests like fruit flies or screwworm in which maggots are the life stages doing the most damage. When it comes to mosquito control,

however, early elimination is not required since only adult females are transmitting diseases. Recent mathematical models show that late-acting lethality (i.e. pupal stage) would be more efficient for mosquito control than early acting lethality: The larvae of RIDL insects would compete for food against wild larvae, therefore having an extra impact on the wild population (Atkinson et al., 2007; Phuc et al., 2007). They would then die due to a late activation of the RIDL lethal gene, before reaching adulthood.

A mosquito transgenic RIDL line with this property has been developed by Phuc *et al* (Phuc et al., 2007) using a positive feedback-type construct. However, the late-acting lethality in this case is due to regulatory effects of the genomic DNA surrounding the construct rather than an engineered late-acting effect. A late-acting promoter used in a two-construct RIDL system might provide a more reliable control over the time of expression of the lethal gene.

b/ Female-specific RIDL technology: genetic sexing

SIT relies on the release of sterile males and therefore separating males from females is a central issue, especially with mosquitoes since any released females would potentially be able to transmit pathogens among human hosts. As discussed in 4.b/ , the genetic sexing strains developed for SIT purposes in the 1960's had several problems. The use of large quantities of insecticides, the poor rearing efficiency and the low stability of the system are issues that could potentially be addressed by transgenesis.

Catteruccia *et al*. produced a transgenic line of *Anopheles stephensi* mosquitoes expressing EGFP under the control of a *Beta2-tubulin* promoter (Catteruccia et al., 2005). This line produces males with green fluorescent testes, a feature that allows sexing by using an automated device that detects fluorescence. This is an efficient way of sexing insects although it requires extra handling. Ideally, a sexing strain would impair the females automatically in the last rearing cycle before release, while leaving the males healthy and fit to compete in the field.

The two-part tetracycline-dependent RIDL system described earlier has been used in *D. melanogaster* to direct lethality against females: The female specificity was provided by using a female-specific promoter (yolk protein 1 (*Yp1*) or yolk polypeptide 3 (*Yp3*)) to drive the lethal genes *hid* or Ras^{V12A}, or a female-specific lethal gene ($msl\text{-}2^{NOPU}$, a mutant allele of the *msl-2* dosage compensation gene) under the control of tetO (Heinrich and Scott, 2000; Thomas et al., 2000). More recently, in the Mediterranean fruit fly *C. capitata*, the sex-specific splicing properties of the sex-determination gene *transformer* have been exploited to disrupt the expression of tTA in males but not in females, inducing the same female-specific lethal phenotype (Fu et al., 2007).

Recently, a genetic sexing strain of *Ae. aegypti* has been produced, based on the RIDL technology (Fu et al., 2010). The female specificity in this case is provided by the female-specific properties of the *Ae. aegypti Actin-4* gene (Muñoz et al., 2004). This system is described further in the 3rd chapter of this thesis.

8. Other innovative vector control strategies

a*/ Wolbachia*-mediated population suppression

Wolbachia is a genus of maternally inherited endosymbiotic bacteria found in arthropods and nematodes. It was first discovered in the mosquito species *Culex pipiens* in 1924 by Hertig and Wolbach (Hertig and Wolbach, 1924) but has recently been estimated to be infecting 66% of all insect species (Hilgenboecker et al., 2008). This high range of hosts is partly explained by an aptitude to interspecific horizontal transfer (Vavre et al., 1999). *Wolbachia* bacteria have the property to manipulate the host reproduction machinery by inducing feminization (Bandi et al., 2001; Fujii et al., 2001), parthenogenesis (Huigens et al., 2000), male killing (Fialho and Stevens, 2000; Hurst and Jiggins, 2000) or cytoplasmic incompatibility (Laven, 1967b; Yen and Barr, 1971, 1973). All of these mechanisms lead to an increased proportion of infected female progeny, which may be viewed as an opportunistic way of multiplying and dispersing.

Cytoplasmic incompatibility (CI) occurs when a male carrying *Wolbachia* inseminates an uninfected female (unidirectional CI) or a female infected with a different *Wolbachia* strain (bi-directional CI). Such crosses are sterile, whereas uninfected males crossed with *Wolbachia*-carrying females result in a fully fertile cross ("rescue" cross) and infected progeny. The molecular mechanisms of CI are not fully understood but many laboratories are focusing on this complex phenomenon.

The CI property of *Wolbachia* was used to develop the Incompatible Insect Technique, which relies on releasing males bidirectionally incompatible with the target population to induce female sterility. A successful trial was performed in Burma in the 1960s against the filarial vector *Culex quinquefasciatus* (Laven, 1967a). The method was deemed unsustainable due to the lack of efficient sexing methods, as for the Sterile Insect Technique previously described.

Ae. albopictus is naturally super-infected with two strains of *Wolbachia*, *w*AlbA and *w*AlbB (Werren et al., 1995; Zhou et al., 1998).

b/ Homing endonuclease-mediated population suppression

Homing endonuclease genes (HEGs) encode an enzyme which recognizes a specific 20-30 bp sequence and creates a double-stranded break. The cell's repair machinery then uses the HEG-bearing chromosome as a template, creating a second copy of the HEG inside of its own recognition site. Chromosomes carrying a copy of the HEG are therefore protected from further cuts. HEGs are active during meiosis, thereby being inherited by more than half of the gametes (Chevalier and Stoddard, 2001; Goddard et al., 2001).

Austin Burt has proposed to use the super-Mendelian inheritance properties of the HEGs to drive pest populations to extinction. The idea is to engineer HEGs to recognize and cleave sequences from essential genes, which would create recessive lethal mutations rapidly inherited by a pest population. The population would eventually crash as crosses between

heterozygous individuals will induce a large proportion of offspring homozygous for the recessive lethal mutation (Burt, 2003).

c/ Population replacement

Other vector control strategies aim at replacing a disease susceptible mosquito population with a refractory one, unable to transmit diseases. Several approaches are being investigated, which would ideally be used synergistically in order to prevent any resistance build-up against one of the strategies.

Reduction of the vector's lifetime:

Most pathogens require a period of development in the mosquito host for it to become infectious. Age is therefore a critical factor in the ability of a mosquito to transmit human pathogens, and only the insects of sufficient age take part in disease transmission. A significant reduction of the mosquitoes' lifetime should therefore prevent some important mosquito-borne diseases (Cook et al., 2008). The life-shortening *Wolbachia* strain *w*MelPop from *Drosophila* was recently introduced into *Ae. aegypti* with the result of halving the host's lifetime (McMeniman et al., 2009) and reducing dengue, chikungunya and *Plasmodium* infection (Moreira et al., 2009) as well as filarial competence (Kambris et al., 2009).

Resistance to parasites:

Olson *et al.* proposed to develop "transgenic *Ae. aegypti* containing heritable, virus-derived, anti-DEN genes or sequences that profoundly alter the mosquitoes' vector competence and prevent virus transmission to new human hosts" (Olson et al., 2002). In this case, transgenic mosquitoes express double-stranded RNAs specific to the dengue virus, which disrupt its replication by RNA interference (Franz et al., 2006).

Several approaches are also investigated in order to engineer *Plasmodium*-resistant mosquitoes for malaria control (reviewed in Nirmala and James, 2003). Those include single-chain antibody fragments directed against parasite ligands (de Lara Capurro et al., 2000); the blocking of

parasite's receptors on the surface of mosquito tissues (Ito et al., 2002); the expression of specific immune response effectors inhibiting the parasite's development in the mosquito (Moreira et al., 2007); and the expression of parasite-killing toxins in the mosquito (Maciel et al., 2008; Moreira et al., 2002). The use of transgenic symbionts of mosquitos to express molecules to reduce vector competence is also investigated (Ren et al., 2008; Riehle et al., 2007), a method known as paratransgenesis (reviewed in Coutinho-Abreu et al., 2010).

As it is unlikely that the engineered population will have a significant fitness advantage, it will not be able to take over the wild population unless the desirable trait follows a "super-Mendelian" rate of inheritance. This problem may be tackled by tightly linking the anti-pathogen gene to a "gene drive" mechanism strong enough to spread it to fixation. Proposed gene drive mechanisms include *Wolbachia* and homing endonuclease genes mentioned earlier (Deredec et al., 2008; Sinkins and Gould, 2006), as well as engineered killer/rescue systems such as under-dominance or Medea elements whereby only the offspring carrying the suitable antidotes – linked to the refractory gene - will be able to survive (Davis et al., 2001; Hay et al., 2010; Magori and Gould, 2006).

9. Conclusion

This thesis presents the development of the gene-transfer technology for *Ae. albopictus* (Chapter 2). Transgenic strains were then generated with a view to exploiting sex-alternate splicing to provide female-specific repressible flightless phenotypes (Chapter 3). A particular candidate strain was further evaluated for its suitability in a RIDL control programme (Chapter 4). Alternative molecular approaches were also developed to produce bi-sex lethal phenotypes, and to widen the possible applications of the on-off tetracycline system (Chapter 5).

Chapter 2

piggybac- and ΦC31-mediated genetic transformation of *Aedes albopictus*

1. Introduction

Aedes mosquitoes are responsible for an estimated 50 to 100 million dengue cases worldwide every year, with nearly half the world's population at risk of being infected (ECDC, 2007; WHO/TDR, 2006). The two main vector species, *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) are also the main vectors of the chikungunya virus, which can cause severely debilitating syndromes lasting up to several months.

In the absence of vaccine or antiviral drugs for either chikungunya or dengue, efficient mosquito control strategies are crucial. Conventional control methods (insecticide spraying and management of breeding sites) have so far failed adequately to control *Ae. albopictus*.

Transgenesis is an essential tool required to develop novel genetics-based control methods. It is therefore highly desirable to establish germline transformation of *Ae. albopictus*. Germline transformation of a number of insect species, including *Ae. aegypti* mosquitoes, is now routine through the use of transposable elements. The most commonly used is *piggyBac,* a class II transposable element that inserts into TTAA sequences (Fraser et al., 1983). *piggyBac* has been used successfully to transform a wide range of insect species from several orders, including Diptera, Lepidoptera, Hymenoptera and Coleoptera (reviewed in (Handler, 2002b)). Among insects, transformation efficiency using *piggyBac* is typically 3-13%, and 4-11% in *Ae. aegypti* in particular (Handler, 2002a; Kokoza et al., 2001; Lobo et al., 2006; Lobo et al., 2002; Nimmo et al., 2006). The short recognition sequence of transposable elements leads to effectively random integrations into the host genome (Thibault et al., 1999).

Positional effects and possible gene disruptions affect both transgene expression and fitness of the transgenic lines, so that a single transgene may lead to a range of phenotypes depending on its insertion site. In some cases it may be useful to have several lines with slightly different phenotypes to choose from; however this random integration pattern makes it difficult to compare two different transgenes as their different phenotypes are an unknown combination of the inherent properties of the transgene and the effects of the insertion sites.

Site-specific transgene integration systems have been developed using recombination systems which target a specific nucleotide sequence that is long enough that it is unlikely to occur naturally in an insect genome. Examples include Cre-*loxP* from bacteriophage P1 (Sauer and Henderson, 1988), Flp-*FRT* from the 2 micron plasmid of *Saccharomyces cerevisiae* (O'Gorman et al., 1991) and phiC31-*att* from a *Streptomyces* bacteriophage (Thorpe and Smith, 1998). In each case one of the target sequences for the recombinase is introduced as a "docking site" into the genome of interest using a transposable element-based "first phase" transgene. Second-phase transgenes can then be inserted repeatedly into the preferred docking site using the appropriate recombination enzyme (Nimmo et al., 2006). Although those integration systems have all been demonstrated in *Drosophila* (Gong and Golic, 2003; Groth et al., 2004), only the ΦC31-*att* system is unidirectional and has been used successfully to integrate a transgene in *Ae. aegypti* (Nimmo et al., 2006). The ΦC31 integrase catalyses a unidirectional recombination between so-called *attB* and *attP* sites, creating *attL* and *attR* junctions (Belteki et al., 2003). Typically, *attP* is used as the docking site for *attB*-carrying transgenes. In *Ae. aegypti*, the transformation efficiency using the ΦC31-*att* system was reported to be 17-32% (Nimmo et al., 2006).

This chapter reports the use of a *piggyBac*-based system to achieve the first successful germline transformation of *Ae. albopictus*. Further, successful site-specific integration into the *Ae. albopictus* genome is described, using the ΦC31-*att* system.

2. Materials and Methods

a/ Plasmid constructs

The OX3860 construct is the pBac[3xP3-ECFPaf]-attP plasmid described by Nimmo *et al.* (Nimmo et al., 2006). The construct OX4105 carries an *attB* site and a 3xP3-DsRed2 marker, and was designed to integrate into the OX3860 construct such that, after integration, the two markers would be in the same orientation (Figure 2-1). This allows the comparison of the expression of the two markers in an equivalent genomic context.

The OX4105 construct was made by modifying pBattB[3xP3-DsRed2nls-SV40]lox66 (Nimmo et al., 2006) to remove the nuclear localisation signal of the DsRed2 protein and change the orientation of the *attB* sequence. The DsRed2-nls-SV40-lox66 cassette was removed using *Age*I/*Not*I and replaced with an *Age*I-*Eag*I DsRed2-SV40 cassette to create pBattB[3xP3-DsRed2-SV40]. The original *attB* cassette was removed from pBattB[3xP3-DsRed2-SV40] using *Xho*I, creating pB[3xP3-DsRed2-SV40]. The *Kpn*I/*Sac*II *attB* fragment from pBattB[3xP3-DsRed2-SV40] was subcloned from pBattB[3xP3-DsRed2-SV40] into pSLfa1180fa and the *Sac*II/*EcoR*V fragment from this plasmid was then cloned into the *Sac*II/*Swa*I sites of pB[3xP3-DsRed2-SV40] creating OX4105.

b/ Insect strains and rearing

The *Ae. albopictus* wild-type strain was colonised in 2006 from Malaysia (Institute of Medical Research, Kuala Lumpur). The strain was reared at 27°C (± 1°C) and 80% (± 10%) relative humidity. Larvae were fed on crushed dry fish food (TetraMin® flake food from Tetra GmbH, Germany) and adults on 10% glucose with 14U / ml penicillin and 14 µg / ml streptomycin. Females were fed on horse blood using a *Hemotek* Insect Feeding System (Discovery Workshops, Accrington, UK) set at 37°C.

c/ Microinjection of *Ae. albopictus*

Pre-blastoderm embryos were injected as described by Morris *et al.* (Morris et al., 1989) except that cover slips of injected embryos were placed vertically into water in order to drain the oil for at least an hour, and then immediately placed vertically in a sealed humid box for 4 days. To produce docking *att*P strains, wild-type embryos were injected with a mixture of the OX3860 construct (300 ng/µl), phsp-Bac plasmid helper (200 ng/µl) (Handler and Harrell, 1999) and *piggyBac* mRNA (300 ng/µl) in injection buffer (5mM KCl and 0.1 mM NaH_2PO_4, pH 6.8). Though, in principle, either mRNA helper or helper plasmid should be capable of mediating

transformation, both were co-injected together to provide a degree of redundancy and in order to increase chances of successful transformation. The *piggyBac* mRNA was transcribed from OX3081 construct (*piggyBac* transposase coding sequence under the control of the T7 promoter (Chamberlin et al., 1970) and the 3'UTR from the *DmVasa* gene (Hay et al., 1988)) using the mMESSAGE mMACHINE® T7 kit (Ambion, Austin, TX). The mRNA was purified using the MEGAclear™ kit (Ambion), precipitated with ammonium acetate and resuspended in 10 µl nuclease-free water. For site-specific integration, embryos from the docking strains were injected with OX4105 (350 ng/µl) and ΦC31 mRNA (600 ng/µl) (Nimmo et al., 2006) in injection buffer. The ΦC31 mRNA was transcribed and purified using the mMESSAGE mMACHINE® T7 and MEGAclear™ kit (Ambion). Construct and helper plasmids were purified using the EndoFree Plasmid Maxi kit (Qiagen, Hilden, Germany). Larvae were screened for fluorescence using a Leica MZ95 microscope with the appropriate filter sets from Chroma Technology (Rockingham, VT) (filters: ECFP: exciter D436/20x; emitter D480/40m; DsRed2: exciter HQ545/30x; emitter HQ620/60m). Pictures of fluorescent larvae were taken with Canon PowerShot S5IS with an MM99 adaptor (Martin microscopes) to fit into the eyepiece.

d/ Inverse PCR

Inverse PCR was performed essentially as described by Handler *et al.* (Handler et al., 1998). Genomic DNA from each line was extracted using the NucleoSpin Tissue kit (Macherey-Nagel). 2.5 µg of gDNA was cut with the restriction enzymes *Hae*III, *Msp*I, *Taq*I and *Dpn*II. PCR was performed using 2 µl of digested genomic DNA, *Taq* DNA polymerase with Thermopol buffer (New England BioLabs, Ipswich, MA) and either the *piggyBac* 5´ or 3´ primer pair (5´ forward: tcttgaccttgccacagagg; 5´ reverse: tgacacttaccgcattgaca; 3´ forward: gtcagtccagaaacaactttggc; 3´ reverse: cctcgatatacagaccgataaaaacacatg). The thermal cycling parameters were 95°C for 5min, followed by 35 cycles of (95°C for 30sec, 55˚C for 1min, and 68°C for 2 min), and a final extension step of 72°C for 10 min.

PCR fragments were extracted using the Minelute Gel Extraction kit (Qiagen), cloned into pJet vectors (GeneJET PCR cloning kit from Fermentas, Vilnius, Lithuania) and transformed into XL-10 cells (Stratagene, La Jolla, CA). Positive clones were purified (GeneJET Plasmid Miniprep Kit, Fermentas) and sent for sequencing (GATC Biotech, Germany).

e/ PCR analysis of site-specific integration

Integration of OX4105 into docking strains was investigated using primers in the 5' flanking genomic sequence (3860C-5'flank1: cacaatggaaccatgaaaacttaaaccag; 3860B-5'flank1: tgagaacaagatggcgattctaggagt) with a primer in the attR sequence (Diag-attBD: tgatggaccagatgggtgagg) or in the 5' *piggyBac* end (PB2: cagtgacacttaccgcattgacaag).

The *attR* junctions were amplified and sequenced using primers in DsRed2 (Diag-DsRed2: ctgggaggcctccaccgagc or Diag3-Dsred: cacctcccacaacgaggactac) and ECFP (Diag2-ECFP: acagctcctcgcccttgctca). The *attL* junctions were amplified and sequenced using primers in the 3' *piggyBac* fragment (pBac3'R: tggacctttctcccttgctactgac; Diag-pb3: ttccgtacaataatgccataggccac) and in the OX4105 backbone (M13-28-R: tgtgagcggataacaatttcacacagga; M13-RP (GATC Biotech): caggaaacagctatgacc). PCR fragments were purified using the Minelute Gel Extraction kit (Qiagen) and sent for sequencing to GATC Biotech.

3. Results

a/ Germline transformation of *Ae. albopictus* with the *piggyBac* transposable element

Five independent transgenic lines were established using the *attP*-containing *piggyBac* construct OX3860 (transgenic lines OX3860A, B, C, D and F). Approximately 6000 eggs were injected and approximately 1500 larvae hatched. This corresponds to 25% survival post-injection, which is comparable to the 20.5% and 23.3% survival obtained in *Ae. aegypti* by

Kokoza *et al.* (Kokoza et al., 2001) and Nimmo *et al.* (Nimmo et al., 2006), respectively. In total, approximately 250 males survived to be crossed with wild-type females in pools of four males for 24 hours before being merged into seven pools, and 300 females were crossed with wild-type males in three pools. In preliminary experiments this seemed to give more reliable production of G_1, though there was also a risk that the G_1 embryos from such pools may not represent all fertile G_0 parents. The five transgenic lines originated from two G_0 male pools and two G_0 female pools. The B and C lines originated from the same male G_0 pool but were easily distinguished by fluorescence phenotype. Mendelian inheritance data are provided in Table 2-1.

Inverse PCR analysis based on their different fluorescent phenotypes of insertions showed that all the lines were independent insertions and showed the typical targeting and duplication of a TTAA sequence by the *piggyBac* element (Table 2-2). These sequences could not be used to directly locate the insertions, as the genome of *Ae. albopictus* has not been sequenced. Inverse PCR results failed to identify a second insertion in the OX3860C line, which was discovered after second-phase insertion (see ΦC31 intergration results below).

Line	OX3860A	OX3860B	OX3860C	OX3860D	OX3860F
WT	264	664	976	7	297
Transgenic	288	583	943	10	327
P value (X^2)	0.307	0.0218	0.4513	0.4669	0.2298

Table 2-1. Mendelian inheritance of the transgene in OX3860 lines.

For each insertion line except OX36860B, the progeny of hemizygotes crossed to wild-type showed Mendelian inheritance of the transgene (not significantly different from 50:50 transgenic versus wild-type ratio). This is consistent with each line carrying a single insertion. For line B, the lower proportion of transgenic progeny may indicate a fitness cost associated with the transgene. Those observations were made on G_2 (line D, hence the small numbers), G_3 (lines A, B, C) or G_6 (line F) generations. Note that other data subsequently showed that OX3860C in fact carries two closely linked transgene insertions.

Strain	5´ Flanking sequence		3´ Flanking sequence
OX3860A	n.d.	TTAA	TCAACTCAACGTACATATGTA
OX3860B	GCGCACAAGCTTAGAGGTACT	TTAA	TCCAAGCAGACAACCGAAATG
OX3860C	CCTGACGTGACTAGATAACCC	TTAA	GGAATGAGTAACTCTTGGTAG
OX3860D	TTTACTAACACAAAATTAGTA	TTAA	CGTCATTCGTTTTGCAGAAGA
OX3860F	CTTCCATGTAGATTGTTTCGT	TTAA	ACGTCCGTGAAATAGTATCGC

Table 2-2. Flanking sequences of integration sites of OX3860 into *Ae. albopictus*.

Genomic sequences immediately flanking the *piggyBac* insertions of OX3860 lines were obtained by inverse PCR. All the insertion sites were unique and occurred at a TTAA site, the canonical recognition sequence for the *piggyBac* transposable element. n.d.: not determined. The 5' inverse PCR for the OX3860A line was not successful but the 3' flanking sequence is sufficient to prove the independence of the A insertion. Full flanking sequences are provided in Appendix 1.

The 3xP3-ECFP marker (Horn and Wimmer, 2000) showed the expected fluorescence in the larval eyes in lines OX3860B, C, D and F. Line OX3860B also showed strong expression in the anal papillae of larvae, which has been previously observed in *Ae. aegypti* (Nimmo et al., 2006). Line OX3860A exhibited an unusual expression pattern, with variable fluorescence intensity between individuals, and between the two eyes of an individual, but PCR analysis confirmed that they all carried the same integration event (data not shown). In addition, progeny from larvae showing weak fluorescence in one eye included individuals with intense fluorescence in both eyes. The variation in fluorescence observed in individuals from the OX3860A line is likely due to unusually strong position effects of adjacent genomic elements, or position effect variegation (Wilson et al., 1990).

The OX3860F insertion was linked to the male-determining locus, the two loci being approximately 8.5 centiMorgans (cM) apart (Table 2-3).

Males	322 (52%)	Fluorescent	298 (93%)
		Wild Type	24 (7%)
Females	302 (48%)	Fluorescent	29 (10%)
		Wild Type	273 (90%)

Table 2-3. The OX3860F insertion is linked to the male-determining locus.

The progeny from a cross between hemizygous OX3860F males (G_6) and wild-type females shows that transgene transmission is highly skewed towards male progeny (93% of male progeny expressed the marker, versus only 10% of the female progeny, n=624). The sex-ratio, however, is normal, indicating a male-linked insertion rather than female lethality. Non-parental phenotype was observed in 8.5% of the progeny, indicating a distance of 8.5 centiMorgans (cM) between the insertion and the male-determining locus.

Transformation efficiency is usually defined as the proportion of fertile (G_0) injection survivors giving at least one transformed (G_1) progeny. In preliminary studies, difficulties had been encountered in getting females to feed and lay when kept individually, so G_0 females were pooled; it is therefore not possible to determine the fertility rate post-injection or to calculate precisely the transformation efficiency. The transformation efficiency was at least 1% in these experiments (six independent insertions from 550 G_0 adults). If we assume that the fertility rate of G_0 adults is similar to *Ae. aegypti* then we can estimate that the transformation efficiency was 2-3%. For comparison, the range of efficiency of *Ae. aegypti* transformation is between 4-11% (Kokoza et al., 2001; Lobo et al., 2002; Nimmo et al., 2006).

b/ ΦC31-mediated site-specific integration

The OX3860 lines carry a ΦC31 *attP* site and therefore allowed us to test the ΦC31 integration system. The OX4105 construct carries a 3xP3-DsRed2 marker and an *attB* site to integrate into attP (Figure 2-1). Embryos from the OX3860A, B and C lines were injected with OX4105 and ΦC31 integrase mRNA. Survivors were mated to wild-type and their progeny was screened for fluorescence. Successful integration was identified as insects expressing DsRed2 in the eyes in addition to the ECFP (cyan) fluorescence. For lines OX3860B and OX3860C, the injected eggs

were derived from a backcross of OX3860[B or C] with wild type and therefore comprised a mixture of hemizygotes (with one copy of the transgene and therefore one *attP* site) and wild-type. For line OX3860A, the injected eggs were derived from a more inbred line and therefore also contained homozygotes. Wild type injection survivors (G_0) – lacking the 3xP3-ECFP marker from the OX3860 construct - were discarded as they lacked an *att*P docking site.

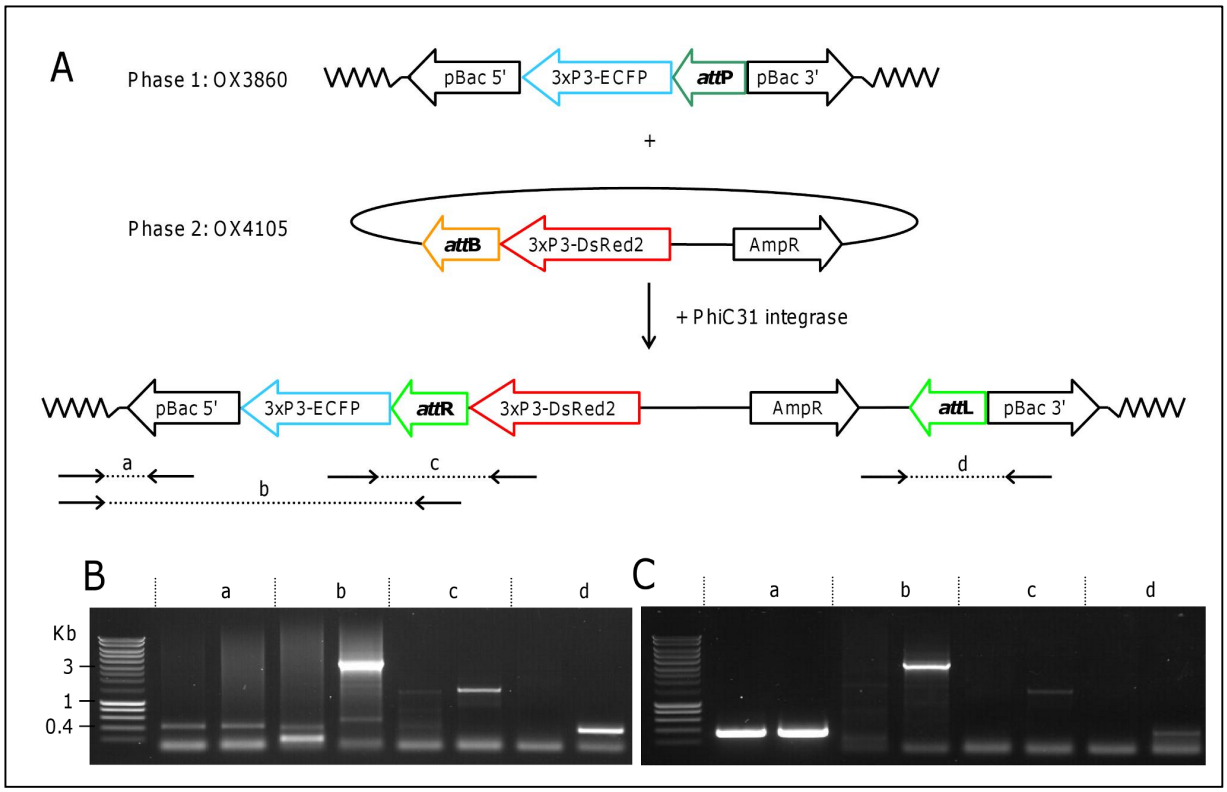

Figure 2-1. ΦC31-mediated site-specific integration of the OX4105 into OX3860 lines of *Ae. albopictus*.

A: The *attP* docking site was inserted into the *Ae. albopictus* genome using the *piggyBac*-based vector OX3860. The OX4105 construct containing an *attB* site was then injected together with mRNA encoding ΦC31 integrase. The expected structure following site-specific integration is represented. The structure of actual insertions was analysed by PCR amplification using primer pairs 'a' (3860B-5'flank1 or 3860C-5'flank1 with PB2; 426bp or 363bp, respectively), 'b' (3860B-5'flank1 or 3860C-5'flank1 with Diag-attBD, approx. 3kb), 'c' (Diag2-ECFP with Diag-DsRed2, 1208bp) and 'd' (pBac-3'R with M13-28-R, 372bp).

B, C: PCR amplifications using primer pairs 'a', 'b', 'c' and 'd' on gDNA from lines OX3860B and OX3860C, respectively. For each primer pair, the left and right lanes correspond, respectively, to gDNA before and after the insertion of the OX4105 construct. In each case the band sizes after insertion correspond to those expected from canonical insertion events as illustrated in panel A. Representative bands were sequenced; these data confirmed that the insertions had the expected structure (data not shown). Equivalent results were obtained for insertion of the OX4105 construct into line OX3860A. The size marker is Smartladder (Eurogentech, Southampton, UK).

For the OX3860A line, 604 eggs were injected, out of which 36 survived to pupae (6%), all of which expressed the cyan fluorescence in the eyes and therefore carried at least one copy of OX3860A. Twelve G_0 males were crossed in one cage with wild-type females and 20 G_0 females in a cage with wild-type males. The male cage produced 72 transgenic larvae out of approximately 1100 larvae screened. The G_0 females gave no transgenic progeny. This corresponds to a minimum transformation efficiency of 3.125% if all of the G_0 were fertile, 6.25% if half were sterile. This assumes that the 72 transgenic larvae are all derived from a single transformation event and G_0 parent. This may well be an underestimate, however, since all the integration events occur into the same docking site, independent events within one pool cannot be distinguished.

For the OX3860B line, 2052 eggs were injected, of which 303 survived to pupa (15%). Of these, 154 were wild-type and discarded, since they did not carry an *attP* site. Thirty-six G_0 males were allowed to mate with wild-type females in three pools of 12 males each, and 50 G_0 females were crossed together with wild-type males in a single pool. One male cross produced one male and three female transgenic offspring which were reared separately. Only one of the transgenic females gave progeny, starting the line OX4105[3860B]. The minimum calculated integration efficiency is 1.16%, although assuming 50% sterility the estimated efficiency is 2.32%.

For OX3860C, 2165 eggs were injected, out of which 477 survived to pupa (22.0%). Of these, 244 were wild-type and discarded. G_0 adults were crossed in pools: 71 G_0 males were crossed in cages of 23, 36 and 12 males with wild-type females, and 90 G_0 females were crossed in cages of 50 and 40 females with wild-type males. High numbers (>20) of second-phase transgenic G_1 individuals were found from the first two male G_0 cages. Within the fluorescent G_1 individuals, two second-phase fluorescence patterns were observed: individuals with bright red eyes and bright blue eyes (found in the progeny of one G_0 pool only, named line 1), and individuals with bright red eyes and weakly fluorescent blue eyes (found in the progeny from both positive male G_0 cages, named lines 2 and 3). Analysis of line 1's progeny (G_2) showed 198 larvae with bright blue and bright red eyes, 220 wild-type larvae, eight with blue eyes only (named line OX3860C1) and 11 with bright red and weakly blue eyes (named line 4). PCR analysis of

genomic DNA from line 1 showed positive amplification of both an empty *attP* site from the OX3860 construct and the *attL* and *attR* junctions characteristic of a site-specific integration into *attP*. Those results led to the conclusion that the OX3860C parent from line 1 had two linked *attP* sites: one that integrated the OX4105 construct and one that stayed free. The linked sites separated in some of the G_2 individuals, giving OX3860C1 (with blue eyes only, *attP* site without integration) and line 4 (red eyes due to the insertion of the OX4105 construct, and weaker blue eyes due to the loss of the C1 insertion). Nineteen progeny with a non-parental phenotype out of 437 indicates a distance of 4.35cM between the two *attP* sites. Further PCR analysis showed that lines 1, 2, and 3 inserted the OX4105 construct into the same one *attP* site for which the flanking sequence was originally found, and are therefore equivalent insertions (data not shown). Lines 1 and 3 come from the same G_0 pool and have the same site-specific integration event: they may come from the same parent. We therefore have evidence of only two independent events, giving a minimum estimated transformation efficiency of 1.24% if all the G_0 were fertile.

In all OX3860 lines, the site-specific integration of 3xP3-DsRed2 showed the same expression pattern as the 3xP3-ECFP of the parental line: variable intensities in OX4105[3860A] individuals, strong anal papillae expression in OX4105[3860B] larvae, eye-only expression in OX4105[3860C] individuals. This is consistent with the two markers being exposed to the same positional effects and was also observed in site-specific integration in *Ae. aegypti* (Nimmo et al., 2006). However the integration of the OX4105 construct appears to have weakened the expression of the 3xP3-ECFP marker in all the lines (Figure 2-2). This may perhaps be due to a titration of the transcription factors by the addition of a second 3xP3 promoter nearby, or to mechanical interference such as promoter occlusion (Adhya and Gottesman, 1982) or dislodgement of a translation-initiation complex by a RNA polymerase transcribing from an upstream promoter (Callen et al., 2004). Mechanical interference would imply imperfect function of the SV40 terminator, which has previously been observed in insect cells (Van Oers et al., 1999).

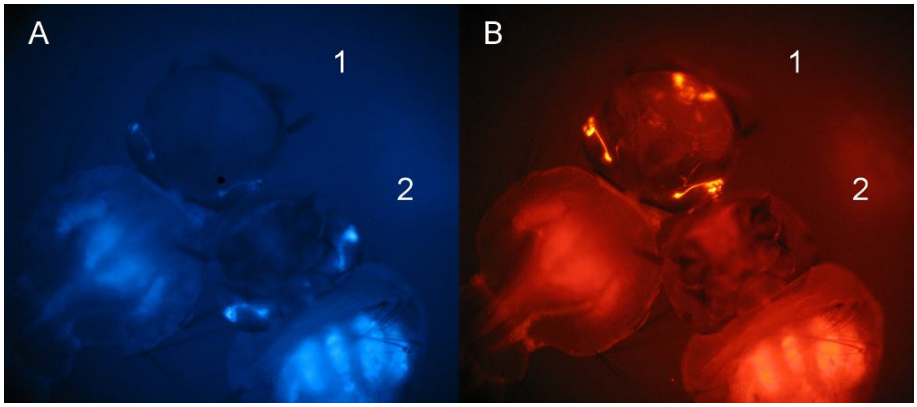

Figure 2-2. Phenotype of transgenic *Ae. albopictus* OX3860B larvae, with and without ΦC31-mediated site-specific integration of the OX4105 construct.

Fluorescence micrographs of two transgenic larvae are shown illustrating the cyan (A) and red (B) fluorescence profiles of each genotype. Larva 1 is OX4105[3860B]; larva 2 is OX3860B. OX3860B larvae carry only the 3xP3-ECFP marker. OX4105[3860B] individuals have both 3xP3-ECFP and 3xP3-DsRed2, giving cyan and red fluorescent eyes. The integration of the OX4105 construct into OX3860 lines appears to reduce the expression of the OX3860 marker (panel A, compare cyan expression of these larvae, each carrying the same 3xP3-ECFP marker). This effect was seen with OX4105 integrations into each of the OX3680 docking sites (data not shown).

PCR characterisation of all three OX4105[3860] lines (Figure 2-1) confirmed the insertion of the OX4105 construct into the *attP* sites from the OX3860 construct, with a canonical *attP*-*attB* recombination verified by sequencing of the *attR* and *attL* PCR fragments.

4. Discussion

This chapter presents the first germline transformation of *Ae. albopictus*. This used a *piggyBac* transposable element, with an estimated transformation efficiency of 2-3%, assuming a post-injection fertility rate similar to *Ae. aegypti* (Nimmo et al., 2006).

Ae. albopictus has proved particularly difficult to suppress using conventional control methods and hopes reside in new technologies. The development of genetically modified strains has the potential to improve the efficiency of the Sterile Insect Technique (Chapter 1/ 4.) by the engineering of transgenic strains carrying a genetic marker that would help monitoring the

program in the field (Chapter 1/ 5.c.), a genetic-sexing system to allow automatic elimination of females before releasing the males (Papathanos et al., 2009), or a RIDL construct inducing conditional lethality without the need for irradiation (Chapter 1/ 7.). Population replacement is another proposed control strategy that could be envisaged (Chapter 1/ 8.c.); population suppression would however have the advantage of controlling both dengue and chikungunya, while reducing the biting nuisance at the same time.

Site-specific integration is an interesting tool allowing direct comparison of two or more transgenes in a particular genomic environment. Out of the five lines produced using the OX3860 construct, three different fluorescence patterns were observed, highlighting the importance of position effects in transgene regulation. Three different lines were successfully transformed by site-specific integration using the ΦC31 integrase and a donor plasmid carrying an *attB* site. The expression profile of the "phase 2" (site-specifically inserted) marker is similar to that of the corresponding "phase 1" marker, indicating that those two elements, separated by 1922 bp, are subjected to similar influence from the surrounding genomic elements. Site-specific integration occurred successfully in only one of the two available *attP* sites from the OX3860C line, albeit with only a small number of independent events detected. This may indicate that the genomic position of the docking site affects the efficiency of ΦC31-mediated integration, as previously observed in *Drosophila* (Bischof et al., 2007). The estimated transformation efficiency with ΦC31 was between 2.3 and 6.3%, depending on the lines. The higher transformation efficiency observed in the OX3860A background is possibly due to the presence of homozygous OX3860A individuals among the population of injected eggs; these have two copies of the *attP* site which may lead to a higher integration frequency for the OX4105 transgene.

Ae. albopictus is quickly growing into a major public health threat throughout the world and consequently the subject of numerous research programs. Genetic transformation and engineering is a key step towards studying and controlling this species using novel molecular techniques and genetic control strategies. The next chapter of this thesis presents the application of the RIDL technology to *Ae. albopictus*.

Chapter 3

Engineering female-specific RIDL strains of *Aedes albopictus*

1. Introduction

After proving that germline transformation could be achieved in *Aedes albopictus* (Chapter 2), the next step was to use this tool towards developing a RIDL strain for genetic control of the species.

Recently, a genetic sexing strain of *Ae. aegypti* has been produced, based on the RIDL technology (Fu et al., 2010). The sex specificity was provided by the female-specific properties of the *Ae. aegypti Actin-4* (*AeAct-4*) gene (Muñoz et al., 2004). *AeAct-4* is expressed in the indirect flight muscles of females, which start developing in late L4 larval stage. The promoter and sex-specific alternative splicing of this gene have been exploited to drive the expression of the tetracycline-repressible transactivator tTA protein, which comprises the tetO-binding tetracycline repressor (tetR) fused to the VP16 transcription activator. The construct also carried a separate VP16 peptide under the control of tetO and the hsp70 minimal promoter. In order to ensure female-specific expression of the tTA, extra stop codons and a frame-shift have been engineered in the male transcripts. In the females' indirect flight muscles, the *AeAct-4* promoter was expected to induce the expression of tTA, which would bind the tetO sequence and enhance the expression of the VP16 peptide. Excessive levels of transcription activator accumulating in cells have a toxic effect, possibly due to transcriptional squelching (Berger et al., 1990; Gill and Ptashne, 1988). In *Ae. aegypti*, this construct was not lethal but rather induced a female flightless phenotype, indicating that the indirect flight muscles are a non-essential and isolated tissue, as previously observed in *Drosophila* (Fernandes et al., 1991; Raghavan et al., 2000). Tetracycline, by preventing tTA from binding the tetO sequence, prevents further production of VP16 and is therefore an antidote to the system: transgenic females reared in tetracycline-supplemented water as larvae were able to fly as adults, allowing the maintenance of the RIDL strain. Males carrying this construct were able to fly as adults even in the absence of tetracycline, confirming that the *AeAct-4* minigene is indeed female-specific (Fu et al., 2010).

This phenotype allows separation of males from females and could be useful in a mass-rearing situation aimed at releasing males only; as mentioned in Chapter 1-4a for the Sterile Insect

Technique, releasing males alone has proven more efficient than both sex releases, and manual sex separation is both time-consuming and damaging. After mating with wild females, all the progeny of the RIDL males released in a control program would carry the RIDL construct and therefore all their female progeny would be flightless. However, both the male and female progeny of transgenic-to-wild matings would survive to adulthood and would therefore provide the extra benefit of larval competition discussed in Chapter 1-7a. Moreover, hemizygous RIDL males would be able to inseminate wild-type females and transmit the RIDL construct to half of their progeny, making half of their female progeny flightless. In the field, flightless females would find great difficulty finding a feeding source or a host, and would be easily predated upon. Considering these factors, the possibility of flightless females transmitting diseases from one human host to another can be considered highly unlikely.

In fact, the properties of such a strain would allow the release of pupae or adults without the need for prior sex-separation, as long as the released generation was reared without tetracycline. One could even envisage the release of eggs, although laboratory rearing offers better control upon the larval density and food regime, both critical factors in the production of large competitive males.

This chapter presents the creation of female-specific RIDL strains of *Ae. albopictus* based on the construct used in *Ae. aegypti* (Fu et al., 2010). The *Ae. albopictus Actin-4* gene was also characterised and used in place of the *Ae. aegypti Actin-4* in a new construct, for which transgenic lines were obtained in both *Ae. aegypti* and *Ae. albopictus* embryos.

2. Materials and Methods

a/ Isolation of the *Ae. albopictus Actin-4* gene*

Ae. aegypti Actin-4 (*AeAct-4*, AY531222), *Ae. aegypti Actin-3* (*AeAct-3*, AY289765) and *Anopheles gambiae Actin-1* (*AnAct-1*, XM315270; *Actin-4* equivalent) sequences were aligned

* This work was done by Sarah Scaife, PhD (Oxitec Ltd)

using ClustalW (EBI). Primers AeA4F and AeA4R were designed in regions that were conserved between *Actin-4* and *AnAct-1* but different from *AeAct-3*. The resulting PCR product was cloned and sequenced. BLAST alignment confirmed homology to *Ae. aegypti Actin-4*. This sequence was extended by a combination of 5'RACE and PCR techniques. 5'RACE was carried out using the Ambion FirstChoice RLM-RACE kit according to the manufacturer's instructions, using primers AlbA4Race and AlbA4RaceN. Vectorette PCR was carried out to extend the sequence from the beginning of the 5'UTR back into the promoter region and from the exon 1 and 2 sequences to fill in the intron sequence. Comparison of cDNA and genomic DNA sequences revealed a large intron in the 5'UTR. 745 bp upstream from the start of the 5'UTR, a coding sequence with BLAST homology to *Ae. aegypti* sensory neuron membrane protein 2 was found, indicating that the maximum promoter fragment had been determined.

b/ Plasmid construction

The OX3688 construct is identical to the OX3604 plasmid (Fu et al., 2010), apart from one of the end markers which was changed from 3xP3-AmCyan into 3xP3-DsRed2 by exchanging a PacI-SpeI cassette (OX3604 carried 3xP3-AmCyan at both ends, by mistake). The markers are 3xP3-DsRed2 and 3xP3-AmCyan for the *piggyBac* ends, and hr5IE1-DsRed2 for the central section.

OX4358 construct[*]: a start codon and Kozak sequence CCACCATG were engineered in the *AealbAct-4* gene's 5' UTR 43 bp before the 5' donor site of the intron by PCR. Two PCR products, promoter-intron and intron-truncated exon 2, were amplified from wild-type *Ae. albopictus* genomic DNA using primer pairs AlbA4proAscF-AlbA4intSpeR and AlbA4intSpeF-AlbA4ex2BglR. The two PCR products were ligated at the SpeI site; the ligated product was cloned in front of the fusion gene ubiquitin–tTAV2–K10 3'UTR which was previously constructed (Fu et al., 2010). The engineered start codon was in frame with the fusion tTAV2 gene. This gene cassette was inserted into a *piggyBac* vector containing Hr5IE1 promoter (Rodems and Friesen, 1993) driving AmCyan (Clontech) expression. tTAV2 is a variant of tTA,

[*] This construct was created by Sarah Scaife, PhD (Oxitec Ltd)

optimised for expression in *Drosophila* by eliminating potential cryptic splice sites and rare codons in the tetR region (Fu et al., 2010).

Table 3-1. Primer sequences and expected product sizes.

F primer name	F primer sequence	R primer name	R primer sequence	Product size
AeA4F	GTGTGACGATGATGCTGGAGCACTAG	AeA4R	CTGGGTACATGGTGGTACCACCAGAC	928 bp
AlbA4Race	GGGATTCAGTGGAGCTTCGGTCAGCAGG			
AlbA4RaceN	TGGATTGGGCTTCGTCACCAACGTAG			
AlbA4proAscF	GGTGTGGGCGCGCCTGATCGGTAAGGTAAGTAAGCATCCGAG	AlbA4intSpeR	GGTGTGACTAGTGTTGACAACTCTTCTAGTTTCTCGGCC	1581 bp
AlbA4intSpeF	GGTGTGACTAGTCCAAAATGAACGTGGACCAGCC	AlbA4ex2BglR	GGTGTGAGATCTGCATGTCGTCACACATTTTGGCGCCGCTTCCAGGTCCGTTGGGTCC	578 bp
AlbA4UTRF	GATTAGTCAAGGACCCAACGGCTC	AlbA4FlR	CACACCCTGGTGACGTGGGC	290, 209, 1749 bp
AlbA4BsmF	GGTGTGCGTCTCACCACCATGTTAGTCAAGGACCCAACGGCTCAAG	UbiR2	CATACCACCGCGCAGGCG	584, 352, 1541 bp
Diag2-ubi	GGATGCCCTCCTTGTCCTGG	Aeact4-ex1	CAATCGGATTTTGACGCTCGCT	495, 252, 2051 bp
Diag2-ubi	GGATGCCCTCCTTGTCCTGG	Aeact4-ex1'	CATGGAAACCGAGGATAACGACGA	446, 203, 2002 bp

c/ Strain background, rearing and transformation

The *Ae. albopictus* and *Ae. aegypti* wild-type strains originate from Malaysia and were colonised by the Institute of Medical Research (Kuala Lumpur) in 2006 and 1977, respectively. The insectary was kept at 27°C (± 1°C) and 70% (± 10%) relative humidity. Larvae were fed on crushed dry fish food (TetraMin® flake food from Tetra GmbH, Germany) and adults on 10% glucose with 14 U/ml penicillin and 14 µg/ml streptomycin. Females were fed on horse blood using a *Hemotek* Insect Feeding System (Discovery Workshops, Accrington, UK) set at 37°C.

Wild-type embryos were injected with a mixture of the OX3688 or OX4358 construct DNA (300 ng/µl), phsp-Bac plasmid helper (200 ng/µl) (Handler and Harrell, 1999) and *piggyBac* mRNA (300 ng/µl) in injection buffer (5mM KCl and 0.1 mM NaH_2PO_4, pH 6.8), as described in Chapter

2-2c. Tetracycline (30µg/ml) was added to the injection mix in order to repress the activation of VP16 expression in injected embryos. Injected G_0 adults were crossed in pools (males in pools of two for 24 hours then merged into pools of 24; females in pools of 100) to wild-type counterparts. G_1 larvae were screened for fluorescence using a Leica (Wetzlar, Germany) MZ95 microscope with the appropriate filter sets from Chroma Technology (Rockingham, VT, USA) (filters: AmCyan: exciter D436/20x; emitter D480/40m; DsRed2: exciter HQ545/30x; emitter HQ620/60m). Transgenic lines were established from single G_1 positive adults and reared on water supplemented with 30 µg/ml chlortetracycline hydrochloride (Sigma-Aldrich, Gillingham, UK). Lines named with different letters have founders from different G_0 pools and are therefore independent genomic integrations.

Pictures of fluorescent larvae were taken with Canon PowerShot S5IS with a MM99 adaptor (Martin microscopes, Easley, SC, USA) to fit into the eyepiece.

d/ Characterisation of transformation events

PCR was performed on G_1 individuals expressing both peripheral markers but not the central one, in order to determine whether the two peripheral elements had inserted as one fragment linked by the plasmid backbone or independently as two separate elements. Presence of the plasmid backbone was investigated with primers Amp-r (CAGTGGAACGAAAACTCACG) and Amp-f (ACGTCAGGTGGCACTTTTCG), and primers M13-28-R (TGTGAGCGGATAACAATTTCACACAGGA) and Diag-transp (CTACCGCTTGACGTTGGCTGCAC). Amplification of sequence from both peripheral markers was performed as positive controls: the 3xP3-AmCyan marker was amplified with primers Su-amcyan-f (CCAGACCTCCACCTTCAAGGTGACC) and Diag-cyan (CGCCGTGGAAGGTGGACT TGTGC), and the 3xP3-DsRed2 marker was amplified with primers Diag2-3xP3 (AGCTGAACAAGCTAAACAATCGG) and Diag7-DsRed (CCATGGTCTTCTTCTGCATCAC). PCR was also performed using primers specific to the central part of the construct, tTAV+365seq- (CGTAGAGAGCATTTTCCAGGCTGAAG) and Diag-ubiq (CGAGCGATACCATCGAGAAC), in case this fragment was present but not expressing the hr5IE1-DsRed2 marker.

e/ Reverse Transcription-PCR

In order to study the endogenous *Actin-4* gene from *Ae. albopictus*, RNA was extracted from pooled samples of 3 wild-type male pupae and 2 wild-type female pupae, using Tri Reagent (Ambion, Austin, TX, USA), according to the manufacturer's instructions. RNA samples were treated with DNase I (Roche, Burgess Hill, UK) and quantified on a Pharmacia Biotech (Little Chalfont, UK) GeneQuant II RNA/DNA calculator. One-step RT-PCR was carried out on 200 ng RNA using SuperScript® III One-step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen, Paisley, UK) and primers in the 5'UTR (AlbA4UTRF) and in exon 2 (AlbA4FlR) (Table 3-1).

Reverse Transcription-PCR was carried out on male and female pupae of OX3688 and OX4358 individuals as above, using primers Aeact4-ex1, Aeact4-ex1' and Diag2-ubi for OX3688, and AlbA4BsmF and UbiR2 for OX4358 (Table 3-1), to confirm that sex-specific splicing was occurring as predicted in this context.

Amplified fragments were loaded on a 1% agarose gel and extracted using the MinElute PCR Purification Kit from Qiagen (Hilden, Germany). Purified products were then cloned into pJet vectors (GeneJET™ PCR Cloning Kit from Fermentas, Vilnius, Lithuania) and transformed into XL-10 cells (Stratagene, La Jolla, CA, USA). DNA from positive clones was purified by miniprep (GeneJET Plasmid Miniprep Kit from Fermentas) and sent for sequencing (GATC Biotech, Konstanz, Germany).

f/ Phenotype analysis

For phenotypic analysis of the transgenic lines, eggs were hatched on day 1. On day 2, "on tet" and "off tet" trays (11 x 19 cm bottom surface) were set up with 300 hemizygous larvae in 300 ml of pure water (1 larva/ml), respectively with or without a supplement of 30 µg/ml chlortetracycline hydrochloride (Sigma-Aldrich, Gillingham, UK). Water on trays was changed on days 6 and 11. Larvae were fed crushed dry fish food (TetraMin® flake food from Tetra GmbH,

Melle, Germany): 12 mg/tray on days 2, 3 and 17; 24 mg/tray on days 4, 9, 11 and 12; 48 mg/tray on day 5; 96 mg/tray on days 6, 7 and 8.

Sexes were separated as pupae, and the number of flying males and females was recorded 24 h after emergence.

3. Results

a/ The OX3688[albopictus] strains

Ae. albopictus embryos were injected with the OX3688 construct (Figure 3-1), similar to the OX3604 construct used by Fu, Lees *et al.* to induce a conditional female-flightless phenotype in *Ae. aegypti* (Fu et al., 2010). Out of the 9468 embryos injected, 1483 survived to pupa stage (15.7%). A total of 1130 adults were crossed (11.9% of injected embryos, 530 males and 600 females).

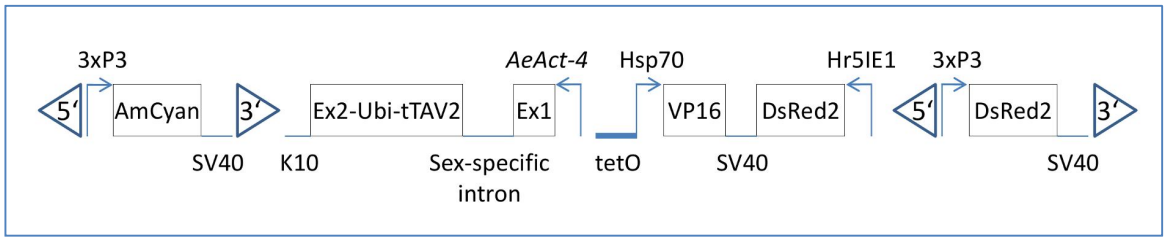

Figure 3-1. Map of the OX3688 transgene.

This is a four-ended *piggyBac* transgene including three different markers: 3xP3-DsRed2 and 3xP3-AmCyan in the *piggyBac* ends, hr5IE1-DsRed2 in the middle part of the transgene. The tTAV2 transactivator is placed under the control of the Ae. aegypti Actin-4 promoter and is therefore expected to be expressed specifically in females' flight muscles. In the absence of tetracycline, tTAV2 is able to bind tetO, thus activating further expression of the VP16 peptide which is a cell toxin. Females carrying this transgene are expected to be flightless in the absence of tetracycline.

As OX3688 is a four-ended *piggyBac* construct (Dafa'alla et al., 2006), four different *piggyBac* elements could be integrated: the 5'-end short element, carrying only the 3xP3-AmCyan marker; the 3'-end short element, carrying only the 3xP3-DsRed2 marker; the two end

elements linked together by the plasmid backbone, characterised by the presence of both the 3xP3-AmCyan and 3xP3-DsRed2 markers; and the intended OX3688 transgene carrying all three markers (Figure 3-1). Transgenic G_1 progeny were found from 5 out of 12 male G_0 crosses, with all the possible integrations listed above being observed and identified by fluorescence phenotype, except for the individuals expressing both side markers. For these, PCR analysis helped to determine whether the two side elements had inserted as one fragment linked by the plasmid backbone or independently as two separate elements (see Materials and Methods):

Larvae with blue eyes only (3xP3-AmCyan marker) were observed in progeny from G_0 male crosses number 6 and 9; larvae with red eyes only (3xP3-DsRed2 marker) were observed in progeny from G_0 male crosses number 5 and 9.

Larvae with both blue and red eyes were observed in the progeny from G0 male crosses number 1, 5, 9 and 10, and PCR analysis on those larvae showed that larvae from G_0 male cross 5 had integrated the two *piggyBac* side elements independently in two separate integration events, whereas larvae from G_0 male crosses 1, 9 and 10 had integrated the two side elements as one fragment linked by the plasmid backbone. PCR on G_1 individuals also confirmed that none of the larvae negative for the hr5IE1-DsRed2 marker was carrying the middle part of the transgene (results not shown).

The central marker (hr5IE1-DsRed2) was never observed on its own but always with the two peripheral markers (3xP3-AmCyan and 3xP3-DsRed2), indicating that none of the peripheral *piggyBac* elements had been remobilised after integration. This phenotype was observed in progeny from G_0 male crosses number 5, 6 and 10.

All the individuals expressing only the peripheral markers were discarded because they did not contain the RIDL component.

G_1 adults expressing all three markers (Figure 3-2) were crossed individually to wild-type insects. Only two males from the G_0 male cage number 5 and two males from the G_0 male cage number 6 gave progeny, respectively starting lines OX3688A, E, C and D. Line C was

discontinued as none of the G_2 survived to adulthood. Line E had flightless females on tetracycline and also did not survive to adulthood in subsequent generations. Line OX3688A carried multiple insertions, indicated by a higher number of fluorescent than wild-type individuals (25 fluorescent and 15 wild-type) in the G_2 generation, as well as several different fluorescent phenotypes (different intensities of red body, with or without red or blue fluorescent anal papillae). Line OX3688D had two insertions recognised by two different intensities of eye fluorescence in G_2 individuals. Insertions were split by separating the different phenotypes and crossing single individuals to wild-type. Line OX3688A was separated into four different lines, out of which only one had healthy females and survived. Out of the D lines, only one made it to the next generation. For simplicity, further mentions of lines OX3688A and D refer to those two surviving lines rather than their multi-insertion parents.

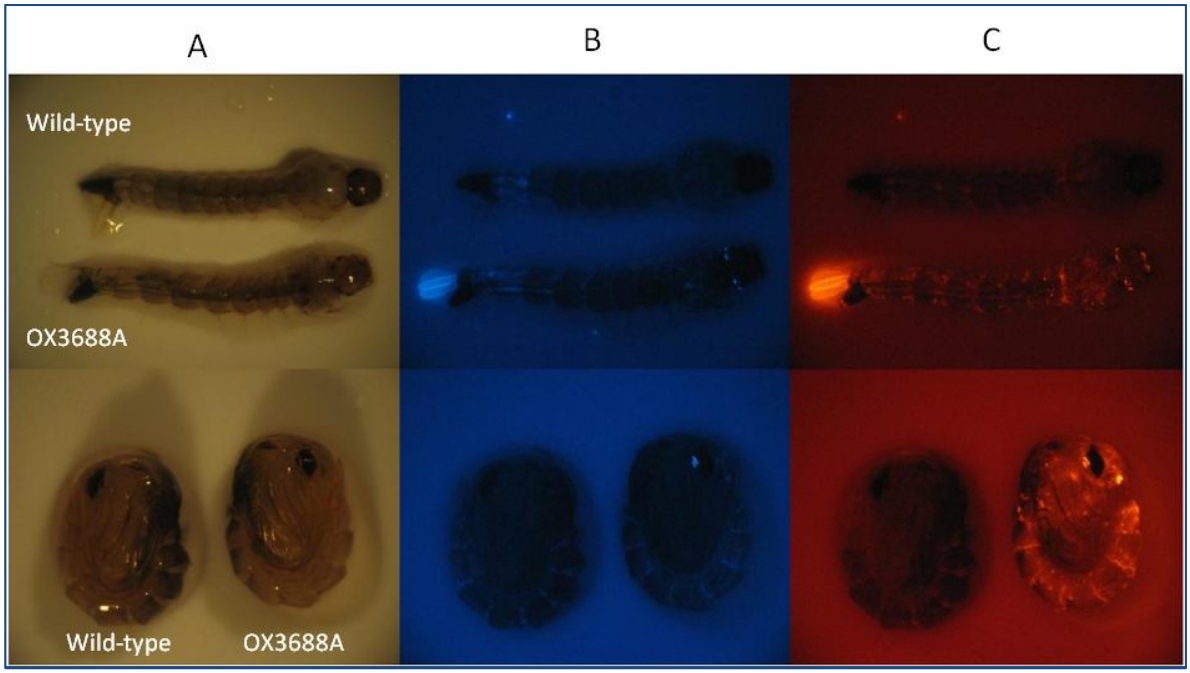

Figure 3-2. OX3688A and WT larvae (top) and pupae (bottom) expressing all three markers: 3xP3-AmCyan (blue eyes and anal papillae), 3xP3-DsRed2 (red eyes and anal papillae) and hr5IE1-DsRed2 (red body).

A: normal light; B: cyan filter; C: red filter.

These results indicate that at least 12 independent integration events (ignoring the multiple integrations) occurred amongst the 530 males crossed. If we assume 30-50% fertility, this corresponds to a transformation efficiency of 4.5-8.6%, which is in the same range as *Ae. aegypti* (Kokoza et al., 2001; Lobo et al., 2002; Nimmo et al., 2006).

b/ Conditional female flightless phenotype in *Ae. albopictus* using the *Ae. aegypti Actin-4* gene

Reverse Transcription-PCR was performed on transgenic OX3688A male and female pupae, showing the expected sex-specific splicing (Figure 3-3). Sequencing of the RT-PCR fragments indicated that the splicing occurred precisely as in *Ae. aegypti* (results not shown).

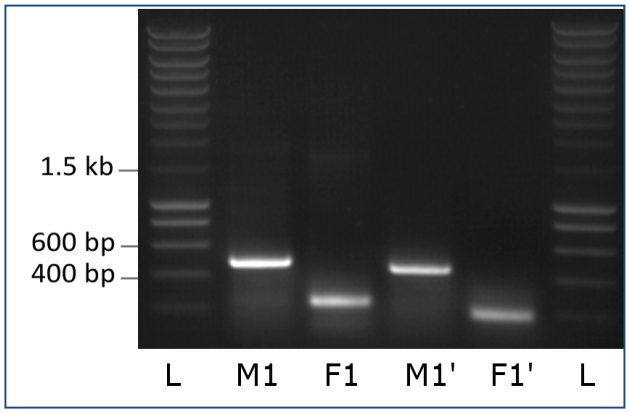

Figure 3-3. RT-PCR on male (M) and female (F) *Ae. albopictus* OX3688A pupae.
Differential sex-specific splicing of the construct is observed. RT-PCR was performed using primer pairs Diag2-ubi and Aeact4-ex1 (M1 and F1) and Diag2-ubi and Aeact4-ex1' (M1' and F1') in ubiquitin and *AeAct-4* exon 1. Ladder (L): Eurogentec's Smartladder. The bands are all of the expected sizes (Table 3-1).

Lines OX3688A and D were reared on and off tetracycline and their flying phenotype assessed:

Line OX3688A showed a repressible female-specific flightless phenotype, with 0% females flying off tetracycline and 61.5% flying on tetracycline, and males flying fine both on and off tetracycline. Line D showed no flightlessness off tetracycline, with 70.2% females flying versus 78.1% on tetracycline (Figure 3-4). The OX3688D line was later discarded, as it did not have the desired phenotype.

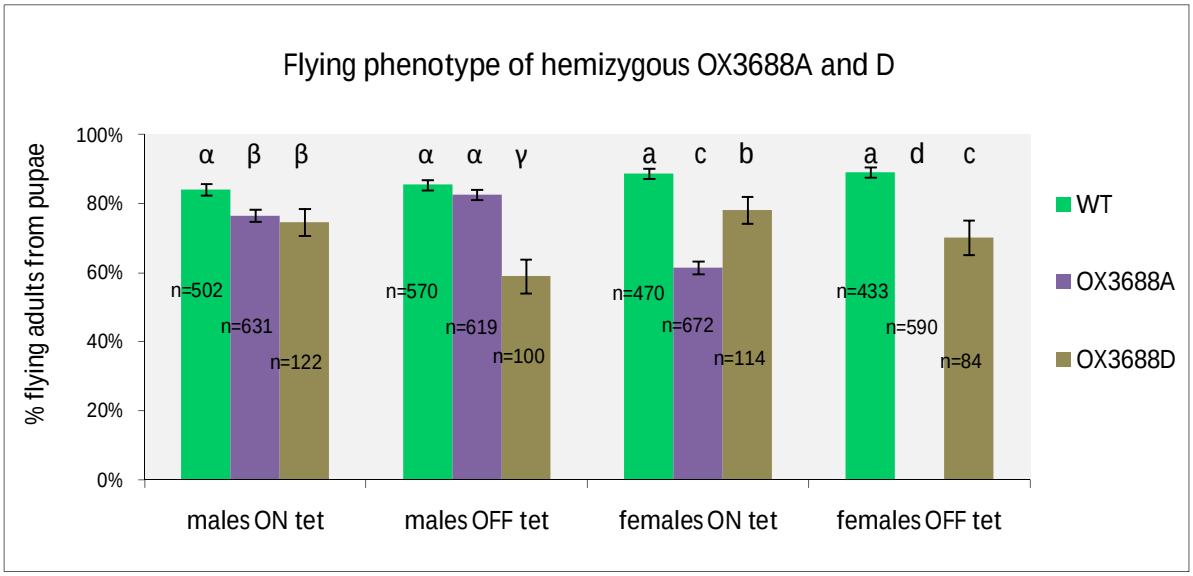

Figure 3-4. Flying ability of transgenic hemizygous OX3688A and D lines of *Ae. albopictus* after being reared with (ON) or without (OFF) a supplement of the tetracycline antidote.

The percentage of flying adults was calculated as a proportion of the number of pupae (n) placed in each cage. OX3688A and D phenotypes were compared to that of same sex wild-type using chi-square tests; significance is indicated above the chart bars: groups with the same letter (Greek letters for the males, Latin letters for the females) are not significantly different from each other at the 5% level.

Fluorescence screening of hemizygous OX3688A in the absence of tetracycline revealed that female pupae OX3688A showed expression of the AmCyan fluorescent protein in the flight muscles, which was not expected as AmCyan is under the control of the eye-specific 3xP3 promoter (Figure 3-5). This observation suggests that in the absence of tetracycline, the binding of tTAV2 on tetO and resulting transcription enhancement may overrule the regulation of nearby elements and induce ectopic expression of the 3xP3-AmCyan cassette.

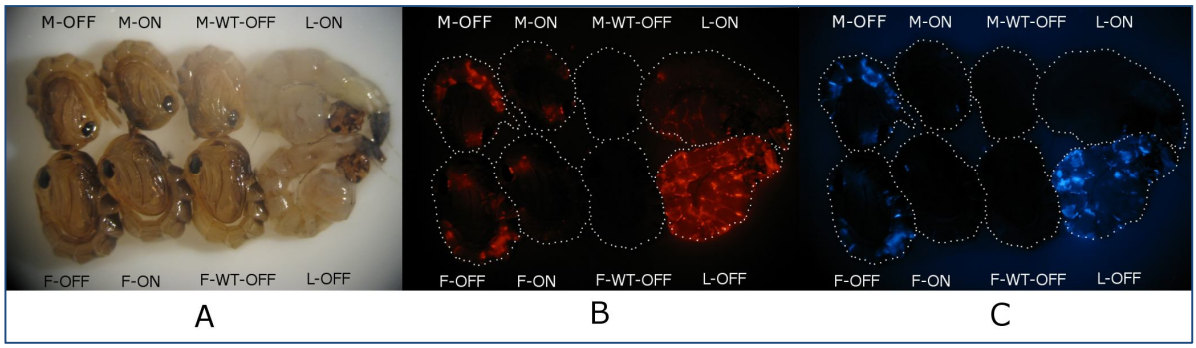

Figure 3-5. OX3688A fluorescent phenotype on and off tetracycline.

A: normal light; B: red filter; C: cyan filter. OX3688A larvae, male and female pupae show brighter expression of the fluorescence markers when reared off tetracycline (L-OFF, M-OFF and F-OFF, respectively) than on tetracycline (L-ON, M-ON and F-ON, respectively). The 3xP3-AmCyan marker is expressed ectopically in the whole body when the strain is reared off tetracycline. Wild-type male and female pupae reared off tetracycline (M-WT-OFF and F-WT-OFF, respectively) are shown for comparison.

c/ Isolation and characterisation of the *Ae. albopictus Actin-4* gene

The *Ae. albopictus Actin-4* gene (*AealbAct-4*) was isolated as described in Materials and Methods. The sequence showed high conservation with *AeAct-4* (and also to *AgAct-1*, results not shown), particularly in the coding sequence (Appendix 2). The positions of the introns are conserved, as is the gene structure with respect to site-specific splicing (Figure 3-6/A). The sex-specific splicing was confirmed by RT-PCR (Figure 3-6/B).

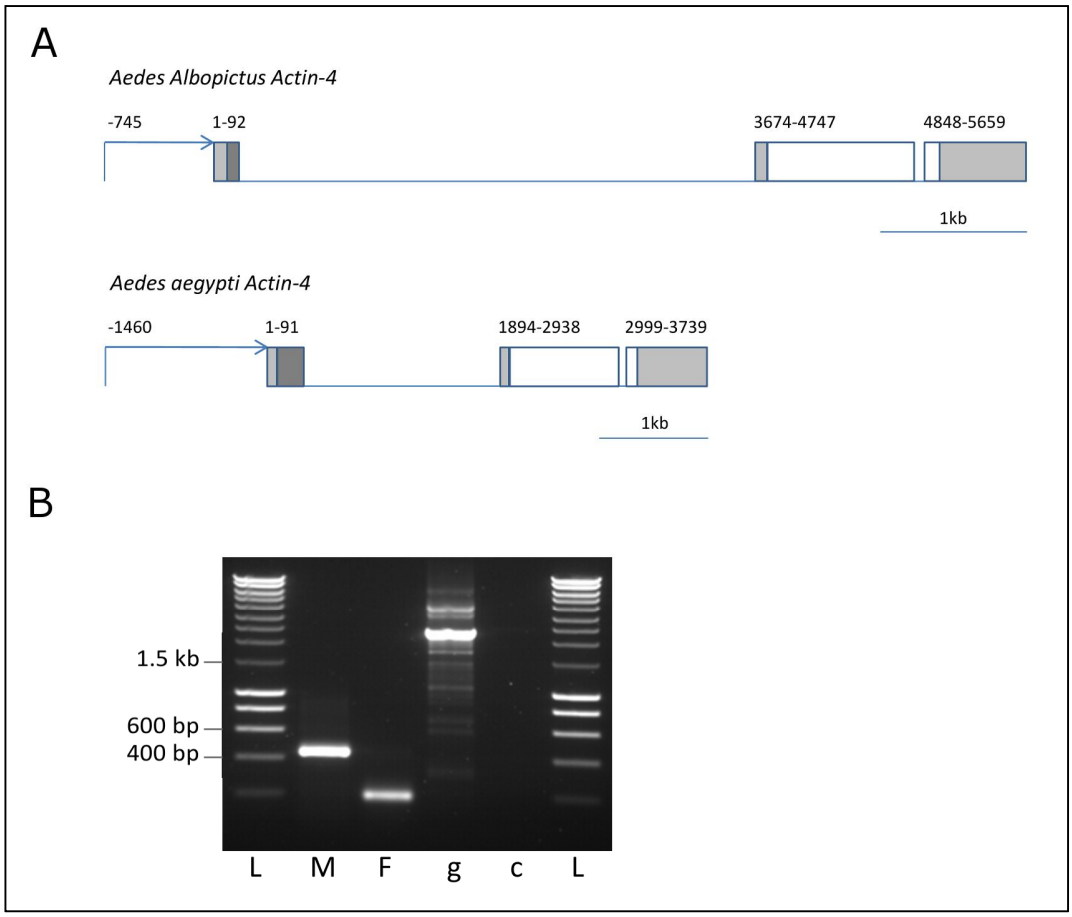

Figure 3-6. Characterisation of the *Ae. albopictus Actin-4* gene.

A: Gene structure of *Ae. albopictus* and *Ae. aegypti Actin-4*. Promoters are indicated by arrows, exons are shown as boxes, introns as lines. Non-coding 5' and 3'UTR are shaded pale grey; the male-specific exons are shaded dark grey. B: RT-PCR confirming differential splicing in male (M) and female (F) *Ae. albopictus* pupae; genomic (g) and no template control (c) are also shown. L: Smartladder (Eurogentec). The bands are all of the expected sizes (Table 3-1).

d/ Conditional female flightlessness in *Ae. aegypti* and *Ae. albopictus* using *Ae. albopictus Actin-4*

The *AealbAct-4* promoter and sex-specific splicing were used to make construct OX4358 (Figure 3-7/A). The OX4358 construct carries a Hr5IE1-AmCyan-SV40 marker cassette leading to expression of the AmCyan fluorescent protein all over the body and allowing easy screening of the transgenics. The *Ae. albopictus Actin-4* (*AealbAct-4*) promoter is placed in front of the *AealbAct-4* exon 1 in which a start codon has been engineered. The *AealbAct-4* sex-specific

intron has been truncated but the male-specific transcript was kept and holds a series of stop codons (Figure 3-7/A, bars below the intron line). The *AealbAct-4* exon 2 was cloned in frame with an ubiquitin-tTAV2-K10 fusion gene. The tetO-Hsp70-VP16-SV40 cassette is activated by the binding of tTAV2 produced in the females' indirect flights muscles (under the control of the *AealbAct-4* promoter) in the absence of tetracycline. Transgenic OX4358 lines were obtained for both *Ae. albopictus* and *Ae. aegypti*.

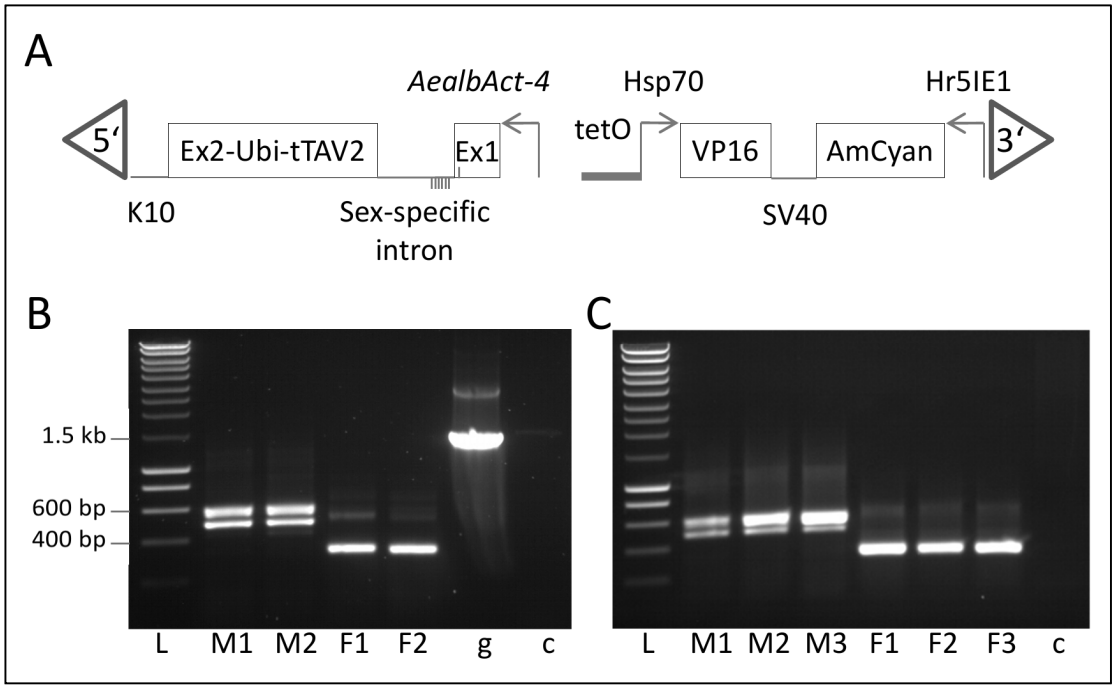

Figure 3-7. The OX4358 construct was inserted into *Ae. albopictus* and *Ae. aegypti*.

A: Map of the OX4358 construct. Promoters are indicated by arrows, exons are shown as boxes, introns as horizontal lines. The engineered start codon is indicated by a bar in Ex1, whilst stop codons in the male exon are shown by bars below the line. B: *Ae. albopictus* OX4358 RT-PCR on two male and two female pupae (M1, M2, F1 and F2, respectively), gDNA amplification and no-template control are shown in lanes "g" and "c" respectively. L: Smartladder (Eurogentech). C: *Ae. aegypti* OX4358 RT-PCR, on three male and three female pupae (M1, M2, M3, F1, F2, and F3 respectively) and no template control (c).

RT-PCR analysis of OX4358 transgenic individuals confirmed sex-specific splicing in both *Ae. albopictus* and *Ae. aegypti* (Figure 3-7/B and /C, respectively). Sequencing of the PCR products revealed that splicing occurs as in the native gene, except for a second male-specific transcript

in which exon 1 has an extra 75 bp. This extra transcript could be a result of truncation of the intron and/or exon 2, disrupting splicing, but as it is still out of frame with the ubi-tTAV2, it does not interfere with the function of the construct. The sequences also confirmed that the ubi-tTAV2 cassette is in frame with the engineered ATG in the female splice but not in the male splice variants, as intended; we therefore expected impairment of the flight muscles of females only.

The flying phenotype of *Ae. aegypti* and *Ae. albopictus* transgenic lines carrying the OX4358 construct was assessed after being reared on and off tetracycline. The *piggyBac*–based OX4358 transgene was inserted randomly and therefore subjected to different positional effects in each transgenic line, leading to a range of phenotypes. Out of the 20 independent lines obtained in *Ae. albopictus*, four exhibited a non-repressible flightless females phenotype - giving flightless females when reared on tetracycline - and five lines were male-linked. Out of the eleven *Ae. albopictus* lines tested on and off tetracycline (Figure 3-8), eight had a repressible female flightless phenotype, with no females flying off tetracycline and between 21.65 and 54.55% of females flying on tetracycline. Three lines showed no specific phenotype, with females able to fly off tetracycline. Ten independent lines were obtained in *Ae. aegypti*, including two male-linked insertion. Out of the eight lines tested on and off tetracycline (Figure 3-8), three had a repressible female flightless phenotype, with no flying females off tetracycline and 57.0 to 94.4% females flying on tetracycline. Four lines did not show a fully penetrant phenotype, with 1.4 to 42.9% females able to fly off tetracycline. The last line showed no specific phenotype, with 92.9% females flying off tetracycline while 83.9% females flying on tetracycline. Males showed no observable detectable impairment of their flying ability when reared off tetracycline, with generally a slightly higher percentage of flying males when reared off tetracycline compared to rearing on tetracycline (Figure 3-8), emphasising the strong female-specificity of the RIDL mechanism.

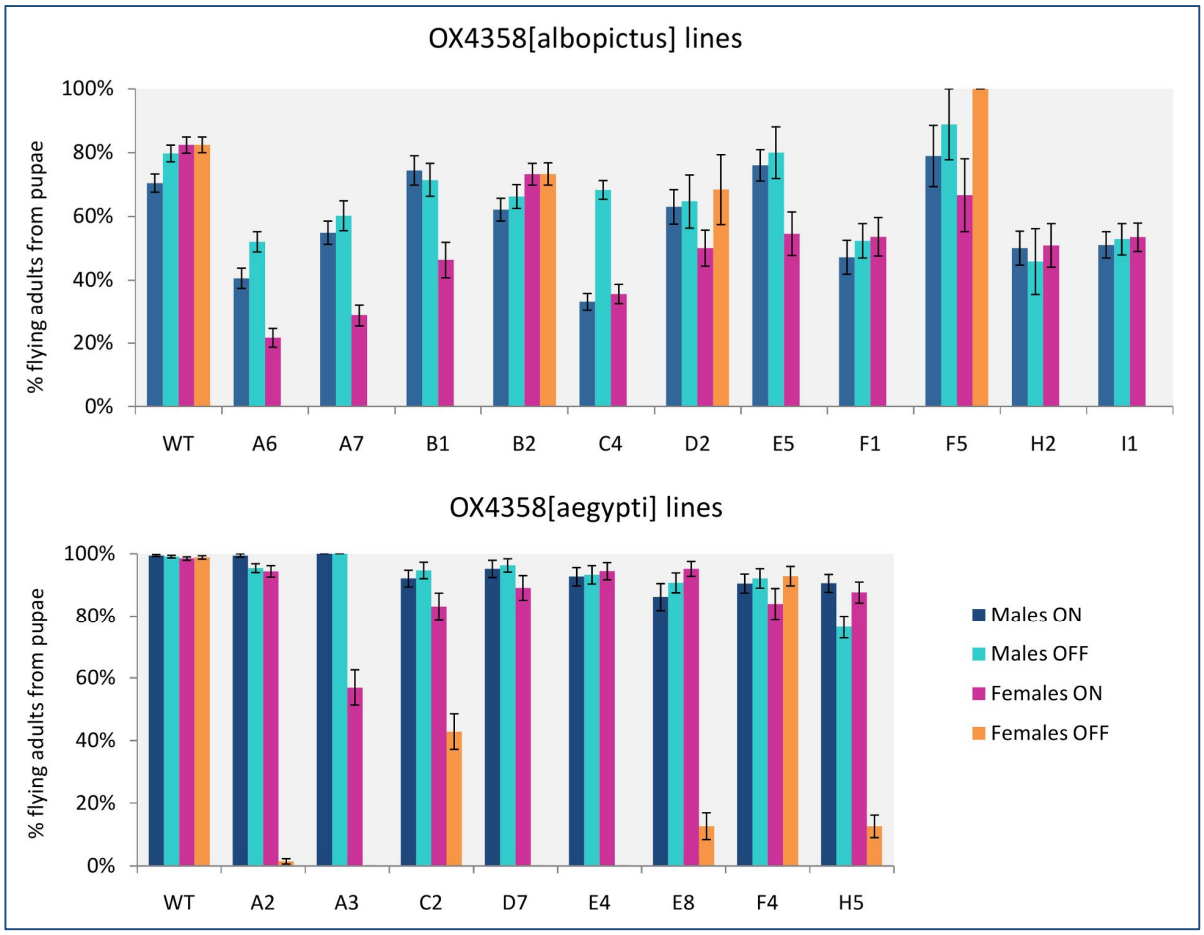

Figure 3-8. Flying phenotype of *Ae. aegypti* and *Ae. albopictus* OX4358 lines after being reared with (ON) or without (OFF) a supplement of the tetracycline antidote.

The percentage of flying adults was calculated as a proportion of the number of pupae placed in each cage. Phenotype of wild-type individuals (WT) is indicated as a control. The phenotypes from the different transgenic lines were assessed as an initial scan and at different times. They are therefore not directly comparable to each other or to the wild-type controls.

4. Discussion

Fu, Lees *et al.* recently reported the engineering of a conditional female flightless phenotype in *Ae. aegypti* using the *Ae. aegypti Actin-4* gene (*AeAct-4*) (Fu et al., 2010). The results presented in this chapter show that *AeAct-4* retains its properties in *Ae. albopictus* and leads to a similar phenotype. Moreover, replacing *AeAct-4* with its *Ae. albopictus* homologue also induced a conditional female flightless phenotype in both *Ae. albopictus* and *Ae. aegypti* species. Despite

some molecular variations between the two homologous genes, the two promoters and the sex-specific splicing appear interchangeable between species.

Noticeably low percentages of flying females on tetracycline were observed in several lines. Moreover, unlike the injections of the OX3860 construct reported in Chapter 2, where transgenic lines were derived equally from male and female G_0 crosses, all the positive G_1's found after injections of the OX3688 construct came from male G_0 crosses; the female G_0 crosses gave extremely few eggs and no transgenic individuals. Those observations may be explained by an excessive production of tTAV2 by the *Actin-4* promoter, or a sub-optimal tetracycline repression of the tetO-VP16 enhancement. This, however, seems to only affect *Ae. albopictus*. This difference between *Ae. albopictus* and *Ae. aegypti* can have several explanations: the *Ae. albopictus Actin-4* promoter may be stronger in an *Ae. albopictus* background; *Ae. albopictus* indirect flight muscles may be more sensitive to over-expression of VP16; *Ae. albopictus* may not accumulate tetracycline as effectively as *Ae. aegypti*; the tTAV2 transactivator may be more efficient in *Ae. albopictus*. Wide phenotypical variations were also noted among the OX4358[albopictus] lines, while the *Ae. aegypti* lines all had good percentages of flying females on tetracycline (except line A3) and healthy males both on and off tetracycline. One would have expected the OX4358 construct to be more tightly controlled in *Ae. albopictus*, as it is based on the *Ae. albopictus Actin-4* gene. On the other hand, the *Ae. aegypti* wild-type strain also performed better than the *Ae. albopictus* wild-type strain, and these differences could be due to the significantly much longer colonisation time of the *Ae. aegypti* strain, which would have tended to homogenise the genetic background of the population over time.

The OX3688A strain was created earlier than the OX4358 strains, as the OX3688 construct had been made earlier. Although the percentage of flying females on tetracycline was sub-optimal (61.5%), suggesting an incomplete repression of the RIDL system, the OX3688A strain showed the expected phenotype off tetracycline, with healthy males and 100% flightless females. This strain was therefore made homozygous and further assessed for its suitability in a control programme. That work is presented in the next chapter.

Chapter 4

Assessing the suitability of a female-specific RIDL strain of *Aedes albopictus* for a control programme

1. Introduction

Chapter 3 reported the development of female-specific RIDL strains. In order for a RIDL strain to be considered suitable as part of a vector control programme, it needs to comply with several key requirements: it should be safe to release; the males should be efficient at finding and mating wild females; the fecundity of females should be suitable for mass production; and ideally the lethality would be late-acting (Phuc et al., 2007; Thomas et al., 2000).

a) The strain must be safe to release: to ensure the safety of the RIDL programme, the transgenic phenotype should be highly penetrant (i.e. virtually all the specimens carrying the transgene should show the corresponding phenotype). For a female-flightless strain, this means that females reared in the absence of tetracycline should not be able to fly (flying females are considered unsafe because they could potentially become disease vectors). A fully penetrant strain would, in addition, remove the need for time-consuming and pupae-damaging manual sex-sorting by rearing the released generation off tetracycline. The likelihood of flightless females transmitting diseases from one human host to another can be considered negligible, and therefore flightless females could be co-released with males without compromising the safety of the programme. The release of RIDL individuals as eggs could even be envisaged.
Even though mathematical modelling for a bisex-lethal control programme suggests that imperfect penetrance would have little adverse effect on the efficiency of a RIDL-based program (Phuc et al., 2007), full penetrance is highly desirable for public approval.

b) The males should not be adversely affected: expression of lethal genes should ideally be restricted to females in order to optimise the competitiveness of homozygous RIDL males, which are already likely to suffer a certain reduction in fitness due to their long history of laboratory colonisation (Benedict et al., 2009; Bush et al., 1976). Released males, reared without tetracycline, must be as competitive as possible. Leaky expression of the toxic gene in those males may impair their ability to fly vigorously and to successfully find and mate wild females.

c) The system should be repressed by tetracycline: incomplete repression of the toxic components of the RIDL system is likely to negatively affect key parameters of female mosquito

biology, such as fecundity and longevity. Failures in previous SIT trials have been largely attributed to insufficient production levels (Benedict and Robinson, 2003); females reared in the presence of tetracycline should have appropriate fecundity in order to allow efficient mass-rearing of the strain.

d) Death should occur after the density-dependent phase: mosquitoes often breed in small containers where the larvae have a limited amount of resources to feed from, resulting in intense competition during the immature stages. Although this larval competition phenomenon may have been over-estimated in previous reports due to the lack of field data (Legros et al., 2009), mathematical models for *Ae. aegypti* suggest that late larval death of RIDL offspring would enhance the efficiency of a control programme as "doomed" transgenic larvae would use some resources before dying (Phuc et al., 2007; Yakob et al., 2008). A late-acting bisex RIDL strain of *Ae. aegypti* was recently developed to meet this requirement (Phuc et al., 2007).

In the work described in this chapter, I attempted to measure the quality of the RIDL OX3688A female-specific flightless strain with regards to those critical parameters. All experiments were performed in a confined laboratory, comparing the transgenic strain to its wild-type background. Clearly, such experiments cannot provide some key information such as males' field performance against wild counterparts or larval competition in the wild. The persistence of the phenotype in different wild-type backgrounds is also a vital parameter that cannot be tested until the release area is determined. The experiments presented here do, however, provide data to assess whether this candidate strain merits further study.

2. Material and Methods

a/ Flanking sequences

Flanking sequences were found using an adaptor-based method. All adaptor sequences are shown in Table 4-1. *Msp*I and *Dpn*II adaptors were made by mixing equal volumes of 100 µM MspI(TaqI)-short or DpnII-short oligonucleotides, respectively, with 100 µM Adaptor-long oligonucleotide and one tenth volume of 10x T4 DNA ligase buffer (New England BioLabs,

Ipswich, MA), heating to 50°C for annealing and cooling down immediately back to room temperature. The *Msp*I and *Dpn*II adaptors should then have a CG or GATC overhang able to ligate to genomic DNA digested with *Nar*I, *BstB*I, *Cla*I and *Msp*I, or *Bcl*I, *Bgl*II and *Dpn*II, respectively.

Genomic DNA was extracted from a pool of OX3688A pupae using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). 500-1000 ng were digested with either *Nar*I, *BstB*I, *Msp*I and *Cla*I for ligation to the *Msp*I adaptor, or *Bcl*I, *Dpn*II and *Bgl*II for ligation to the *Dpn*II adaptor (Table 4-1; all enzymes produced by New England BioLabs). Ligations to the adaptors (0.5 µg/µl) were performed overnight at room temperature using T4 ligase (New England BioLabs) and then used as templates for PCR amplification.

5' and 3' flanking sequences were amplified by PCR using primer sets PRIMER/PB2 nested with PRIMER/PB1 or PRIMER/PB4 nested with PRIMER/PB3, respectively (Table 4-1). For the primary PCR reaction, 5 mM primers were mixed at a 1:10 ratio of PRIMER:PB2 or PRIMER:PB4, in order to favour the *piggyBac*-specific amplification. PCR reactions were performed in a 25 µl final volume, using *Taq* polymerase from New England BioLabs and the following thermal profile: an initial denaturation step of 1 min at 94°C, 35 cycles of 10 sec at 94°C, 45 sec at 55°C and 1 min 30 sec at 68°C, and a final elongation step of 9 min at 68°C.

Amplified fragments were then extracted and purified from an agarose gel and sent for sequencing to GATC Biotech (Konstanz, Germany) using primers MID and PB6 or MID and PB5 for the 5' and 3' flanking amplifications, respectively. Primers 3688-5-2A, 3688-gtyp-R1 and 3688-gtyp-R2 were designed in the 5' and 3' flanking sequences to be used for genotyping (Table 4-1).

Table 4-1. Primers and probes sequences, and expected product sizes.

Name	Direction	Sequence 5'-3'	Specificity	Amplicon size
Adaptor-long	Adaptor	GTGTAGCGTGAAGACGACAGAAAGGGC GTGGTGCGGAGGGCGGTG		
MspI(TaqI)-short	Adaptor	CGCACCGCCCTCCG		
DpnII-short	Adaptor	GATCCACCGCCCTCCG		
PRIMER		GTGTAGCGTGAAGACGACAGAA	Adaptor	
PB1	Reverse	GGCGACTGAGATGTCCTAAATGCAC	*piggyBac* 5' end	
PB2	Reverse	CAGTGACACTTACCGCATTGACAAG	*piggyBac* 5' end	
PB3	Forward	CAGACCGATAAAACACATGCGTCA	*piggyBac* 3' end	
PB4	Forward	GTGCCAAAGTTGTTTCTGACTGACTA	*piggyBac* 3' end	
PB5	Forward	CACATGCGTCAATTTTACGCATGATTATC	*piggyBac* 3' end	
PB6	Reverse	CAGCGACGGATTCGCGCTATTTAG	*piggyBac* 5' end	
MID		GACGACAGAAAGGGCGTGGTG	Adaptor	
3688-5-2A	Forward	GCTTTGCAAGCCATGTGGGAAATCA	5' flanking sequence	260 bp with PB2 if transgene present
3688-gtyp-R1	Reverse	CGATTACGAGTCGAGTGACTCC	3' flanking sequence	207 bp with 3688-5-2A if no transgene; 485 bp with PB4 if transgene present
3688-gtyp-R2	Reverse	CACTGTACACTGTTGTGCAC	3' flanking sequence	354 bp with 3688-5-2A, if no transgene; 632 bp with PB4 if transgene present
AedesF	Forward	CTGCAGTAGTGATGAAGATGAACCA	*Ae. albopictus* IAP1	96 bp with AedesR
AedesR	Reverse	GGGCGAAAATGCCGTATTGTACTCA	*Ae. albopictus* IAP1	
AedesPro	Probe (Forward)	AGACACCAGTCGGACTTGCAAAATCTG	*Ae. albopictus* IAP1	
K10F155	Forward	CTCTGCTGACTTCAAAACGAGAAGAG	K10 3'UTR	109 bp with K10R266
K10R266	Reverse	ATTGGGTTTCACCGCGCTTAGTTACA	K10 3'UTR	
K10Bea2	Probe (Forward)	GACCACCGACGGCTCATTAGGGCTCGTG TGGTC	K10 3'UTR	
328F	Forward	CCAGCAGATACTATTGCG	*Wolbachia w*AlbA	379 bp with 691R
183F	Forward	AAGGAACCGAAGTTCATG	*Wolbachia w*AlbB	501 bp with 691R
691R	Reverse	AAAAATTAAACGCTACTCCA	*w*AlbA and *w*AlbB	
SVNP2F2	Forward	TGCGGTTTGTGGCGTATTCTCAGT	*Ae. albopictus* synaptic vesicle protein gene	497 bp with SVNP2R2
SVNP2R2	Reverse	CCTCCACGGGTTCGATTGTTTTG	*Ae. albopictus* synaptic vesicle protein gene	

b/ Comparative real-time PCR

Due to the transgene being inserted in a repeated region, it was impossible to assess the genotype of individuals by simple PCR. Comparative real-time PCR was therefore used for this purpose, with a transgene-specific primer/probe set targeting the *fs(1)K10* 3'UTR and an endogenous primer/probe set specific to the *Ae. albopictus* housekeeping gene *IAP1* (Inhibitor of Apoptosis 1) which acted as normaliser (Table 4-1). Relative quantities of *fs(1)K10* 3'UTR versus IAP1 were used to distinguish OX3688 hemizygous and homozygous genotypes, as previously described (Yi et al., 2008).

Genomic DNA was extracted from single headless adults using the NucleoSpin Tissue kit (Macherey-Nagel) with a final elution volume of 80 µl. Head were removed to avoid PCR inhibition by eye pigments (Eckhart et al., 2000). A primer mastermix was prepared with 0.8 µl of K10F155 and K10R266 primers (10µM), 0.6 µl of AedesF and AedesR primers (10 µM), 0.6 µl of K10Bea2 and AedesPro probes (10µM), and 6 µl of pure water per reaction. An equal volume of TaqMan® Gene Expression Master Mix (Applied Biosystems, Warrington, UK) was then added to the primer mastermix. Each reaction was set up with 19 µl of final mix and 1 µl of purified gDNA.

Quantitative PCR reactions were performed on a Mx3005P thermal cycler (Stratagene, La Jolla, CA, USA) with an initial denaturation step of 10 minutes at 95°C followed by 43 cycles of the following steps: 11 seconds at 94°C, 15 seconds at 60°C, 30 seconds at 54°C, 30 seconds at 60°C. Data were analysed using the MxPro-QPCR software (Stratagene).

c/ Phenotype analysis

For phenotypic analysis, eggs were hatched on day 1. On day 2, "on tet" and "off tet" trays (11 x 19 cm bottom surface) were set up with 300 larvae in 300 ml of pure water (1 larva/ml), respectively with or without a supplement of chlortetracycline hydrochloride (Sigma-Aldrich, Gillingham, UK). Chlortetracycline hydrochloride concentration was 30 µg/ml unless stated

otherwise. Water on trays was changed on days 6 and 11. Larvae were fed finely ground fish food (TetraMin®, Tetra GmbH, Melle, Germany) as follows: 12 mg/tray on days 2, 3 and 17; 24 mg/tray on days 4, 9, 11 and 12; 48 mg/tray on day 5; 96 mg/tray on days 6, 7 and 8. Sexes were separated as pupae, and the number of flying males and females was recorded 24 hours after emergence.

Wings were digitalised using a Discovery™ VMS-004 USB microscope (Veho, Eastleigh, UK) and measured from the distal end of vein 3 to the distal end of the allula (Figure 4-1) using ImageJ 1.42q software (http://rsbweb.nih.gov/ij/).

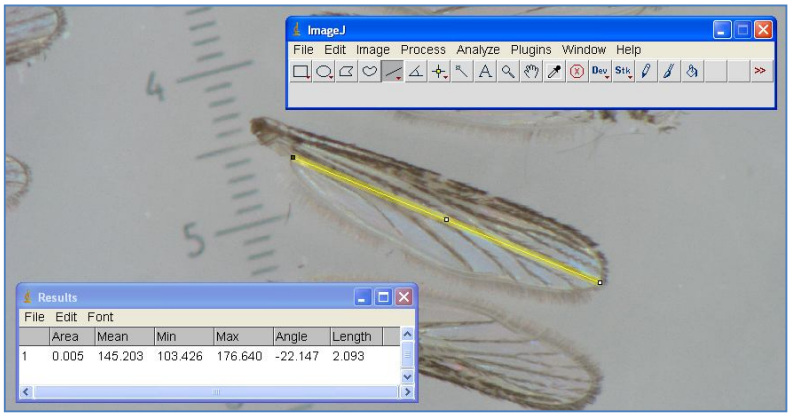

Figure 4-1. Wing length measurement using the ImageJ software.
Wings were measured from the distal end of vein 3 to the distal end of the allula (yellow line).

Please note that for practical reasons, “chlortetracycline hydrochloride” is referred to as “tetracycline” or “tet“ in this thesis.

d/ Mating competition

Wild-type *Ae. albopictus* and OX3688A homozygous specimens were reared without tetracycline at a density of 600 larvae per litre of water. In a first experimental setting, wild-type and OX3688A larvae were reared together in a 1:1 ratio and separated by fluorescence phenotype as pupae. In a second experimental design, wild-type and OX3688A larvae were

reared in separate trays and did not require fluorescence screening. Specimens of each genotype were sexed as pupae to ensure virginity and placed into small cages for emergence and maturation until the start of the mating competition. Following the emergence of adults, fifty sexually-mature males (2-4 days-old) of each genotype were placed in large mating cages (75 cm x 75 cm x 115 cm; BugDorm-2400F, MegaView, Taichung, Taiwan) together with fifty wild-type females (1-3 days-old). Adults were recovered after 48 hours. Males' wings were measured to compare the size of wild-type and homozygous specimens; females were blood-fed and transferred into individual tubes four days later for egg laying. Eggs were hatched four days after oviposition, and larvae were screened for fluorescence to determine the paternal genotype.

e/ Males' longevity

Wild-type and OX3688A homozygous G_8 were reared off tetracycline at 0.6 larvae/ml and one-day old adults were distributed in small cages (15 x 15 x 15 cm). For each genotype, five cages were set up with 10 males each. A 10% sucrose solution was available *ad-libitum* throughout the experiment. Dead males were recorded daily.

f/ *Wolbachia* PCRs

DNA from 11 pools of 10 OX3688A G_{10} female adults (heads removed) was extracted using the GeneJET Genomic DNA Purification kit (Fermentas). DNA from a wild-type female was used as positive control. Infection status was determined using primers specific for either *w*AlbA (328F and 691R, Table 4-1) or *w*AlbB (183F and 691R, Table 4-1) as previously described (Zhou et al., 1998). DNA quality of all the extractions was checked using primers specific of the synaptic vesicle nuclear protein gene, as previously described (SVNP2F2 and SVNP2R2, Table 4-1; (Kittayapong et al., 2002)).

g/ Statistical analysis

Data were analysed using PASW Statistics software (version 18.0; SPSS Inc., Chicago, IL, USA). Assumptions of normality were tested with Kolmogorov-Smirnov tests. When variables met the normality assumption, treatments were compared using one-way ANOVAs or t-tests, as indicated in the results section. When required, *post hoc* tests were used for pairwise comparisons: Tukey's HSD *post hoc* test were used when homoscedasticity was observed between groups; Dunnett's T3 *post hoc* test was used for unequal variances. Arcsine square root transformation was applied to the pupation percentage and female flying percentage under different tetracycline concentrations to achieve normality. For the wing length sets of data that did not achieve normality by transformation, non-parametric alternatives to ANOVA and t-tests were used: Kruskal-Wallis and Mann-Whitney U-test, respectively. Mann-Whitney U-tests with Bonferroni correction were also used for pairwise comparison after a significant result with Kruskal-Wallis. Development rates were analysed by Cox-regression; Kaplan-Meier survival curves were used to plot males' longevity, and compared using the log-rank test.

3. Results

a/ Making the OX3688A strain homozygous

5' and 3' flanking sequences were found as described in the methods section and primers designed in each sequence for genotyping (Table 4-2). PCR genotyping involves two types of primer pairs: a) one pair to detect the presence of the transgene, amplifying from one of the flanking sequences into the transgene; and b) one pair to detect the absence of transgene, amplifying from 5' into 3' flanking sequence (Figure 4-2). Hemizygous individuals possess one chromosome with the transgene and one without, and are expected to produce a positive band with both the a) and b) primer pairs. Homozygous individuals carry an insertion on both homologous chromosomes and are therefore expected to produce a positive amplification with primer pair a) only.

5' flanking sequence		3' flanking sequence
GCTTTGCAAGCCATGTGGGAAATCACACTGTTGAAAA TGCTTTGACATCCATGTGCACAACAGAACATGTGCTC GATGAACAAATTGAGATTTGAATTA	TTAA	AAGAAATCCACGTACTCCGGTGGAGACTCGAACTCACGACTC CCAATTTGCTAGACGGGCGCTTTCTATTCCTTCAAGCTACGGA GTCACTCGACTCGTAATCGGTCGGCCGAGGACCGACCCGTC GAGAGCCCGACTGCACACTAATTGTCATACCAATAAGTGATT GGGATGCAGTGCCCGAAACTCTTGATAATTAAAAATAATTTCC TCTCCCGCTTTGACACACAAGTGCACAACAGTGTACAGTG

Table 4-2. Flanking sequence from the OX3688A integration site. Primers' binding sequences underlined.

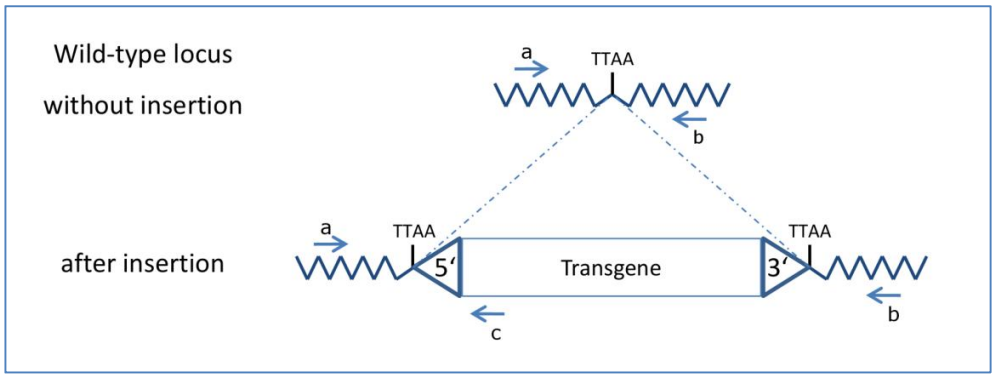

Figure 4-2. Genotyping using PCR.

Wild-type, hemizygous and homozygous individuals would be identified by positive amplification with primer pair a+b only, a+b and a+c, or a+c only, respectively. Insertion of the large sized transgene prevents successful amplification with primer pair a+b after insertion.

However, two problems may be encountered with such PCRs: Firstly, if the transgene has inserted into a highly polymorphic region of the genome, polymorphisms in the flanking sequences may prevent primer's annealing and PCR amplification. Secondly, if the transgene has inserted in a repeated region of the genome, the b) primers will anneal to that alternative template and produce a misleading band from homozygous genomes which will therefore not be identified as such.

Numbers of fluorescent and wild-type pupae of a hemizygous x hemizygous cross suggested no homozygous lethality, with an observed number of fluorescent pupae not significantly different from 75% (551 fluorescent pupae out of 758, X^2=2.155, p=0.1421). Fifty fluorescent progeny from this cross were used to test our genotyping primers (primers 3688-5-2A and PB2 to detect

the insert; primers 3688-5-2A and 3688-gtyp-R1 to detect the absence of insert). According to the Mendelian laws of inheritance, this meant that a third of the fluorescent individuals - about 16 - were expected to be homozygous. However, positive amplification was observed with the b) primer pair from every single individual, suggesting that the OX3688A line carries the transgene in a repeated region. Later real-time PCR on the same individuals diagnosed 10 homozygous individuals in this pool.

Single male crosses were set up with fluorescent individuals (initially with one female per male, then three females per male). After allowing mating for two days, males were removed and genotyped by real-time PCR as described in the methods section. The females which had mated homozygous males were pooled together for blood-feeding, and then split again into individual egg laying tubes. Females were then genotyped by real-time PCR.

Progeny from hemizygous females mated with homozygous males were screened for fluorescence. No wild-type progeny was observed, confirming that the males were correctly genotyped.

Families from homozygous parents were amplified separately for two generations and screened to confirm 100% fluorescence. Furthermore, all the G_1 individuals from those families were genotyped by real-time PCR in case any incorrect genotyping had occurred in the G_0 selection process.

In total, 979 G_0 males and 198 G_0 females were genotyped, but only 8 families produced enough G_3 individuals in order to participate in the creation of the final homozygous line, which was started with 6 females and 5 males from each of those 8 families.

As a final control, two males from each of those eight families were individually crossed to wild-type females. All progeny were fluorescent, confirming that the fathers were indeed homozygous.

b/ Effect of tetracycline on development and flying phenotype

The following experiment had two aims: 1/ to determine the minimum tetracycline concentration that would achieve the optimum phenotype, to avoid using unnecessarily high amounts of antibiotic; 2/ to determine the level of tetracycline contamination that would allow this line to establish in the wild, by finding the lowest tetracycline concentration able to repress the flightless phenotype and produce flying females.

The initial assumption was that 30 µg/ml was already a relatively high concentration, which is why it was used as the highest value in a set of decreasing concentrations (30, 10, 3, 1 and 0.5 µg/ml) tested experimentally. Only one tray of 300 larvae was set up for each concentration, aiming at narrowing down the concentrations meriting further investigations.

The maximum percentage of flying females was obtained at 30 µg/ml of tetracycline (Figure 4-3) but was only 28.28%, which was unlikely to be enough for the successful mass-rearing of this line. To test whether the percent of flying females could be increased by using higher tetracycline concentrations, the following experiment was set-up: Six trays of control wild-type larvae were set up without antibiotic and 5 trays of OX3688 homozygous G6 were set up with three different tetracycline concentration: 30, 60 and 100 µg/ml. All trays contained 300 larvae at a density of 1 larva/ml. The effects of these different tetracycline treatments on several fitness parameters were recorded, and analysed as mentioned in the Methods section. Males and females were observed separately to account for the female-specificity as the OX3688 construct.

Homozygous males and females OX3688A did not develop significantly slower or faster than their wild-type counterparts when reared at 30 and 60 µg/ml, but took significantly longer than the wild-type to reach pupation when reared at 100 µg/ml (Table 4-3).

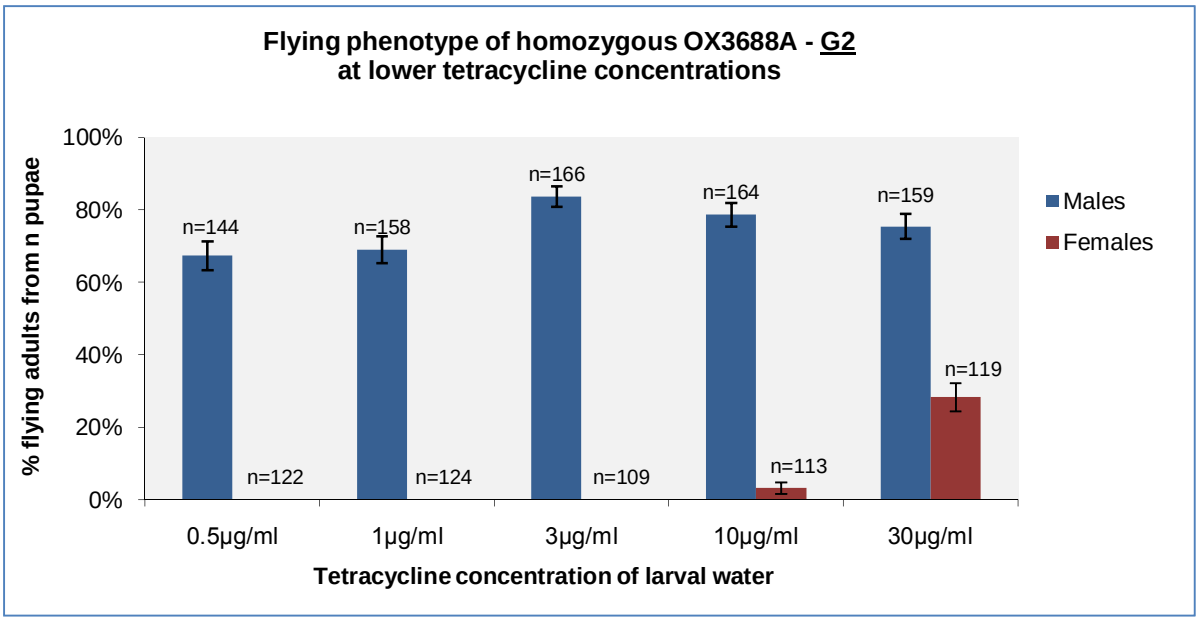

Figure 4-3. Flying phenotype of OX3688A homozygous G2 males and females from pupae, when reared at different concentrations of chlortetracycline hydrochloride.

Error bars represent the standard error of single percentages obtained from single experimental trays. Note that at generation G2 only 28.28% of the homozygous OX3688A females were able to fly when reared on 30 µg/ml of chlortetracycline.

	Treatment	Days to pupation	Statistical Groups
Males	WT off tet	9.55 ± 0.085	A
	30 µg/ml	9.56 ± 0.079	A
	60 µg/ml	10.18 ± 0.180	A B
	100 µg/ml	10.93 ± 0.256	B
Females	WT off tet	10.80 ± 0.087	A
	30 µg/ml	10.59 ± 0.092	A
	60 µg/ml	11.04 ± 0.228	A B
	100 µg/ml	11.76 ± 0.202	B

Table 4-3. Mean pupation time in days (± standard error), for homozygous males and females OX3688A (generation G_6) when reared at different concentrations of tetracycline, compared to wild-type reared off tetracycline.

Groups denoted by the same letter are not significantly different from each other (One-way ANOVA followed by Dunnett T3 *post hoc* test).

Pupation percentage (proportion of larvae reaching pupation) were not significantly different between OX3688A reared at 30, 60 and 100 μg/ml and wild-type reared off tetracycline (Figure 4-4; One-way ANOVA p=0.896 on transformed data). The increase in tetracycline concentration significantly decreased the males' eclosion percentage (Figure 4-4; ANOVA p<0.0001, followed by Tukey HSD *post hoc* test), but the males' flying percentage was not reduced in any of the treatments compared to the wild-type males (Figure 4-4; One-way ANOVA p=0.152). Females' eclosion percentage of OX3688A was significantly lower than wild-type when reared at 30 and 100 μg/ml but not when reared at 60 μg/ml (Figure 4-4; One-way ANOVA p=0.003, followed by Tukey HSD *post hoc* test). OX3688A females' flying percentage was lower than the wild-type only at 30 μg/ml (Figure 4-4; One-way ANOVA p=0.001 on transformed data, followed by Tukey HSD *post hoc* test), indicating that higher concentrations are more efficient at suppressing the RIDL phenotype. Note that in Figure 4-4 the flying percentage represents the number of flying individuals out of the number of eclosed adults rather than out of the number of pupae placed in the cage.

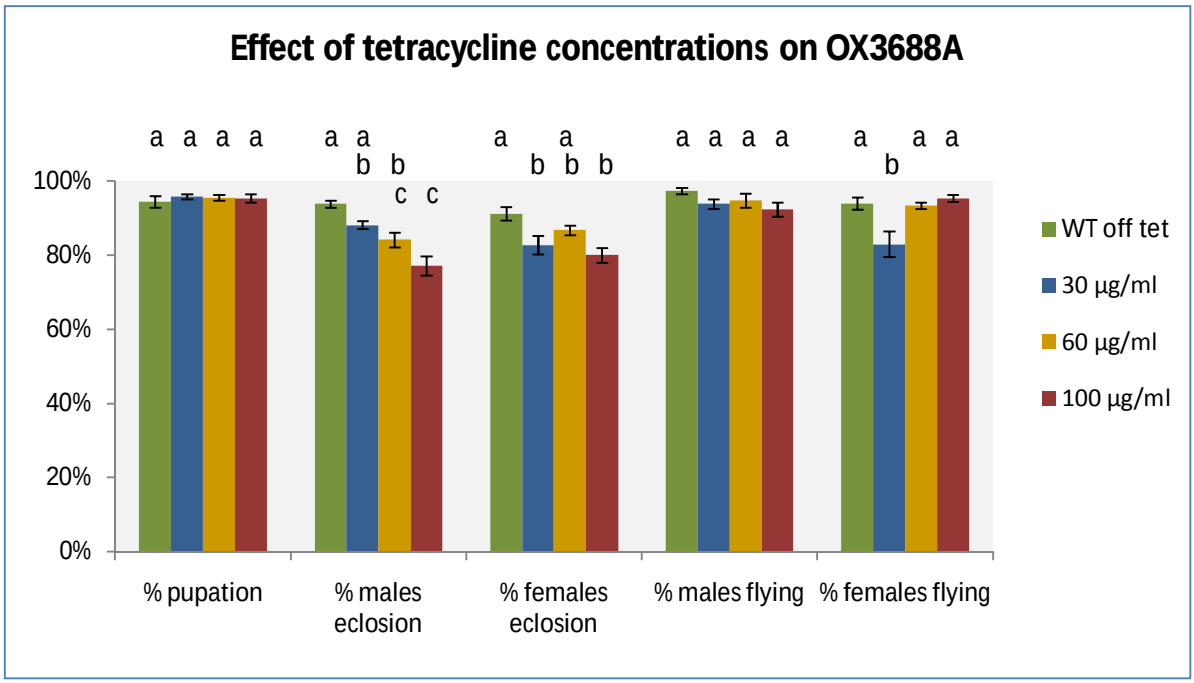

Figure 4-4. Effect of different tetracycline concentrations on the survival and flying ability of OX3688A homozygous G6. Note that here the flying percentage represents the number of flying individuals out of the number of eclosed adults rather than out of the number of pupae placed in the cage. Groups denoted by the same letter are not significantly different from each other. Error bars: standard error of the mean.

The fitness of the G_6 generation of homozygous was significantly higher than that of the G_2 generation, especially for females. For better comparison, Figure 4-5 shows the percentage of pupae giving flying adults, for G_2 reared at 30 µg/ml and G_6 reared at 30, 60 and 100 µg/ml. Besides, Figure 4-5 illustrates more clearly than Figure 4-4 the negative effect of high tetracycline concentration in males (One-way ANOVA $p<0.0005$, followed by Tukey HSD *post hoc* test) being balanced in females at 60 µg/ml by the repression of the RIDL system (One-way ANOVA $p=0.001$, followed by Tukey HSD *post hoc* test). When reared at 30 µg/ml of tetracycline, the percentage of flying females from pupae raised from 28.28 ± 4% in the G_2 generation of homozygous to 68.87 ± 3% in the G_6 generation.

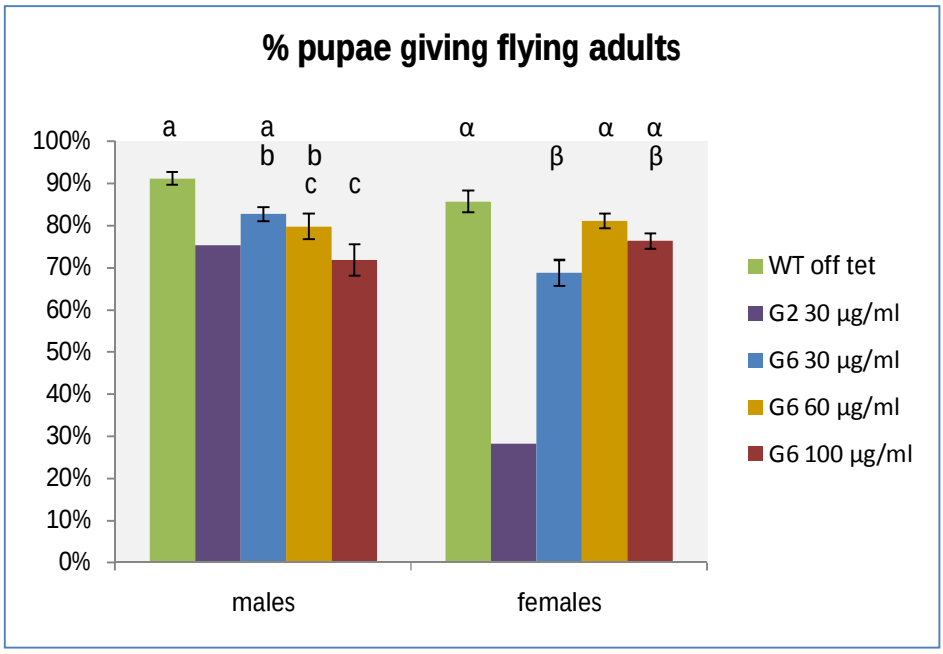

Figure 4-5. Percentage of pupae producing flying adults, for the wild-type strain reared off tetracycline, homozygous OX3688A strain G_2 generation reared at 30µg/ml of tetracycline, and homozygous OX3688A strain G_6 generation reared at 30, 60 and 100 µg/ml of tetracycline.

Groups denoted by the same letter (Latin for males, Greek for females) are not significantly different from each other (One-way ANOVA followed by Tukey HSD *post hoc* tests). Error bars: standard error of the mean. G_2 male and female data are presented for reference but result from single repeats and therefore were not included in the statistical analysis. Error bars represent the standard error of the mean.

The only difference in the way the two experiments (on G_2 and G_6 individuals) were carried out was that in the G_2 pupae were left off tetracycline while the G_6 pupae were kept in the same tetracycline concentration as the larvae (30, 60 or 100 µg/ml). The next experiment was therefore set up in order to test whether the increase in flying ability of the females observed between the G_2 and G_6 females reared at 30 µg/ml was due to the presence of tetracycline in the G_6 pupae water. We also tested the effect of adding the tetracycline at day 6 instead of day 2, with the intent of reducing the amount of tetracycline required to rear the strain. Day 6 was chosen for being approximately the time when the antidote theoretically would start to be needed; the *Actin-4* promoter - and consequent expression of tTAV2 - is induced in female pupae (Muñoz et al., 2004) so tetracycline should be absorbed before that stage. We also tested the addition of tetracycline (30 or 100 µg/ml) at pupa stage only, after larvae were

reared off tetracycline. Males' flying phenotype was excluded from this experiment as previous experiments showed it was not dramatically affected by this range of tetracycline concentrations. Furthermore, in a control programme released males would have been reared without tetracycline, which limits our interest for males' fitness in these experimental conditions.

Seven trays of 300 larvae in 300 ml were set up for each treatment, and pupae split into the two treatments (30 or 100 µg tetracycline/ml water) after sexing.

	Treatment	Mean days to pupation	Statistical Groups
Males	Off tet	9.5816 ± 0.04205	A
	30 µg/ml on day 2	9.4660 ± 0.05543	A
	30 µg/ml on day 6	9.4824 ± 0.04264	A
Females	Off tet	10.6757 ± 0.07862	B
	30 µg/ml on day 2	10.3133 ± 0.05387	A
	30 µg/ml on day 6	10.6224 ± 0.08032	B

Table 4-4. Mean pupation time in days (± standard error), for homozygous males and females OX3688A reared off tetracycline, with 30 µg/ml tetracycline added at day 2 (day 1=hatching), and with 30 µg/ml tetracycline added at day 6. Groups denoted by the same letter are not significantly different from each other.

Different timings of tetracycline addition did not significantly affect males' development (One-way ANOVA p=0.200) but females' development time was significantly increased by rearing off tetracycline and late addition of tetracycline (One-way ANOVA p=0.005, followed by Tukey HSD *post hoc* test; Table 4-4).

The results presented in Figure 4-6 showed the addition of tetracycline at pupal stage did not influence females' flying ability (One-way ANOVA p=0.312). We therefore concluded that the increased flying ability of homozygous females observed at 30 µg/ml of tetracycline between the G_2 and G_6 generations was due to the selection of flying females at each generation.

Selection did not occur while the line was maintained as hemizygous, since wild-type females were regularly added in order to limit inbreeding and to boost egg production. Late addition of tetracycline to the rearing water (day 6 rather than day 2) significantly decreased the pupation percentage (One-way ANOVA p=0.009, followed by Tukey HSD *post hoc* test) but had no significant effect on the percentage of flying females from pupae (One-way ANOVA p=0.312; Figure 4-6).

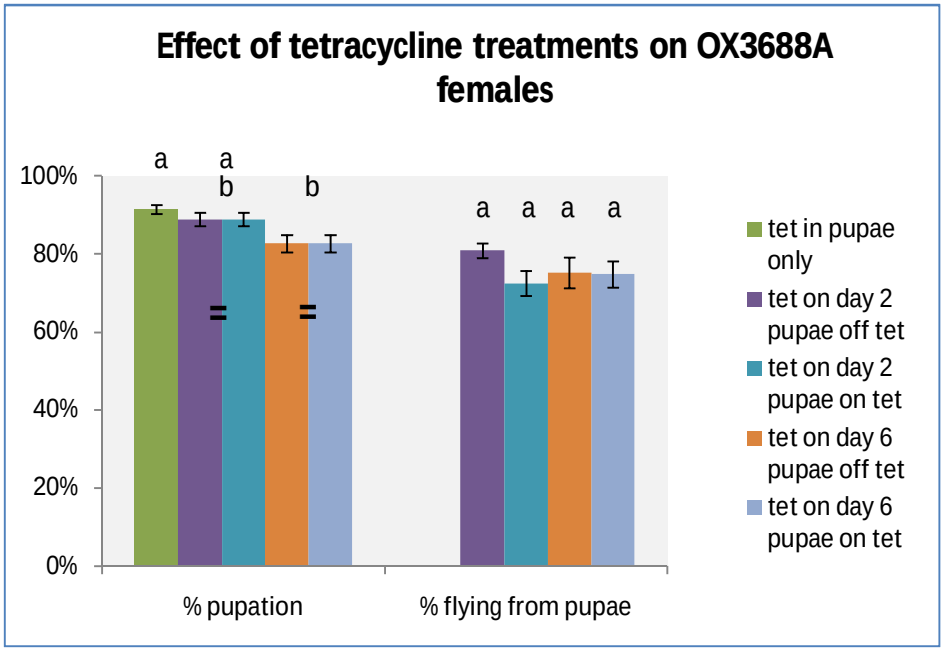

Figure 4-6. Effect of late (day 6 post-hatching) versus early (day 2 post-hatching) addition of tetracycline to the rearing water, and maintaining pupae off tetracycline versus on 30 µg/ml of tetracycline.

The addition of tetracycline (30 and 100 µg/ml) only at pupa stage was not sufficient to suppress the flying phenotype and did not produce any flying females. Groups denoted by the same letter are not significantly different from each other. Error bars represent the standard error of the mean.

A last experiment was performed in order to assess the repressibility of the flightless phenotype by low tetracycline concentrations. Five trays of 300 larvae in 300 ml were set up for each tetracycline concentration (0, 0.3, 1 and 30 µg/ml), using OX3688A homozygous G_7 generation. Rearing without tetracycline did not produce any flying females. At 0.3 and 1 µg/ml,

respectively 1.66 ± 0.58% and 12.17 ± 1.55% of the eclosed females were able to fly, compared to 90.79 ± 1.74% at 30 µg/ml (Figure 4-7).

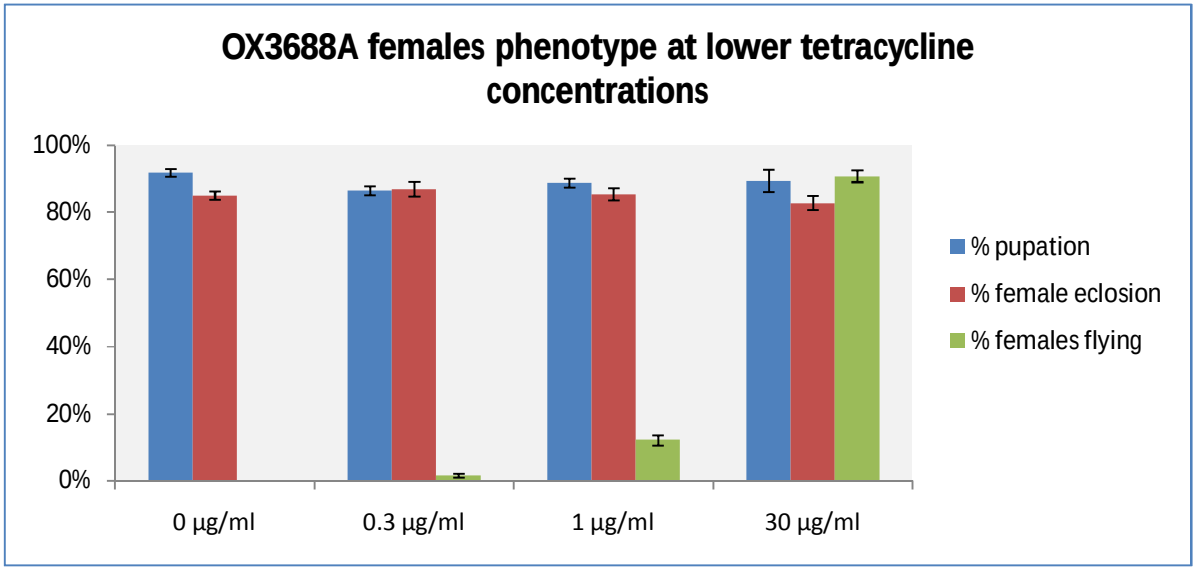

Figure 4-7. Phenotype of OX3688A homozygous G_7 females reared at 0, 0.3, 1 and 30 µg/ml tetracycline.

Tetracycline concentration of the rearing water did not significantly affect the pupation and eclosion percentages (ANOVA p=0.346 and p=0.514, respectively). Error bars represent the standard error of the mean.

c/ Fecundity of homozygous females

The aim of this experiment was to identify any crippling effect of high concentration of chlortetracycline on the fecundity of OX3688A homozygous females that would counterbalance the potential benefit of an increased percentage of flying females.

Females homozygous OX3688 G_6 reared in media containing tetracycline at 30, 60 and 100 µg/ml were used for this experiment, as well as wild-type females reared off tetracycline (females from Figure 4-4). For each treatment, five cages (15x15x15cm) were set up with 30 females. Twenty homozygous males reared at the same concentration were added into each cage.

Dead females were dissected and their wing length measured. The size of OX3688A females reared at 30 and 60 µg/ml was not significantly different from that of wild-type females reared off tetracycline; transgenic females reared at 100 µg/ml however were significantly smaller (Kruskal-Wallis $p<0.001$, followed by Mann-Whitney U-tests with Bonferroni correction; Table 4-5).

Treatment	N	Wing length (mm)	Groups
WT off	41	2.5428 ± 0.01534	A
30 µg/ml	51	2.5349 ± 0.01292	A
60 µg/ml	40	2.5325 ± 0.01810	A
100 µg/ml	65	2.4101 ± 0.01687	B

Table 4-5. Mean wing length in mm (± standard error) of homozygous females OX3688A G6 reared at 30, 60 and 100 µg/ml of tetracycline, compared to wild-type females reared off tetracycline.

Groups denoted by the same letter are not significantly different from each other.

Cages were blood-fed and eggs collected twice a week for the first 3 weeks, which corresponds to the time that cages would be kept in a mass rearing environment. Experiments looking at individual egg laying where females of similar sizes have taken similar size bloodmeals would provide more specific information on fecundity, but be less indicative of egg production in a mass-rearing context. The egg production of OX3688A females was significantly lower than that of wild-type females when reared at 100 µg/ml, but not at 30 or 60 µg/ml (One-way ANOVA $p=0.005$, followed by Dunnett T3 *post hoc* test; Figure 4-8).

Hatch rates were compared based on 14 egg papers for the different treatments. Hatching was induced in a vacuum chamber for one hour before counting the number of larvae. Hatch rates from the different treatments were not significantly different (One-way ANOVA $p=0.692$; Figure 4-9). All hatch rates were very low, indicating either a low fitness of the wild-type background strain or an inadequate storing or hatching of the eggs.

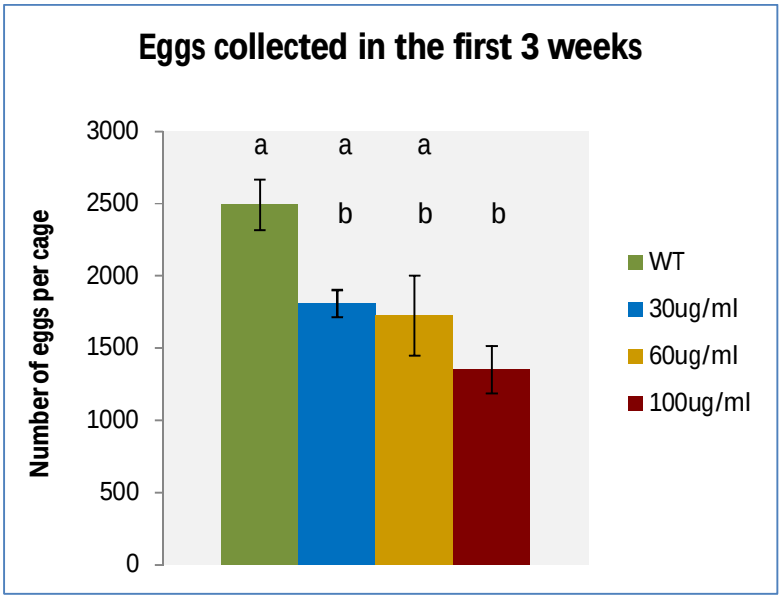

Figure 4-8. Egg production from cages of OX3688A homozygous females reared at 30, 60 and 100 µg/ml of tetracycline compared to cages of wild-type females reared off tetracycline.

Cages were set up with 30 females and 20 males. Groups denoted by the same letter are not significantly different from each other. Error bars represent the standard error of the mean.

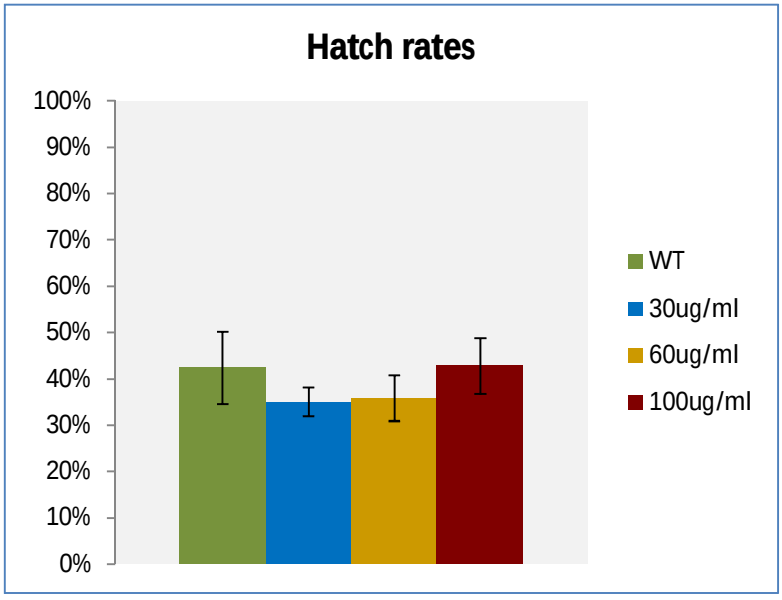

Figure 4-9. Hatch rate of homozygous OX3688A eggs from females reared at 30, 60 and 100 µg/ml of tetracycline compared to wild-type females reared off tetracycline.

Average from 14 egg papers. There is no significant difference between groups (one-way ANOVA, p=0.692). Error bars represent the standard error of the mean.

d/ Mating competitiveness of homozygous males

Males' mating competitiveness is a key parameter for the success of a control programme. This experiment was designed to identify any loss of competitiveness due to the RIDL construct as the homozygous males were set to compete with wild-type males of the same genetic background.

Males reared together:

In an attempt to exclude any confounding effects caused by rearing experimental specimens in separate trays, wild-type and OX3688A G_6 homozygous larvae were mixed and reared in the same trays throughout their larval development. Larvae were reared at relatively low density (300 wild-type plus 300 homozygous larvae in 1 litre of water) and fed an excess of food (enough for 700 larvae) in order to limit larval competition. Upon pupation, OX3688A and wild-type specimens were identified and segregated from each other by fluorescence screening, and sexes were separated by morphological differences observed under microscope.

Seven repeats were carried out with this experimental design; results are presented in Table 4-6. Despite a p value of 0.052 from the repeated G-test, wild-type males inseminated more females than the OX3688A males in all but one repeats, indicating an overall better performance of the wild-type strain.

As adults body size is an indication of fitness, it was critical that competing wild-type and OX3688A males were the same size in order to compare exclusively the effect of genotypes. We used wing length as a body size indicator. Wings of the males used in the mating competition experiments were not measured as many were damaged and unusable, which could have skewed the comparison. Extra trays were therefore set up and reared in the same conditions in order to produce males for wing length measurements. Results showed that rearing wild-type and OX3688A together resulted in significantly delayed pupation of OX3688A males and females compared to their wild-type counterparts (W=23.965 for males and W=28.769 for

females; df=1; p<0.001; Figure 4-10). Although development time was not a critical parameter of this experiment, this rearing setting also resulted into significantly smaller OX3688A males compared to wild-type males (t=3.820; p<0.001, Table 4-7). To address this limitation, an experiment was set-up rearing wild-type and OX3688A in separate trays and keeping the same larval density and food regime. In those conditions, OX3688A males and females developed at similar rates than WT specimens (W=1.920 for males' development; df=1; p>0.05; W=3.453 for females' development; df=1; p>0.1; Figure 4-10); most importantly, the size of wild-type and OX3688A males was not significantly different (U=1053.000; p=0.700, Table 4-7). A second set of experiments was consequently set up.

Rep	Egg batches	Unhatched	WT father	RIDL father	Both	G value	P value
1	26	0	15	10	1	0.931292	0.334527
2	24	2	13	9	0	0.731334	0.392451
3	31	2	18	9	2	2.650905	0.103491
4	30	0	10	17	0	1.835713	0.175454
5	24	0	11	10	0	0.047637	0.827227
6	30	4	16	7	3	2.839761	0.091958
7	35	0	20	14	1	1.004682	0.316181
	Heterogeneity values					6.251704	0.395593
		Pooled values	103	76	7	3.78962	0.051571
C.I. (95%)		Low limit	53.70%	38.85%			
		High limit	68.52%	48.29%			

Table 4-6. Mating competition between males reared together.

50 homozygous G_6 OX3688A males competed against 50 wild-type males for mating 50 wild-type females during 48 hours in a 75x75x115cm cage. Data was analysed using a replicated G-test without Yates correction. No heterogeneity was found between replicates (p=0.4), indicating that the pooled G value can be used for this set of experiments.

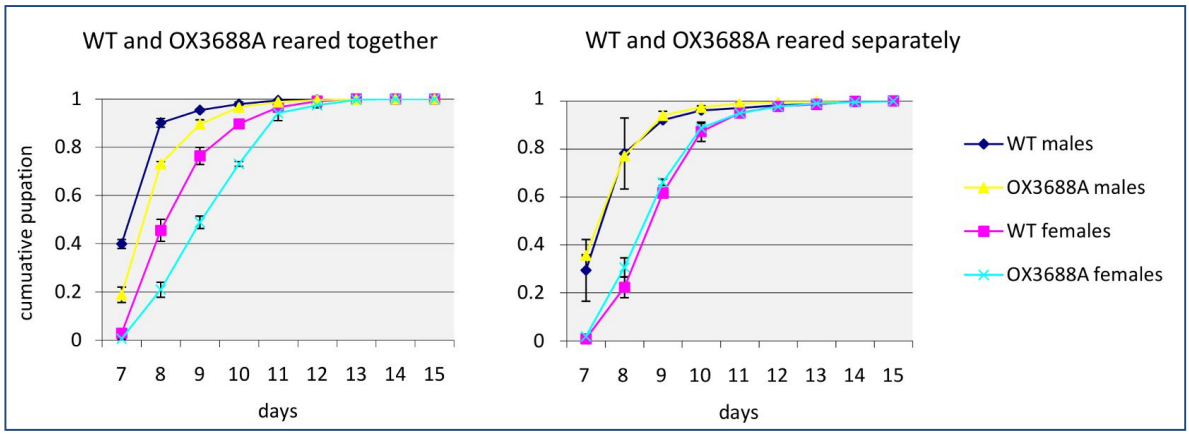

Figure 4-10. Average cumulative pupation over time of males and females from the wild-type and homozygous OX3688A G_6 strains reared together in the same tray or separately in different trays, at 0.6 larvae/ml.

Error bars represent the standard error of the mean.

Rearing	Strain	N	Mean ± SEM (mm)	Significance
Together	WT males	78	2.142 ± 0.0085	p<0.001 (t=3.820)
	OX3688A males	72	2.098 ± 0.00763	
Separate	WT males	48	2.086 ± 0.0112	p=0.700 (U=1053.000)
	OX3688A males	46	2.081 ± 0.0100	

Table 4-7. Mean ± SEM wing length (mm) of wild-type and homozygous OX3688A G_6 males reared off tetracycline together or in separate trays, at 0.6 larvae/ml.

When reared together, the wild-type males develop into bigger adults than the homozygous transgenics. In comparison, when reared in separate trays, male adult size wasn't significantly different between strains. Note that the "together" and "separate" experiments were not performed at the same time and therefore should not be directly compared.

Males reared separately:

As rearing both types of males together appeared to produce significantly bigger wild-type males than RIDL males, but rearing each type separately didn't (Table 4-7), a second set of experiments was set up using wild-type and OX3688A reared separately at 0.6 larvae/ml (600 larvae in 1 litre of water) with enough food for 700 larvae. OX3688A G_8, G_9 and G_{10} generations were used for these experiments. Development times were not significantly different between wild-type and OX3688A (Figure 4-11; W=0.554; p>0.1 for males G_8 versus wild-type males;

W=0.446; p>0.1 for females G_8 versus wild-type females; W=2.367; p>0.1 for males G_{10} versus wild-type males; W=0.521; p>0.1 for females G_{10} versus wild-type females), confirming previous observations.

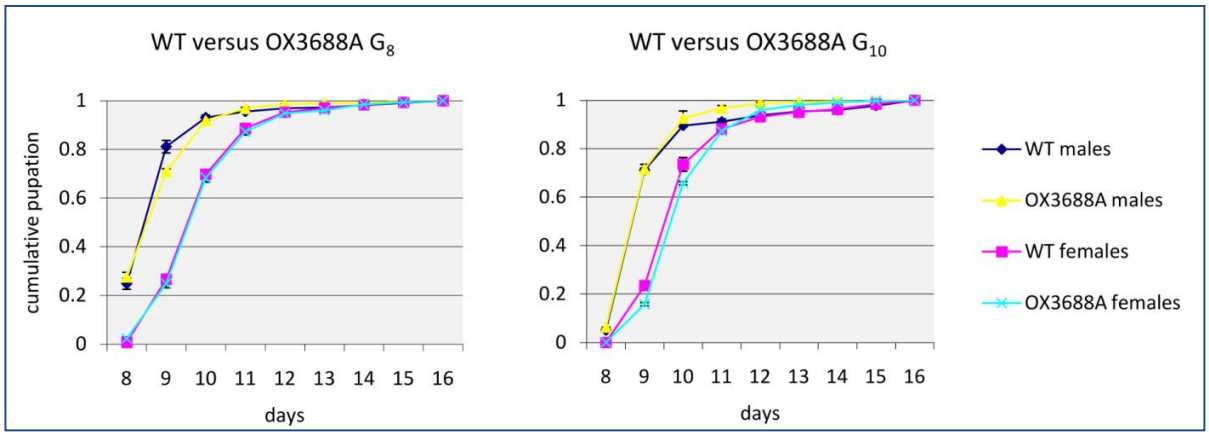

Figure 4-11. Average cumulative pupation over time of males and females from the wild-type and homozygous OX3688A G_8 or G_{10} reared at 0.6 larvae/ml.

Error bars represent the standard error of the mean.

Mating competition results are presented in Table 4-8, showing a highly significantly lower mating success of the homozygous OX3688A males compared to their wild-type counterparts: wild-type males inseminated an average of 69.13 ± 2.77% of the females, against 36.21 ± 3.19% inseminated by OX3688A males (including multiple matings in both categories). Replicated G-tests of goodness-of-fit were performed on the outcome of the mating competition repeats, showing significantly less success of the OX3688A compared to wild-type males in 6 out of 12 repeats. For each generation used, the heterogeneity value was not significant between replicates, indicating that the pooled G values can be used. A highly significant difference was found between the performances of wild-type and RIDL males of the G_9 and G_{10} generations ($p=2x10^{-7}$ and $p=8x10^{-8}$, respectively). RIDL males of the G_8 generation showed no significant difference compared to the wild-type (p=0.1386) although they inseminated less females in all but one repeats.

Exp	Rep	Egg batches	Unhatched	WT father	RIDL father	Both	G value	P value
G8	1	38	1	22	12	3	2.5267	0.1119
	2	36	3	18	11	4	1.3323	0.2484
	3	41	1	19	17	4	0.0909	0.763
	4	41	1	18	18	4	0	1
			Heterogeneity values				1.757	0.6243
			Pooled values	77	58	15	2.193	0.1386
G9	5	41	0	25	15	1	2.404	0.121
	6	45	2	28	15	0	3.992	0.0457
	7	36	0	26	9	1	8.112	0.0044
	8	40	0	31	5	4	16.413	5E-05
			Heterogeneity values				3.941	0.2679
			Pooled values	110	44	6	26.98	2E-07
G10	9	40	3	29	7	1	13.566	0.0002
	10	36	2	23	9	2	5.591	0.0181
	11	41	1	30	10	0	10.465	0.0012
	12	38	1	23	14	0	2.211	0.137
			Heterogeneity values				2.918	0.4044
			Pooled values	105	40	3	28.915	8E-08
C.I. (95%) for successful matings			Low limit	63.71%	29.95%			
			High limit	74.55%	42.47%			

Table 4-8. Mating competition between males reared separately.

The three sets of repeats used generation G_8, G_9 and G_{10} of the homozygous OX3688A line, respectively. 50 homozygous OX3688A males competed against 50 wild-type males for mating 50 wild-type females during 48 hours in a 75x75x115 cm cage. Data was analysed using a replicated G-test without Yates correction, counting multiple matings as success for both wild-type and OX3688A males.

Wings of competing males were measured for each set of experiments; no significant size difference between wild-type and OX3688A males was noticed (Table 4-9).

The number of hatched offspring produced by females mated with either wild-type or OX3688A males was compared for each set of repeats (Table 4-10); in repeats 1 to 4, OX3688A males (G_8) gave significantly more larvae than wild-type males, a difference that was not observed in repeats 5-12 (G_9 and G_{10}).

Experiment	Strain	N	Mean ± SEM (mm)	Significance
G8 (rep 1-4)	WT males	72	2.13 ± 0.007	p=0.583 (t=-0.550)
	OX3688A males	88	2.14 ± 0.007	
G9 (rep 5-8)	WT males	88	2.05 ± 0.008	p=0.309 (t=-1.020)
	OX3688A males	107	2.06 ± 0.006	
G10 (rep 9-12)	WT males	19	2.14 ± 0.017	p=0.083 (t=1.790)
	OX3688A males	16	2.10 ± 0.013	

Table 4-9. Mean ± SEM wing length (mm) of wild-type and homozygous OX3688A males used for the mating competition experiments.

Experiment	Strain	N	Mean ± SEM (mm)	Significance
G8 (rep 1-4)	WT males	77	22.27 ± 1.835	p=0.018 (U=1700.5)
	OX3688A males	58	30.53 ± 2.799	
G9 (rep 5-8)	WT males	110	46.43 ± 2.362	p=0.949 (t=-0.064)
	OX3688A males	44	46.73 ± 4.544	
G10 (rep 9-12)	WT males	105	43.92 ± 2.001	p=0.980 (t=0.025)
	OX3688A males	40	43.83 ± 3.449	

Table 4-10. Mean ± SEM larvae produced by females mated with wild-type or OX3688A males.

e/ Longevity of homozygous males

No significant difference between the lifetime of wild-type and OX3688A males was observed (Figure 4-12; Kaplan Meier Log Rank p=0.331).

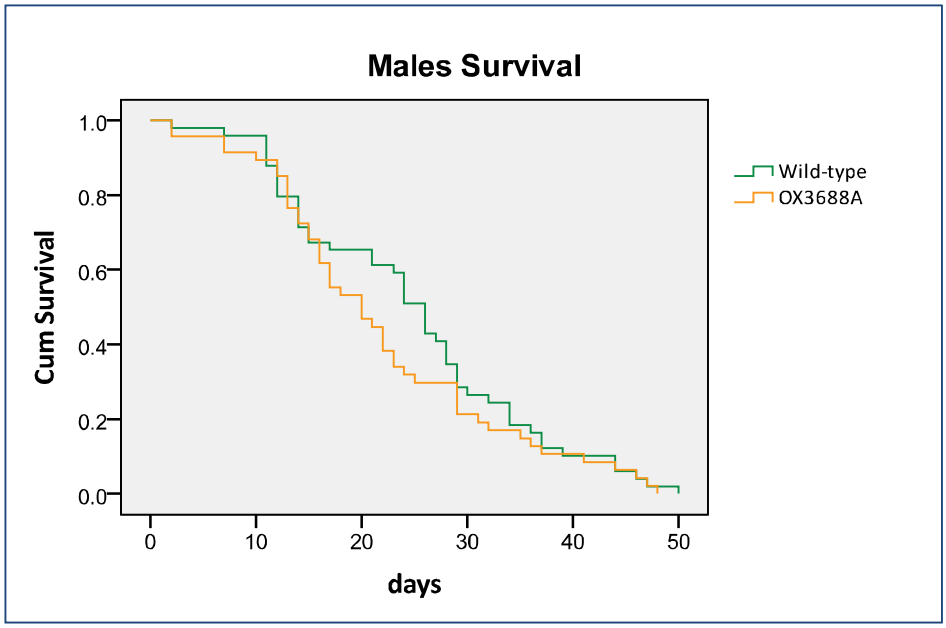

Figure 4-12. Survival of homozygous OX3688A (G_8) and wild-type males reared off tetracycline.
No significant difference observed (Kaplan-Meier Log Rank p=0.331).

f/ *Wolbachia* status

As mentioned in the introduction, *Ae. albopictus* is super-infected with two strains of *Wolbachia*, *w*AlbA and *w*AlbB. The tetracycline antibiotic used to suppress the flightless phenotype of OX3688A is also used to eliminate *Wolbachia* from insect hosts (Dobson and Rattanadechakul, 2001). We therefore investigated the infection status of the homozygous line after several generations.

PCR analysis on OX3688A homozygous G_{10} individuals (110 females in pools of 10) indicated that the line was cured of both *w*AlbA and *w*AlbB. PCR on 24 wild-type individuals (12 males and 12 females) showed that they were all super-infected (results not shown).

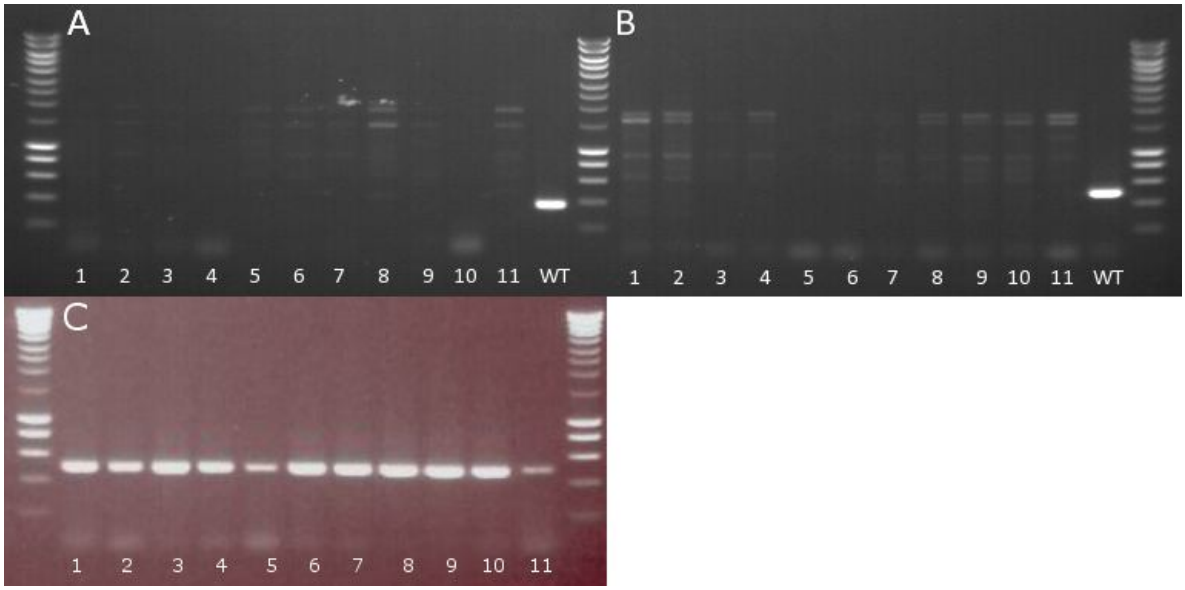

Figure 4-13. OX3688A G10 is cured of *w*AlbA and *w*AlbB.

PCR was performed on 11 pools of 10 OX3688A G_{10} females (1-11) and a wild-type control (WT) for the presence of *w*AlbA (A: primers 328F and 691R) and *w*AlbB (B: primers 183F and 691R), showing positive amplification of wild-type genomic DNA only. All genomic DNA extractions gave positive amplification with endogenous primers, indicating good DNA quality (C: primers SVNP2F2 and SVNP2R2).

4. Discussion

High penetrance was observed for the OX3688A phenotype, with no females able to fly in the absence of tetracycline at the G_7 generation (Figure 4-7). 1.66% females were able to fly when the strain was reared at 0.3 µg/ml (Figure 4-7). In the environment, *Ae. albopictus* breeds in clean water containers, filled either by rainfall or humans, which are highly unlikely to contain tetracycline. Even if they did come into contact with tetracycline-polluted water, the concentration would likely be lower than 0.3 µg/ml (the highest published record of tetracycline contamination in the environment that I was able to find in the literature mentioned a maximum of 0.065 µg/ml of tetracycline, in a municipal sewage in China (Liu et al., 2009)). Based on these observations, OX3688A should be unable to breed in the environment, and therefore should be safe to use in a control programme. However, considering the substantial improvement of the females' flying ability between generations G_2 and G_6 of the homozygous line (Figure 4-5), it seems essential to regularly verify that OX3688A females

remain sensitive to the RIDL system, i.e. flightless off tetracycline. It would also probably be safer to maintain the strain at a concentration of tetracycline higher than the 30 μg/ml standard used at Oxitec for RIDL mosquito strains, for instance 60 μg/ml, in order to limit the selection pressure for least sensitive females. No such selection was occurring in the hemizygous state as transgenic males were usually crossed to wild-type females.

Safety considerations aside, increasing the tetracycline concentration from 30 to 60 μg/ml offered greater repression of the RIDL construct in the OX3688A strain, as shown by the improved eclosion and flying ability of females (Figure 4-5). Increasing further the tetracycline concentration to 100 μg/ml did, however, have a negative impact on the females' eclosion (Figure 4-5), which is most probably due to tetracycline toxicity at high concentration, as observed more clearly in males (Figure 4-4 and Figure 4-5). The released generation from a female-specific RIDL strain such as OX3688A would be reared off tetracycline in order to separate out the females; the negative impact of tetracycline on males' fitness would only occur in laboratory and mass rearing conditions where it should not have a significant effect on productivity.

The size and fecundity of OX3688A females reared at 60 μg/ml of tetracycline was similar to OX3688A females reared at 30 μg/ml and wild-type females reared off tetracycline (Table 4-5, Figure 4-8 and Figure 4-9). OX3688A females reared at 100 μg/ml were significantly less fecund (Table 4-5, Figure 4-8), probably a direct consequence of the significantly smaller females. Both of these observations confirm the harmful effect of tetracycline at this concentration, and corroborate the direct relationship between body size and fecundity previously reported (Armbruster and Hutchinson, 2002).

Considering these results it is clear that, of the three tetracycline concentrations tested, 60 μg/ml would be optimal for the OX3688A strain.

The constant requirement for tetracycline of the RIDL strains was expected to have an impact on *Wolbachia* densities. Results presented here show that the OX3688A strain was cured of *Wolbachia* at the G_{10} generation (Figure 4-13). The loss of *Wolbachia* was shown not to affect males' fitness (Calvitti et al., 2009), but to reduce females' fecundity and egg hatch rate (Dobson et al., 2004), an effect that did not appear in the results presented here (Figure 4-8 and Figure 4-9). The poor egg hatch rates however may prevent efficient mass-production of the OX3688A strain, possibly a consequence of an inbred genetic background rather than an effect of the transgene. Cytoplasmic incompatibility between the OX3688A strain and wild-type populations offers added protection in case of a loss of penetrance of the flightless phenotype, as any OX3688A females mated with wild-type males would produce non-viable progeny.

Results from rearing off tetracycline suggest that the female-specific RIDL phenotype from the OX3688A strain is induced after the density-dependent phase, with no significant difference in the pupation time and similar male sizes between OX3688A and wild-type (Figure 4-10, Figure 4-11, Table 4-7 and Table 4-9). However, when homozygous OX3688A and wild-type larvae were reared together in a 1:1 ratio for the first set of mating competition experiments, OX3688A homozygous larvae developed significantly slower and homozygous males came out significantly smaller than their wild-type counterparts (Figure 4-10 and Table 4-7). These results indicate a significantly lower larval competitiveness of the homozygous OX3688A compared to wild-type larvae, even in a relatively non-competitive environment (0.6 larvae/ml with a small excess of food). However, the competitiveness of homozygous RIDL larvae is only relevant if the control strategy involves the release of homozygous eggs rather than homozygous adult males or pupae. In the case of an adult/pupae release strategy, only hemizygous RIDL larvae would be involved in resource competition with their wild-type siblings. It would be interesting to study the outcome of such competition, ideally using insects from the wild or recently colonized to cross and compete with the OX3688A strain.

OX3688A homozygous G_8 males did not perform significantly worse than their wild-type counterparts in the mating competition experiment, despite inseminating fewer females in all but one repeat (Table 4-8). They had similar sizes and development time as their competitors (Figure 4-11, Table 4-9), and did not have a significantly shorter lifetime (Figure 4-12). Males from generations G_9 and G_{10} of the OX3688A homozygous strain (used in the second and third set of mating competition tests) also had similar sizes than their wild-type competitors (Table 4-9), but performed significantly worse in almost every repeat (see individual p values in Table 4-8). The development time of G_{10} homozygous was also not significantly different from that of the wild-type strain. It would be interesting to compare later generations of the OX3688A homozygous strain to wild-type in the future to see whether the strain is getting worse over time. All repeats combined, and including multiple matings as both OX3688A and wild-type success, 36.21 ± 3.19% of the females which produced hatching offspring were inseminated by OX3688A males, against 69.13 ± 2.77% inseminated by wild-type males. This corresponds to a Relative Sterility Index (RSI) around 0.36 for the OX3688A strain. A strain equally competitive to wild-type would have a RSI of 0.5; the threshold RSI for medfly (*Ceratitis captiata*) SIT trials is 0.2 against recently colonised individuals from the target population in field cages (FAO/IAEA/USDA, 2003). Here, the OX3688A males are were competing against a relatively well adapted laboratory strain in a laboratory environment, and would likely perform worse in more challenging conditions. Although lower male competitiveness can be compensated by releasing larger numbers, this may be an important issue with the OX3688A strain considering the low hatch rate (Figure 4-9) and consequently low predicted mass rearing yield.

The evident background issue arising from those results could possibly be overcome by introgressing the strain into a better wild-type background and made homozygous once again. However, such an approach would take at least five generations of introgressing in order to change >95% of the genetic background ($1-0.5^5=0.96875$), plus the highly time-consuming homozygous-selection process using comparative PCR. It would also not guaranty that the new strain would be better, as we can not predict the phenotype of OX3688A into a different

genomic background. At the moment, there is no reason to think that any OX4358 strain (Chapter 3) would have a better fitness than OX3688A, although if PCR genotyping was possible on one of those insertions it would greatly facilitate the homozygous-making process and subsequent quality control of the strain. There is no doubt that new *Ae. albopictus* injections should take place in a different, better wild-type background strain.

Overall, results from Chapter 3 and 4 suggest that the Actin4-tTAV2 constructs are too toxic and not adequately repressed by tetracycline in *Ae. albopictus*, and the high selection pressure, which allowed the OX3688A homozygous strain to develop a fitness comparable to the wild-type strain, is not a desirable phenomenon. A less toxic construct would allow creating more candidate strains to compare and choose from. With the aim of tailoring RIDL constructs for successful application in this species, the next chapter of this thesis is looking at alternatives to tTAV2.

Chapter 5

Decreasing the toxicity of the female-specific RIDL system for *Aedes albopictus*

1. Introduction

The results presented in chapters 3 and 4, and in particular the low percentage of hemizygous *Aedes albopictus* RIDL females able to fly when reared on tetracycline, show that sub-optimal phenotypes and fitness levels were achieved by the Actin4-tTAV2/tetO-VP16 transgenes (OX4358 and OX3688) in *Ae. albopictus*. This is likely due to excessive basal toxicity of such transgenes in this species.

Excessive toxicity in females reared on tetracycline can be due to an incomplete repression of the system by tetracycline, or an excessive basal expression of either tTAV2 or VP16. The lower mating competitiveness observed in OX3688A males (chapter 4) may be due solely to the insertion site, but could also be due to some leaking of the Actin4 promoter or basal expression of VP16 in males. Several elements of the Actin4-tTAV2/tetO-VP16 transgene can be suspected of causing excessive toxicity: the Actin4 promoter; the tTAV2 transactivator; and the tetO-VP16 effector. Work by Fu *et al.* indicated that the Actin4-tTAV2 cassette is able to cause female flightlessness by itself (Fu et al., 2010), and therefore a sensible target for further investigations. Reducing the negative impact of the construct on males' fitness could in theory be achieved by preventing the Actin4 promoter from leaking in males, although in practice our understanding of the structure-function relationship of insect promoters in general is too poor to allow a rational-design approach to this potential problem. Lowering the basal toxicity of the tTAV2 transactivator seems a more feasible approach, and should help improve the fitness of females reared on tetracycline. The transactivator needs however to retain high affinity for the tetO sequence in order to achieve 100% of flightless females off tetracycline.

The sequence of the original tetracycline-controlled transactivator, tTA (Gossen and Bujard, 1992), has been optimised for codon usage in insects in order to enhance its expression; both the sequences of the tetracycline repressor (tetR) and VP16 transcription activator have been optimised, resulting in "tTAV2" used in OX3688 and OX4358 constructs. tTAF3 is yet another variation of the tTA transactivator, which consists of the optimised tetR used in tTAV2, fused with 3 minimal activation domains derived from VP16 (Baron et al., 1997). Baron et al.

suggested that tTAF3 might have lower toxicity than tTA. The aim of the experiments described in this chapter was to compare tTAV2 with tTA and tTAF3, in terms of toxicity, effectiveness and repressibility by tetracycline.

The site-specific integration system, described in chapter 2, is an ideal tool for an accurate comparison of transgenes without confounding position effects. Lines OX3860B and OX3860C (Chapter 2) each carry an attP docking site in obviously different genomic surrounding, revealed by the different expression patterns of the 3xP3-ECFP marker (blue eyes and blue anal papillae in OX3860B, blue eyes only in OX3860C). However, the flightless phenotype produced by Actin4-tTAV2 transgenes is hard to quantify. Driving the transactivator with a promoter specific to more essential tissues was anticipated to induce death rather than flight impairment and therefore a better tool for estimating the variations in toxicity of the different transactivators.

Literature investigation led to the *Ae. aegypti* hexamerin-1 gene (*AaHex1*). Hexamerins are storage proteins accumulated during larval stages; they are synthesised in the fat bodies, secreted in the hemolymph and stored in the fat bodies shortly before pupation as nutrient reserves (Telfer and Kunkel, 1991). Work by Korochkina *et al.* showed high expression levels of *AaHex1* restricted to the 4th larval stage (Korochkina et al., 1997a). The AaHex1 protein has a tighter peak of expression than the AaHex2 protein, with hemolymph levels declining from 5 hours after larval/pupal ecdysis (Korochkina et al., 1997b). The AaHex-1γ subunit is, in addition, more highly expressed in females. The *AaHex-1γ* promoter was chosen to drive tTAV2, tTA and tTAF3, hoping that it would induce different degrees of late-acting lethality after site-specific integration into OX3860B and OX3860C.

2. Material and Methods

a/ Plasmid construction

OX4427, OX4429 and OX4436 constructs: The *AaHex-1γ* promoter was amplified from *Ae. aegypti* genomic DNA with primers AeHex1-bbs-f and AeHex1-bbs-r (see Table 5-1 for primer sequences), purified on agarose gel and MinElute column (Qiagen, Hilden, Germany) and digested with *Bbs*I (FastDigest enzyme from Fermentas, Vilnius, Lithuania). tTAF3, tTA and tTAV2 were amplified respectively from construct OX396 with primers tTAF3-Bbs-f and tTAF3-pac-r, construct OX3335 with primers tTA-bsmb-f and tTA-pac-r, or construct OX3737 with primers tTAV2-bbs-f and tTAV2-pac-r (unpublished constructs, Oxitec Ltd); PCR products were purified on agarose gel and MinElute columns, then digested with *Pac*I and respectively *Bbs*I, *BsmB*I or *Bbs*II (FastDigest enzymes from Fermentas). Construct OX3515, containing a 3xP3-DsRed2 (red eyes) marker and an *attB* site for site-specific integration into *attP*, was used as backbone for the three final constructs: it was digested with *Eag*I and *Pac*I (FastDigest enzymes from Fermentas) and purified on MinElute column (Qiagen) to discard the short 25 bp product while keeping the 4421 bp fragment. OX4427, OX4429 and OX4436 were completed by ligating the digested PCR products (*AaHex-1γ* promoter and respectively tTAF3, tTA or tTAV2) into the digested backbone using Rapid Ligase from Fermentas.

OX4499 construct (attB-hr5ie1-DsRed2-tetO-Michelob_x) was obtained by replacing the 3xP3-DsRed2 marker from the OX3582 construct (Fu et al., 2010) with the hr5-ie1-DsRed2 cassette from the OX3978 construct (attB-hr5ie1-DsRed2-tetO-AmCyan; Oxitec Ltd, unpublished). Michelob_x is a pro-apoptotic, “killer” gene (Zhou et al., 2005). Both donor constructs were digested with *BamH*I and *Pac*I (FastDigest enzymes from Fermentas); the 4616 bp restriction fragment from OX3582 and the 2347 bp restriction fragment from OX3978 were gel-purified using Minelute column (Qiagen) and ligated using Rapid Ligase (Fermentas).

Table 5-1. Primer sequences, and expected product sizes.

Name	Direction	Sequence 5'-3'	Specificity	Amplicon size
AeHex1-bbs-f	Forward	ACGCGAAGACTAGGCCGTCAGCCAGTTTGCCG TCATG	*Ae. aegypti Hex1γ* promoter	1457 bp
AeHex1-bbs-r	Reverse	TCGCGAAGACATCCATTGACGGTAGTTCTCGC TGGAAG	*Ae. aegypti Hex1γ* promoter	
tTAF3-Bbs-f	Forward	AGCGGAAGACTAATGGGCAGCCGCCTGGATAA G	tTAF3	771 bp
tTAF3-pac-r	Reverse	TCGCTTAATTAATACCAGCATGTCGAGATCAAA GTCG	tTAF3	
tTA-bsmb-f	Forward	AGCGCGTCTCACTACCCCACCGTACTCGTCAA TTCC	tTA	1031 bp
tTA-pac-r	Reverse	ACGCTTAATTAACCCACCGTACTCGTCAATTCC	tTA	
tTAV2-bbs-f	Forward	AGCCGAAGACACATGGGCAGCCGCCTGGATAA GTC	tTAV2	1041 bp
tTAV2-pac-r	Reverse	AGGCTTAATTAATTAGCCGCCGTACTCATCGAT G	tTAV2	
Diag-AeHex1pro	Forward	GCGCCCCATACCTAGAAAGTG	*Ae. aegypti Hex1γ* promoter	820 bp with Diag2-VP16new
Diag5-dsred	Reverse	ACCATCGTGGAGCAGTACGAG	DsRed2	with Diag-AaHex1pro: 1189 bp for OX4427 and 1450 bp for OX4429
3'cent-3	Reverse	AAACCTCCCACACCTCCC	SV40 3'UTR	sequencing primer
Diag2-VP16new	Reverse	CGATGGTGCTGCCGTAGTTG	tTAV2	820 bp with Diag-
3860B-5'flank1	Forward	TGAGAACAAGATGGCGATTCTAGGAGT	OX3860B 5' flanking sequence	426 bp with PB2
3860C-5'flank1	Forward	CACAATGGAACCATGAAAACTTAAACCAG	OX3860C 5' flanking sequence	363 bp with PB2
PB2	Reverse	CAGTGACACTTACCGCATTGACAAG	*piggyBac* 5'-end	
AmCyTaqF	Forward	GGCGACGACATGAAGATGACCTAC	AmCyan	122 bp
AmCyTaqR	Reverse	GTCACCTTGAAGGTGGAGGTCTGG	AmCyan	
AmCyTaqprobe	Probe	GCGTGAACGGCCACTACTTCACCGTGA	AmCyan	
18StaqF	Forward	ACGCGAGAGGTGAAATTCTTG	*D. melanogaster* rRNA	69 bp
18StaqR	Reverse	GAAAACATCTTTGGCAAATGCTT	*D. melanogaster* rRNA	
18StaqProbe	Probe	6-FAM-CCGTCGTAAGACTAAC-MGB	*D. melanogaster* rRNA	

b/ Strain background, rearing and transformation

The *Ae. albopictus* OX3860B and OX3860C carry the an *attP* docking site in two different genomic locations, as described in Chapter 2. The *Ae. aegypti* wild-type strain originate from Malaysia and was colonised by the Institute of Medical Research (Kuala Lumpur) in 1977. The insectary was kept at 27°C (± 1°C) and 70% (± 10%) relative humidity. Larvae were fed on crushed dry fish food (TetraMin® flake food from Tetra GmbH, Germany) and adults on 10% glucose with 14 U/ml penicillin and 14 µg/ml streptomycin. Females were fed on horse blood using a *Hemotek* Insect Feeding System (Discovery Workshops, Accrington, UK) set at 37°C.

Embryos from *Ae. albopictus* OX3860B and OX3860C strains were injected for site-specific integration with OX4427, OX4429 or OX4436 (350 ng/µl) and phiC31 mRNA (600 ng/µl) (Nimmo et al., 2006) in injection buffer. Similar concentrations of OX3978 and OX4499 were injected into line OX3860B only. The *piggyBac* and phiC31 mRNAs were transcribed and purified using the mMESSAGE mMACHINE® T7 and MEGAclear™ kit (Ambion, Austin, TX). Construct and helper plasmids were purified using the EndoFree Plasmid Maxi kit (Qiagen). Injected G_0 adults were crossed in pools to wild-type counterparts. G_1 larvae were screened for fluorescence using a Leica (Wetzlar, Germany) MZ95 microscope with the appropriate filter sets from Chroma Technology (Rockingham, VT, USA) (filters: ECFP: exciter D436/20x; emitter D480/40m; DsRed2: exciter HQ545/30x; emitter HQ620/60m). OX4427, 4429 and 4436 lines were started with 3 G_1 positive adult males, which were subsequently analysed by PCR to confirm their genotype.

Pictures were taken with Canon PowerShot S5IS with a MM99 adaptor (Martin microscopes, Easley, SC, USA) to fit into the eyepiece.

c/ Characterisation of transformation events

Transgenic G_1 individuals used to start all the lines were characterised by PCR to ensure that insertions were as expected. tTAF3 (from OX4427) and tTA (from OX4429) were amplified using primers Diag-AeHex1pro and Diag5-dsred, and the resulting fragments were sequenced with

primer 3'cent-3. tTAV2 (from OX4436) was amplified using primers Diag-AeHex1pro and Diag2-VP16new, and the resulting fragments were sequenced with primer Diag-AeHex1pro. Site-specific integration into the correct *attP* site was checked using primers PB2 and respectively 3860B-5'flank1 or 3860C-5'flank1 for insertions into line OX3860B or OX3860C.

d/ Test crosses

Eggs were collected from crosses of hemizygous G_3 males from lines OX4427[B], OX4429[B], OX4436[B], OX4427[C], OX4429[C] and OX4436[C] (carrying the red eyes marker 3xP3-DsRed2) with hemizygous G_3 females from lines OX3978 or OX4499 (carrying the red body marker hr5ie1-DsRed2). The progeny from those crosses, according to Mendel's laws of inheritance, was expected to be a mixture of parental and non-parental phenotypes: red eyes, red body, wild-type, and "red eyes with red body" individuals, in equal proportions.

Eggs were hatched on day 1. On day 2, "on tet" and "off tet" trays (11 x 19 cm bottom surface) were set up with 300 larvae in 300 ml of pure water (1 larva/ml), respectively with or without a supplement of 30 µg/ml chlortetracycline hydrochloride (Sigma-Aldrich, Gillingham, UK). Larvae were fed finely grinded TetraMin® as follows: 12 mg/tray on days 2, 3 and 17; 24 mg/tray on days 4, 9, 11 and 12; 48 mg/tray on day 5; 96 mg/tray on days 6, 7 and 8. Pupae of different genotypes were separated by fluorescence phenotype. Sexes were also separated as pupae. Each category was transferred into recognised cages and emergence into adults was recorded.

For rearing in wells, 12 well plates (Nunc A/S, Roskilde, Denmark) were used, with or without a supplement of 30 µg/ml chlortetracycline hydrochloride (Sigma-Aldrich). Larvae were fed a solution of ground TetraMin® dispensed using a repetitive pipette.

e/ Comparative RT-PCR

Comparative RT-PCR was performed on RNA samples of 3 pooled female pupae with an AmCyan-specific primer/probe set (AmCyTaqF, AmCyTaqR and AmCyTaqprobe, Table 5-1) and an endogenous primer/probe set specific to the 18S ribosomal RNA (18StaqF, 18StaqR, 18StaqProbe, Table 5-1) which acted as normaliser.

RNA was extracted using Tri Reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and pellets were resuspended in 60 µl Tris 10mM. RNA samples were quantified on a Pharmacia Biotech (Little Chalfont, UK) GeneQuant II RNA/DNA calculator, and 0.5 µg were treated with DNase I (Roche, Burgess Hill, UK) in a final volume of 11.5 µl. cDNA was synthesised using the RevertAid™ First Strand cDNA Synthesis Kit from Fermentas (random hexamer primers), according to the manufacturer's instructions. A mastermix was prepared with 2.5 µl of each primer (10 µM), 0.625 µl of each probe (10µM), and 0.25 µl of pure water and 12.5 µl of TaqMan® Gene Expression Master Mix (Applied Biosystems, Warrington, UK) per reaction. Each reaction was set up with 24 µl of mastermix and 1 µl of cDNA. Each sample was analysed in triplicate.

Comparative RT-PCR reactions were performed on a Mx3005P thermal cycler (Stratagene, La Jolla, CA, USA) with the following programme: step 1 - 2 minutes at 50°C; step 2 – 10 minutes at 95°C; step 3 - 15 seconds at 95°C; step 4 - 1 minute at 60°C; cycle steps 3 and 4 fourty times. Data were analysed using the MxPro-QPCR software (Stratagene).

3. Results

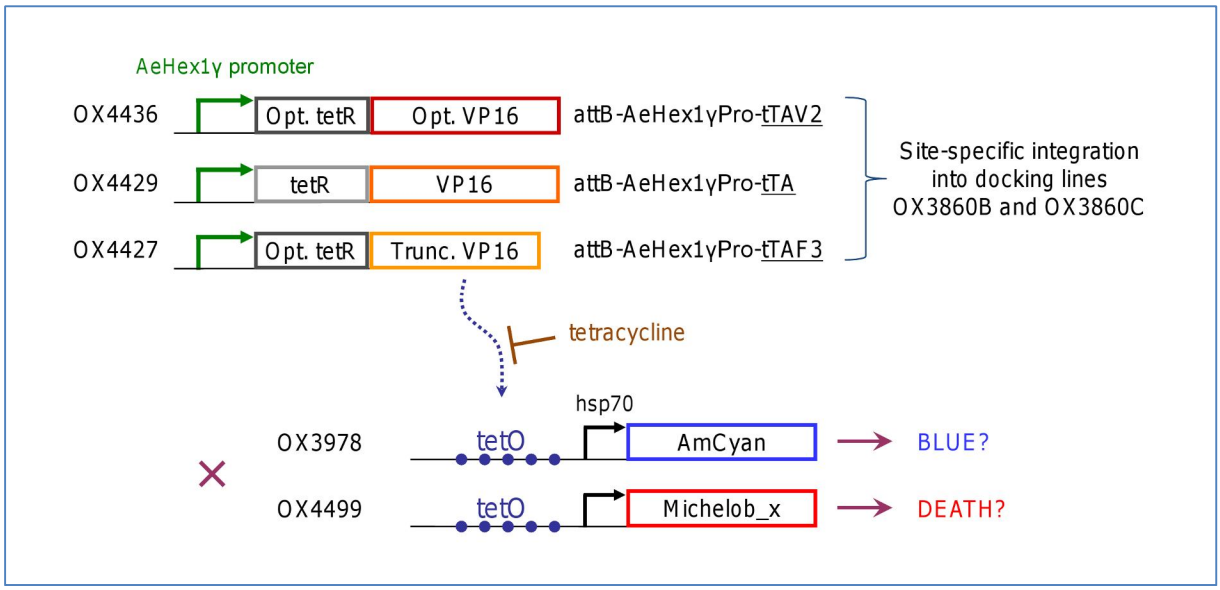

Figure 5-1. Experimental design for direct comparison of the tTAV2, tTA and tTAF3 transactivators.

The transactivators tTAV2 (optimised tetR + optimised VP16), tTA (original tetR + VP16) and tTAF3 (optimised tetR + truncated VP16) were placed under the control of the *Aedes aegypti* Hexamerin-1γ promoter (AaHex1γPro). The corresponding constructs, respectively OX4436, OX4429 and OX4427, were inserted site-specifically into lines OX3860B and OX3860C using the *attP*/*attB* system. Resulting transgenic lines were crossed individually to the OX3978 and OX4499 effector lines. Efficacy and repressibility of each transactivator was assessed by comparing levels of AmCyan expression in crossed progeny and their survival off and on tetracycline.

The transactivation capacity of tTAV2, tTA and tTAF3 were compared as described in Figure 5-1. Each transactivator was placed under the control of the Hex1γ promoter, expected to induce expression in late developmental stages. Corresponding constructs, OX4427 (Hex1γ-tTAF3), OX4429 (Hex1γ-tTA), and OX4436 (Hex1γ-tTAV2), all carried the 3xP3-DsRed2 (red eyes) transformation marker and an *attB* site. In order to negate position effects, these constructs were inserted site-specifically into the *attP* docking sites from lines OX3860B and OX3860C described in Chapter 2. Six transgenic lines were generated: OX4427[B], OX4429[B], OX4436[B] (insertions into OX3860B line), OX4427[C], OX4429[C] and OX4436[C] (insertions into OX3860C line).

Each transactivator line was crossed to tetO-AmCyan (OX3978) and tetO-Michelob_x (OX4499) effector lines, both carrying a hr5ie1-DsRed2 (red body) marker. Progeny was reared off and on tetracycline (as described in the methods section) to observe both the efficacy and repressibility of each transactivator. Pupae carrying both a transactivator and an effector construct were identified as expressing DsRed2 both in the eyes and the body.

a/ Crosses to tetO-AmCyan (OX3978)

Progeny from transactivator lines crossed with tetO-AmCyan and carrying both constructs (i.e. with red eyes and red body) were screened under a cyan filter to observe induction of the AmCyan reporter gene. In the absence of tetracycline, AmCyan expression was observed in L3, L4 and pupa stages from crosses to OX4427[B] OX4427[C], OX4436[B] and OX4436[C], with OX4436[B] being brightest and OX4427[C] dimmest (Figure 5-2). This indicates that tTAF3 is functional and weaker than tTAV2, and that the *attP* insertion locus of line OX3860B is more transcriptionally active than that of line OX3860C. When reared on tetracycline, AmCyan expression was significantly reduced but still noticeable. In the absence of tetracycline, none of the progeny from crosses to OX4429[C], and only some of the progeny from crosses to OX4429[B] showed visible levels of AmCyan expression, precluding tTA as an alternative to tTAV2.

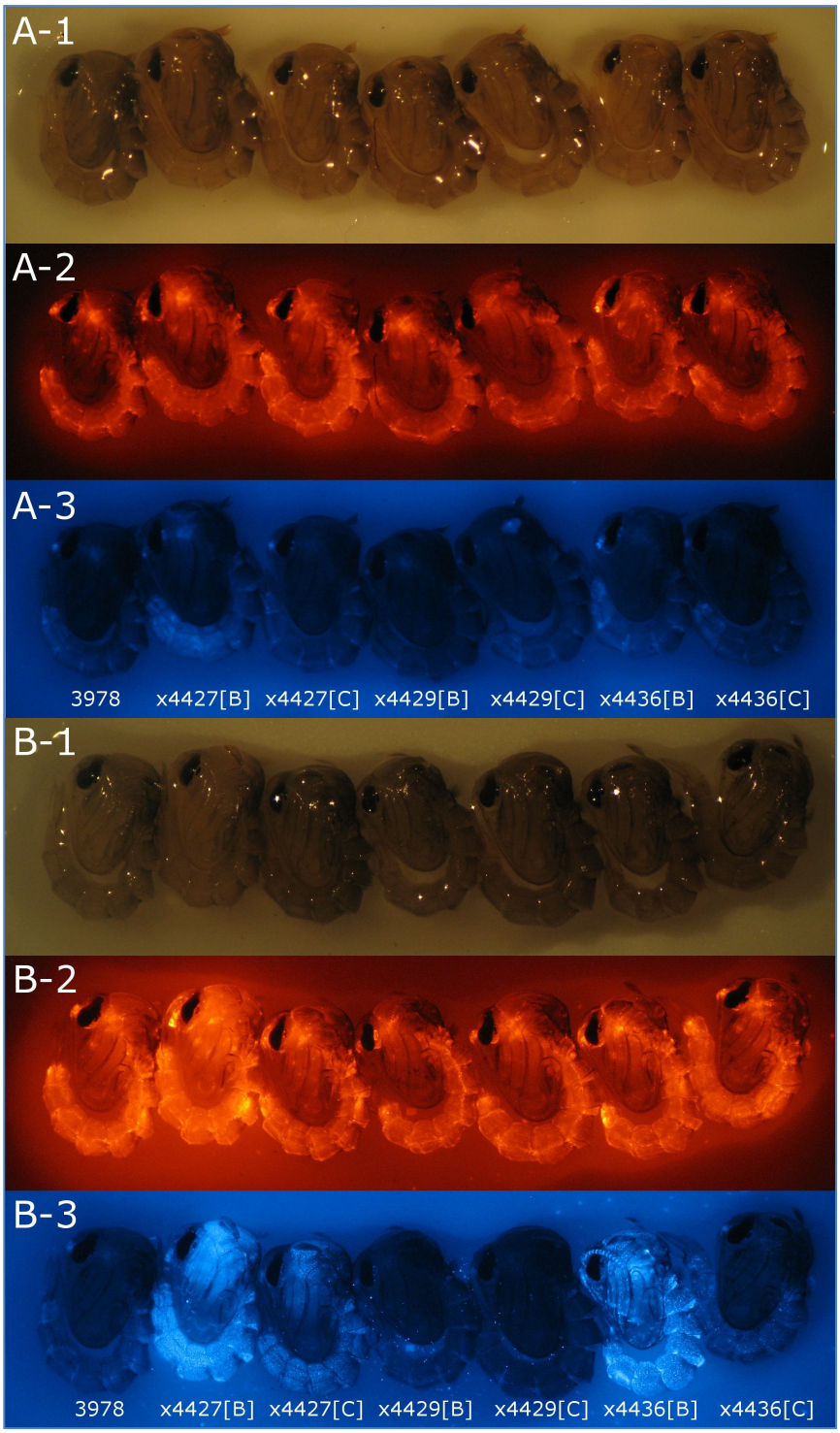

Figure 5-2. Pupae of the tetO-AmCyan reporter line (OX3978, marker hr5ie1DsRed2), alone or crossed to Hex1γ-tTAF3 (OX4427[B] and [C], marker 3xP3-DsRed2), Hex1γ-tTA (OX4429[B] and [C], marker 3xP3-DsRed2), and Hex1γ-tTAV2 (OX4436[B] and [C], marker 3xP3-DsRed2).

A: reared On tetracycline; B: reared Off tetracycline; 1: white light; 2: red filter; 3: blue filter.

A pool of three female pupae from each type of cross, carrying both the transactivator and the tetO-AmCyan constructs, were harvested from two separate hatching events. Both sets were analysed by comparative RT-PCR in order to compare AmCyan expression levels, using the OX3978 sample (tetO-AmCyan without transactivator) as calibrator. Despite differences in expression levels between the two sets of samples, possibly due to pupae being harvested at different ages, results from both sets confirmed previous observations that tTAF3 is a weaker transativator than tTAV2 (Figure 5-3).

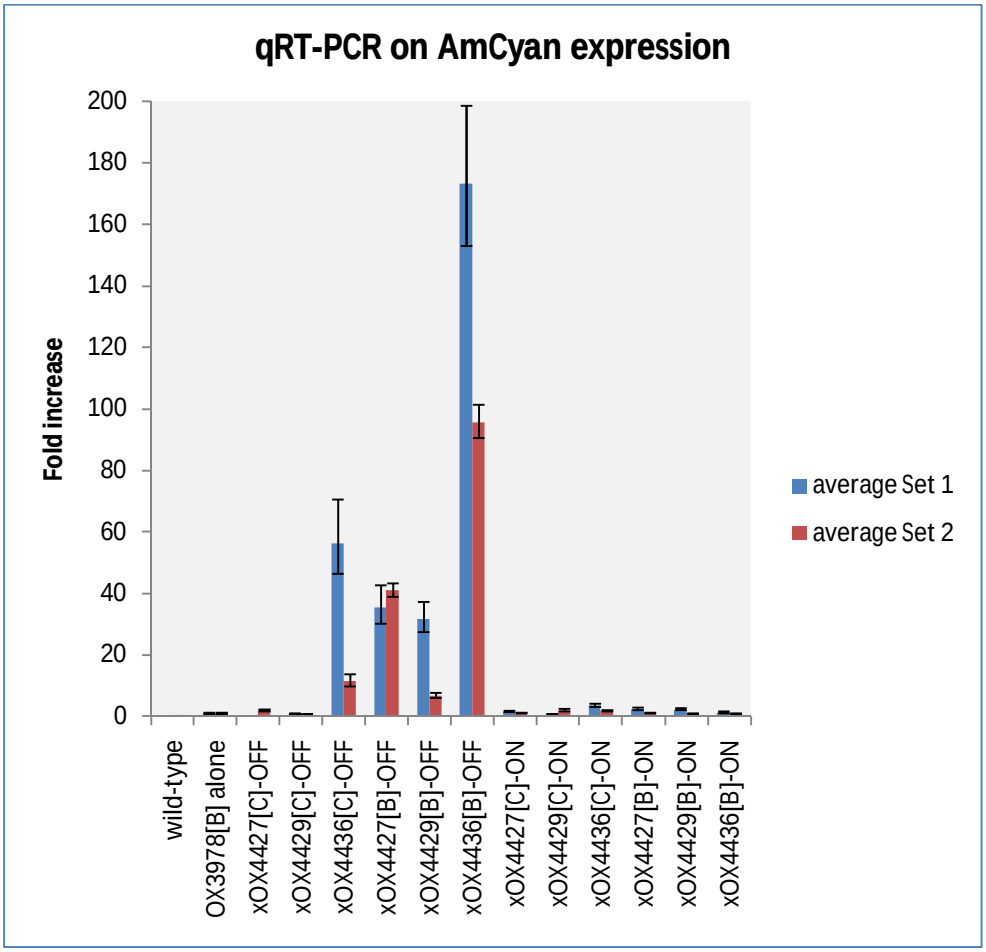

Figure 5-3. Comparative RT-PCR on AmCyan expression from the tetO-AmCyan reporter line (OX3978) crossed to Hex1γ-tTAF3 (OX4427[B] and [C]), Hex1γ-tTA (OX4429[B] and [C]), and Hex1γ-tTAV2 (OX4436[B] and [C]), when reared On and Off tetracycline.

Uncrossed OX3978 sample was used as the calibrator. Error bars are the standard error of the average fold increase (based on the standard deviation of the $\Delta\Delta C_T$ values). Each sample contained 3 female pupae.

b/ Crosses to tetO-Michelob_x (OX4499)

Progeny from transactivator lines crossed with tetO-Michelob_x were screened as pupae and numbers from each phenotype were recorded, as well as the numbers of adults eclosed (Figure 5-4). In accordance with previous results from the tetO-AmCyan crosses, insertions into OX3860C showed weaker phenotypes than into OX3860B, and the transactivator ability of tTA appeared insufficient. In the absence of tetracycline, only 6 individuals carrying both OX4427[B] (tTAF3) and OX4499 survived to pupa stage, compared to 60 pupae carrying OX4499 only; none of the individuals carrying OX4436[B] (tTAV2) and OX4499 survived to pupa stage in the absence of tetracycline, compared to 45 pupae carrying OX4499 only. In the presence of tetracycline, 49 individuals carrying OX4427[B] and OX4499 survived to pupa stage, compared to 56 pupae carrying OX4499 only; 20 individuals carrying OX4436[B] and OX4499 survived to pupa stage, compared to 56 pupae carrying OX4499 only.

In order to determine the time of death of individuals carrying both constructs and not surviving to pupa stage, some progeny from the OX4427[B], OX4429[B] and OX4436[B] crosses to OX4499 were screened as L1 and individuals with both red eyes and red body, i.e. carrying both the transactivator and tetO-Michelob_x constructs, were reared in individual wells. As a control, some individuals carrying only a transactivator constructs or OX4499 were also reared in wells. The stage of death was recorded and results are presented in Figure 5-5. The OX4499 construct induces significant toxicity by itself, with only 46% survival to adulthood. The transactivator constructs alone appeared largely innocuous, with around 90% survival to adult. In the presence of tetracycline, the survival of OX4429[B] crossed to OX4499 was quite similar to OX4499 alone, but the survival to adult was not much lower in the absence of tetracycline despite an overall earlier time of death. In the absence of tetracycline, all of the individuals carrying both OX4499 and OX4436[B] died before reaching L3 stage, whereas 8 out of 52 individuals carrying OX4499 and OX4427[B] survived to pupa stage, with 4 reaching adulthood. Tetracycline seemed to generally delay the time of death, with 28 out of 53 individuals carrying OX4499 and OX4436[B] surviving to L4 stage (4 reaching adulthood), and 19 out of 36 individuals carrying OX4499 and OX4427[B] surviving to L4 stage (10 reaching adulthood).

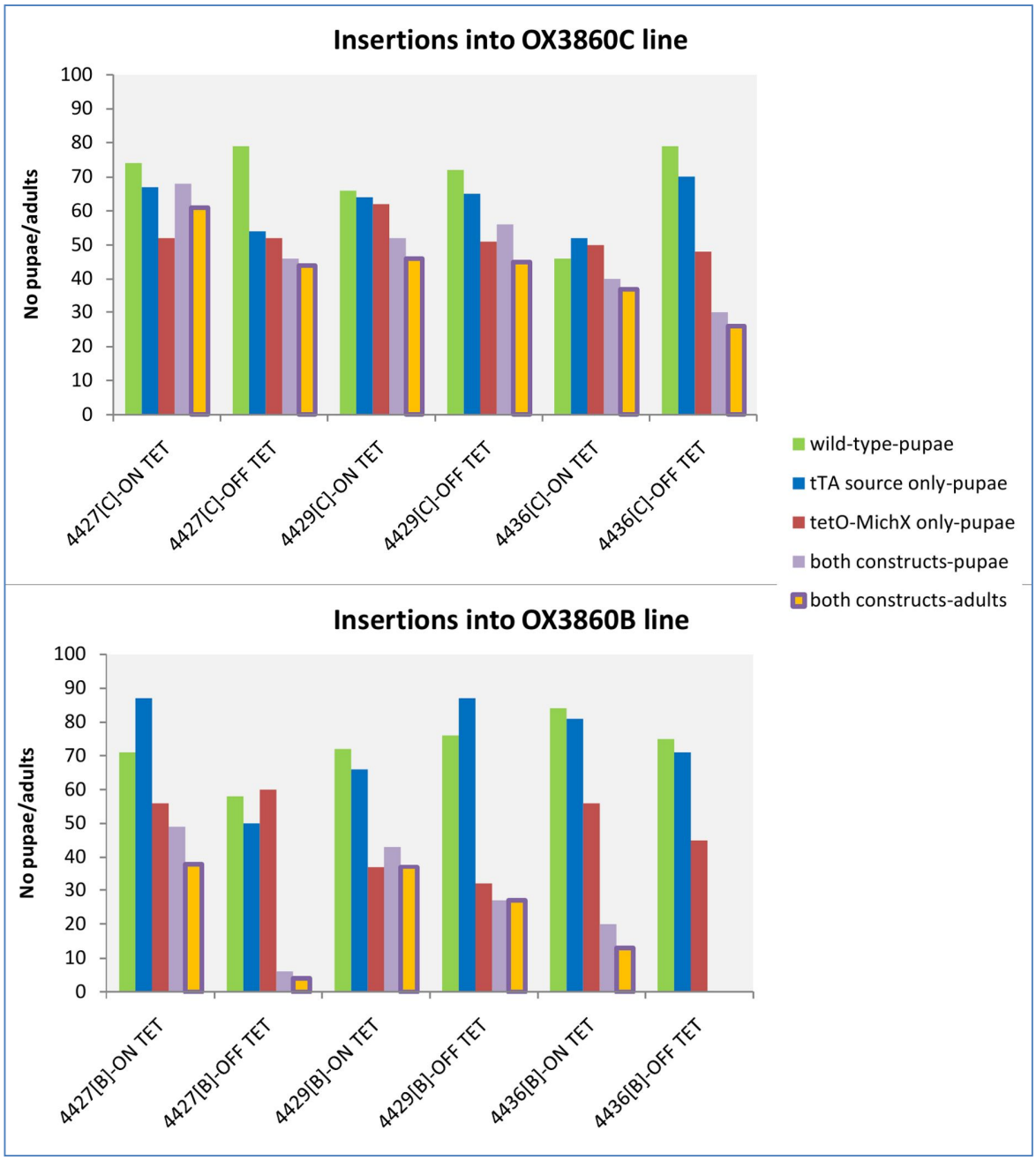

Figure 5-4. Progeny from crosses between OX4499 (tetO-Michelob_x) and OX4427 (Hex1γ-tTAF3), OX4429 (Hex1γ-tTA) and OX4436 (Hex1γ-tTAV2) constructs inserted site-specifically into OX3860B ("[B]") or OX3860C ("[C]") *attP* docking lines.

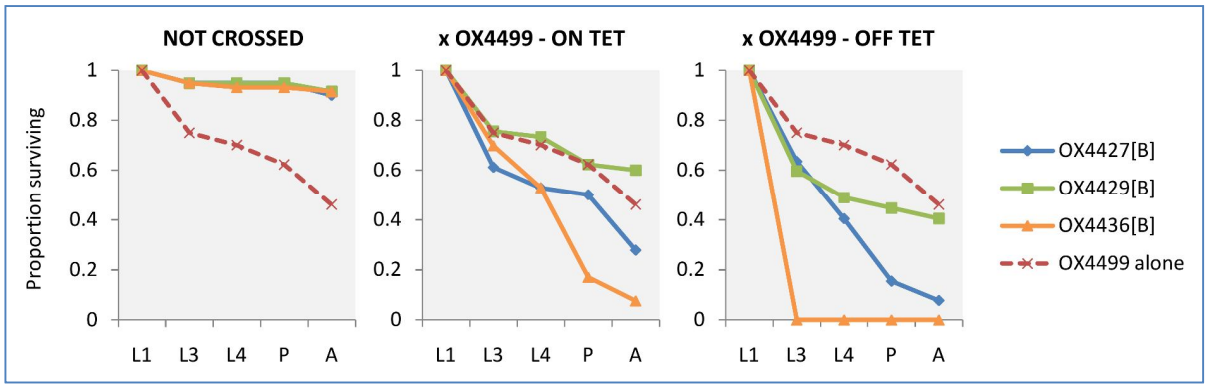

Figure 5-5. Proportion of progeny from transactivator lines OX4427[B] (tTAF3), OX4429[B] (tTA) and OX4436[B] (tTAV2) crossed to the tetO-Michelob_x effector line (OX4499) reaching L3, L4, pupa (P) and adult (A) stages, compared to OX4499 alone.

Individuals were screened at L1 stage and reared individually in 5 ml wells. n=204 for OX4499 alone; n=60, 59 and 59 for OX4427[B], OX4429[B] and OX4436[B] not crossed, respectively; n=36, 45 and 53 for OX4427[B], OX4429[B] and OX4436[B] x OX4499 ON TET, respectively; n=52, 47 and 60 for OX4427[B], OX4429[B] and OX4436[B] x OX4499 OFF TET, respectively.

4. Discussion

Even though the levels of expression in the OX3860C docking site were too low to induce a lethal phenotype when crossed to tetO-Michelob_x, the tTAF3 and tTAV2 transactivators did allow the production of visible levels of AmCyan. The tTA transactivator, on the other hand, didn't, and also showed low and inconsistent activity in the OX3860B docking site compared to tTAF3 and tTAV2. Those results suggest that the optimisation of the tetR sequence is critical in *Ae. albopictus*, an unexpected result considering that tetR originates from *E. coli* and retains its function in mammalian cell lines (Gossen and Bujard, 1992) and *Drosophila* (Thomas et al., 2000). However, the need for optimisation of the tetracycline-repressible system arose before this work was initiated and indeed the RIDL constructs used in *C. capitata* and *Ae. aegypti* contained not tTA but tTAV, which holds the optimised tetR sequence found in tTAF3 and tTAV2 (Fu et al., 2007; Gong et al., 2005; Phuc et al., 2007).

The tTAF3 transactivator appeared effective yet weaker than tTAV2 in both the OX3860B and OX3860C docking sites, as indicated by the levels of expression of AmCyan and the survival of the tetO-Michelob_x crosses. The better repression by tetracycline is a particularly attractive

feature as it would permit the creation of healthier RIDL lines of *Ae. albopictus*. On the other hand, tTAF3 failed to produce a fully penetrant phenotype when crossed to the OX4499 effector line, with some individuals able to reach adulthood. Phenoype penetrance off tetracycline is a key safety requirement for RIDL lines and tTAF3 may possibly be too weak an alternative to tTAV2. It would be interesting to see how tTAV compares to tTAV2 and tTAF3 in the same conditions and this will certainly be done in the near future.

The phenotypes observed here relate only to the OX3860B docking site and the Hexamerin-1γ promoter; levels of tTAF3 expression in a different locus or under the control of a different promoter may lead to a fully penetrant phenotype. Under the very strong Actin-4 promoter, for example, tTAF3 may perhaps be a good choice of transactivator. Having a range of transactivators to choose from would allow the development of more finely tailored phenotypes in *Ae. albopictus*.

Chapter 6

Summary and Conclusions

In view of the significant re-emergence of dengue fever in large parts of the world and traditional control methods failing to suppress the disease (see Chapter 1), it has been necessary to develop new strategies aimed at vector control. In response to this need, *Ae. aegypti* have been genetically engineered for use in SIT-like control programmes. This novel technology, called "Release of Insects carrying a Dominant Lethal gene" or RIDL®, proposes to replace the need for irradiation by engineering tetracycline-repressible sterility in insects using a dominant lethal system (Alphey, 2002; Alphey and Andreasen, 2002; Alphey et al., 2010; Alphey et al., 2008; Thomas et al., 2000) controlled by the Tet-Off system (Gossen et al., 1994; Gossen and Bujard, 1992). Two RIDL strategies have been applied to *Ae. aegypti*: the late-acting bi-sex lethal phenotype (Phuc et al., 2007), and the female-specific flightless phenotype (Fu et al., 2010).

The secondary vector of dengue, *Ae. albopictus*, is expanding its geographic range and recently caused important chikungunya outbreaks in the Indian Ocean islands (see Chapter 1). This species is therefore perceived as an important public health threat and current control methods have proved inadequate for controlling it. This thesis focuses on the development of transgenic strains of *Ae. albopictus* for vector control using the RIDL technology.

The first step was to ensure the feasibility of the gene transfer technology in this species. Successful transformation of *Ae. albopictus* was achieved using the *piggyBac* transposable element (Chapter 2). Rearing *Ae. albopictus* in the laboratory, however, proved more demanding than doing so with *Ae. aegypti*, especially post-injection rearing which involves blood-feeding small pools of females on artificial membranes.

The site-specific integration technique allows the comparison of transgenes in the same genomic locus, avoiding variations in expression caused by position effects. This system was shown to function in *Ae. albopictus* (Chapter 2) using the PhiC31 integrase, which catalyses recombination between *attP* and *attB* sequences (Thorpe et al., 2000). It was also exploited to compare expression of different transgenes, with a view to tailoring the RIDL components to *Ae. albopictus* (Chapter 5).

The potential control tool investigated in this thesis is the tetracycline-repressible female-specific flightless phenotype, as recently developed in *Ae. aegypti* using the promoter and sex-specific splicing sequences from the *Ae. aegypti actin-4* gene (Fu et al. 2010). The desired phenotype was engineered in *Ae. albopictus*, using the regulatory regions from both the *Ae. aegypti* and *Ae. albopictus actin-4* genes (Chapter 3). One strain, OX3688A, has been made homozygous for the transgene insertion to assess its suitability for a RIDL control programme (Chapter 4). Although the high phenotype penetrance observed in OX3688A suggest that it would be safe to release, there may be some limiting features, some of which may not be specific to this particular strain:

1/ The low hatch rate observed for OX3688A, which seems to reflect a weakness of the genetic background rather than a direct consequence of transgenesis, implies that this strain has low productivity in rearing and would be expensive to mass-produce for a potential release programme. This may be a significant issue, considering that any deficit in males' mating competitiveness would have to be compensated for by releasing larger numbers. We have recently started working with a new, potentially fitter, wild-type strain to use as background for future transgenic lines.

2/ The unavailability of standard PCR genotyping means that controls for maintaining a transgene-homozygous colony are not straightforward. Unfortunately, this seems an unavoidable downside of transposon-mediated germline transformation. Insertions into coding regions can cause prohibitive fitness losses, making insertions into non-coding genomic regions more desirable. However, these areas can tend to be repetitive or polymorphic, and therefore often not amenable for PCR genotyping. For this reason, it could be helpful to have a set of well-characterised docking lines (i.e. carrying an *attP* site at a known genomic location) with flanking sequences known to be suitable for PCR genotyping, where RIDL constructs could be inserted in a site-specific fashion. However, this approach would require subsequent removal of the antibiotic resistance gene and bacterial origin of replication from the inserted construct.

3/ Once the strain was made transgene insertion-homozygous, backcrossing appears to have caused strong selection for healthier females, i.e. the females with a weaker phenotype (i.e. less detrimental) in the presence of tetracycline. Although this process improved the rearing efficiency of the strain, with more females able to contribute to the next generation, it seems essential to ensure that the females remain 100% flightless off tetracycline. If this strain was to be released in the field, regular and stringent phenotypic controls should be implemented every couple of generations to ensure that the number of flying females is maintained at an acceptably low level. In order to limit this selection process, the tetracycline rearing concentration of the OX3688A strain has been increased from 30 to 60 µg/ml. According to the results presented in Chapter 4, this concentration offers better repression of the flightless phenotype without significantly slowing down development. It might be advisable for all RIDL strains to be reared at a tetracycline concentration that reduces such selection pressure, at least in the filter colony (i.e. a closely controlled 'mother colony' that feeds into mass-rearing (Dyck et al., 2005; Fisher and Caceres, 2000)). With the female-specific RIDL approach, the release generation would be reared off tetracycline in order to create flightless females; the released males would therefore not be affected by any possible detrimental effect of such tetracycline concentrations.

On the other hand, the loss of *Wolbachia* observed in the transgene insertion-homozygous strain due to continuous tetracycline rearing (Chapter 4) confers an extra level of safety: if some transgenic females were able to fly in the absence of tetracycline, their mating with wild males would not yield progeny as a result of Cytoplasmic Incompatibility. They would, however, still be able to bite and transmit disease, and be compatible with co-released RIDL males.

The RIDL construct based on the *Actin-4* gene (either from *Ae. aegypti* or *Ae. albopictus*) and the tTAV2 transactivator generally seemed too toxic in *Ae. albopictus*, with sub-optimal repression by tetracycline (Chapter 3). Reducing the efficiency of the transactivator was one possible way to decrease this toxicity, and the tTA and tTAF3 transactivators were compared to tTAV2 in a site-specific approach (Chapter 5). In order to facilitate the comparison, the transactivators were driven by the promoter from the *AaHex-1γ* gene (Gordadze et al., 1999),

active in the fat bodies and therefore expected to induce more obvious phenotypes than the indirect flight muscle-specific *Actin-4* promoter (Muñoz et al., 2004). The tTA transactivator appeared largely ineffective in *Ae. albopictus*. The tTAF3 transactivator was effective yet significantly weaker than tTAV2; possibly too weak to be used as an alternative to tTAV2 in a RIDL context, which demands near-complete phenotype penetrance in the absence of tetracycline. It will nevertheless be tested in an *Actin-4* context in the near future, but another tTAV2 alternative, tTAV, will also be investigated as a possible intermediate between tTAV2 and tTAF3. This experiment demonstrates the usefulness of the phiC31-mediated site-specific integration technique.

Even though the female-specific approach has several advantages (discussed in Chapters 1 and 4), it may also be associated with regulatory hurdles and poor public acceptance, as the released males would not be effectively sterile (their male offspring would survive in the wild). A late-acting bi-sex lethal phenotype has indeed been engineered in *Ae. aegypti* using the RIDL technology (Phuc et al., 2007), but the late-acting phenotype of the OX513A strain is mainly attributable to the genomic surroundings of this particular insertion site. The OX513 construct used to produce this strain is not specifically designed to confer late-acting lethality, and no similar lines have been produced in *Ae. aegypti* despite extensive injections of OX513 and similar constructs. The creation of *Ae. albopictus* strains with a phenotype similar to OX513 would therefore require identification of a late-acting bi-sex promoter. The *AaHex-1γ* promoter mentioned above was reported as conferring late-acting and bi-sex expression in *Ae. aegypti*, and may therefore be a good candidate to create OX513-like strains. Even though this promoter did not show a specifically late activity in the experiments presented in this thesis (Chapter 5), it seems worth investigating the expression pattern under different position effects. Alternatively, an *Ae. albopictus* homologue to *AaHex-1γ* may offer a solution to this problem. The combination of a late-acting promoter expressing in essential tissues, such as *AaHex-1γ*, with the sex-specific splicing elements of *Actin-4*, may also provide a lethal alternative to the female-specific flightless phenotype.

The successful application of the RIDL technology in *Ae. aegypti* suggested that its transfer into *Ae. albopictus* would be fairly straightforward. Nevertheless, some unforeseen challenges arose from substantial rearing hurdles and apparent genomic differences.
Despite their demanding blood-feeding habits slowing the creation and maintenance of new transgenic lines, recently colonised genetic backgrounds might be preferred for RIDL transformations in order to limit genetic differences with the future target population which might impair males' behaviour and mating competitiveness. The use of live animals as blood sources rather than artificial membranes would likely improve egg production, but is associated with undesirable and impractical aspects. Conceivably, a more lab-adapted background may provide high enough production levels to counterbalance the associated males' deficiencies.

It may be possible to overcome the sub-optimal performance of female-specific RIDL constructs in *Ae. albopictus* by tuning the RIDL components for this species, a process greatly facilitated by the availability of the site-specific integration system.

The recent open-field releases of the *Ae. aegypti* OX513A strain in Grand Cayman confirmed the potential of RIDL for mosquito control, and several *Ae. aegypti* control programmes may soon occur across the world. In places where *Ae. aegypti* and *Ae. albopictus* co-habit, pressure on *Ae. aegypti* populations might allow *Ae. albopictus* to thrive. The development of an effective *Ae. albopictus* RIDL strain is therefore urgent in order to prevent further spread of this highly opportunistic species.

Future such work with *Ae. albopictus* should include the comparison of the tTAV transactivator with tTAV2 and tTAF3 in a site-specific context, followed by the development of repressible female-specific flightless strains using a weaker transactivator under the control of the *Actin-4* regulatory regions. Candidate strains for release programmes should be selected not only according to their phenotype, but also to the genomic sequences surrounding the transgene insertion. A reliable and straight-forward PCR genotyping protocol would greatly facilitate the selection of homozygous individuals, allowing the generatin of homozygous strains from a greater number of founder individuals. PCR genotyping also ensures a reliable quality control

for the filter colony in mass-rearing. The potential of the *AaHex-1γ* promoter, or its *Ae. albopictus* homologue, to drive bisex late-acting lethality should be further investigated as an alternative to the female-specific RIDL approach. Furthermore, the combination of the late-acting *AaHex-1γ* promoter with the sex-specific splicing properties of *Actin-4* would potentially drive late-acting female-specific lethality rather than flightlessness. The effectiveness of candidate RIDL strains relies on their mass-rearing productivity and male field performance, which should be tested promptly. But safety should remain the central concern: RIDL strains should be maintained on high, non-selective tetracycline concentrations (to prevent selection against the desired phenotype) and regular off-tetracycline phenotype checks should be conducted.

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Appendices

Appendix 1.

Full flanking sequences of integration sites of OX3860 into *Aedes albopictus*.

Genomic sequences flanking the *piggyBac* insertions of OX3860 lines derived from inverse PCR. The canonical TTAA insertion site is double-underlined. Annealing sites for the primers used to investigate second phase integration into lines OX3860A, B and C are underlined.

Strain	5´ flanking sequence – TTAA insertion site – 3´ flanking sequence
OX3860A	TTAATCAACTCAACGTACATATGTACAGAGGGATACATCATTAGTGAAATTACGAAAGAATCCACAGCTCAGG TGAGATTTGAACTCACGACCCTTTTACGCTAGACAAGTGCTTTTCCAACTAAGCTACCGAGCTAATTAATGACA AATGACATGGCATTTTGGTTGGTACAAGCTAATTCAAATCTCACCGATC
OX3860B	GGCCGCCAGATCTTCCGGATGGCTCGAGTTTTTCAGCAAGATTCTTGACCTTGCCACAGAGGACTATTAGAGG TAAGAATAAACATTGTTGGTCAACTTCAAAGTCCACGAGGCGTAGCCGAGTCTCTGCACTGAACATTGTCAGA TCTCAAAGAGGTCCGACGCGTATGTGCCGCAATATATATGACCCACTTTTATGCTTCAAACTATTTTTTACTGAT GAGATAATTTCGGAAATTGTAAAATGGACAAATGCTGAGATATCATTGAAACGTCGGGAATCTATGACAGGTG CTACATTTCGTGACACGAATGAAGATGAAATCTATGCTTTCTTTGGTATTCTGGTAATGACAGCAGTGAGAAAA GATAACCACATGTCCACAGATGACCTCTTTGATCGACACTCCTTTGTGACTTGGGTGCGGCTTGGGTTGGGCTG AGAACAAGATGGCGATTCTAGGAGTTCTTTTAGAGGTTTCTTCAAGAATTACTCCAGAGATTCTCTCAGGAATA CACCAAATATTTCTTCTGGAATCCATCAAGGGGATTCTTAAGGAATTCTTTCTGAAATTCCATCAAGATTTCATT CTCCAAAAAACCTCTAATGGAAGCTTCAGAAATTCTAACAAGCTATCCAGGGCTTTAGCAATTTCTCTCCTTTGG CAGACACCTGCATACGGTGCGCACAAGCTTAGAGGTACTTTAATCCAAGCAGACAACCGAAATGATAGGTAAC ATTGTCAACGAAAGATAGATGATAACGAACACGAGCTGACACAAACTTCAACGAACGTTTAGTTTAACTCGAA ATTAATTAAGTTAAAGATAAAGAAAGTTTTAACGCTAATTCTTTTTTGAATATCAGGTATATCATATTATAAGGA ATACGCCTTGAAGCTAGATGCGAAATTGGCC
OX3860C	TCGATATTTGTATGGAAAAATCGCCGATTATTATTTAGGTATTGCCATACCTGATGTCATTATTCACGCGTTATG CACAGAATACACACCAGTTATTATTTTAGATATTTTACCTCTTATGCAGGGCTACTTAATACCTCATTCAGGTTG TAGGTATTCGGTTTTCCATACCTGAGTTAGTTATTCTTCAGCTATTTTCTCCTGCTCGGGTGAGGATATCCAAAA TGCATTAAGGACAAGCTTGTTGGTATCCAAAGATGGGCGCCACAATGGAACCATGAAAACTTAAACCAGCGCA CCGCACCTTCTTATGACAAATGAGCAAGAACAAACTAAGAATTACACTATTGCCTGTTGTTTACATTTTAAGATT TGTGCATTTGAACGGTGTATTGTTGAACTGGGATTCAAAGAGAGTTGACCTTAGTTTCCGAATTTCTATTGAGC CTGACGTGACTAGATAACCCTTAAGGAATGAGTAACTCTTGGTAGGATC
OX3860D	TCGATTTTATTCTCTGTCGCATTCGCTCCGAAGATAATTTATTTCAAGGCACACTTAGGTGCCAGCCAGAGTGG ACGCGTCACGCGGCGCGGCGCGGCGCAATGCGGCATTTGACAGGTCGCGCGGCGACGCGCGGCAGAACTCC GTTCATTGGAATACAGGGAAAACAATCATAGCATGCCAGAGTGCGCGCGGAACGATGCGAAGCGGAATGCGT TTTGCCGCGCGGCGCGCGCCAGAACAGTGCATGCACTGTTTTTGATGCGAAATTCGTGCGTTTCTTGTTTGTTT ACCTCGGTGATTCTCAAAGTTGTAATTGAAATGGCAGAGGAAAAAAAAGAAATTGAGCAGCTAATCGGGTCC

	GTATTTTTACGGAAAGAGTTGTGGGATCAAACTTCTCGGGGATACCGAAACAGAGTGTTGGTGGACAATTGTT GGAAGGAGCTTGCCGAAGAATTCAAAGTATCTGGTGAGCATCTCATTTTACTAACACAAAATTAGTATTAACG TCATTCGTTTTGCAGAAGATTTTTTGAAGAAAAAGTGGAAGAGCCTGCGGGACAAATATGGAAAGATTTTGAG GAATCTTCCCGTATCAAGGTCCGATGAATATCGTACTCTCTGAATTCACCCAAAAAATATCTTCGAACGCCTTCA GACATGAAATATTCATCATCTGAATCCATTTCGGCAACTTTAATGTTCAAATACGATGAAACAATTGGTTTGCTT TTGTTTTGAACCCGCACACTTTTGCTTCGCATCAAATAGGCCTTTTCAGTTGACAGGTCCGTGTATAGAGATAA GTGACATTTCAACACACGAACACTAGCGCGAATTTTTCCTCACACAAAAATGCACGCCAATTCAATGGCTACTC AGATTGCATATGCAAATATTACCCAATCGA
OX3860F	TCGACAGACTTGTCTGAACTTCGCAGGTACGCCATCTGGAACTTCCATGTAGATTGTTTCGTTTAAACGTCCGT GAAATAGTATCGCCGATGTACACCGACTGGCAAAACGGTCCTGATCGTAGCCAGCCGAGCTACCTGGGCGTA GGTCTTGTCCTTGCTTTTGGATGTATCCTTTCGCCACAAGCCGAGTTTTGTAGCGAACCGGTCTGCCGTTTTCAT CTTCCTTTATCCAATACACCCATTTGGATTTGAGAGGTTTCACCCTAAGACAGGTAGCCAGCTGCCAGACGTCG TTTTTCTTCAGCGACATTGACCGATAGAGCTGCCGTTGGACTCCGGTTTCATAGGTAGGTACATACCATGGTAT ACAAGTCAACGCAGCACCACATCCTTTTCGGTAGCCGAGAATCCAGAAGCATTGCACGAACCTTTTCGATGAG CGTACAATTAAACCTCTCTGCCAATCCATTCTGTTGTGGAGAATACGCAACCGTCGCTTTAATCTGGAATTCTTG CGCTTTGTACCAGTTTTTCTGATTGTTCGAGCCATACTAAGTACATTGATCCACCGTAAGTTTTGAAATATTCTT ACCGAATGCTGCTGTCGCCATCGGTTCATATTCACGAAACCGTTCAAAAACTTGAGACTTCTTTTTCATCAAGTA TATTACCGAGAAATGGCTATAATCATCAATAAACGAAATGAAATAGCGGGAGCCATCCCACGATAGAGGATC GATTGGCC

Appendix 2.

Clustal alignment of *Ae. aegypti* (Aeg) and *Ae. albopictus* (Alb) *Actin-4* cDNA sequences.

Positions of introns are marked with a vertical line, translation start and stop are underlined, the male specific exon is shown in italics.

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AlbAct4cDNA      -ACAATCGGATTTCGACGTTCGCTCTGGTGCAGTTCGATACGGTCTAGTGATTAGTCAAG 59
AegAct4cDNA      CACAATCGGATTTTGACGCTCGCTCTGGTACAGTTCGATACGGTCTAGTGA--AACCGAG 58
                  ************ **** ********** ********************  *  * **

AlbAct4cDNA      GACCCAACGGCTCAAGTTTTTTCCCTCTTTGATCCAG|GTCGGTGCGTTGTGGTCGGCGGT 119
AegAct4cDNA      GAT--AACGACGAAGGTTTTTCCCCA--TTGATCCAG|GTCGGTGT-TTGTGATTGGTGGA 113
                 **   **** *  * ****** ***   ***************  ***** * ** **

AlbAct4cDNA      GATAAGTGGAAAAGTTGTGAAAAGT-CTACC--ATCCGT-GGAAATGTGCCGTCCTGCTG 175
AegAct4cDNA      AA------AAGAGCTCGAGAAAAGTTCCATCGAAGCCGTTGGAAATGTGCCGTCTTCCTG 167
                  *       * *  * * ******* * * *  * **** ************** * ***

AlbAct4cDNA      TGACGTCCTGTGGAGTCCGTCCGTTGTCTTCGTCTGGTTATGATGTGAAATGTGCTGTCC 235
AegAct4cDNA      TGATGTCGTGTGGATCCGGTTCCTTGTCCACGTCTGGTGATCGTGTAAAATGTGCTGTCT 227
                 *** *** ******  * ** * *****  ******** **  *** *************

AlbAct4cDNA      TGTGGCGTCTTGAATATGGTAGATCCTGTGAATATGACCCGACG-AACGTTGATCCCTTG 294
AegAct4cDNA      TGTGGCGTCATATGTGTTCCAGATCCAGTGATTACGATCCGATGTGATGTTGATCCCTTG 287
                 ********* *   * *   ****** **** ** ** **** *  * *************

AlbAct4cDNA      TGAACGTCTTGTGTTGTTCCATGTGTGTTGTTC----------TCAG|GACCCAACGGAC 343
AegAct4cDNA      TGAACGTCTTATCCTGTTCCGTGTGCACCATGCATAATGTCGTATTAC|GACCTAACGGAC 347
                 ********** *  ****** ****     * *           * * **** *******

AlbAct4cDNA      CTGGAAGCGGCGCCAAAATGTGTGACGACATGCAGATCTTGATGCTGGAGCACTAGTCAT 403
AegAct4cDNA      CTTGAAGCGGCGCCAAAATGTGTGACGA----------TGATGCTGGAGCACTAGTCAT 396
                 ** ************************          ********************

AlbAct4cDNA      CGACAATGGATCCGGTATGTGCAAAGCCGGTTTCGCTGGTGATGACGCCCCACGTGCCGT 463
AegAct4cDNA      TGACAACGGATCCGGCATGTGTAAGGCCGGTTTCGCTGGTGATGATGCCCCACGTGCCGT 456
                  ***** ******** ***** ** ******************** **************

AlbAct4cDNA      GCTTCCCGTCCATTGTCGGCCGCCCACGTCACCAGGGTGTGATGGTCGGTATGGGTCAGA 523
AegAct4cDNA      -CTTCCCGTCCATTGTCGGCCGCCCTCGCCACCAGGGTGTGATGGTCGGTATGGGTCAAA 515
                  ********************** ** ***************************** *

AlbAct4cDNA      AGGATGCCTACGTTGGTGACGAAGCCCAATCCAAACGTGGTATYCTCACCCTGAAGTACC 583
AegAct4cDNA      AAGATGCCTACGTCGGTGATGAAGCCCAATCGAAGCGAGGTATCCTCACCCTGAAATATC 575
                 * ************ ***** ************ ** ** ***** ************ ** *

AlbAct4cDNA      CGATAGAGCACGGTATCATCACCAATTGGGATGAYATGGAGAAGATYTGGCATCAYACCT 643
AegAct4cDNA      CCATAGAGCACGGTATCATCACCAACTGGGATGATATGGAGAAGATTTGGCATCACACCT 635
                 * ********************** ******** ************ ******** ****

AlbAct4cDNA      TCTACAATGAGTTGCGAGTGGCTCCTGAAGAACATCCWGTCCTGCTGACCGAAGCTCCAC 703
AegAct4cDNA      TCTACAACGAGTTGCGAGTAGCTCCTGAAGAACATCCAGTATTGCTGACTGAGGCTCCCT 695
                 ******* *********** ***************** **  ******* ** *****

AlbAct4cDNA      TGAATCCCAAGTCCAACCGTGAGAAGATGACTCAGATCATGTTTGAGACGTTCGCTTCGC 763
AegAct4cDNA      TGAATCCAAAGTCCAATCGCGAGAAGATGACTCAGATCATGTTTGAAACATTCGCTTCGC 755
                 ******* ******** ** ************************** ** **********
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AlbAct4cDNA      CAGCTGTGTATGTTGCCATCCAGGCTGTCCTGTCCCTGTACGCCTCCGGTCGTACCACTG 823
AegAct4cDNA      CAGCTGTGTATGTTGCCATCCAAGCTGTTCTGTCCCTGTACGCCTCCGGTCGTACTACTG 815
                 ********************** ***** ************************* ****

AlbAct4cDNA      GCATTGTGTTGGATTCTGGAGATGGTGTCTCCCACACCGTCCCAATCTACGAAGGTTATG 883
AegAct4cDNA      GTATTGTTCTGGATTCCGGAGATGGTGTCTCCCATACCGTCCCAATCTACGAAGGTTATG 875
                 * *****  ******* ***************** ************************

AlbAct4cDNA      CCCTGCCACATGCCATACTCCGTATGGACTTGGCTGGTCGCGATCTGACCGACTACCTGA 943
AegAct4cDNA      CTCTGCCACATGCCATCCTCCGTATGGATTTGGCTGGTCGTGATCTGACCGATTACCTGA 935
                 * ************** *********** *********** *********** *******

AlbAct4cDNA      TGAAGATCCTGACTGAACGCGGTTACTCGTTCACCACCACCGCTGAACGTGAAATCGTTC 1003
AegAct4cDNA      TGAAGATCTTGACCGAACGTGGATACTCTTTCACCACCACCGCTGAACGTGAAATCGTTC 995
                 ******** **** ***** ** ***** ******************************

AlbAct4cDNA      GTGACATCAAGGAAAAGCTGTGCTACGTCGCCCTGGACTTCGAGCAGGAAATGCAGGCTG 1063
AegAct4cDNA      GTGACATCAAGGAGAAGCTGTGCTACGTCGCTCTGGACTTCGAGCAGGAAATGCAAGCCG 1055
                 ************* ***************** ********************** ** *

AlbAct4cDNA      CCGCCGCTACCTCGTCGTCCGAGAAGTCCTATGAACTTCCCGACGGTCAGGTCATCACCA 1123
AegAct4cDNA      CTGCCGCTACGTCTTCATCCGAGAAGTCTTATGAACTTCCCGATGGCCAAGTCATCACAA 1115
                 * ******** ** ** *********** ************** ** ** ******** *

AlbAct4cDNA      TCGGTAACGAACGTTTCCGTGCCCCAGAAGCCCTCTTCCAGCCATCCTTCTTGGGTATGG 1183
AegAct4cDNA      TCGGCAACGAACGTTTCCGTGCTCCAGAAGCCCTTTTCCAGCCATCCTTCCTGGGAATGG 1175
                 **** ***************** *********** *************** **** ****

AlbAct4cDNA      AATCCACTGGCATTCACGAGACCGTCTACAATTCCATTATGCGTTGCGATGTCGACATCC 1243
AegAct4cDNA      AATCAACTGGCATTCATGAAACGGTCTACAACTCGATCATGCGTTGCGATGTCGACATCC 1235
                 **** *********** ** ** ******** ** ** **********************

AlbAct4cDNA      GTAAGGATCTGTACGCTAACAGCGTCTTGTCCGGTGGTACCACCATGTATCCAG|GTATTG 1303
AegAct4cDNA      GCAAGGATCTCTATGCTAACAGCGTCTTGTCTGGTGGTACCACCATGTACCCAG|GTATTG 1295
                 * ******** ** ***************** ***************** **********

AlbAct4cDNA      CCGATCGTATGCAGAAGGAAATCACTTCCCTGGCTCCATCCACCATCAAGATCAAGATCA 1363
AegAct4cDNA      CTGATCGTATGCAGAAGGAAATCACTTCCCTGGCTCCATCCACCATCAAGATCAAGATCA 1355
                 * ************************************************************

AlbAct4cDNA      TTGCACCACCAGAACGTAAATACTCCGTCTGGATCGGTGGATCCATCTTGGCTTCCCTGT 1423
AegAct4cDNA      TTGCCCCACCGGAACGTAAATACTCCGTCTGGATCGGTGGATCCATCCTGGCCTCGCTGT 1415
                 **** ***** ************************************ **** ** ****

AlbAct4cDNA      CCACCTTCCAGGCCATGTGGATCTCCAAGCAGGAATACGACGAAGGCGGCCCAGGAATCG 1483
AegAct4cDNA      CTACCTTCCAAGCTATGTGGATCTCCAAGCAGGAATACGACGAAGGTGGCCCAGGAATTG 1475
                 * ******** ** ******************************** *********** *

AlbAct4cDNA      TCCACCGCAAGTGCTTCTAAGCTGAACCACCCTTTGTACTGATTACCATAAGCGACATTG 1543
AegAct4cDNA      TCCACCGCAAGTGCTTCTAAGCCGATC--CCGATTGTACTGATTACCATAAGCGACATTG 1533
                 ********************** ** *  **  **************************

AlbAct4cDNA      CCAGTGAAAGCGACAACAGCAGCATCAAAGTACATTTGTCATTCTGATTCGGCTAGTACC 1603
AegAct4cDNA      CCAGTGAAAGCGACAACAGCAGCATCAAAGTACATTTGTCATACTGATTCGGCTACTACC 1593
                 ****************************************** ************ ****

AlbAct4cDNA      ACCATCCGGAATCAGCTTGCATCGAACATCAATTCACGTTATTCAATGTATCTGTCATCC 1663
AegAct4cDNA      ACCATCCGGAATCAGCTTGCATCGAACATCAAATCACGTTATTCAATGTATCTGTCATCC 1653
                 ******************************** **************************

AlbAct4cDNA      AGCTCAGACAACACGTGGTGCATTCTCGGCCTACGAAGACCTACTACACCCGCGGAGAAA 1723
AegAct4cDNA      AGCTCAGACAAGTCG--GAGCTTTTCCAGTC-GCGAAAATCTGCGACTCCAGCGGA--AA 1708
                 ***********  **  * ** **  * * *  **** * ** * ** ** ***** **

AlbAct4cDNA      GCACTGCACCCCAGAGAGGACTCGTATGATAGCCAGGGAGGAGAACATCAACTTACCTTG 1783
AegAct4cDNA      GCACCGAACCACAGAGAGGACTCGTATGAAAGCCAGGGAAGAAACCATCA-TTCACCTTG 1767
                 **** * *** ****************** ********* ** * *****  * ******
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AlbAct4cDNA      TGGCAAATCTTCATTAAAAAAACGGACATCTTCA-TGACTAAGAGCCCATGTGCTTAGCT 1842
AegAct4cDNA      CAGCAAATAGGAA--AAAAAAACGGACATCTTCAACAAACAAAAGCCCATGCGCT-AACT 1824
                   ******    *  ******************   *  ** ******** *** * **

AlbAct4cDNA      TGGTTTAGGAGTTTAGTGTGACACCATGACCCCGCTGATGATCTTATCTTAGCAC--CAC 1900
AegAct4cDNA      TGGTTTAGGAGTTTAGTGTGACACCATGACCCCGCTGATGATCTTTACTTAGCACACCAT 1884
                 *******************************************  ********  **

AlbAct4cDNA      AACCACCTTTATGCGTTCGTTCATCCAAAA-CAAGAGGACATCACTGCAGCCGCGAGAAG 1959
AegAct4cDNA      AACCACCTTTATGCGTTCGTTCATCCAAAAACTACAGGATATCACTGCAGCCGCGAGAAG 1944
                 ****************************** * * **** ********************

AlbAct4cDNA      AACTCGCGAACCATCCTGTTTTCTTTTTTATAATATTCTTACTTTTAGTTTCAAATTATT 2019
AegAct4cDNA      AACTCGTGAACCATCCTGTTTTCTTTTTTATTATATTCTTACTTTTAACTTCAAATTATT 2004
                 ****** ********************** **************  ***********

AlbAct4cDNA      TTCAGTAATAAAAAACGTCTCAAAACATTAAAAAAAAAAAAAAACCTATAGTGAGTCGTAT 2079
AegAct4cDNA      TTCAGTAATAAAA--CGTCTCAAAATAAT------------------------------ 2031
                 *************  ********** * *

AlbAct4cDNA      TAATTCGGATCCGCG 2094
AegAct4cDNA      ---------------
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