Development of strains and procedures for genetic control of *Aedes aegypti* (Diptera: Culicidae)

Department of Zoology
Christ Church College

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

November 2013

Abstract

The mosquito *Aedes aegypti* is responsible for 50 million dengue fever infections in humans each year. A novel control strategy, named RIDL (Release of Insects carrying a Dominant Lethal) relies on releasing large numbers of genetically sterile male insects in order to control pest populations. This thesis describes the development of new tools to improve the efficiency of RIDL against *Ae. aegypti* and assessment of candidate strains for field release. **Chapter 3** describes a new selection system for *Ae. aegypti* based on ethanol susceptibility conferred by the alcohol dehydrogenase gene (*Adh*) from *Drosophila melanogaster*. I observed that the susceptibility of *Ae. aegypti* larvae to ethanol can be triggered by expression of *Adh* in larvae**. Chapters 4** and **5** focus on RIDL strains with a genetic sexing mechanism, for easy and stringent selection for males before mass-releases, eliminating disease-transmitting females. In **Chapter 4**, I describe the creation of a late-acting sexing strain of *Ae. aegypti* based on the *Ae. aegypti doublesex* (*Aedsx*) alternative splicing system. In **Chapter 5**, I describe an attempt to create an early-acting sexing system. Killing the females of the release generation early would free space and resources for the production of males. This was done by combining the *Adh* gene and the *Aedsx* alternative splicing system described in **Chapters 3** and **4**. **Chapter 6** reports the results of a comparison, in terms of quality and productivity, between an existing *Ae. aegypti* RIDL strain and a wild-type control. Results showed equivalent female quality and productivity between the two strains, while RIDL males were less fertile in comparison with wild-type males. RIDL eggs also seemed more susceptible to long-term storage. The results of this work show promise for development of novel RIDL strains that may be used in the field to control disease-transmitting mosquitoes.

Acknowledgements

I would like to thank my supervisor Luke Alphey, and my joint supervisors Sebastian Shimeld and Steven Sinkins for their supervision, guidance, and for giving me the great opportunity of completing a PhD study at Oxitec Limited and Oxford University. I would also like to thank Dr Derric Nimmo, Dr Marco Neira and Dr Kelly Matzen for their supervision in the laboratories at Oxitec and their critical comments during the completion of this study. Thank you to my examiners Stuart Wigby and Robert Saunders who gave me a very agreeable hard time during my viva.

I am very grateful to the molecular team at Oxitec: Tarig Dafa'alla, Guoliang Fu, Sarah Scaife, Andrea Miles, Caroline Phillips and Claire Harris for their guidance with construct making and their extreme patience in the molecular biology lab. I would also like to thank the mosquito team staff at Oxitec Ltd. for all their support and such friendly and hospitable atmosphere: Pam Gray, Sian Morgan, Heather Haines, Louise New, Jill Renmant, Neil Naish, Zoe Curtis, and Jessica Stevenson.

I greatly appreciated the friendly 'PhD student natural selection' atmosphere at Oxitec with those who are already Drs: Geneviève, Irka, Emma, Clare, Oliver, Sara, Lili; and the rest of the students: Romisa, Tom, Michal, Isaac, Mike, Roya, Markus and newbie Ed! Thanks for the 'Oxitec-R-group': Matt, Pete, Tim and Isaac. Thanks guys.

I would never thank Mister Moustapha Sy enough for his continuous moral support, for providing me the greatest bibliographical support on how to write properly in the beautiful and efficient language that is English, for proof reading my thesis as well as formatting it! Thanks to my flatmates Mark and Sarah who handled very well my nervous break-downs.

Thanks to my parents, both of them, for bringing me to this world full of hopes and possibilities and … mosquitoes.

Finally, I would like to thank the Biotechnology and Biological Sciences Research Council for awarding me the funding that made this study possible.

This document contains confidential and proprietary information belonging to Oxitec Ltd, the CASE partner. This report must be maintained in strict confidence at all times. It is to fulfil specific DPhil requirements of Amandine Collado and may not be used for any other purpose. No part of this document, or the information contained herein, may be published in any form without the prior written approval of a Director of Oxitec Ltd.

Author’s declaration

I declare that all the work presented here is my own original research subject to the following acknowledgement: the design of the OX4346 and OX4489 plasmid constructs presented respectively as part of Chapter 3 and 4 was done by Tarig Dafa’alla and the design of the OX4597 plasmid construct (Chapter 5) was designed jointly with Tarig Dafa’alla, as indicated in a foot note.

Table of Contents

List of figures

List of tables

Chapter 1: General introduction

Mosquitoes (family: Culicidae) represent a major socio-economical and health threat to human populations as they can transmit diseases such as malaria, dengue, chikungunya, West Nile, Japanese encephalitis or lymphatic filariasis. More than 3,200 species of mosquito exist worldwide, three quarters of which live within tropical latitudes (Becker et al., 2010). While in temperate climates, i.e. in Western Europe and North America, mosquitoes are mostly a nuisance (the bite of the mosquito provokes itchy skin rashes), they occasionally transmit pathogens to humans like the West Nile virus epidemic in the USA or the outbreak of chikungunya fever in Italy in 2007 (Rezza 2009).

1.1. *Aedes aegypti*

1.1.1. *Ae. aegypti* as a disease vector

Of the diseases transmitted by mosquitoes, dengue fever (DF) is transmitted primarily by *Ae. aegypti*, which infect about 50 million humans each year. The symptoms of DF can range from flu-like (high fever accompanied with severe headache) to the more severe (eye, muscle and bone pain, rash, haemorrhaging and low white cell count) or it can even be fatal (WHO 2012). An estimated 50,000 people develop annually the potentially lethal form, dengue hemorrhagic fever (DHF) for which no specific treatment exists (WHO 2012). DF has become the most important arboviral human disease since the beginning of the 21st century, having serious social and economic impacts in the areas where it is endemic (Gubler 2012) (**Figure 1.1.**). The causal agent of DF is the dengue virus, a member of the family Flaviviridae (genus Flavivirus) and there are four different serotypes (DEN-1, DEN-2, DEN-3 and DEN-4). Although infection with any one of these confers immunity against that particular serotype, it does not confer immunity against any of the other three. For this reason, the development of a tetravalent vaccine that protects against all four serotypes simultaneously—a task not yet successfully achieved (Sabchareon et al., 2012; Schmitz et al., 2011)—is needed to provide full protection against the disease (Webster 2009). In the absence of such a vaccine, vector control is the most effective way to prevent transmission.

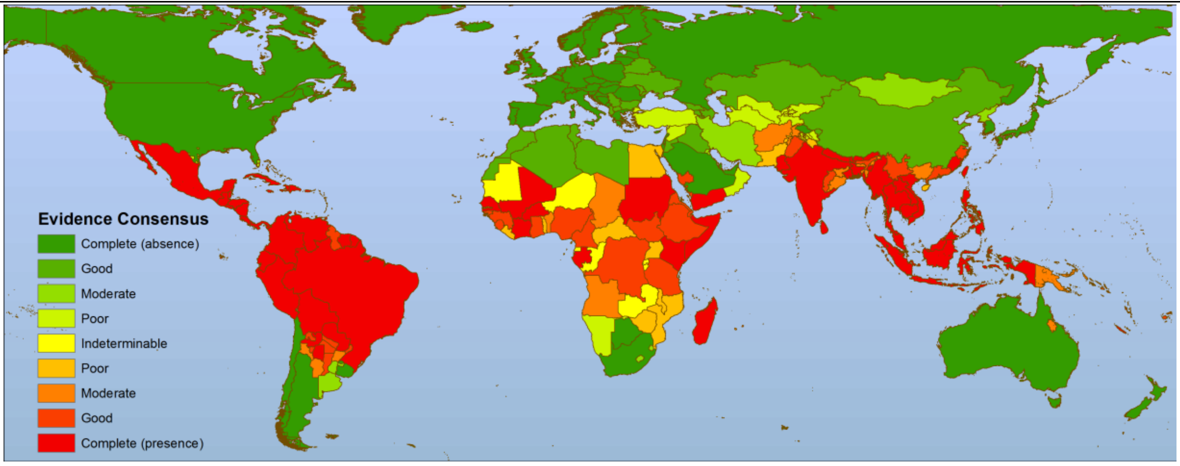
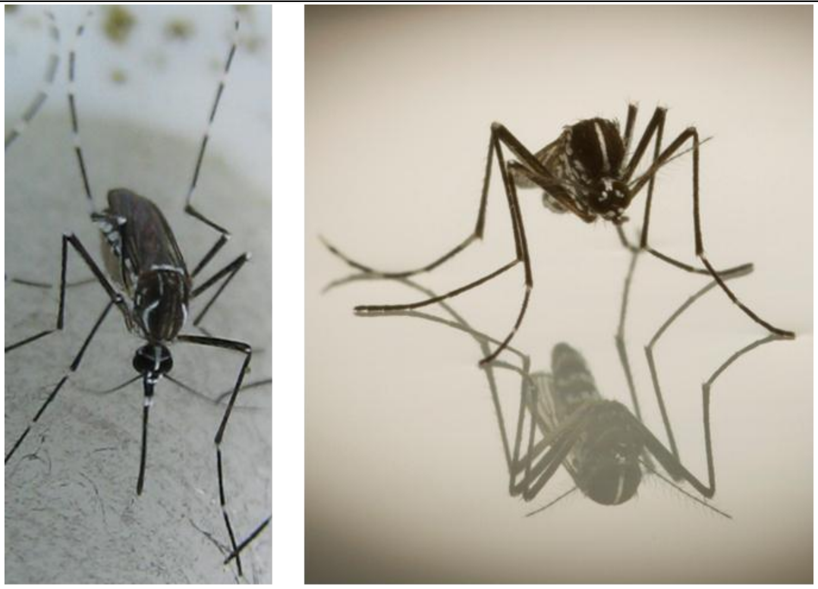


Figure 1.1. World-wide distribution where dengue fever cases have been reported in 2010 from Brady *et al.* (2012) (with the permission of Oliver Brady).
Colouring is based on evidence based consensus with green representing a complete consensus on dengue absence and red a complete consensus on dengue presence.

Additionally, *Ae. aegypti* is the most important vector of yellow fever, a Flavivirus endemic to countries of Sub-Saharan Africa and Latin America. There are estimated 200,000 cases each year causing 30,000 deaths. Current control measures include a vaccine providing up to 35 years of immunity within a week for 95% of those people vaccinated (WHO 2011), in addition to traditional mosquito control methods. *Ae. aegypti* is also one of the main vectors of chikungunya virus, a member of the genus Alphavirus (family Togaviridae). Although the symptoms of infection with this virus are rarely lethal, they can be severely debilitating. The word chikungunya means ‘to walk bent over’ in Swahili because the virus provokes strong incapacitating arthralgia when contracted. Other symptoms include fever, headaches and skin rashes, sometimes making the disease hard to distinguish from DF (Pialoux et al., 2007).

1.1.2. Distribution and Ecology

Ae. aegypti (Linnaeus) (**Figure 1.2.**) is part of the sub-family Culicinae, one of three in the Culicidae family which includes the genera *Culex* and *Aedes*, among others. It is believed to have first evolved in Africa and is currently distributed worldwide, occupying both tropical and sub-tropical latitudes between *35° N and 35° S* (Gibbons and Vaughn 2002). *Ae. aegypti* is a highly anthropophilic species, well adapted to semi-natural and artificial breeding sites. Its eggs, resistant to desiccation soon after oviposition, are deposited on damp substrates and can remain viable for several months waiting for rain, having entered diapause. In the egg stage, they are more resistant to variations in temperatures than larvae or adults, which cannot survive temperatures below 8°C (Clements 1963). Females exhibit multiple and opportunistic host-biting behaviour, usually during the day and at the dusk (Canyon and Muller 1999). *Ae. aegypti* often breeds in sympatry with the ‘Asian tiger mosquito’ *Aedes albopictus* (Skuse) (**Figure 1.2.**), and their females are also responsible for the transmission of chikungunya, yellow fever and DF, among other diseases.



Aedes aegypti Aedes albopictus

Figure 1.2. The main vector of Dengue fever and its sympatric species, *Ae. aegypti* and *Ae. albopictus*.
The female *Ae. aegypti* presents a lyre-shaped white patern on the thorax as well as an *Ae. albopictus* female with a large white band on the thorax (Image courtesy of Oxitec Ltd and Jake Galson).

1.2. Controlling *Ae. aegypti* populations

1.2.1. Larval control

Reduction of mosquito populations can be achieved by the management or removal of larval breeding sites when they are accessible (**Figure 1.3.**). The treatment of water surfaces with oil or other monomolecular films can eliminate larval breeding sites by preventing the larvae and pupae from breathing normally. In most countries, chemical control of larval mosquitoes is implemented using organophosphate chemicals such as temephos (American Cyanamid Co., Princeton). Immediate results make these products popular; however the development of resistance is a problem (Hemingway and Ranson 2000).

Mosquito larvae can also be controlled using biological agents. The most commonly used organisms in this category are bacteria such as *Bacillus* *sphaericus* (*Bs*) and *Bacillus thurigiensis* var. *israeliensis* (*Bti*). When ingested by the mosquito larva, these bacteria produce toxins that fatally damage the insect's digestive tract. *Bti* and *Bs* formulations have been regularly applied for the past 20 years in urban and flooding waters in Europe (Becker et al., 2010) and in the USA (Atkinson 2009), to control *Aedes* and *Culex* populations. Since they are specific to mosquitoes, blackflies and chironomid larvae, they are less likely to be a threat to the environment than non-specific chemical agents (Glare and O'Callaghan 1998). However, like chemicals, the persistence of *Bti* spores has been observed in the environment, which could lead to resistance in the field, as it has been artificially be induced in *Ae.aegypti* (Paris et al., 2011b; Tetreau et al., 2012b).

Predators of mosquito larvae, like the mosquito fish, *Gambusia affinis*, can be used for vector control simply by introducing them into ponds where mosquitoes breed (Prasad 1993). This control method is an easy way for individuals to treat standing waters on their own properties that are infested with mosquito larvae, however, as with the introduction of exotic species in general, the foreign species itself may cause unforeseen problems. The mosquito fish, for example, has been shown to displace native fish species through resource competition and egg predation (Lacey and Orr 1994).

Larval control strategies rely on a long-term commitment of strong centralized governments and community-based programmes (Gubler 2011; Gubler 1989). They require the constant education and training of local staff in managing and monitoring *Ae. aegypti* larval breeding sites. These strategies are expensive and time consuming, and have failed in the past mostly because of political and economical problems (i.e. in Puerto Rico and in Cuba (Gubler 2005).

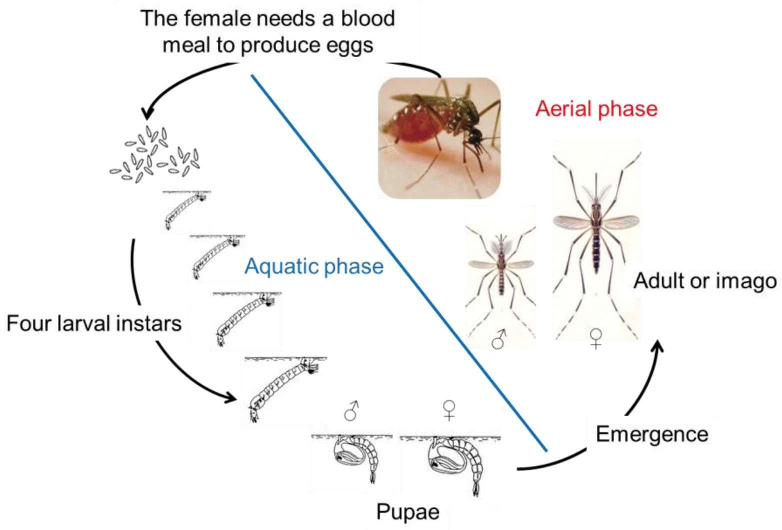


Figure 1.3. A simplified mosquito life cycle.

The life cycle of a mosquito is predominantly aquatic: after the female ingested a blood meal, she will deposit her eggs on water or on a dampen surface at the edge of the water and substrate; After rainfall or after the water tank is filled and the eggs are submerged, larvae hatch and develop throughout 4 instars and finally metamorphose into pupae. The mosquito adult will emerge at the surface of the water (Arial phase).

1.2.2. Adult control

Common techniques for adult mosquito control focus mainly on the application of chemical insecticides by thermal fogging, ultra low volume (ULV) spraying, and indoor residual sprays in urban areas. *Ae. aegypti* had been successfully eradicated from most of the Americas by the 1960s using dichlorodiphenyltrichloroethane insecticides (DDT) (Gubler 2005). However, after the Pan American Health Organization (PAHO) eradication programme stopped the treatments, the mosquito re-invaded the continent in the early 1970s (Gubler 2002). DDT is a non species-specific insecticide, killing insects indiscriminately, and is highly persistent in the environment (Spencer et al., 1996). Moreover, treatments with DDT have to be maintained at high levels to prevent re-infestation (Gratz 1991).

Thermal fogging and ULV trials applied with truck-mounted units or aeroplanes—using organophosphorous concentrates such as Malathion—have shown good results in *Ae. aegypti* reduction in Asia and South America (Gratz 1991). This is not always the case as contradictory results show that similar applications seemed to have a low impact on reducing dengue transmissions in the Caribbean, probably because of the behaviour of local *Ae. aegypti* adults, that rested indoors and were therefore not easily reached by the treatments (Gubler 2011). Insecticide-impregnated bed-nets are often used to prevent human contact with Anopheles mosquitoes (which are nocturnally active), and therefore inhibit malaria transmission. Unfortunately, this technique is largely ineffectual against *Ae. aegypti* due to its diurnal activity.

As mentioned earlier with biological control agents, mosquitoes develop resistance to prolonged chemical interventions, compromising their efficiency (Hemingway and Ranson 2000). Though effective in reducing disease transmission, chemical interventions can also pose a threat to human health (Teixeira et al. 2003; Perry et al. 2007; Koureas et al. 2012), as chemical residues accumulate in the environment and may enter the food chain. Efforts must therefore be focused on other environmentally friendly techniques, such as genetic control, to prevent and combat vector-borne diseases.

1.2.3. Novel techniques to control *Ae. aegypti* based on the sterile insect technique (SIT)

Principle and applications of SIT

First proposed by Knipling in 1959, SIT involves the sterilization by irradiation of mass-reared male insects, and their subsequent release into the wild. These sterile males seek and mate with wild females. Since the offspring of these matings are not viable, a reduction in the local insect population will, ideally, be achieved over time. For an SIT program to be effective, several conditions need to be met, including a) the rearing and sterilization of large numbers of individuals of the targeted species, and b) the separation of males from females prior to release, as SIT programmes with all-male releases are more efficient (Rendon et al. 2004). If both sterile males and females are released simultaneously, they mate with each other instead of seeking partners in the wild, reducing their overall effectiveness. Stringent sex separation is particularly important for organisms such as mosquitoes whose females can transmit diseases or for crop pests where oviposition by females destroys fruits.

This technique has been used since the 1960s to tackle pests responsible for economical damage such as larvae of the New World Screwworm (*Cochliomyia hominivorax,* a fly which feeds on the flesh of wounded cattle and other livestock), the Mediterranean fruit fly (*Ceratitis capitata,* which deposits eggs inside fruits of commercial interest) and caterpillars of the pink bollworm (*Pectinophora gossypiella*, a moth which devastates cotton crops). In addition, SIT has been used to control disease vectors such as *Glossina austeni* (tsetse fly) and mosquitoes (Dyck 2005).

Several trials involving sterile mosquitoes have been performed since the 1960s, targeting *Culex, Anopheles* and *Aedes* species (Benedict and Robinson 2003; Lofgren et al., 1974; Patterson et al., 1970). Trials in India and in El Salvador, although interrupted for political reasons in the mid 1970s, were useful for identifying the strengths and weaknesses of this control strategy, which include:

- Limited levels of sterility achieved in eggs laid by wild females in a release programme of *Cx. quinquefasciatus* in India (Klassen and Curtis 2005).
- Difficult mechanical sex separation with *Anopheles* species as opposed to *Aedes* and *Culex* (Papathanos et al., 2009).
- Insufficient production of males (Benedict and Robinson 2003).
- Immigration of mated females (Dame 1981).

Recent advances in molecular biology provide the tools to address some of the problems encountered during previous SIT trials, and improve the technique by producing fitter insects and providing stable genetic sexing systems.

RIDL: the Release of Insects carrying a Dominant Lethal

One of the potential applications of transgenic technologies in the field of vector biology is the replacement of natural mosquito populations with genetically modified strains that would be refractory to dengue virus or malaria parasites. Such strains are currently being developed (Ito et al., 2002; Olson et al., 1996). Another strategy is population suppression using a genetic alternative to the sterile insect technique, where sterility is induced by a repressible dominant lethal gene rather than irradiation called Release of Insects carrying a Dominant Lethal gene (RIDL) (Thomas et al., 2000).

RIDL technology is based on the tetracycline-dependent repression of a dominant lethal gene (Heinrich and Scott 2000; Thomas et al., 2000). A transgenic mosquito RIDL line (*Ae. aegypti* OX513A) with one-component positive feedback system has been developed by Phuc *et al* (2007). In this line, the tetracycline-repressible transcriptional activator gene (tTAV) is driven by a minimal non sex-specific promoter (hsp70) and the regulatory element tetO (**Figure 1.4.**). In the absence of tetracycline, tTAV initially expressed via the minimal promoter binds to the tetO element, driving the expression of more tTAV in a feedback loop system. The tTAV protein accumulates and induces cell death, likely due to interferences of its VP16 domain with key transcription factors (Phuc et al., 2007). In presence of tetracycline (provided as part of the diet), any tTAV expressed binds to tetracycline with high affinity, preventing it from binding to tetO sites, which in turn prevents the establishment of the aforementioned feedback loop. As a result, mosquitoes reared in the presence of tetracycline survive to adulthood. When released, RIDL males mate with wild females, their progeny inherit the lethal cassette, and since no tetracycline is normally present in natural breeding sites, their progeny will die.

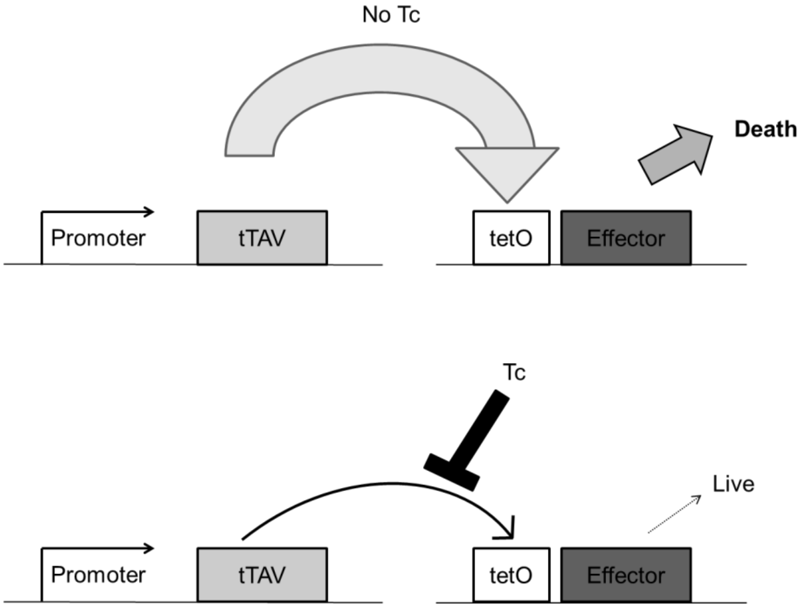


Figure 1.4. The tetracycline repressible system (Modified from Gong et al., 2005)
In the tetracycline repressible system, tTAV is expressed in absence of tetracycline (No Tc) and binds to tetO inducing the expression of more tTAV and, when levels of tTAV produced are too high, it becomes lethal. In presence of tetracycline (Tc), basal levels of tTAV are prevented from binding tetO element hence producing high levels of lethal effector molecule tTAV.

One of the advantages of a transgene-based technology such as RIDL is that it is easily transferable to other pest insects or disease vectors to match specific species requirements. In the case of mosquitoes, an additional advantage lies in the time of death induced by the RIDL system: In the absence of tetracycline, lethality in the OX513A strain (**Figure 1.5.**) occurs at late 4th instar larval or pupal stages. This late-acting lethality provides the extra benefit to the progeny of released RIDL males competing for food against their wild counterparts during the early larval stages. This is an advantage because mosquitoes are a density-dependant species and larval competition will put additional stresses on the wild population (Atkinson et al. 2007; Phuc et al. 2007).

Competitive performance of released males is a major criterion for the success of a release programme. Fitness of irradiated males has been shown to be variable during previous SIT field studies (Benedict and Robinson 2003). Although recent work has shown reduced male performances of the strain OX513A compared to its laboratory wild-type counterpart (Wilke et al., 2009; Bargielowski, Alphey, & Koella, 2011) this reduction was not as significant as that for irradiated males and the line still showed promise for a field release. And indeed, this strain showed promising results in Cayman Island in 2010 (Harris et al., 2012) where the mosquito population in a 16 ha area was reduced by up to 80% when compared with a control zone.

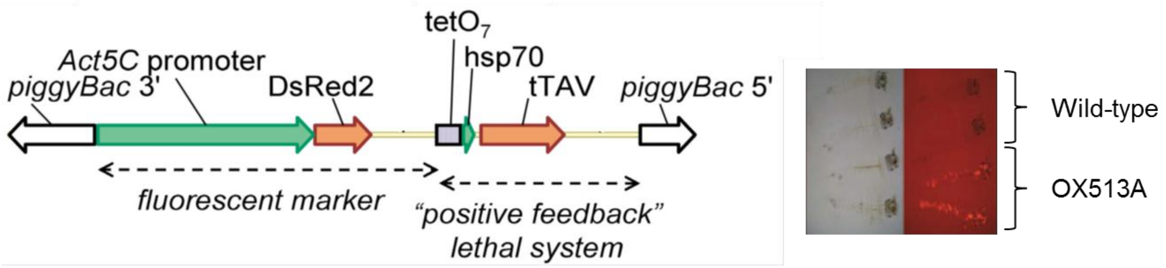


Figure 1.5. The *piggyBack* plasmid construct OX513A from Phuc *et al.* (2007) **and its fluorescence pattern in second instar *Ae. aegypti* larvae from Harris *et al.*** (2012)**.**

The tetracycline-repressible transcriptional activator gene tTAV is driven by a minimal promoter (hsp70) and the regulatory element tetO. The transformation marker DsRed2 is driven by Act5C promoter leading to a red fluorescent spotted pattern all over OX513A the larvae and pupae (not shown) in comparison with no red fluoresrence in the wild-type larvae.

Genetics-based mosquito control strategies such as RIDL are modeled on traditional SIT and require the release of fit, engineered males at high ratios compared to the wild population in order to achieve reduction of the targeted population. The OX513A strain, and RIDL technology in general, have great potential as means to control dengue, however, the area initially targeted in 2010 was almost 3.5 times larger than the area that OX513A was able to control (55 ha). The trial was conducted without complementary classical mosquito control methods, and the production of RIDL mosquitoes was difficult with the team struggling to release more than 2,000 RIDL males during the first 2 months of the trial. There is now no doubt that RIDL males were successful in seeking and mating with the wild-type females once released (Harris et al., 2011; Harris et al., 2012b), however, what remains a challenge is the capacity to scale up the production of mosquito males in order to target larger areas. In 2012, similar projects in Brazil led to the rise of the mosquito male production from 33,000 to 550,000 per week with improvements of an egg production from 1 million to approximately 4 million per week, and this number will keep increasing up to over 10 million (Andrew McKemey and Danilo Carvalho, personal communication).

Mosquito production therefore needs to reach high quality and numbers as standard.

1.3. The mass release of a male-only population

1.3.1. Classical sex sorting systems

As previously explained, sex separation prior to release is seen as essential for mosquito SIT because of the female's ability to bite and transmit disease. Sex dimorphism in *Culex* and *Aedes* genus allows sex separation at the pupal stage, the female pupa being bigger than the male pupa (Bellini et al. 2007; Papathanos et al. 2009). In past SIT trials, pupae were sexed using mechanical sex separators (McCray 1961; Sharma 1972). Once collected, pupae were pushed through a grid with a gentle stream. Smaller male pupae could pass through the slits of the separator, while larger female pupae were retained on the screen (Ansari et al., 1977b; Fay 1963). Although this technique allows removal of 99.9% of female *Ae. aegypti* pupae, it is not optimal, as up to 60% of the male pupae were also retained on the sieve with the females and are therefore lost (Ansari et al. 1977a; Papathanos et al. 2009). As there is a revival of interest in SIT-based programmes for controlling mosquito populations, mass rearing procedures need updating and improving.

1.3.2. Developing Genetic sexing systems

Genetic approaches in the form of Genetic Sexing Strains (GSSs) can improve the efficiency of male mosquito mass production prior to field release by selectively killing the females early in the development, halving the cost and space requirements for rearing (Caceres 2002).

Anopheles mosquitoes carry a sexual chromosome pair as opposed to other Culicine mosquitoes such as Aedes and Culex. It allowed physical linkage of a gene of resistance to an insecticide (e.g. propoxur) to the male Y-chromosome by chromosomal translocation. Using such technique requires first the creation of strains carrying a translocation on the male-determining chromosome by inducing mutations so genes conferring resistance to insecticides can be linked to the male trait. Females, sensitive for the chosen insecticide, can be eliminated by poisoning them. This step, creating strains by trial and test for translocation is a long process, unpredictable and time consuming (Franz 2005) and needs repeating and tried for each species that SIT programmes are targetting; adding the condition that no genetic recombination event occurs in males. If so, this leads to risking releasing wild-type females the next generation.

That way, Seawright et al (1978) obtained the elimination of 99.7% of *Anopheles albimanus* females after treating the eggs with propoxur, without any significant male loss (Seawright et al., 1978). Unfortunately, this system proved to be unstable under mass rearing conditions, as in generations, 0.2% more susceptible males as well as resistant females (through genetic recombination) were generated (Bailey et al., 1980).

Transgenic technology allows for the creation of genetically engineered GSS, which can easily be transferred from one species to another with minor changes in the genetic constructs used for transformation. Such technology has already been developed in *Anopheles stephensi* through the expression of green fluorescent protein in gonads so that males can be separated from females by hand (time consuming) or using automated sorting machines (currently prohibitively expensive) (Catteruccia et al., 2005).

In *Ae. aegypti*, a female-specific adult flightless system (**Figure 1.6.**), repressible in presence of dietary tetracycline (similar to the original RIDL design, but targeted to the female flight muscle - IFM) has recently been created (Fu et al., 2010). Unfortunately, such a transgenic sexing system only allows the separation of males and females at either late larval or adult stages (fluorescent gonads and flightless females, respectively). This strategy allows for the release of both males and females, but requires rearing of both. From an economic point of view, an earlier discrimination between the sexes is desirable, and can be achieved as previously shown with a female specific *C. capitata* temperature sensitive lethal strain (TSL) used in SIT (Caceres 2002; Robinson 2002).

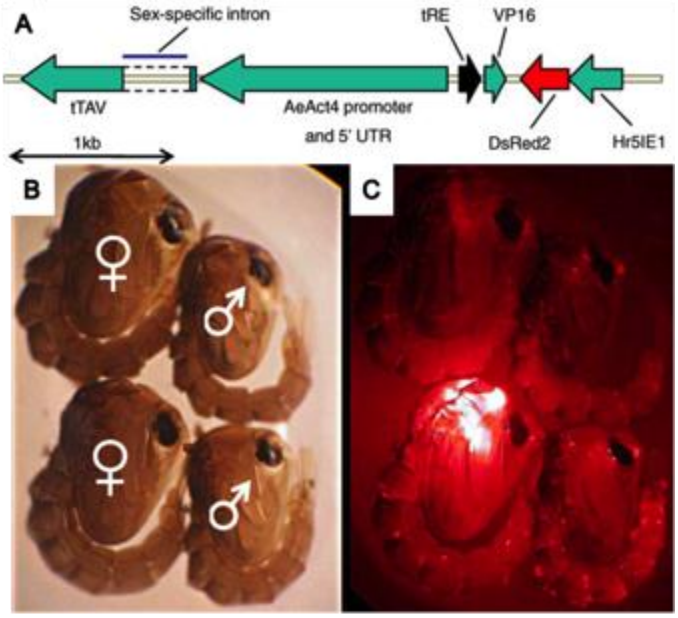


Figure 1.6. Diagram of part of the flightless female plasmid construct OX3604 taken from Fu *et al.* (2011) **and its fluorescence pattern in *Ae. aegypti* pupae.**

The *pigggyBac*-based OX3604 flightless plasmid (**A**) and the divergent fluorescence pattern of the OX3604 male and female pupae alone (top) and OX3604 + tetO-DsRed2 (bottom) under white (**B**) and fluorescent light (**C**). The tTAV2 protein is produced under the control of the promoter and alternative splicing element of the *AeAct4* gene and therefore expressed in a female-specific manner. The tetracycline response element (tRE), comprising multiple copies of *tetO* and driving VP16, is activated by tTAV2. The DsRed2 marker is driven by the Hr5*ie1* promoter resulting in red spots all over the OX3604C individuals. The bottom OX3604 pupae (**B** and **C**) were crossed to a reporter line tetO-DsRed2 hence a stronger female specific red fluorescence where IFM develop in female pupae before emergence of the female adult.

1.3.3. The use of inducible expression of genes

The discrimination of the females from the males as early in development as possible is a major improvement to the RIDL strategy and is possible through genetic engineering. The system used to discriminate between the sexes needs also to be easy to use, inexpensive and, most importantly, non-toxic.

Physical stimuli can induce genes expression in animals and in plants: heat shocking to 37°C for about 30 minutes would induce the expression of a gene linked to the endogenous promoter hsp70 in the vinegar fly *Drosophila melanogaster* (Lis 1983)*,* while at room temperature the gene would be inactive. In plants, hydrogen peroxide treatment, wounding or ultra-violet light can induce a peroxidase gene promoter in the sweet potato *Ipomoea batatas* (Kim et al., 2003). Blue-light induces the dimerization of a fungal light sensor protein Vivid (VVD) and, if combined with the Gal4/UAS binary system (an analogue to the tetO/tTAV system), can induce the expression of a gene of interest in mice (Wang et al. 2012) (**Figure 1.7.**).

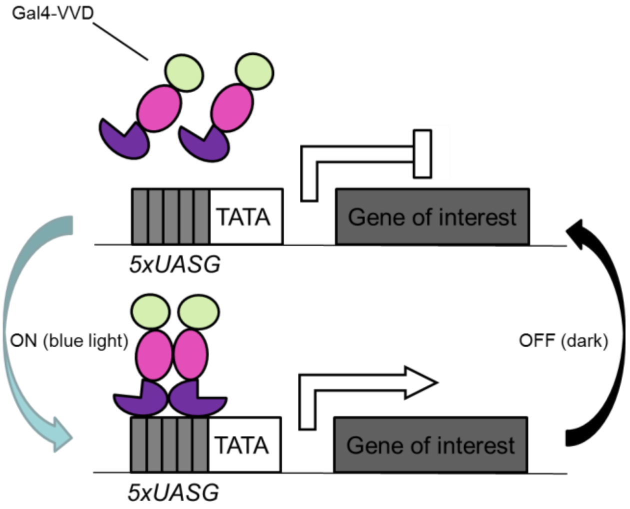


Figure 1.7. Diagram of the light inducible construct for the expression of a gene of interest modified from Wang *et al.* (2012).

Blue-light activation induces the dimerization of the Vivid protein (VVD) that has previously been fused to the transactivator protein Gal4 and consequently initiates transcription of the gene of interest by binding to its binding site UAS (5x UASG).

Two visible markers can be used in combination, one as a transformation marker and another one divergently expressed in one sex only (Marois et al., 2012; Robinson and Van Heemert 1981) allowing discrimination between the sexes during early developmental stages (from the first instar larval stage). Male-specific resistance to insecticides as suggested in Sinkins and Hastings (2004) or to alcohol as proposed by Robinson and Van Heemert (1981) can be achieved by making use of resistant alleles to chemicals under the expression of a male-specific promoter or a sex-specific splicing system.

Among the mentioned inducible systems that can be used in *Ae. aeygpti*, ethanol resistance, engineered specifically in males is the most attractive. Ethanol is a common product used in biology laboratories, and as a consequence is cheap, readily available, and has low human toxicity.

Insects from the Drosophilidae and Tephritidae families are routinely exposed to environmental alcohol throughout their immature stage (larval). The larvae feed principally on fermenting fruit or fungus, and can face ethanol concentrations of 4% or more (McKechnie and Morgan 1982). Alcohol dehydrogenase (ADH) is responsible for 90% of the degradation of ethanol (EtOH) in *D. melanogaster.* ADH catalyses the oxidation of ethanol to acetaldehyde, a highly toxic by-product that is subsequently converted into acetate that *D. melanogaster* uses as energy resource (Carvalho et al., 2009 and Heinstra et al., 1983). The ADH locus was successfully exploited to create a genetic sexing strain in *D. melanogaster* (Robinson and Van Heemert 1981). To create such an Adh-GSS system, first, males homozygous for the Adh active allele and carrying a translocation between the male-determining chromosome and the Adh-chromosome were crossed to virgin females homozygous for the Adh-null allele.

The male progeny F1 was then crossed again to virgin females showing no ADH activity, and, finally, the resulting F2 progeny was reared on ethanol (4%) to confirm the desired phenotype: producing a male-only population in presence of ethanol in the larval medium. The females, homozygous for the null allele were sensitive to alcohol. In Drosophila, there is absence of genetic recombination in the males so that the translocated locus remains on the male-determining locus, a characteristic which does not seem to be so true when male-linked translocations were induced in *Ceratitis capitata* (Robinson and Van Heemert, 1982).

A first attempt at the same scheme was unsuccessful with the Mediterranean fruit fly *Ceratitis capitata,* as none of the null mutants generated at the *Adh* locus proved to be viable when made homozygous (Robinson et al., 1986). The same species was then transformed using the *D. melanogaster Adh* gene and a male specific promoter (MSSP-alpha2) to drive expression in the male fat body (Christophides et al., 2001; Christophides et al., 2000). Unfortunately, the expression of ADH in the male individuals was only two-fold upregulated compared to that of the females, insufficient for a functional genetic sexing system. The ADH system needs more adjustments to successfully generate male-only resistance to alcohol in *C. capitata*.

Mediterranean fruit fly populations are naturally exposed to high doses of alcohol which might explain the difficulties in generating resistance to alcohol in those insects. Perhaps the use of a stronger male-specific promoter might help solving the problem. Happily, the scientific literature does not mention the presence of naturally-occurring alcohols in the larval mosquito diet, and the ADH system might be easier to implement in *Ae. aegypti* (Chapter 2).

Once a marker is chosen (e.g., divergent fluorescence, insecticide resistance), it would then need to be expressed by a sex-specific promoter, and functional at early developmental stages (embryonic or first instar larvae). Most promoters shown to have divergent expression in males or females are activated late during the development (fluorescent gonads – **Figure 1.8. -** or female flightless – **Figure 1.6.**), and few characterised sex-specific promoters are suitable for early expression in development. Fortunately, female-specific lethal systems or male-specific resistance mechanism might be engineered using sex-alternate splicing systems.

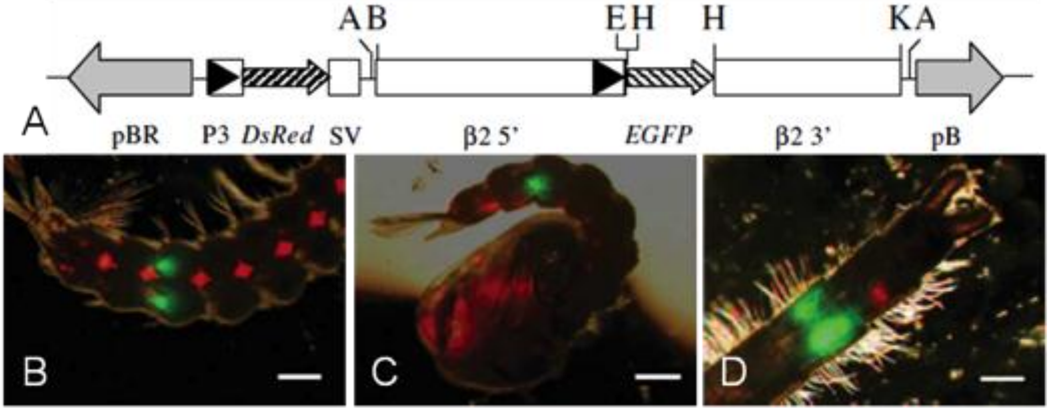


Figure 1.8. Diagram of part of the male-green-testis plasmid construct (A) and its fluorescence pattern in *Ae. aegypti* pupae and adult (B, C and D) from Catteruccia *et al.* (2005) (with the permission of Flaminia Catteruccia)**.**

Pictures above show the fluorescence pattern of the end of the abdomen of a male pupa (B and C) as well as the end of the abdomen of a male imago under fluorescent light (D). The Red marker is the transformation marker DsRed under the influence of the synthetic Pax-6 promoter (P3). The green fluorescence expressed through the eGFP gene is driven by the male gonads specific ß2-tubulin promoter (A).

1.3.4. Using alternative splicing systems in pest control

Alternative splicing systems are very common in higher eukaryotes and allow the production of more than one protein out of the same gene (Weaver 2011). In insects, such systems are particularly studied in genes involved in sex-determination such as *transformer* and *doublesex*. The key advantage of using the alternative splicing systems in GSS are the following: a gene involved in sexual determination such as *doublesex* has been shown to be expressed from very early embryonic stages in *Ae. aegypti* (Salvemini et al., 2011); genes such as *transformer* and *doublesex* are differentially spliced in males and females are hence perfect candidate elements for engineering genetic sexing systems.

In insects, genes switched on in a cascade determine the sex of an individual. It is the way those genes are primarily induced that varies between different groups of insects: for example, in XX/XY systems, *D. melanogaster* sex is regulated by the X-chromosome dosage (Erickson and Quintero 2007), whereas in the malaria mosquito *Anopheles gambiae* the Y-male-chromosome contains a dominant male determining factor (Clements 1992). In *Ae. aegypti*, as well as in other Culicine mosquitoes, heteromorphic sex chromosomes are absent, and, instead, sex is controlled by an autosomal locus carrying a male determining gene, M, acting as a dominant male determiner (Hickey and Craig Jr 1966). While various systems are responsible for the determination of the sex in insects some genes or functions have been conserved (*doublesex* and *transformer*).

Recently, the properties of *C. capitata transformer* (**Figure 1.9.**) have been exploited to lead to a successful GSS in the Mediterranean fly *C. capitata* itself (Fu et al., 2007). *C. capitata* female individuals produce three different transcripts F1, M1 and M2. The F1 transcript is the only one that produces a functional protein, and males only produce transcripts M1 and M2 that contain additional exons with in-frame-stop codons and, consequently, result in non-functional proteins. To generate a female-specific RIDL strain in *C. capitata*, Fu *et al.* (2007) inserted the tTAV gene into an engineered *Cctra* cassette containing two segments: the tTAV expression cassette and the genetic marker (*Hr5iE1* – DsRed2) allowing detection of transgenic individuals by fluorescence. The 3’ end native transformer intron sequence is flanking the 5’ end of minimal promoter *Dmhsp70* and its 5’ end with the fused sequences of the repressor tetR and the transcriptional activation domain VP16 forming the tTAV expression cassette (tetO$_{21}$ - *Dmhsp70* minimal promoter –Cctra:tTAV) (see construct diagram in **Figure 4.3.**). The lay-out of the RIDL cassette with the elements of the native *tra* transcript allows the expression of tTAV in females only, triggering the lethal feedback loop in absence of tetracycline.

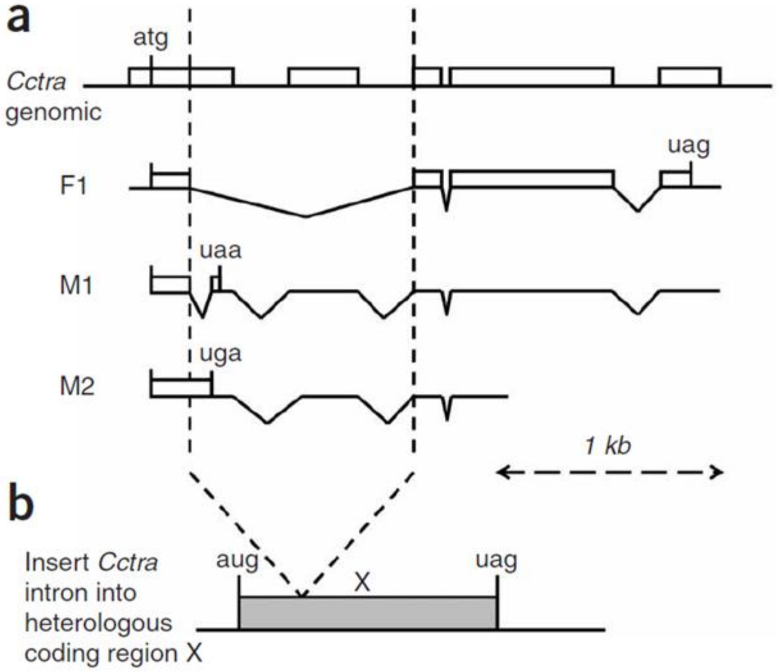


Figure 1.9. Diagram of *C. capitata transformer* gene organization (*Cctra*) and female splice variant (F1) as well as two male splice variants M1 and M2 from Fu *et al.* (2007)**.**

In *C. capitata* females, the three transcripts F1, M1, and M2 will be produced **(a)**. F1 is the only transcript encoding a functional *Cctra* protein. M1 and M2 do not as they contain additional exons with in-frame-stop codons. *C. capitata* males will only produce the two transcripts M1 and M2 leading to non functional *tra* product. Part of the *tra* gene can be extracted **(b)** (the area marked in between the dashed black lines) and placed in the coding region of the gene X. Only females will therefore produce functional protein X.

The alternatively spliced gene *doublesex* has also been exploited to lead to a successful GSS in two moth species *Pectinophora gossypiella* (*Pg*) and *Plutella* *xylostella* (*Px*) (Jin et al. 2013) (**Figure 1.10.**). The *dsx* alternative splicing results in different products than with *C. capitata transformer*: both male and female transcripts result in functional male-specific or female-specific *dsx* proteins. A female-specific RIDL system was successfully engineered when the tTAV coding sequence was inserted into the female specific exon 3. Moreover lethality in transformed moths was induced at early larval stage, confirming the suitability of *dsx* in the conception of an early-acting, sex-specific genetic sexing strain.

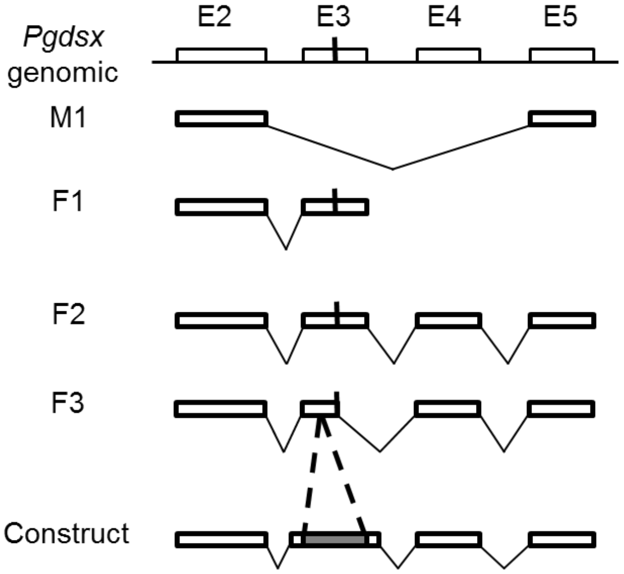


Figure 1.10. Diagram of *Pectinophora gossypiella doublesex* gene organization (*Pgdsx*), the splice variants in males and females and sex specific splicing and *Pgdsx* use in sex specific expression of genes modified from Jin *et al.*, (2013)**.**

The exons E2 and E5 are shared in male and females transcripts. The exons E3 and E4 are spliced in females only. The male transcript (M1) will produce a functional, male-specfic *Pgdsx* protein. In females, three transcripts F1, F2 and F3 are produced. The insertion of a coding sequence of interest (grey box) in the female specific exon resulted in a successful female-specific RIDL strain in the moths' *P. gossypiella* and *Plutella xylostella*.

Insect sex determination is triggered differently in different insects, but there are conserved genes (*dsx*) and functions (*tra*) that are alternatively spliced, and can successfully produce genetic sexing strains as shown in the Mediterranean fruit fly as well as two moth species. Genetic engineering experts predicted success when combined with the RIDL construction (Dafa'alla et al., 2010), and the same can be tried in *Ae. aegypti* as *dsx* (*Aedsx*) was been fully characterized in 2011 by Salvemini et al. (2011). The organisation and alternative splicing system of *Aedsx* (**Figure 1.11.**) is similar to *P. gossypiella dsx*: the *Aedsx* gene contains a female-specific exon (5) spliced into the female *Aedsx* transcripts only. More detailed information is provided in Chapter 3 on the use of *Aedsx* to generate sex-specific RIDL expression in the dengue fever mosquito.

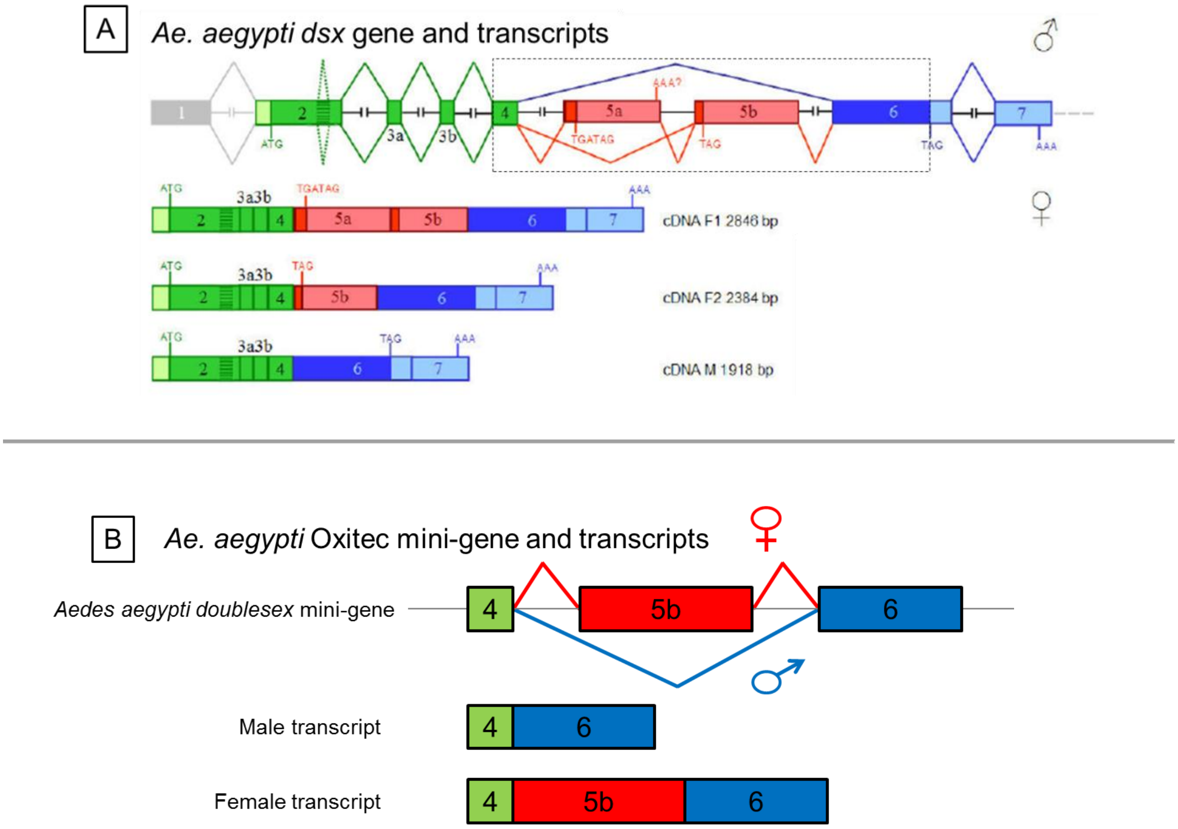


Figure 1.11. Diagram of endogenous *Ae. aegypti doublesex* gene modified from Salvemini *et al.,* (2011) **and mini-gene organization as well as their female and male splice variants.**

A: Green boxes represent the exons 2, 3a, 3b and 4 that are common in both females and the male *dsx* transcripts (F1, F2 and M). Red and blue boxes respectively represent the female specific exons 5a and 5b as well as male specific exons 6 and 7. **B:** Oxitec Ltd. mini-gene version comprises of the shared exon 4, the female specific exon 5b and the male specific exon 6 as well as engineered shortened introns between exon 4 and 5b and between exon 5b and exon 6.

1.4. The mass production of *Ae. aegypti*

There are two principle and competing pre-requisites usually focused on for a successful SIT (or RIDL) programme: on the one hand, large quantities of mosquitoes must be produced in a space and cost limited environment; on the other hand, these mosquitoes must attain high standards of quality regarding male mating competitiveness and longevity into the wild. Less often highlighted is the need for sufficient female fecundity to provide the egg supply for the male-only mass release.

One way to produce large quantities of insects in a facility with limited space is to use high larval rearing densities. However, increased larval densities tend to produce specimens of low quality, and previous studies show that larval overcrowding has an impact on larval mortality, development time, longevity, size and fecundity in *Ae. aegypti* (Barbosa and Peters 1973). In studies focused on RIDL strain OX513A, increasing larval rearing density were shown to delay pupation, decrease adult longevity and reduce adult size in both transgenic OX513A and a wild-type laboratory strains, the decrease in adult size being more pronounced in OX513A individuals than in their wild-type counterparts (Bargielowski et al., 2011a). While OX513A female productivity was not the focus of this study, it is likely that larval rearing densities, known to impact the size of the out-coming imago, might equally impact the fecundity of the mass reared individuals.

Male performance of the RIDL strain OX513A has extensively been studied and compared to that of a wild-type laboratory strain (Bargielowski et al., 2011b; Bargielowski et al., 2012). While OX513A males can inseminate the same number of females over the first three days as their wild-type competitors, they inseminate fewer of them over the course of their lifetime (Bargielowski et al., 2011b). OX513A and wild-type males of equivalent adult size exhibit the same teneral energy reserves, however, OX513A covers 38% less distance than the wild-types under artificial conditions (Bargielowski et al., 2012).

These studies are important since they permit evaluation of possible fitness costs associated with the RIDL construct, and help to better calibrate the RIDL release strategy in the field. The same evaluation needs to be carried out on the productivity of the RIDL strain females, as OX513A males have proven their performances in the field (Harris et al., 2011 and Harris et al., 2012b) and larger release areas are planned.

1.5. Conclusion

Controlling *Ae. aegypti* populations remains the most efficient way to prevent dengue transmissions to humans. While the traditional mosquito control methods listed above are inefficient on their own, combining existing mosquito control methods, i.e. targeting aquatic breeding sites and adults (aerial phase), with new technologies is promising (Becker et al., 2010 and Gubler 2011). SIT-based techniques such as RIDL have recently proven their efficiency on the field (Harris et al., 2012a). However, the RIDL method, like classical SIT based techniques, strongly relies on the synchronous production of a large quantity of high quality mosquitoes. The release of a male-only population requires an efficient and cheap sexing system that would, ideally, discriminate between the sexes very early in development. If millions of engineered sterile males are to be released to seek females in the wild, egg production needs to be optimised. Assessment of quality and productivity of RIDL strains and their variants is needed to validate their utility in a mass release programme.

1.6. Aim of the thesis

The aim of the thesis is twofold: Firstly, to develop new tools for an efficient mass release of a male-only population of *Ae. aegypti* using genetic engineering, and secondly, to evaluate the quality and productivity of a transgenic candidate strain for field release, OX513A. Transgenic strains were generated with a view to exploiting the *D melanogaster Alcohol dehydrogenase* (*DmAdh*) gene to provide a suitable marker-system for a GSS in *Ae. aegypti*, induced by ethanol (Chapter 3). Sex-alternate splicing was evaluated using two genes, *Ceratitis capitata transformer (Cctra)* and *Ae. aegypti doublesex* (*Aedsx*), in combination with a one-component tetracycline repressible system (**Figure 1.4.**) to generate female-specific expression in *Ae. aegypti* (Chapter 4). The initial attempt to combine both sex-alternate mechanisms and the *DmAdh* gene is reported in Chapter 4, and possible solutions are suggested for further investigation into the development of a GSS using the ADH system. Finally, a particular candidate strain, bi-sex RIDL called OX513A was further evaluated for its suitability in a RIDL-SIT programme, i.e. its propensity to mass rear successfully under artificial conditions (Chapter 6).

Chapter 2: General Materials and Methods

2.1. *Ae. aegypti* strains and rearing procedures

Mosquitoes used in this set of experiments were maintained in artificial breeding conditions under a 12h/12h artificial light/darkness photoperiod with temperatures varying from 24.5 °C to 29.8°C and relative humidity from 37 % to 90.5 %. Immature stages (mosquito larvae) were fed with finely ground Tetramin® fish food (Tetra GmbH, Germany) under a strict regime (**Table 2.1.**). When needed, pupae were screened for fluorescence using a Leica MZ95 microscope mounted with fluorescence unit containing filters from Chroma Technology (Rockingham, VT, USA) specific for the AmCyan fluorescent protein (exciter D436/20x; emitter D480/40m) and DsRed2 (exciter HQ545/30x; emitter HQ620/60m). Pupae were manually sex-sorted under dissecting microscope by observing the terminal end of the abdomen (**Figure 2.1.**). Mosquito cages (BugDorm-4005, Megaview MegaView, Taichung, Taiwan; dimension: 17.5 x 17.5 x 17.5 cm) were provided with 10 % sugar bottles with dentist cotton wicks supplemented with 14 $\mu g.ml^{-1}$ of both streptomycin and penicillin to prevent from bacterial growth. Female mosquitoes were provided with defibrinated horse blood (TCS Biosciences Ltd., UK) 2 days after being mated and eggs were collected 2 days later on wet white filter paper (Whatman, UK) or cotton wool.

Day	*Food (mg) per larva*
1	0.08
2	0
3	0.16
4	0.32
5	0.64
6 and above	0.32

Table 2.1. Feeding regime for *Ae. aegypti* fed on Tetramin® tropical fish food following Irka Bargielowski's.

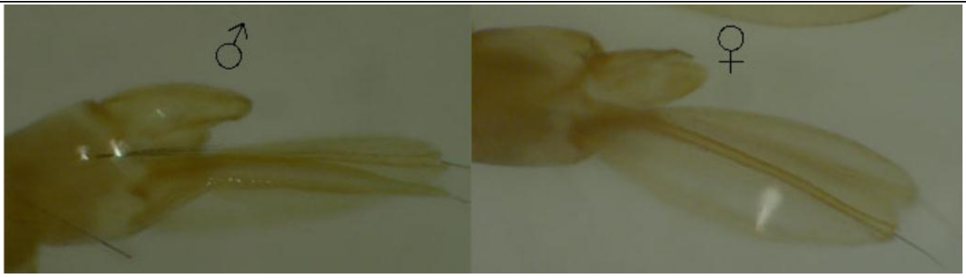


Figure 2.1. Anatomical differences between *Ae. aegypti* males and females at pupal stage. The male pupa (left) can be differentiated from the female pupa (right) by observing the end of the abdomen, in form of a hook in males (left) and round-shaped in females (right).

2.1.1. Description of the *Ae. aegypti* wild-type strain used in this study

The *Ae. aegypti* wild-type strain originates from field-caught females from Jinjang, Selangor, Malaysia. It was colonised in 1975 and has been reared in the laboratory since then for many generations. This strain was chosen because of its genetic similarity to the modified RIDL females they were compared to (see below) as well as being well adapted to the artificial blood feeding system which makes them suitable for such a designed fecundity assay (see below). This strain may not respond the same way to recently colonised strains (see Discussion for more details) but was chosen because of its genetic similarity to the OX513A with which it has been compared.

2.1.2. Description of the *Ae. aegypti* transgenic strains

Throughout the thesis, various *Ae. aegypti* transgenic strains were created and analysed in search for a specific phenotype such as a non bi-sex selection for the ADH marker (OX4346 in **Chapter 3**); a female specific lethality within a tetracycline repressible system (OX3097 and OX4489 in **Chapter 4**), and, finally, a female specific selection for the ADH marker (OX4597 in **Chapter 5**). Other transgenic lines were created before this study and were used in my thesis for diverse reasons: the OX3599 strain was used as a 'genetically-transformed-control' strain in the 'Chemical selection' study (**Chapter 3**) in comparison with the *Adh*-transformed lines (OX4346); the bi-sex *Ae. aegypti* RIDL line (OX513A) was used for fecundity and fertility experiments (**Chapter 6**). The list of each transgenic line used in this thesis as well as their main genetic elements are presented in **Table 2.2.**.

Plasmid constructs	*Marker*	*Features*	*Chapter discussed*
OX4346	3xP3 – DsRed2	Aehsp83 – DmAdh*****	3
OX3599	3xP3-DsRed2	teto$_{21}$-Dmhsp70- AeCalmodulin	3
OX3097	Hr*iE1* – DsRed2	tetO$_{21}$ – Dmhsp70 – tTAV1	4
OX4489	3xP3 – DsRed2	tetO$_{7}$ – Dmhsp70 – Aedsx– tTAV1	4
OX4597	Hr*iE1* – AmCyan	Aehsp83 – Aedsx – DmAdh******	5
OX513A	Act5C – DsRed2	tetO$_{7}$ – Dmhsp70 –tTAV	6

Table 2.2. Listing of each plasmid construct and transgenic strains studied in the thesis.

***** Full *Drosophila melanogaster* intron and exon sequence of the *Adh* gene; ****** Full exon sequence of the *Dm Adh* gene without the intron sequence.

OX513A, bi-sex lethal

One RIDL candidate lines is OX513A (Chapter 6), is the result of the injection of a *piggyBac* plasmid construct (**Figure 2.2.**) in *Ae. aegypti*. OX513A includes a red fluorescence gene (DsRed2) driven by a *D. melanogaster* non-muscle actin gene promoter (Act5C), allowing transgenic larvae to be discriminated from wild-type by observation under a fluorescence microscope. The construct further includes a tetracycline-dependent dominant lethal system (**Chapter 1**), allowing transgenic specimens to develop normally throughout their life cycle when reared in the presence of tetracycline, while killing specimens reared in the absence of tetracycline. This transgenic line was originally transformed in the Rockefeller strain and subsequently out-crossed into a Mexican line of *Ae. aegypti*. It has since been crossed to the wild-type line described above for several generations so both lines share most of their genome.

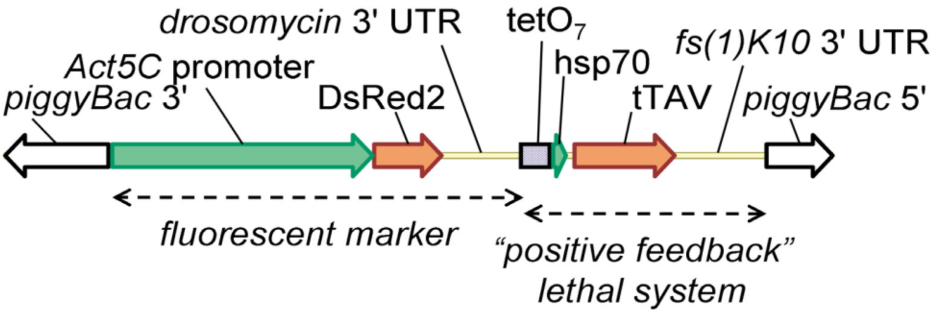


Figure 2.2. The *piggyBac* plasmid construct OX513A from Phuc *et al.* (2007)**.**
The tetracycline-repressible transcriptional activator gene tTAV is driven by a minimal promoter (hsp70) and the regulatory element tetO. The transformation marker DsRed2 is driven by Act5C promoter leading to a red fluorescent spotted pattern all over the larvae and pupae body.

2.1.3. Plasmid construction

All digest and PCR reactions were loaded on a 1% agarose gel and extracted using the MinElute PCR Purification Kit from Qiagen (Hilden, Germany). PCR and digest reactions Products were ligated together using T4 DNA ligase (New England Biolabs) and cloned into XL-10-Gold Ultra-competent Cells (Stratagene, Agilent Technologies). Bacterial colonies were PCR screened and individual colonies picked for screening were also used to inoculate LB broth with 100 µg.ml^{-1} ampicillin and grown in a shaking incubator for several hours at 37 °C. Positive colonies were selected, 20 µl of the positive inoculate was used to inoculate 3 ml of LB with 100 µg.ml^{-1} ampicillin. The bacteria were grown overnight at 37 °C in a shaking incubator. DNA from positive clones was purified by miniprep (GeneJET Plasmid Miniprep Kit from Fermentas) and sent for sequencing (GATC Biotech). Bacteria expressing the final construct were used to inoculate 3 ml of LB with 100 µg.ml^{-1} of ampicillin. The cultures were grown for 8 hours at 37 °C at 200 rpm, these cultures were then was used to inoculate 250 ml of LB with 100 µg.ml^{-1} of ampicillin and grown overnight at 37 °C at 200 rpm. DNA was extracted from 1 ml of the culture by using the GeneJet^{TM} Plasmid Miniprep kit and eluting in 50 µl of elution buffer and 1 ml was removed for a glycerol stock. DNA was extracted from the remaining bacterial cells using the Endofree Plasmid Maxiprep kit (Qiagen, Crawley, West Sussex, UK) and eluting in 50 µl of endotoxin-free water.

2.2. Microinjections and establishements of transgenic lines

2.2.1. Preparation of injection solutions

The injection mixture consisted of a final concentration of 450 µg.µl^{-1} of plasmid DNA construct together with 300 µg.µl^{-1} mRNA helper encoding for *piggyBac* transposase and suspended in injection buffer (5 mM KCl and 0.1 mM NaH_2PO_4, pH 6.8). 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 MEGAclearTM kit (Ambion), precipitated with ammonium acetate and resuspended in 10 µl nuclease-free water.

2.2.2. Microinjections of *Ae. aegypti* eggs

Freshly laid *Ae. aegypti* eggs (1 – 2h) were injected with the help of a femtoJet microinjector (Eppendord, UK) following the procedure from Morris et al. (1989) with an extra set to the published procedure: the injected eggs mounted on slides were immediately transferred vertically in water to remove the carbon oil. After the whole microinjection process, injected eggs were left at 100% relative humidity (RH) for 3 days before being submerged in deionised water with one drop of Liquifry No. 1 (Interpet Ltd., Dorking, Surrey, UK) added to induce hatching.

2.2.3. Establishement of transgenic lines

Upon pupation, surviving specimens from the microinjections (called generation zero or G_0) were separated by sex at pupal stage (**Figure 2.1.**) to ensure virginity. Crosses were then performed as follows: transgenic females were pooled with wild-type virgin males (usually a 3:1 ratio), and transgenic males were pooled with wild-type virgin females (usually a 1:10 or 1:15 ratio). Specimens in crosses were allowed to mate for 2 days before they were blood fed two to three times to obtain a next generation of eggs (G_1), which were collected on a weekly basis. G_1 individuals were subsequently separated by sex and phenotype (transgenic or wild-type), with transgenic females being then out-crossed to wild-type males, and transgenic males out-crossed to wild-type females. This out-crossing process was repeated in the next generations, allowing for the selection of transgenic lines which contain a single insertion of the plasmid.

2.3. General statistical methods

Statistical analyses were performed using R software version 2.12.2 (R Development Core Team 2011). Age at pupation, wing length and female fecundity (number of eggs per gonotrophic cycle) were analysed with an ANOVA including treatments (strains, ethanol, sexes) and their interaction.

Unless stated otherwise, *Ae. aegypti* adult survival was analysed using the Kaplan-Meier method (Kaplan & Meier 1958) with non censored data (**Chapter 3** and **Chapter 6** experiment 1) as well as with censored data (**Chapter 6** experiment 2). Homogeneity of survival functions between treatments was tested with a log-rank test.

Ae. aegypti adult longevity was also analysed as an ANOVA in **Chapter 3** including ethanol treatments, sexes as well as their 2-way interaction as no mosquito was censored and the distribution of longevity was normal and gave similar results to the Kaplan-Meier analysis (Kaplan & Meier 1958).

Proportions (sex ratio and hatch rate) were analysed with a general linear model (GLM) with a binomial or a quasibinomial error family including treatments (strains, ethanol, sexes) as well as their interactions.

Chapter 3: Chemical selection for an inducible lethal system in *Aedes aegypti*

3.1. Introduction

Traditionally, the Sterile Insect Technique (SIT) (Knipling 1959) involves the release of mass-reared irradiated male insects into the wild, which will seek and mate with wild-type females. Since few or none of the offspring of these matings will be viable, a reduction in the local insect population will, ideally, be achieved over time.

The success of SIT relies on the mass-release of sterile males. Single-sex release of males requires removal of females (sex separation); this would preferably be at some early developmental stage (i.e. embryonic or first instar larvae) to minimise the cost of rearing such females prior to their elimination. In the particular case of mosquitoes, removal of females before release is critical for two reasons: a) sterile females would distract released males from mating wild females, thus reducing the efficiency of the programme, and b) female mosquitoes, even if sterile, would still be able to bite and transmit diseases.

Several approaches have been historically used to achieve sex-separation: Trials of the SIT in El Salvador for control of *Anopheles albimanus* used an insecticide in artificial blood meals to kill up to 93% of the females prior to release. Since mosquito males do not blood feed, they should not be affected, however, a 25% of the adult males were killed too from contact with contaminated blood or other unknown factor (Lowe et al., 1981). As previously mentioned in Chapter 1, other approaches (i.e. differences in cephalothorax size, sex-linked insecticide resistance genes, etc) have been also been explored, always with mixed results (Ansari et al., 1977b and Seawright et al., 1978).

Transgenic technology has allowed for the creation of genetically engineered sexing strains (GSS), which can easily be transferred from one species to another with minor changes in the genetic constructs used for transformation (Jin et al. 2013). Tetracycline-repressible female-specific lethality has been implemented using lethal genes under control of female-specific promoters (Gossen and Bujard 1992) in *D. melanogaster* (Heinrich et al., 2002 and Thomas et al., 2000) and the Mediterranean fruit fly *C. capitata* (Gong et al., 2005). Similarly, a tetracycline repressible female-specific flightless phenotype has recently been developed in *Ae. aegypti* using the promoter region from the AeAct-4 gene to express a toxic protein in the indirect flight muscles of adult females (Fu et al., 2010), which effectively constitutes a late acting lethal phenotype since these flightless females are unable to forage for a blood meal (and therefore cannot reproduce), or escape from predators. If released into the wild, the female progeny of transgenic individuals and wild individuals will be flightless due to the absence of tetracycline. The late acting phenotype is advantageous as larval competition between transgenic and wild-type larvae in breeding sites enhances the efficiency of an engineered male mosquito release (in comparison with classical SIT), leading to a quicker suppression of the targeted population (Phuc et al., 2007). However the female specific late-acting phenotype as a GSS can be improved by adding a system to kill females at an early stage (e.g. as 1st instar larvae), therefore reducing the cost of mass rearing in the release generation by halving the space required for larval rearing, staffing and food supply (Caceres 2002). Such a system would need to be independent of the tetracycline-regulated late-acting lethal, and probably inducible, so that female larvae can be killed by a specific treatment applied to cohorts intended for release.

The *Drosophila melanogaster* alcohol dehydrogenase gene (*Adh*) is a candidate for an alternative GSS in *Ae. aegypti*. The ADH pathway has been extensively studied because it plays a major role in environmental alcohol resistance in Drosophilidae and Tephritidae populations, the larvae of which are exposed to high concentrations of alcohol during development through their diets, which consist of fermenting fruit or fungus (Ashburner 1998; Gibson et al., 1981). The ADH system is responsible of 90% of the degradation of ethanol (EtOH) in *D. melanogaster,* catalyzing first the oxidation of ethanol to acetaldehyde, and subsequently the conversion of this highly toxic product into acetate, a common endogenous substance (Carvalho et al., 2009; Heinstra et al., 1983).

It has been proposed that alcohol resistance constituted a good selective marker when linked to a male specific trait in *D. melanogaster* (Robinson and Van Heemert 1981). In this study, sensitivity to environmental ethanol was created by inducing null mutations at the *Adh* locus. Then, an *Adh* allele linked to the Y-chromosome resulted in a male only line when 4% v/v ethanol was added in the larval diet (Robinson and Van Heemert 1981). However, in *C. capitata*, the creation of *Adh* null lines proved to be difficult, as none were viable as homozygotes preventing the achievement of a GSS (Robinson et al., 1986). In different Drosophila populations, the ADH protein is polymorphic for 2 different allozymes, Fast ADH (ADH-F) and Slow ADH (ADH-S) (Fletcher et al., 1978). Advantages of the Fast ADH (greater catalytic activity and higher amounts of ADH protein in Fast allozyme populations) over the Slow ADH led Christophides et al. (2001) to enhance resistance in male *C. capitata* creating transgenic insects carrying the former Adh-f allele under the control of a male-specific promoter. Although this method managed to increase ADH activity in the males by two fold (compared to the females), it did not allow for useful discrimination between the sexes.

Mosquito larvae feed on microorganisms, algae, protozoa, invertebrates and detritus present in the water. They can breed in water with very poor food supply but also in heavy organic matter content waters (Christophers 1960 and Becker et al., 2010). It has not come to our knowledge that mosquito larvae are naturally exposed to environmental sources of alcohol like Drosophilidae and Tephritidae larvae are (Ashburner 1998; Gibson et al. 1981). Also, in the animal kind, from insects to mammals, the two key enzymes known to metabolise ethanol and acetaldehyde are ADH and ALDH (Guo and Ren 2010), both present in the dengue fever mosquito (Park & Kwak 2009; Somwang et al. 2011). *Ae. aegypti* larvae populations are particularly exposed to various xenobiotics and can develop a panel of detoxification processes to combat insecticides. A recent study reported that both ADH and ALDH enzymes were involved in the metabolic pathway of the insecticide permethrin’s metabolites *in vitro* (Somwang et al., 2011). Transcriptomic studies showed a significant up-regulation of the alcohol dehydrogenase gene transcription (AAEL011130) in *Ae. aegypti* strains exposed to xenobiotics (the pyrethroid insecticide permethrin or the polycyclic aromatic hydrocarbon fluoranthene) compared to the non exposed strains (Poupardin et al., 2012); but a down-regulation of the same gene was observed when *Ae. aegypti* was artificially selected for *Bacillus thurigiensis israelensis* (*Bti*) resistance (LiTOX strain) in comparison with the *Bti* sensitive wild-type reference strain Bora-Bora (Tetreau et al., 2012c). It has then been thought possible that even moderate levels of increase in ADH expression could lead to the successful development of a GSS in the dengue fever mosquito *Ae. aegypti*.

The strategy adopted in this study for a successful development of a GSS using *D. melanogaster Adh* gene was to drive the latter under a strong, ubiquitous promoter to confer enough resistance to alcohol so transgenic larvae carrying a copy of the transgene would be efficiently discriminated from wild-type when exposed at low doses to the chemical. The *D. melanogaster* minimal promoter hsp70 is generally used in most lethal repressible systems in moths, medflies and mosquitoes at Oxitec (Fu et al., 2010; Labbé et al., 2012 and Phuc et al., 2007). While the engineered hsp70 shows minimal basal expression in Drosophila, hsp83 proteins have been shown to be strongly ubiquitously expressed and only a few fold induced following heat shock (Xiao and Lis 1989).

In this study, *Ae. aegypti* was then transformed on two separate occasions with a *piggyBac* vector carrying the *D. melanogaster Adh* gene driven by the *Aedes* hsp83 promoter creating 4 independent transgenic lines (OX4346A, B, D and F) the first time, and 12 the second time (the following four lines were kept as they all confirmed the phenotype observed the first time round: OX4346G[1], OX4346H, OX4346N and OX4346O). One of the first 4 initial transgenic strains created was studied with RT-PCR (OX4346F) to determine the expression levels of the *Adh* gene at different development stages. Ethanol bioassays were also used to determine the existence of a possible resistance phenotype of the transgenic strains (all initial 4 *Adh*-lines) in comparison with the wild-type strain (long term as well as short term exposure). The phenotype observed the first time was confirmed when tested on the four new *Adh*-lines (out of 12) created with the same

---

[1] The transgenic line OX4346G is referred as OX4346F1 in the laboratory book OXI-116.

construct 2 years later after the loss of the 4 initial lines: long term exposure to 0.5 and 1% ethanol is sufficient for a safe discrimination between the *Adh*-transformed larvae and the wild-type ones. In order to validate the *DmAdh* gene as a selective marker and its potential use in a GSS, the wild-type laboratory strain was submitted to the same ethanol treatments to assess its impact on several fitness parameters: adult longevity, mortality of the immature stages, sex ratio distortion, fecundity and fertility of the strain when the females were mated with males treated with ethanol.

3.2. Materials and Methods

3.2.1. Construct OX4346

Construct OX4346 [2] (12,106 bp) was engineered following reports by Christophides et al. (2001) on *C. capitata* (**Figure 3.1.**). The coding region and intron of the gene encoding the FAST isoform of *Drosophila* ADH, driven by the *Aedes* hsp83 promoter, was inserted into a *piggyBac* vector with DsRed2 fluorescent marker driven by an eye specific promoter (3xP3) (Berghammer et al. 1999).

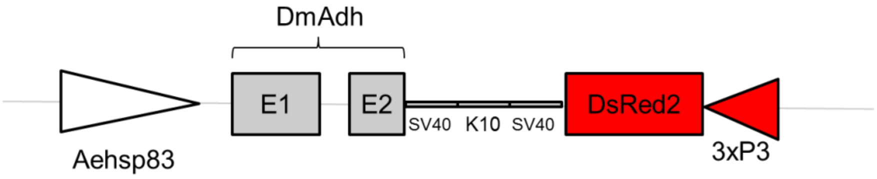


Figure 3.1. Construct OX4346 carries the Aedes hsp83 promoter linked to the *D. melanogaster Adh* intron and coding sequence.
The red eye transformation marker is translated with the three times artificial *Pax-6* promoter (3xP3) driving the *Discosoma* DsRed2 gene. The alcohol resistance mechanism is induced with the full intron and coding sequence of *D. melanogaster Adh* gene driven by the *Ae. aegypti* hsp83 promoter. Three prime untranslated regions (3'UTR) are placed downstream the *Adh* gene such as K10 (Keratine 10) and SV40 (Simian Virus 40) to enhance post translation of the gene of interest (here *Adh*).

---

[2] Construct designed by Tarig Dafa'alla, PhD at Oxitec Ltd.

3.2.2. Analysis of transgenic lines

Expression level of the *Adh* transgene in *Ae. aegypti* OX4346:

Using TRIzol® Reagent (Invitrogen), RNA was extracted from samples of 18 h, 46 h, 89 h and 165 h old OX4346F (OX4346 line F) larvae, as well as male and female pupae (one individual extracted for each sample). Extracted RNA was resuspended in 30 µl Milli-Q pure water (Millipore, Billerica). RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase from Invitrogen (**Table 3.1.**). Primers for detection of the *D. melanogaster Adh* gene were chosen to anneal on each side of the inserted gene that included a 70 bp intron sequence (**Table 3.2.**).

As a control for DNA contamination in RNA samples, genomic DNA was extracted from one OX4346 line F male pupa. The resulting amplicon of the inserted *Adh* gene gDNA is bigger by 70 bp than its RNA counterpart (respectively 406 bp and 336 bp) due to the presence of an intron. Additionally, a control experiment in which no reverse transcriptase was added to the reaction mix was performed to test for the presence of DNA contamination. Finally, we used the *Ae. aegypti* muscle actin (AeMuAc) as positive endogenous control (**Table 3.2.**) as it is expressed throughout development (Sarah Scaife, personal communication).

Relative RNA concentrations were estimated by running equal volumes of each sample on an agarose gel, and comparing the intensity of fluorescence of the migrated bands. RNA templates for RT-PCR were then levelled by increased dilutions: L1 and L2 (2 µl), L3 (0.5 µl RNA + 1.5 µl H_2O), L4 and male pupae (0.2 µl RNA + 1.8 µl H_2O) and female pupae (0.2µl after a 1:1 dilution of original RNA sample + 1.8 µl H_2O). Equal volumes of normalised RNA template (2 µl) were added in the RT-PCR one step procedure with both *Adh* and AeMuAc primers.

Reaction mix	***Final concentrations***	***Volume in µl***
2X Reaction mix	1X	12.5
RNA template	0.01 pg to 1 µg	2
Sense primer	10 µM	0.2
Anti-sense primer	10 µM	0.2
Super Script III RT / Platinum® *Taq* Mix	-	1.0
H_2O	-	10.1
Total volume	**-**	**25.0**

Table 3.1. Reverse Transcription PCR volume reaction mix.

F primer name	***F primer nucleotide sequence***	***Product size (bp)***
DmAdh-1	CACCAAGCTGCTGAAGACCATCTTC	336 bp (cDNA)
DmAdh-3	ATGCCGGGGTTCACAGTGTAAG	406 bp (gDNA)
AeMuAcRTF	GGCATTCATTCTAGTTCCGAAACCG	1066bp (cDNA)
AeMuAcR	CCCAGGAAGGATGGCTGGAAGAG	3141bp (gDNA)

Table 3.2. Primer sequences and expected products.

3.2.3. Ethanol bioassays

Mosquito larvae were raised at temperatures between 25.9 °C and 29.1 °C and Relative Humidity varying between 40 and 80%. Stock solutions of 8.4 ml absolute ethanol (0.6 % v/v, Fisher Scientific) were prepared in a 1,400 ml final volume adding deionised water. Batches of 300 first instar larvae were repeated 3 to 5 times for each treatment and previously counted into 50 ml of deionised water. Those 50 ml L1 aliquot were poured at random into 250 ml of deionised water (control batches) or 0.6 %v/v ethanol solution (stock solution) bringing the final ethanol concentration to 0.5 %v/v and the final volume larvae were reared in 300m ml. After addition of the larvae, the rearing vessels were covered to reduce evaporation of the ethanol during the experiment.

In order to test whether the response to ethanol observed in the OX4346 strain was the result of the general fitness cost associated with genetic transformation - as opposed to being the direct effect of expression of the *Adh* transgene - ethanol bioassays were performed on the non-*Adh* transgenic line OX3599, which is an inbred transgenic line carrying the DsRed2 marker under control of the 3xP3 promoter. As an extra control, the same ethanol bioassays were performed on the original wild-type laboratory strain to measure the impact of long term ethanol exposure on survival to pupae. Ethanol bioassays exhibiting more than 20 % larval mortality in the controls (0 % ethanol) were discarded from the analysis (Abbott 1925).

Long-term exposure

Larvae were reared in ethanol solutions of initial concentrations of 0 %, 0.5 %, 1 %, 1.5 % and 2 % v/v, which were not renewed over the course of the experiment. Each replicate was fed daily with a standard amount of Tetramin® fish food (Tetra Werke, Melle, Germany) per larva (**Table 2.1.**). Pupae were picked daily and numbers of transgenic and wild-type male and female individuals were recorded.

Short-term exposure (24 h)

First instar larvae were raised in ethanol solutions of concentration 0, 1, 2, 3 and 4 % v/v for 24 h, after which the larvae were transferred into 300 ml deionised water and fed with the same standard feeding regime as for ‘long-term exposure’ experiments. Pupae were again picked daily and numbers of transgenic and wild-type male and female individuals recorded.

3.2.4. Impact of ethanol on the *Ae. aegypti* wild-type laboratory strain fitness

During the longevity and fecundity experiments, temperatures in the laboratory were varying between 26.8 and 29.8°C and Relative Humidity between 37 and 72%.

Wing length measurements

Ae. aegypti wing length from alular notch to apex (Nasci 1990) was chosen as the traditional measure of size for mosquitoes. Females' wings were removed and mounted on microscope slides with 70% ethanol. A photograph of the wings together with a graticule was taken for measurement, using a Canon powershot S5IS camera and a 99mm adapter (S/N:3754, Martin Microscope Company). Wings were then measured using ImageJ software (http://rsbweb.nih.gov/ij/).

Adult mosquito survival assay

Male pupae were collected on pupation days 6, 7, 8 and 9 and female pupae on day 8 only and placed into separate cages for eclosion. For each ethanol treatment (0, 0.5 and 1 %) and each pupation day, four cages were set up with 5 males or 4 females each. A 10% sucrose solution supplemented with 14 μg.ml^{-1} penicillin and 14 μg.ml^{-1} streptomycin was available *ad-libitum* throughout the experiment. Dead mosquitoes were recorded on alternating days until all died. Adult survival was measured as the mean number of days between egg hatching and the death of the adult.

Fecundity and fertility assay

Three different types of single-pair mating crosses with 40 repeats of each were set up in tubes four days later, as follows: 1 wild-type female reared in deionised water only with 1 wild-type male reared in deionised water only, or with 1 wild-type male reared in 0.5 % v/v ethanol, and finally with 1 wild-type male reared in 1 % v/v ethanol. Females were blood fed and eggs collected through 3 gonotrophic cycles, females being placed in fresh plastic tubes for egg laying. Eggs were counted for each female of each cross as well as for each gonotrophic cycle. Female's time of death as well as wing length were recorded. Eggs collected from each gonotrophic cycle and cross were tested for their hatch rate (i.e., 90 females in total) 4 to 7 days after being laid. Live larvae were counted 24 hours after hatch stimulus.

2.3.5. Statistical analysis

Statistical analyses were performed using R software version 2.12.2 (R Development Core Team 2011). Adult longevity was assessed as the mean number of days male and female mosquitoes lived between the four groups and was analysed as an ANOVA as well as with a Kaplan-Meier analysis which gave similar results. Larval survival was estimated as the proportion of transgenic individuals to wild-type surviving to pupation in each pot, was ArcSin transformed and was analysed as an ANOVA including ethanol treatment (0%, 0.5% and 1%), strain (*Adh*-A, *Adh*-F, *Adh*-H, *Adh*-N, *Adh*-O, OX3599 and wild-type) and their 2-ways interaction. The proportion of wild-type individuals surviving to pupation in each pot was used as a control.

3.3. Results

3.3.1. Production of transgenic lines

Out of 1,715 wild-type eggs injected during the first injection period, 375 (22%) survived to adulthood, and 4 transgenic lines were obtained from separate adult pools: OX4346A, OX4346B, OX4346D and OX4346F (Group 1). Transgenic larvae from all four lines expressed the 3xP3 DsRed marker in the eyes and in the anal papillae. OX4346D and OX4346F additionally expressed the marker in the ganglionic connectives (**Figure 3.2.**). Another 1,634 wild-type eggs were injected 2 years later (Group 2) since the four former transgenic lines had been lost. Seven percent survived to adulthood and 12 transgenic lines were obtained from separate adult pools. All 12 transgenic lines expressed the same phenotype mentioned above and 4 were chosen for further analysis (long term ethanol exposure) as they were exhibiting a 1:1 transgenic to wild-type ratio as well as male to females at the third generation (called lines OX4346G, H, N and O), indicating a non-sex-linked single insertion.

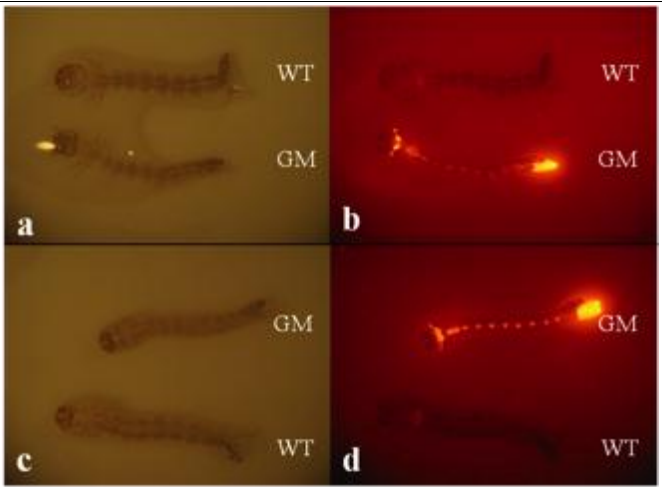


Figure 3.2. Phenotype of piggyback transformed *Ae. aegypti* OX4346F larvae.
Fluorescence micrographs of heterozygous OX4346F (GM) and wild-type (WT) larvae from dorsal view (a, b) and ventral view (c, d) expressing 3xP3-DsRed2 marker (red eyes; anal papillae and ganglionic connectives) under normal light (a,c) and red filter (b, d).

3.3.2. Characterization of the lines

OX4346 lines, single insertion and sex bias

OX4346 heterozygous individuals crossed to wild-type and bearing a single insertion of the construct should result in a marker segregation of 1:1 in the progeny. Screening progeny from these outcrosses at the 4th generation showed a 1:1 segregation of marker for all lines excepting line D, suggesting the lines carried a single insertion of the construct. Segregation of the marker in line D showed a significant difference from the expected 1:1 ratio (out of 1,053 screened pupae, 46% were transgenic and 54% were wild-type). However, a double insertion of the plasmid would exhibit a ratio of 75% transgenic compared to 25% wild-type, therefore OX4346 line D could also be considered as a putative single insertion, perhaps with a modest dominant fitness cost.

The OX4346B insertion was linked to the male-determining locus with the estimated distance between the two loci of approximately 11.4 centimorgans (cM) (**Table 3.3.**).

A				**B**			
Males:	702 (50%)	GM	625 (89%)	Males:	770 (54%)	GM	377 (49%)
		WT	77 (11%)			WT	393 (51%)
Females:	696 (50%)	GM	82 (12%)	Females:	661 (46%)	GM	316 (48%)
		WT	614 (88%)			WT	345 (52%)

Table 3.3. The OX4346B insertion is linked to the male-determining locus.
The screening and sexing of pupae from a cross between heterozygous OX4346B (GM) males and wild-type (WT) females (**A**) showed a transmission of the transgene skewed towards male progeny (89% of male progeny expressed the marker, versus only 11% of the female progeny, n=1,398). When heterozygous OX4346B females (GM) were crossed to wild-type (WT) males (**B**) the proportions of the progeny were normal (51:49 and 48:52 transgenic to wild-type) as well the sex-ratio in both crosses (50:50 and 54:46 male to female ratio) indicating a male-linked insertion rather than female lethality. Non-parental phenotype was observed in 11.4% of the progeny, indicating a distance of 11.4 centiMorgans (cM) between the insertion and the male-determining locus.

A weaker male linkage seems to be observed by screening and sexing 1,348 pupae from a cross between OX4346F males with wild-type females (**Table 3.4.**), with an estimated distance of 31.7 cM between the insertion of the OX4346F from the male-determining locus.

A		GM:	480 (68%)	**B**		GM:	344 (49%)
Males:	703 (52%)	WT:	223 (32%)	Males:	696 (51%)	WT:	352 (51%)
		GM:	204 (32%)			GM:	355 (53%)
Females:	645 (48%)	WT:	441 (68%)	Females:	668 (49%)	WT:	313 (47%)

Table 3.4. There is a weak linkage of the OX4346F insertion to the male-determining locus. The screening and sexing of pupae from a cross between heterozygous OX4346F (GM) males and wild-type (WT) females (**A**) showed a transmission of the transgene slightly skewed towards male progeny (68% of male progeny expressed the marker, versus 38% of the female progeny, n=1,348). When heterozygous OX4346F females (GM) were crossed to wild-type (WT) males (**B**) the proportions of the progeny were normal (51:49 transgenic to wild-type) as well the sex-ratio in both crosses (52:48 and 51:49 male to female ratio) indicating a male-linked insertion rather than female lethality. Non-parental phenotype was observed in 31.7% of the progeny, indicating a distance of 11.4 cM between the insertion and the male-determining locus.

Screening and sexing 1,053 pupae from a cross between OX4346D males with wild-type females showed no significant difference in the proportions of male and females (sex ratio) (**Annexe 8, Table 8.1.**); 25 and 21% were respectively transgenic males and females and 27 and 27% were wild-type males and females. No sex ratio distortion was recorded for the newly injected lines OX4346 H and N (**Annexe 8, Table 8.1.**).

Very few eggs were collected from the OX4346A heterozygous strain. Out of 906 pupae analysed, the result of the crossing from OX4346A heterozygous females with wild-type males gave a 50:50 transgenic to wild-type ratio as well as a 58:42 male to female ratio (**Annexe 8, Table 8.1.)**. Sex ratio of the heterozygous OX4346A individuals was not significantly different from 50:50, whereas the proportion of males and females within the wild-type individuals was (P-value < 0.001, n=906).

Out of 730 analysed pupae, the heterozygous OX4346G strain showed a skewed proportion toward males (59:41 male to female ratio, P-value < 0.007) whereas the heterozygous OX4346O individuals showed a skewed proportion towards heterozygous transgenic females (43:57 heterozygous OX4346O male to female ratio, n=959, P-value = 0.005). Those results are scored as departing the 50:50 ratio when Chi-square tested (59:41 and 43:57 for the respective OX4346G and OX4346O heterozygous strains), however, the distortion of the sex ratio might just be a reflection of the low number of larvae available for each test (n=730 and n=959 respectively, one repeat each only).

DmAdh* gene is expressed from early larval stage in *Adh*-transformed *Ae. aegypti

To evaluate the expression of the *Adh* transgene in immature stages, RT-PCR analysis was performed on line OX4346F using 1st to 4th instar larvae, as well as pupae. This *Adh*-line from Group1 was chosen since it presented single insertion pattern, no sex bias (**Table 3.5.**) and responded strongly to ethanol bioassays (**Figure 3.5.**). The expected *Adh* amplicon (336 bp) was observed in all life stages of the transgenic line, shown alongside with the corresponding plasmid control band, containing the 70 bp intron, at 406 bp **(Figure 3.3.**). A faint band can be seen at 406 bp in all the samples, suggesting some gDNA contamination in the RT-PCR template. The control samples (Water, wild-type samples and ‘No RT’) showed no evidence of *Adh* transcripts. DNA amplification from the gDNA template has clearly migrated above the 400 bp band, suggesting that the smaller (336 bp) amplicon observed following RT-PCR is the result of the splicing of the 70 bp intron following transcription.

Adh transcripts were detected in 18 h old larvae, suggesting that the *Aedes* hsp83 promoter is active at early larval stages. This agrees with previously reported expression profiles of the *hsp83* gene in *Ae. aegypti* (Zhao et al., 2010). The

Aedes muscle actin gene was chosen as positive control and was expected to be expressed at all life stages. However, the expected 881 bp cDNA bands were only observed from the 3rd larval instar (respectively, sample **5, 6, 7, 8, 10** and **11** in **Figure 3.4.**).

It is possible that expression of the endogenous muscle actin gene on single first and second instar larvae (L1 and L2) chosen as control in this experiment only reaches detectable levels on single third instar larva (L3) (sample **5** in **Figure 3.4.**). However, AeMuAc transcripts were recently amplified from 100-first-instar-pooled-larvae's cDNA from 25 PCR cycles (M. Conway, personal communication).

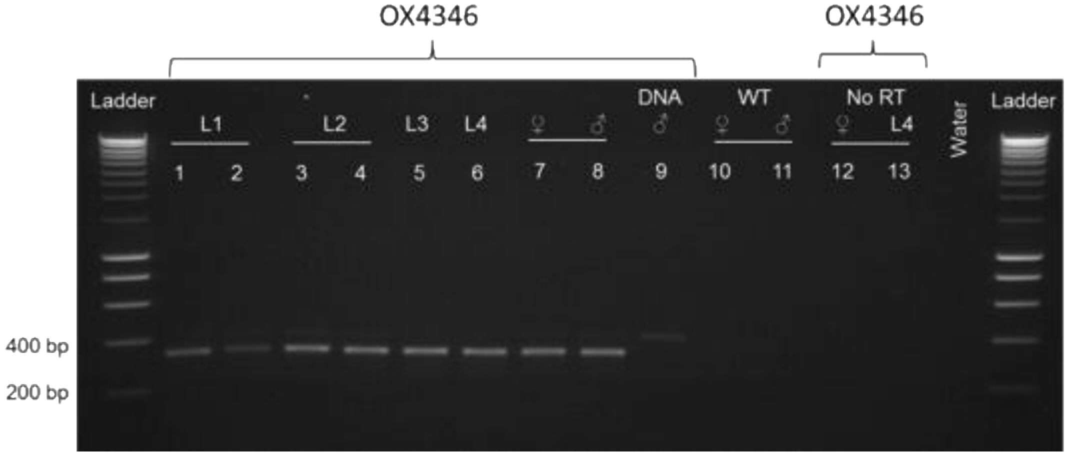


Figure 3.3. RT-PCR on *Ae. aegypti* OX4346F larvae and pupae using primer pairs DmAdh-1 and DmAdh-3.

RT-PCR was performed on heterozygous OX4346F samples (lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13) as well as wild-types (lanes 10 and 11) using primer pairs DmAdh-1 and DmAdh-3. A constant expression of the *DmAdh* transcripts throughout the immature stages of the heterozygous OX4346 is observed. Ladder: Smart ladder from Eurogentech, 200 to 10,000bp; Lanes 1 and 2: 18 h old larvae (L1); Lanes 3 and 4: 46 h old larvae (L2); Lane 5: 89 h old larva (L3); Lane 6: 165 h old larva (late L4); Lanes 7 and 8: resp. Female and Male pupae; Lane 9: Male pupa gDNA control; Lanes 10 and 11: resp. Female and Male wild-type pupae; Lanes 12 and 13: same samples as in lanes 7 and 6 but run without Super Script III reverse transcriptase for negative control; Water: water only as template for negative control. The bands are of the expected size (Table 3.2.).

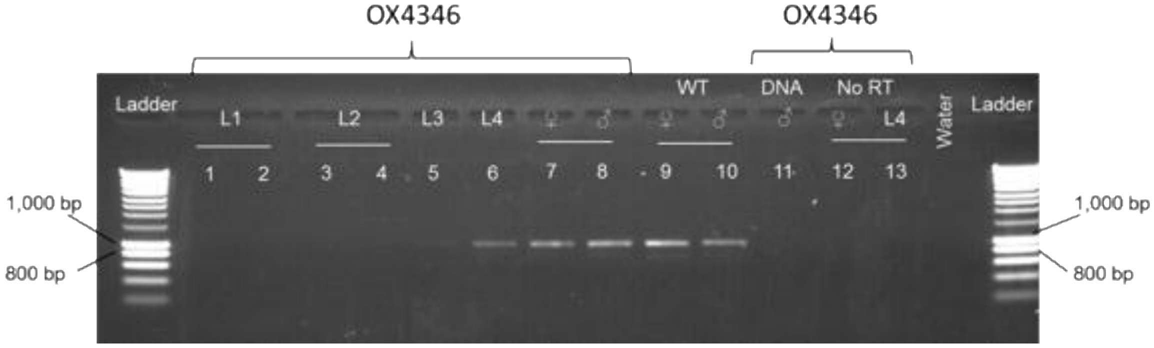


Figure 3.4. RT-PCR on *Ae. aegypti* OX4346F larvae and pupae using primer pairs AeMuAcRTF and AeMuAcR.

RT-PCR was performed on heterozygous OX4346F samples (lanes 1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 13) as well as wild-types (lanes 9 and 10) using primer pairs AeMuAcRTF and AeMuAcR. A differential expression of *Aedes muscle actin* transcripts are observed faintly from L3 larval stage to pupae. Ladder: Smart ladder from Eurogentech, 200 to 10,000bp; Lanes 1 and 2: 18 h old larvae (L1); Lanes 3 and 4: 46 h old larvae (L2); Lane 5: 89 h old larva (L3); Lane 6: 165 h old larva (late L4); Lanes 7 and 8: resp. Female and Male pupae; Lanes 9 and 10: resp. Female and Male wild-type pupae; Lane 11: Male pupa gDNA control; Lanes 12 and 13: same samples as in lanes 7 and 6 but run without Super Script III reverse transcriptase for negative control; Water: water only as template for negative control. The bands are of the expected size (Table 3.2.).

Long-term exposure to ethanol kills the larvae carrying the OX4346 transgene

The *Adh*-transformed lines were reared in deionised water containing varying concentrations of ethanol to test for any tolerance to alcohol conferred by the *Adh* transgene. The proportion of *Adh*-transformed and wild-type individuals was evaluated as explained in the Materials and Methods section.

Survival to pupation with long-term exposure to ethanol

Ae. aegypti has successfully been transformed with the *D. melanogaster Adh* gene, with the aim to confer resistance to transgenic larvae when exposed to ethanol. However, unexpectedly, results clearly have shown the opposite: *Adh*-transformed larvae of all strains exhibit increased susceptibility instead of resistance when ethanol is supplemented in the larval water in comparison with wild-type.

Out of 1,000 L1 tested, 90% OX4346A and 91% wild-type survived to pupae when no ethanol was supplemented in the larval rearing water (**Figure 3.5.**). At 0.5% ethanol, fewer OX4346A survived (73%) than wild-type (85%) (**Annexe 8, Table 8.2.**). Finally, at 1% ethanol, no OX4346A**/+** survived whereas 89% wild-type did.

Out of 1,500 L1 tested, 77% of OX4346B larvae survived to pupae in absence of ethanol, whereas 88% of wild-type did. At 0.5% ethanol only 4% of OX4346B survived to pupae, while 84% wild-type did. No OX4346B individuals survived when exposed to 1% ethanol, whereas 77% of the wild-type survived.

Comparing OX4346D to wild-type, 85% and 88% of larvae respectively survived to pupae when no ethanol was supplied out of 1,500 L1 tested. However, only 19% OX4346D larvae survived when exposed to 0.5% ethanol, compared to 85% survival for wild-type under similar conditions. Furthermore, 0% survival of transgenic specimens was observed at a concentration of 1% ethanol, compared to 74% survival for wild-type. These results suggest that OX4346D larvae were more sensitive to ethanol than wild-type.

When 1,500 OX4346F larvae were raised in the absence of ethanol, 91% of OX4346F and 89% of wild-type specimens reached the pupal stage (**Figure 3.5.**). However, at 0.5% ethanol, only 1% of OX4346F larvae survived to pupae, as opposed to 36% of wild-type. At 1% ethanol concentration, 37% of wild-type larvae survived compared to 0% of OX4346F. The ethanol bioassay was repeated three times using the same OX4346F line to confirm the results (**Annexe 8, Table 8.2.**). Each time, the same pattern is observed: OX4346F larvae do not survive at 1%

ethanol (0% survival) whereas wild-type survive better (60% and 38%). This variation in survival rate of the wild-type specimens might be due to experimental error or variation in the dosage of the ethanol solutions prepared, as bioassays were performed on different days and stock solutions were prepared independently for each bioassay (**Annexe 8, Table 8.2.**).

The other four *Adh*-transformed lines (Group2) were assessed with long term ethanol exposure at 0, 0.5 and 1% v/v with three repeats each of 300 L1 in 300 ml medium. Results were consistent with the first 4 *Adh*-transformed lines: on average, 0%, 5%, 19% and 0% of the transgenic individuals survived (lines OX4346G, H, N and O respectively) as opposed to 52%, 48%, 56% and 46% for the wild-type individuals when submitted to 1% ethanol dose (**Annexe 8, Table 8.2.**).

More bioassays were then performed to confirm that the inserted *Adh* gene alone is responsible for this increase in susceptibility rather than the transformation of *Ae. aegypti*. For that purpose, another transgenic line was bio-assayed (which did not contain the *Adh* gene). Either in absence or in presence of ethanol in the larval media, a proportion of 75% OX3599[3] and 25% wild-type individual was expected, the generation tested being the product of the first inbreeding of single inserted transgenic line (the offspring of heterozygote OX3599 individuals crossed with heterozygote OX3599 will be composed of 25% wild-type, 25% homozygote OX3599 and 50% heterozygote OX3599). In the absence of ethanol and at both ethanol concentrations tested (0.5% and 1%), the proportions of transgenic and

---

[3] The *Ae. aegypti* OX3599 line is a *piggyBac* transformed line carrying the marker DsRed2 under the control of 3xP3 promoter.

wild-type surviving to pupae did not deviate from the expected frequencies (**Figure 3.5.** and **Annexe 8, Table 8.2.**).

As advised in Abbot 1925, toxicological assays with over 20% mortality in the control treatments (deionised water only) were discarded from the analysis. Were then discarded *Adh* lines B, D and G. For the rest of the *Adh* lines all three parameters had a significant impact on larval survival: ethanol treatments (0, 0.5 and 1% v/v), line (*Adh* lines, A, F, H, N, O and OX3599) and the 2-ways interaction EtOH : line (F=227.016 df=1, p<0.001; F=25.32, df=6, p<0.001; F=9.59, df=6, p<0.001). Bioassays reavealed that ethanol had a particular effect on *Adh*-F larvae in comparison with the other strains (**Figure 3.6.**) (t=-3.731, df=6, p<0.001). However, toxicological assays were set up for each strain in 5 replicates staggered throughout the week (i.e. line *Adh*-F five replicates were set up on day 25 May 2010, line *Adh*-D on the 26 May, line *Adh*-A on the 27th etc). Fresh ethanol solutions (0, 0.5 and 1% v/v) were then prepared for each strain every day and the fact that line *Adh*-F was responding more strongly to ethanol treatments could be an artefact (somehow more concentrated 0.5% v/v ethanol stock solution on that day). The interaction of ethanol treatments (0, 0.5 and 1% v/v) and the lines (*Adh* lines, A, F, H, N and O) were however significantly lower for all the *Adh*-transformed lines only in comparison with the non-*Adh*-transformed line (OX3599) and the wild-type control line (**Figure 3.6.C**).

This suggested that the *Adh* transgene alone was responsible for the increased susceptibility to ethanol observed in the transgenic specimens, rather than this effect being a by-product of transformation. Also, based on the multiple independent insertions, the effect is not due to position effect, either.

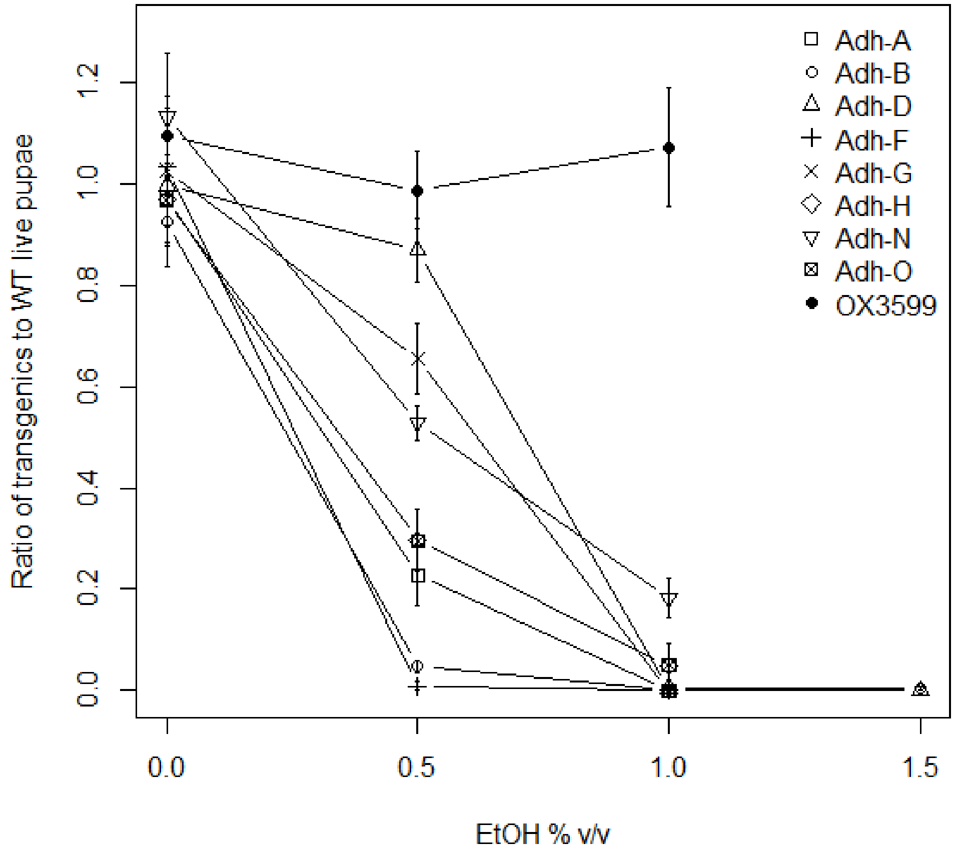


Figure 3.5. Ratio of *Adh*-transformed to wild-type siblings from various heterozygous *Adh*-transformed lines when exposed to long term ethanol.

Heterozygous *Adh*-transformed lines were tested for long term ethanol exposure and the ratio between wild-type individuals to *Adh*-transformed siblings was calculated. The *Adh*-transformed heterozygous lines were OX4346 line A, B, D, F, G, H, N and O. A non *Adh*-transformed line (OX3599) was also tested to long term exposure to ethanol. The ratio of transgenic versus wild-type siblings has been corrected for the non *Adh*-transformed line OX3599 (divided by 3) to bring the ratio around 1 as the line was ‘inbred’ (75% are transgenic and 25% wild-type, see Materials and Methods and Results for more details). Error bars represent standard error of the mean ratio. N=5 with 300 starting first instar larvae per replicate (except line *Adh*-A: 200 starting first instar larvae).

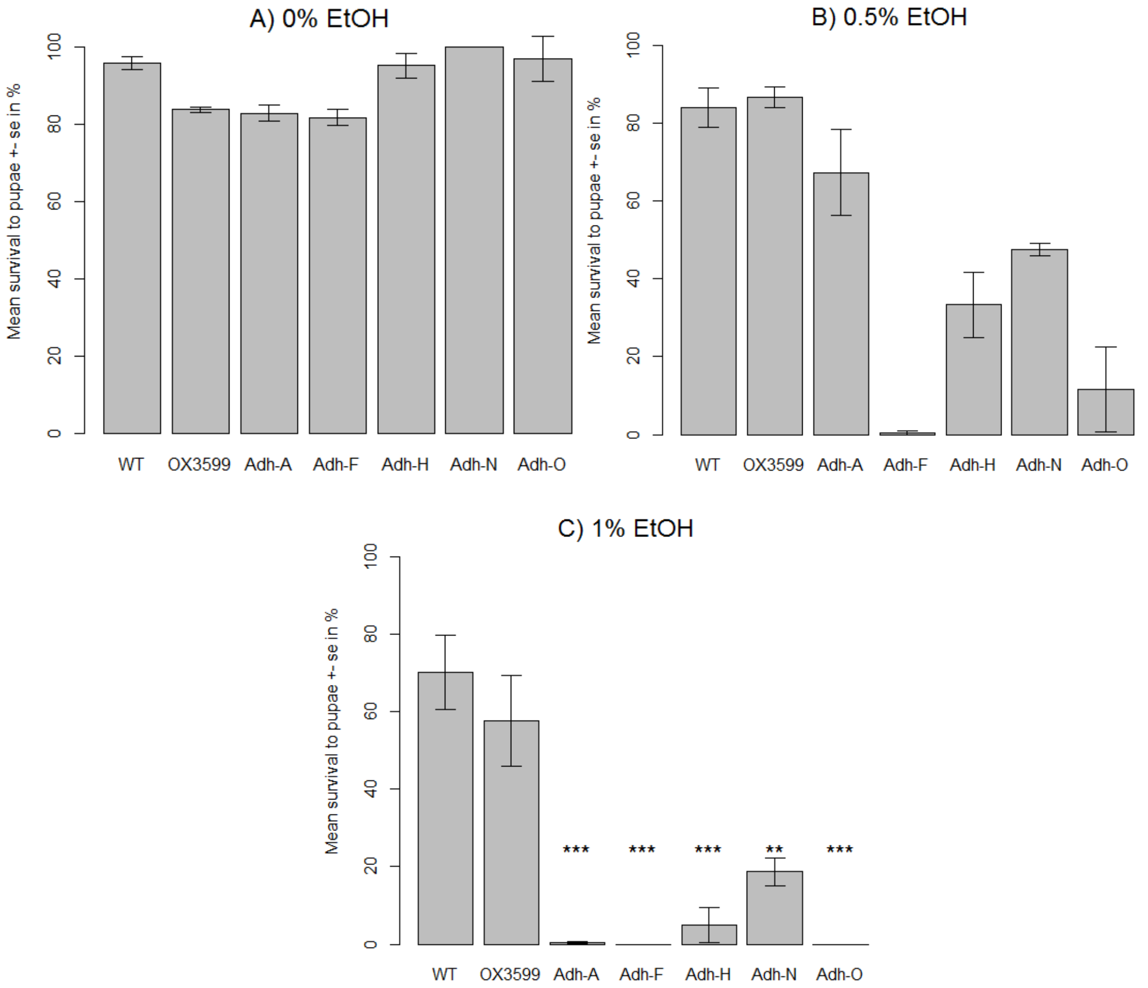


Figure 3.6. Survival from first instar larva to pupa of heterozygous *Adh*-transformed *Ae. aegypti* lines in comparison with a non *Adh*-transformed and a wild-type laboratory line.

Percent survival from first instar larvae to pupae of *Adh*-transformed *Ae. aegypti* lines (*Adh*-A and F from Group1 and *Adh*-H, N and O from Group2) in comparison with the wild-type laboratory strain (WT) as well as a non-*Adh*-transformed *Ae. aegypti* strain (OX3599). The different strains were reared in standard conditions (deionised water only with fish food) (A); submitted to long term exposure to 0.5% ethanol (EtOH) (B) and 1% ethanol (C). Error bars represent the standard error of the mean. Number of replicates are N=5 with 300 L1 per replicates for *Ae. aegypti* strains OX3599, *Adh*-F and wild-type; N=5 with 200 L1 per replicate for line *Adh*-A; N=3 with 300 L1 per replicates for lines *Adh*-H, N and O. Differences between the interaction of ethanol treatments and line is denoted by stars on graph C only (p value <0.001 = *** and p value <0.01 = **) on the graph C) in comparison with the controls (WT strain).

Short-term exposure to ethanol (24 h)

Long-term exposure to ethanol led to a higher mortality of *Adh*-transformed larvae in comparison with wild-type or non-*Adh*-transformed ones. One possible explanation is that highly toxic by-products of the metabolisation of ethanol by ADH accumulate and eventually lead to the death of the larvae following long-term exposure to ethanol. One hypothesis was that if more OX4346 larvae survive to pupae than wild-type following a shorter exposure period (24 h) from first instar larvae (24h post hatching stimulus), this would indicate an improved detoxification of ethanol with the help of the extra *Adh* gene present in transgenic larvae than in wild-type, but with exposure time not being long enough to accumulate lethal levels of toxic by-products.

Overall, ethanol treatments (0, 1, 2, 3 and 4% v/v) significantly reduced larval survival of *Adh*-transformed lines as well as wild-type (F=589.99, df=1, p<0.001) (**Table 3.5.**). There was a significant difference of larval survival of the *Adh*-transformed individuals in comparison to the wild-types (F=16.78, df=3, p<0.001). Larval survival was lower for the *Adh*-line D (t=-0.307, df=3, p<0.001) and higher for *Adh*-B and F (t=0.23 and 0.17, df=3, p<0.01 and 0.05). However, none of the interactions between the line and the ethanol treatments were significant.

Bioassays performed on *Adh*-D progeny showed a low survival in absence of ethanol: significantly fewer transgenic than wild-type survived to pupae in the absence of ethanol (65% transgenic against 76% wild-type). This was not consistent with previous bioassays performed on *Adh*-D showing a survival above 85% for both transgenic and wild-type (**Annexe 8, Table 8.2.**) and might be due to experimental error (e.g. containers used for larval bioassays might have been contaminated, therefore affecting larval fitness). In the literature, a bioassay showing more than 20% mortality in the controls is discarded (Abbott 1925) as it does not truly reflect the impact of the insecticide or other toxin on the mosquito larva. In that case, when *Adh*-transformed line D was removed from the analysis, results revealed a significant reduction of larval survival due to ethanol treatments (F=458.36, df=1, p<0.001), as well as a significant difference of survival between strains (F=3.29, df=2, p=0.04292), both *Adh*-transformed larvae B and F surviving better than their wild-type counterparts (respectively p=0.0096 and p=0.048). The interactions of ethanol treatments with the lines were not significant, however, the differences of response to ethanol treatments between the different *Adh*-transformed lines cannot be trusted as bioassays were performed separately in time (this was for logistic reasons: to perform 3 repeats of 300 first instar larvae each for 6 lines simultaneously would require the manual count of a total of 5,600 first instar larvae in one go).

These preliminary results suggest that a 24 h exposure to ethanol at low concentrations might not be enough to cause a toxic effect to larvae. However, at higher concentrations (2% for line F and 3% for line B), wild-type larvae seem to survive better than transgenic, showing that the *Adh* transgene does not seem to have a ‘protective’ effect against ethanol exposure. To show that acetaldehyde accumulates and kills *Adh*-transformed larvae, shorter exposure to ethanol might need testing.

Strain	EtOH % v/v	N	GM % ± SE	WT % ± SE	df	t-value	P value
Adh-B males	0	1,398	94% (0.4)	92% (0.3)	7	2.837	0.0056
	1	1,383	90% (0.2)	94% (0.4)			
	2	1,323	90% (0.4)	87% (0.4)			
	3	390	22% (0.5)	30% (0.3)			
Adh-D males	0	1053	65% (0.8)	76% (0.4)	7	-3.782	0.000277
	1	922	55% (0.9)	68% (0.7)			
	2	732	45% (1.3)	52% (1.3)			
	3	163	7% (1.2)	14% (1.1)			
Adh-F females	0	1,364	93% (0.3)	89% (0.5)	7	2.146	0.034
	1	1,415	95% (0.2)	94% (0.3)			
	2	1,145	68% (0.4)	85% (0.5)			
	3	198	11% (0.6)	15% (0.3)			
	4	2	0% (0.4)	0% (0.4)			

Table 3.5. Number of surviving *Adh*-carrying transgenic individuals (GM) in comparison to the wild-types (WT) after 24h exposure to ethanol at larval stage.

Pupae resulting from crosses between *Adh*-transformed heterozygote and wild-type individuals were exposed during 24 h to 0, 1, 2, 3 and 4% ethanol at first instar larval stage. Number of replicates = 5 with 300 starting first instar larvae per replicate. The three heterozygous *Adh*-transformed lines (B, D and F) were tested against the response to 24h exposure of various ethanol treatments (0, 1, 2, 3 and 4% v/v) of their wild-type siblings. N is the sample size, df is the degree of freedom and a p-value < 0.05 indicates a proportion of transgenic to wild-type significantly different from expected (1:1) as analysed by a two-way ANOVA.

3.3.3. Impact of ethanol on *Ae. aegypti* wild-type fitness

Wing length

Wild-type females were generally larger (2.82 ± 0.022mm, N=16) than the wild-type males (2.16 ± 0.034mm on average, N=14, 18 and 17) (F=1,092.33, df=1, p<0.001) and ethanol treatments (0%, 0.5% or 1% v/v) applied at larval stage did not affect males' wing length.

Longevity

Adult *Ae. aegypti* lived an average of 57 days from the moment the eggs were hatched (from 17 to 91 days) irrespective of sex (F=1.255, df=1, p=0.264) (**Figure**

3.7.). None of the parameters tested did impact adult wild-type longevity: ethanol treatments at 0, 0.5 and 1% ethanol (F=0.284, df=2, p=0.753) and the day of pupation (i.e. adults coming from pupae eclosed on pupation day 1) (F= 0.645, df=3, p=0.587). None of the 2-ways interactions (EtOH:sex and EtOH:Day of pupation) were significant: EtOH:Sex (F=0.085, df=2, p=0.919) and EtOH:Day of pupation (F=0.635, df=4, p=0.638).

Wild-type males and females both lived on average 58.6, 62.3 and 58.1 days when respectively subject to 0%, 0.5% and 1% ethanol treatments respectively. A Kaplan-Meier survival analysis gave similar results to the previous analysis that is, survival was not significantly significant between the ethanol treatments (wild-type females: X^2=0.7, df=2, p=0.712; wild-type males: X^2=0.8, df=2, p= 0.656).

Ethanol treatments were clearly not the only parameters affecting *Ae. aegypti* wild-type longevity tested in laboratory conditions: other laboratory settings can explain such results i.e. variation of temperatures and humidity (see Material and Methods) as well as the experimental design (inevitably, some cages were closer to the heater or humidifier).

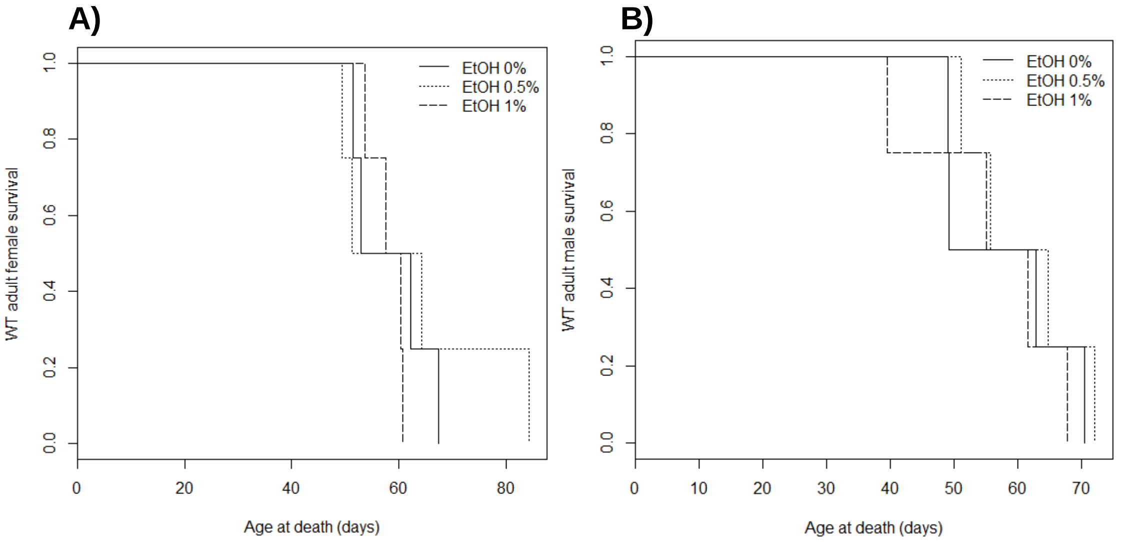


Figure 3.7. Mean survival curves (days) of wild-type females (A) and wild-type males (B) mosquitoes reared at different ethanol concentrations.

All treatments were repeated 5 times with 5 males or 4 females in each replicate. Each line represents the mean of groups to avoid pseudoreplication. Solid black line represents ethanol concentration at 0%; dotted black line represents ethanol concentration at 0.5% and dashed black line represents ethanol concentration at 1%.

Larval survival to pupation and sex ratio

Two long-term ethanol exposure bioassays were performed separately on the wild-type laboratory strain. On average, 85% (between 81 and 89%) of the larvae in each container survived to pupation during the first bioassay. During the second experiment, 89% (between 80 and 98%) of the larvae survived to pupation (**Annexe 8, Table 8.2.**). In both experiments, both sex ratio and larval survival to pupation did not vary between repeats (N=5) as well as between ethanol treatments (0, 0.5 and 1% v/v). None of the interactions were significant ($p>0.0941$).

Age at pupation

Age at pupation was analysed separately for males and females wild-type exposed to 0, 0.5 and 1% v/v ethanol in their larval water. Two experiments were conducted at different times, and on average, wild-type females pupated after males: 7.92 and 7.56 days (experiment 1), 8.82 and 7.97 days (experiment 2) (**Figure 3.8.**). Ethanol treatments at 0, 0.5 and 1% did not impact female larvae development which pupated after 7.88, 7.88 and 8 days during the first experiment; 9.19, 8.13 and 9.14 during the second experiment. Wild-type males were not affected by ethanol treatments either, pupating after 7.52, 7.40 and 7.76 days during the first experiment; 7.96, 7.54 and 8.41 days during the second experiment.

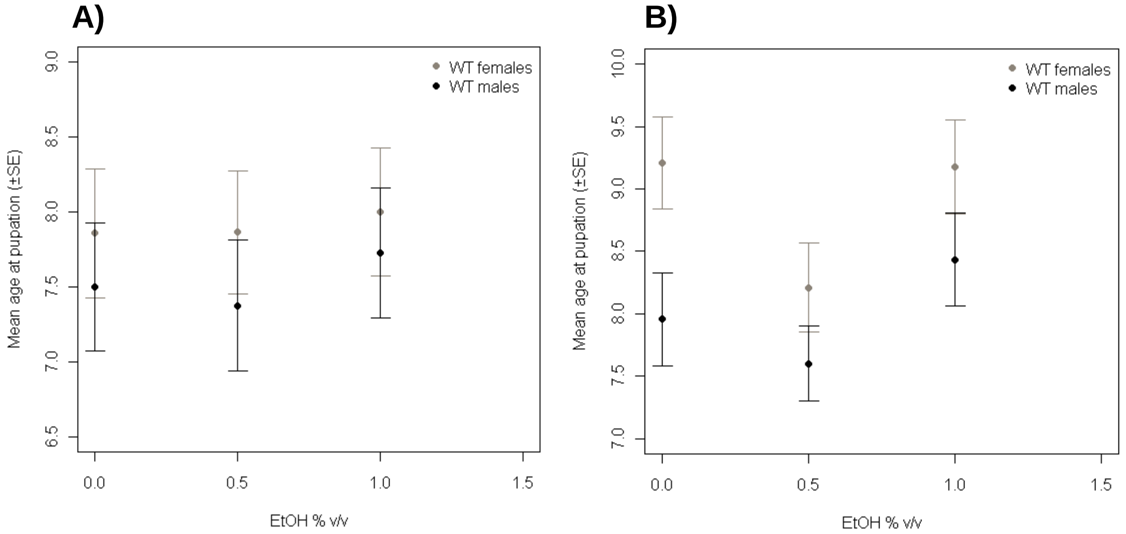


Figure 3.8. Comparison of mean age at pupation between wild-type (WT) females and male in response to long term ethanol exposure at immature stage.

Comparison of mean age at pupation of wild-type (WT) females and males during a first (**A**) and a second (**B**) repeat of the experiment when reared with 0, 0.5 and 1% v/v ethanol in their larval water; error bars showing standard error of the mean. N=5 with 300 starting first instar larvae in each replicate in each experiment (**A** and **B**).

Fecundity and fertility of wild-type females when mated with males exposed to 0, 0.5 and 1% v/v of ethanol

Wild-type *Ae. aegypti* males were exposed to long term 0%, 0.5% and 1% ethanol in the larval water and mated with wild-type females reared in deionised water only (N=30). Results showed no significant difference of egg production for each of the three gonotrophic cycles tested between a wild-type female mated with either male types (between 74 and 78 eggs were produced, on average) (**Figure 3.9.**). The eggs collected out of those crosses throughout 3 gonotrophic cycles were hatched for fertility testing between 4 to 7 days after being laid. No significant difference was found between the hatch rate of a female mated with either type of males during first (between 82 and 85%), second (between 79 and 86%) and third gonotrophic cycle (between 80 and 82% hatch rate). During the first gonotrophic cycle, out of 30, 1 female that mated with a wild-type male reared without ethanol did not lay eggs, and the same for 1 female that mated with a wild-type male reared with 0.5% ethanol in the larval water, and 1 female that mated with a wild-type male reared with 1% ethanol. Those samples were removed from the fecundity and fertility analysis.

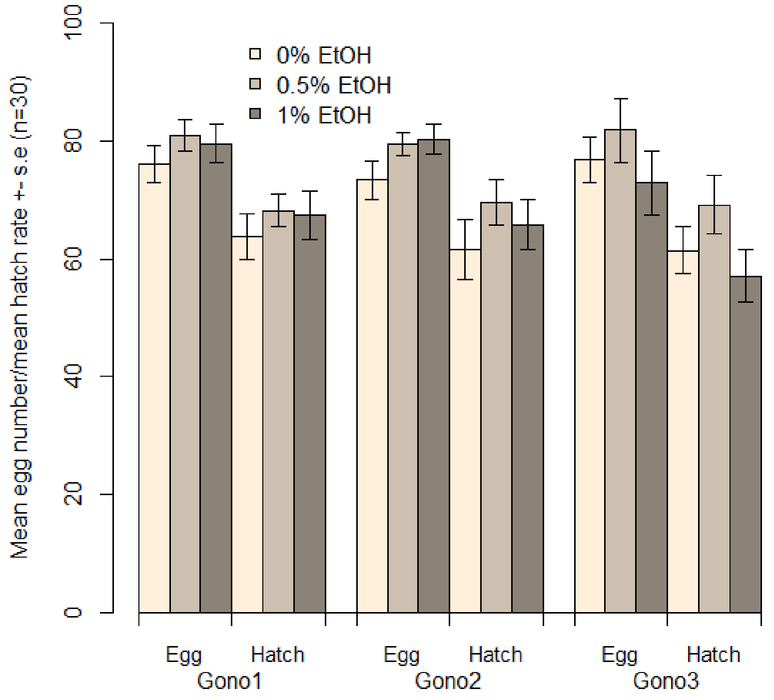


Figure 3.9. Comparison of egg production and hatch rate when wild-type females were mated with wild-type males exposed to long term ethanol (0, 0.5 and 1% v/v).
Mean number of eggs produced (Egg) as well as mean number of hatched eggs (Hatch) per female (N=30) per cross for the first 3 gonotrophic cycles (Gono1, 2 and 3). Females were mated with wild-type males reared in deionised water (0%), with 0.5% ethanol (EtOH) added in the larval water (0.5%) or 1% ethanol. Error bars represent standard error of the mean.

3.4. Discussion

3.4.1. *DmAdh* sensitises *Ae. aegypti* larvae to ethanol

As mentioned previously in the introduction, the *DmAdh* gene coding for the Fast allozyme looked promising for desensitizing *Ae. aegypti* larvae to ethanol. Instead, surprisingly, our data suggest that this *D. melanogaster Adh* gene driven with Aehsp83 promoter renders *Ae. aegypti*'s larvae far more susceptible to ethanol than their wild-type counterparts. Survival to pupation is similar between *Adh*-transformed strains and *Adh*-lacking strains, as well as the wild-type laboratory strain, when reared under standard conditions (no ethanol). Lethality is induced when *Adh*-transformed larvae are submitted to long-term exposure to ethanol from first instar larvae, whereas 24 h exposure to ethanol resulted either in a non-significantly-higher survival of *Adh*-transformed in comparison with wild-type larvae, or a significantly-reduced survival of *Adh*-transformed in comparison with wild-type as the concentration of ethanol was increased. These assays were performed on heterozygous transgenic larvae carrying one copy only of the *Adh* transgene. This means that, potentially, the system could be even more sensitive to ethanol in insects homozygous for the *Adh* cassette, which would be the relevant situation for practical use, at least in the context of chemical selection of mass-reared insects.

Although we have not investigated the precise biochemical basis of the response of *Ae. aegypti* to ethanol with or without *DmAdh*, as explained in the Results section, one possible explanation is that higher dosage and shorter exposure to ethanol might confer an advantage to *Adh*-transformed larvae as opposed to the wild-type larvae, allowing them to survive in such a toxic environment. Experiments performed using 24 h exposure to ethanol did not allow convincing rejection of such a hypothesis, and more experiments would provide a better understanding of the detoxification processes involving the ADH protein in the dengue fever mosquito.

The ADH-F has a higher catalytic activity to ethanol than the ADH-S and a BLAST analysis against its nucleotide sequence found no equivalent in *Ae. aegypti*. The endogenous *Ae. aegypti Adh* gene has shown to exhibit differential transcription profiles when *Ae. aegypti* laboratory populations were exposed to xenobiotics over many generations in comparison to sensitive strains (Poupardin et al., 2012; Tetreau et al., 2012a).

In *Drosophila melanogaster*, ADH is a key enzyme for both ethanol detoxification and metabolism utilisation. However, while primary alcohols like ethanol are efficiently used as energy sources, secondary alcohols like isopropanol are transformed into ketones, which can be more harmful for the fly than the precursor alcohol. Secondary alcohols are even routinely used to select ADH null mutants that survive after exposure to the alcohol in comparison with the ADH producing flies (David et al., 1981).

DmAdh converts ethanol to acetaldehyde which is toxic. In humans acetaldehyde is converted to acetate by acetaldehyde dehydrogenase. Possibly, *D. melanogaster* has a much higher ability than *Ae. aegypti* to metabolise acetaldehyde; this would be reasonable given the higher exposure to ethanol and high ADH activity of Drosophila. Then in the *Adh*-expressing lines of *Ae. aegypti*, higher concentrations of ethanol lead to higher production of acetaldehyde, saturating the ability to metabolise it (unlike in Drosophila). Incidentally, that would imply that acetaldehyde is not detoxified by *DmAdh*, as it has been speculated.

Further bioassays using secondary alcohols might help answering the questions raised.

3.4.2. Wild-type larvae tolerate the ethanol doses required to kill sensitized larvae

The doses required to kill sensitized larvae are tolerated by wild-type larvae and an *Adh*-lacking transgenic line. Ethanol can be supplemented at low dosage, up to 1%, without immediate impact on *Ae. aegypti* wild-type fitness (adult male longevity and fertility). If such system is used to create a genetic sexing strain, one would not anticipate resistance development in the *Adh*-transformed strain as only the ‘release generation’ will ever be exposed to selective concentrations of ethanol as opposed to the ‘mass reared’ generations that will always be reared under standard conditions (no exposure to ethanol). A male mating competitiveness study would be useful however.

3.4.3. The ‘*Adh*-inducible-lethal-system’ is a good marker for a Genetic Sexing Strain

Ethanol is a cheap, well understood and relatively safe selection agent. Alcohol dehydrogenase enzyme is part of the Short-chain Dehydrogenase Reductases (SDR) protein family (Jörnvall et al., 1995). The *Adh* gene is present through the animal kingdom from bacteria to humans as well as the higher plants (Ashburner 1998) hence its use in genetic engineering might be more easily accepted. Also, whether the *Adh*-system does confer resistance or sensitivity to ethanol matters little, since it can be induced by a sex-specific promoter or combined with an alternatively spliced gene such as *transformer* or *doublesex* (see Chapter 4 and 5) to generate an ‘inducible sex specific lethal’.

Chapter 4: Use of alternative splicing to engineer female-specific lethality in *Aedes aegypti*

4.1. Introduction

As detailed in section **1.3.1.** and **1.3.2.** in the general introduction of this thesis, mosquito sex separation can be achieved at pupal or adult stages using different approaches, such as sorting by differences in pupal cephalothorax size for Aedes and Culex species (Fay & Morlan 1959; Focks 1980) or sex-linked insecticide resistance genes for Anopheles species (Bailey et al. 1980; Curtis et al. 1976), but either with mixed results or time consuming manual quality control (Ansari et al. 1977b; Papathanos et al. 2009). Transgenesis tools in pest crop or disease vectors are soon to offer improvements to sexing process through the generation of engineered sexing strains (Catteruccia et al. 2005; Labbé et al. 2012; Tan et al. 2013; Jin et al. 2013).

The *Ae. aegypti* RIDL strain OX513A operates by a bi-sex dominant lethal system: male and female larvae and pupae die when tetracycline is absent in the larval rearing water (Phuc et al. 2007). Linking RIDL to a female-specific system, via a sex-specific promoter or alternative splicing or both (Fu et al. 2010), would considerably improve the mass-release of a male-only population by reducing production costs of the engineered sterile mosquitoes if females are eliminated during early developmental stages (Caceres 2002). A female specific RIDL (fsRIDL) on the other hand, i.e. late acting female lethality repressible with the addition of tetracycline in the larval diet, would accelerate the suppression process of the targeted population (Thomas et al. 2000; Phuc et al. 2007). The two female specific lethal systems described above are distinct: on one hand a female specific lethal could be induced independently of the RIDL system so females are killed at early developmental stage which helps the release of RIDL male only in the field; this might be described as an early-acting genetic sexing system (GSS) (Yamada et al. 2012). It would need a separate 'sterilisation' system, which could be provided by the OX513A insertion, for example. An fsRIDL on the other hand might not allow for discrimination at early stage between the sexes but would accelerate the suppression process of the targeted population post release (Thomas et al. 2000). An example of such a system one could imagine a transgene-induced repressible dominant female-specific flightless phenotype: once the fsRIDL males have mated with the wild type females, 100% of the female progeny will be unable to fly when there is no tetracycline available to repress the feedback loop system (**Figure 1.4.**), and 100% of the male progeny will carry on, passing on the dominant fsRIDL component of their genome. Such a system would elegantly provide both genetic sexing and 'sterilisation', but with some degree of trade-off – the female-specific lethal should kill late in development for optimal population control (given density-dependence at the larval stage), but early for production efficiency. Flightless-ness, a late lethal phenotype, is clearly not optimal for genetic sexing.

Such a fsRIDL strains exists: coupled to the *AeAct-4* promoter, the tetracycline-repressible transactivator tTAV2 (a variant of tTA optimised for expression in Drosophila **Figure 4.1.**) is produced in the indirect flight muscles of adult females) (Fu et al. 2010); in the absence of tetracycline this drives even more tTAV2 to such levels leading to a female flightless phenotype in the strain OX3604C (Fu et al., 2010). In the absence of tetracycline, an adult female OX3604C emerges into an adult unable to fly, therefore unable to mate or – in the field – forage for food (i.e., blood meal or sugar) and consequently incapable of producing offspring.

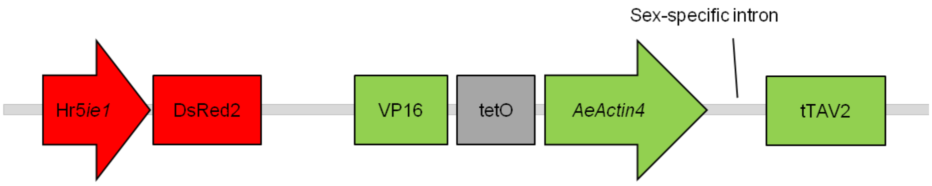


Figure 4.1. Diagram of part of the flightless female plasmid construct OX3604, modified from Fu et al., 2010.

The tTAV2 protein is a variant of tTA optimised for expression in Drosophila by eliminating potential cryptic splice sites and rare codons in the tetR region (Fu et al. 2010). tTAV2 is produced under the control of the promoter and alternative splicing element of the *AeAct4* gene and therefore expressed in a female-specific manner. The tetracycline response element (tRE), comprising multiple copies of *tetO* and driving VP16, is activated by tTAV2. The DsRed2 marker is driven by the Hr5*ie1* promoter resulting in red spots all over the OX3604C individuals.

Few characterised female-specific promoters are suitable for RIDL applications, especially for expression early in development. An alternative to generate female-specific lethal systems is to use sex-alternate splicing to regulate the RIDL phenotype (Fu et al., 2010). To date, however, attempts to use the sex-specific spliced intron part of the AeAct4 only to create a female-specific expression in *Ae. aegypti* have failed (**Figure 4.1.**).

Genes such as *transformer* (*tra*) and *doublesex* (*dsx*) are alternatively spliced, and play a role in sex determination in many insects (Gempe and Beye 2011). The first intron of *tra* has been successfully engineered in the Mediterranean fruit fly (medfly) *Ceratitis capitata* (*Cctra*) (Fu et al., 2010), producing tTAV proteins in medfly females only, switching on the tetracycline repressible feedback loop system in the absence of tetracycline in the larval diet. Since no *tra* homologue has been discovered yet in *Ae. aegypti* (Saccone et al. 2011), *dsx* represents a promising alternative: it has been identified in this mosquito (Salvemini et al.,

2011) and a functional mini-gene version was engineered by colleagues at Oxitec, Ltd. in 2008 (Patent GB2443186A). Moreover, inserted together with the tetracycline repressible lethal system in the moth, pink bollworm (*Pectinophora gossypiella*), *dsx* alternative splicing achieved female-specific lethality in the absence of tetracycline (Jin et al. 2013). In that fsRIDL construct, tTAV was inserted into the *Pgdsx* female specific exon such that tTAV is present on a female-specific exon and therefore present in mature mRNA only in females but not in males; in absence of tetracycline, females die from an over-expression of tTAV but males are unaffected (**Figure 4.2.**). Similar constructs injected in *Ae. aegypti* have failed to confer female-specific lethality, as the alternative splicing originally achieved with a *dsx*-mini-gene appears to be disrupted when combined to the *tetO*-tTAV cassette (T. Dafa'alla and G. Labbé, personal communication).

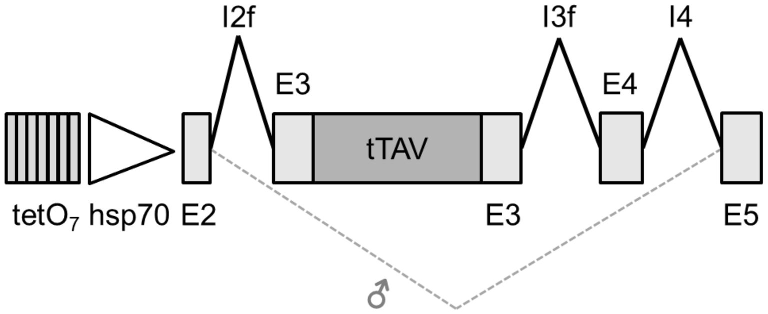


Figure 4.2. Diagram of a tetO-hsp70-tTAV cassette inserted into *Pgdsx* mini-gene modified from Jin *et al* (2013).

Exons are E2, E3, E4 and E5. Introns are I2f, I3f and I4. E3 and E4 are spliced in female only whereas E5 is spliced in both males and females (see the only male transcripts, splicing out tTAV making the transformed males not expressing tTAV in comparison with females).

This chapter describes two attempts at creating the first *Ae. aegypti* fsRIDL strain based exclusively on sex specific splicing. Firstly, *Ae. aegypti* was transformed with the same construct used to generate sexing strains in medfly – OX3097 – which carries an alternative splicing cassette derived from *Cctra*. As detailed in section **1.3.4.** of the general introduction in this thesis, no *transformer* homologue has not been found in mosquitoes at all (Saccone et al. 2011). In the medfly *Ceratitis capitata* the *transformer* gene is spliced so functional TRA proteins are produced in females only (**Figure 1.9.**). Splice variants of engineered *Cctra* in transformed males and females were analysed (line OX3097), and female specific lethality assessed on and off tetracycline. Secondly, *Ae. aegypti* was transformed with a new *dsx* construct exploiting the sex specific splicing properties of the *Ae. aegypti dsx* in order for tTAV to read in frame in females only, while males would produce a non-functional *dsx*-tTAV transcript. Male and female splice variants of *dsx*-tTAV transcripts were analysed in transgenic individuals, and on/off tetracycline assays were performed.

4.2. Material and Methods

4.2.1. Plasmid constructs

The construct OX3097 has previously been described by Fu *et al.* (2007), and was formerly referred to as pLA3097. This *piggyBac*-based construct carries the *C. capitata tra* first intron (*Cctra* intron) inserted into a tetracycline positive feedback system (**Figure 4.3.**). Expression of the gene coding for the red fluorescent protein, DsRed2, is driven by the Hr5*ie1* promoter (Rodems and Friesen 1993) to provide a transformation marker.

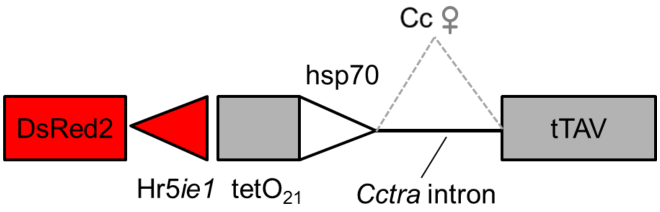


Figure 4.3. Map of the OX3097 plasmid construct modified from Fu *et al* (2007).
The *piggyBac* OX3097 plasmid construct is detailed in the general introduction in figure 1.9. and is shown above without the ‘red body’ transformation marker (Hr5*ie1* promoter driving DsRed2). The main elements are the tTAV gene and its response element tetO (sequence which is repeated 21 times) placed upstream the minimal Drosophila hsp70 promoter driving tTAV in a female specific manner in *C. capitata*. In the mediteranean female flies only the *transformer* intron splicing allows the production of functional tTAV.

Construct OX4489[4] is a *piggyBac*-based construct derived from OX3097 (previously described and carries the same tetracycline repressible feedback loop system with the exception of carrying seven repeats of *tetO* (Heinrich and Scott 2000; Thomas et al., 2000) instead of 21. The transformation marker was also replaced with a 3xP3- DsRed2 cassette (driving DsRed2 expression in the eyes), and the *Ae. aegypti dsx* mini-gene was also added (**Figure 4.4.**), replacing the *Cctra*-derived alternative splicing cassette of OX3097.

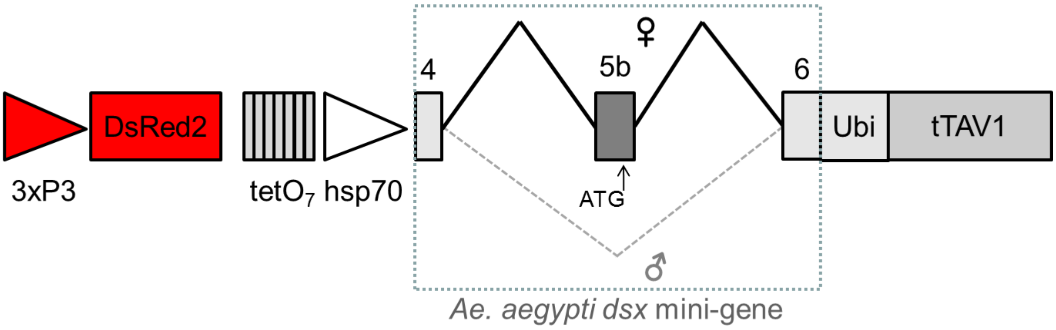


Figure 4.4. Diagram of the engineered *piggyBac*-based construct OX4489.
A tetO-tTAV1 cassette is inserted into *Ae. aegypti doublesex* (*Aedsx*) mini gene (refer to **Figure 1.11.B**) and adjoins a transformation marker (3×P3-DsRed2).

---

[4] Plasmid construct designed by Tarig Dafa’alla, PhD at Oxitec Ltd.

4.2.2. Reverse-Transcription PCR and sequencing

In order to study the putative splice variant forms of *Cctra* and *Aedsx* minigenes in OX3097 and OX4489 transgenics, respectively, RNA was extracted from single male and female pupae from each line using the RNeasy kit (Qiagen, Hilden, Germany). RNA samples were treated with DNase I (Roche, Burgess Hill, UK) and quantified on a Pharmacia Biotech GeneQuant II RNA/DNA calculator (Little Chalfont, UK). 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) with construct specific primers pairs (**Table 4.1.**). Another reaction was run specifically with the primer pairs for actin 1 (coordinates for *Bombyx mori* actin 1), used as a positive control (Canning et al., 1996).

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 sequenced by GATC Biotech (Konstanz, Germany) using pJET primers (**Table 4.1.**).

Primer name	***Primer nucleotide sequence***
226) Hsp70-seq	AAGTGAACACGTCGCTAAGCG
735) tTAV+365seq	CGTAGAGAGCATTTTCCAGGCTGAAG
630) Agdsx-e3-f	GAGATGATGCCCCTGATGTACG
1128) Diag2-SV40	GATGAGTTTGGACAAACCACAACTAGA
1319) AeCytL	TTCACCACCACCGC(C/T)GAG
1320) AeCytR	ATACCGGGGTACATGGTGG
1156) pJET-FP2	ATCAACTGCTTTAACACTTGTGC
1157) pJET-RP2	AAAGAAGAACATCGATTTTCCATG

Table 4.1. Primer pairs sequences for analyzing OX3097 and OX4489 mRNA transcripts using Reverse Transcription PCR.

Primer pairs for pJET and Actin 1 sequences as well as primer pairs specific for *Drosophila hsp70* and tTAV (used to analyse OX3097 splice forms in male and female individuals) and finally *Aedsx* and SV40 primer pairs to analyse OX4489 transformed individuals.

4.2.3. Phenotype analysis

Assays in the presence and absence of tetracycline (on and off tetracycline) were performed to test for the female-specific lethal phenotype. Eggs from the respective transgenic lines were hatched in deionised water, and after 24 h three replicates of 300 first-instar larvae were counted and placed into 300 ml of deionised water (1 larva.ml^{-1}). Control larvae were put in 300 ml of deionised water supplemented with 30 µg.ml^{-1} chlortetracycline hydrochloride (tetracycline) (Sigma-Aldrich, Gillingham, UK). The cohorts of larvae were reared using a standard feeding regime (**Table 2.1.**). Transgenic and wild-type pupae were separated by phenotype, sexed and counted and kept in separate plastic pots for each repeat, type and sex, covered with a net and cotton wool pad dampened with a 10% sucrose solution on top. Twenty-four hours after emergence, the number of dead pupae and semi-emerged males and females was recorded.

4.2.4. Test crosses

The assumption of such crosses was to check whether female individuals carrying both constructs *Aedsx* (OX4489) and *tetO-Reaper* (OX4393) would die in absence of tetracycline added in the larval diet. Heterozygous females from lines OX4489B, D and H (carrying the red eye marker 3×P3-DsRed2) were crossed with heterozygous males from a tetO-effector OX4393 line 12 (carrying the red body marker Hr5*ie1*-DsRed2) and eggs were collected. According to Mendel's laws of inheritance, the progeny from those crosses were expected to be in equal proportion (25%) of each parental phenotype (red-eyed OX4489 and red-bodied OX4393) as well as 25% of wild-type (non fluorescent individuals) and both red-eyes with red-bodied individuals (**Figure 4.5.**).

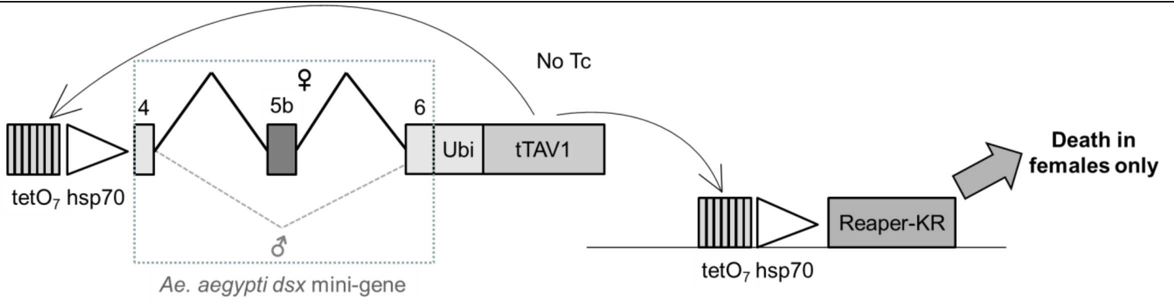


Figure 4.5. Crosses scheme of two heterozygous *piggyBac* transgenic lines 'red eye' *Aedsx* (OX4489) and an effector 'red body' *Reaper-KR* (OX4393).

Heterozygous *Aedsx* females (top construct) of three different 3xP3-DsRed2 lines (*Aedsx*-B, D and H) were crossed to tetO-effector males (bottom right construct namely OX4393) carrying Hr5*ie1*-DsRed2 transformation marker. The progeny of the crosses could be screened for differential fluorescence pattern: red eyes *Aedsx* only, red body effector only, both red eyes and red body or non fluorescent wild-type phenotypes. If the *Ae. aegypti* dsx mini-gene works, then only functional tTAV1 is being produced in females and, females carrying both *dsx* and effector construct shall express Reaper-KR and die when tetracycline is absent in their larval diet (no Tc).

4.2.5. Statistical analysis

Statistical analyses were performed using R software version 2.12.2 (R Development Core Team 2011). Larval survival was estimated as the proportion of transgenic individuals, relative to wild-type counterparts, surviving to pupation in each pot. Data was ArcSin-transformed and analysed using ANOVA to examine the impact of on/off tetracycline treatments, line and sex as well as their interactions on survival of larvae to pupation. The strain OX3097E was estimated separately, the insertion of the transgene being linked to the sex determination locus: larval survival to pupation was the proportion of male and female pupae obtained from the initial 300 first-instar larvae (L1) placed in each pot and was analysed with a general linear model (GLM) with a binomial error family including tetracycine treatments, sex and repeats and their interactions.

4.3. Results

4.3.1. OX3097 *Ae. aegypti* strains

The OX3097 construct was micro-injected into 1,223 wild-type *Ae. aegypti* eggs, of which only 86 survived to adulthood only (7%, 37 males and 49 females). Four OX3097-transformed lines came from separate adult male G_0 pools: OX3097A, B, D and E; and one, OX3097C, came from a G_0 female pool. Lines OX3097A, B, C and D were established from a single transgenic G_1 male crossed to 10 wild-type females. All five transgenic lines expressed a spotted red fluorescent pattern all over the body, as expected from the Hr5*ie1* DsRed2 marker, although intensity varied between the lines (lower in OX3097D and E). Of the progeny from the cross between OX3097B males and wild-type females 97% were transgenic males and only 6% were transgenic females. Out of 2,897 screened pupae, only 4.6% exhibited a non-parental phenotype (48 wild-type males and 85 transgenic females) suggesting that the insertion of the OX3097 construct was located at 4.6 centimorgan (cM) from the sex-determining locus in line B. Also, the cross between heterozygous OX3097E females and wild-type males suggested the insertion of the construct at about 17.3 cM of the sex-determining locus (**Table 4.2.**), however this time, the insertion was linked to the female-determining allele.

OX3097B	Males:	1,470 (51%)	GM	1,422 (97%)	**OX3097E**	Males:	493 (48%)	GM	102 (21%)
			WT	48 (3%)				WT	391 (79%)
	Females:	1,427 (49%)	GM	85 (6%)		Females:	524 (52%)	GM	450 (86%)
			WT	1,342 (94%)				WT	74 (14%)

Table 4.2. The OX3097B and OX3097E insertion is linked to the sex-determining locus. The progeny proportions when larvae were reared with tetracycline from a cross between heterozygous OX3097B males (left panel, n=2,897) and wild-type females showed that the insertion was linked to the male-determining allele (4.6 cM). The proportions of the OX3097E progeny (right panel, n=1,017) showed a transmission of the transgene skewed towards the female progeny (17.3 cM from the female-determining allele).

4.3.2. OX4489 *Ae. aegypti* strains

Out of 1,207 *Ae. aegypti* embryos injected with OX4489, 131 survived to adulthood (11%), constituting a proportion of 44% female and 56% male. Six transgenic lines were obtained from separate adult male pools: OX4489A, B, C, D, E and F, as well as one transgenic line, OX4489H, from the adult female G_0 pool. Three lines exhibited red fluorescence similar to that described in Chapter 2 (OX4346 transgenic lines): red eyes plus red ganglionic connectives (OX4489A, C and F). The other four OX4489 lines exhibited red fluorescent eyes only (OX4489B, D, E and H) (data not shown). All seven lines were then tested for on/off tetracycline assays, and, as mendelian inheritance indicated that three lines carried one copy of the transgene (OX4489B, D and H), and also exhibited no linkage of the transgene to one sex in particular, they were kept for test crosses to tetO-Effector lines when the others were discarded. All three transgenic lines chosen expressed red eyes only from the 3×P3-DsRed2 marker, although intensity varied between the lines (data not shown).

4.3.3. Effect of tetracycline on fluorescence pattern of OX3097 and OX4489 pupae

The positive feedback loop system initiates when tTAV is produced under the control of the *hsp70* minimal promoter and binds to *tetO*, which enhances further expression of tTAV, in the absence of tetracycline. Another phenomenon takes place which has been anecdotally reported (R. Lee and M. Conway, personal communication): *tetO*'s activity is bidirectional and, in absence of tetracycline, seems to drive the elements of the transformation marker within transgenic pupae exhibiting brighter fluorescence in the absence of tetracycline than when they are reared with tetracycline. In our case, this happened to be a useful tool for sexing OX4489 pupae: heterozygous females exhibited a much brighter red fluorescence than heterozygous males in the absence of tetracycline, a difference not seen when OX4489 larvae were reared with tetracycline (**Figure 4.7.**). The same phenomenon occurred also when OX3097 larvae were reared in the absence of tetracycline; however the pattern was not gender-specific (**Figure 4.6.**).

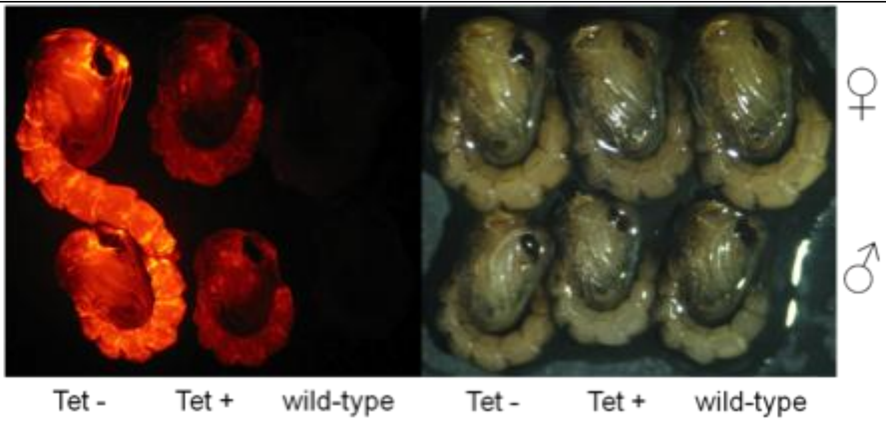


Figure 4.6. Phenotype of transgenic OX3097 and wild-type male and female pupae. Expression of DsRed2 gene under fluorescent light (left panel) and white light (right panel) driven by Hr5*ie1* promoter in OX3097A female and male pupae (symbols) when tetracycline is absent (Tet-) and present (Tet+) in the larval rearing water. Wild-type male and female pupae are shown as a control.

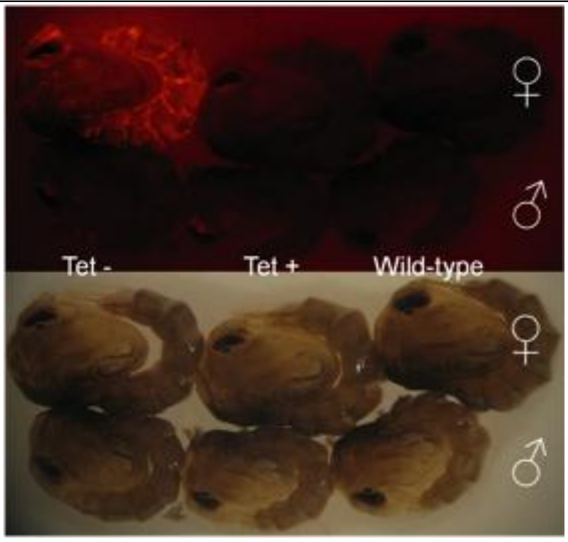


Figure 4.7. Phenotype of transgenic OX4489 and wild-type male and female pupae. Expression of DsRed2 gene under fluorescent light (top panel) and white light (bottom panel) driven by 3xP3 promoter in OX4489B female and male pupae (symbols) when tetracycline is absent (Tet-) and present (Tet+) in the larval rearing water. Wild-type male and female pupae are shown as a control.

4.3.4. The effect of tetracycline on survival to adulthood

Both OX3097 and OX4489 transformed individuals carry a repressible lethal system based on a tetracycline rescue system. In the presence of the antibiotic, the killing system composed of tetO-*hsp70*-tTAV switches off, and the mosquito larvae survive. In the absence of the antibiotic, the killing system is triggered by constitutively producing tTAV under the control of the *hsp70* minimal promoter, the former binds to *tetO* since no tetracycline is present, and the positive feedback loop is activated leading to death. Moreover, both OX3097 and OX4489 carry alternatively spliced engineered genes (*Cctra* in OX3097 and *AeDsx* in OX4489) aimed at producing tTAV transcripts in female individuals only.

Bi-sex lethality is achieved with OX3097 construct

Due to the strong linkage of the OX3097 transgene to the male determining locus (4.6 cM), line OX3097B was not tested for sex specific lethality of the tetracycline repressible system coupled to the *Cctra* gene. Survival to pupation of the 3 transgenic lines OX3097A, C and D when reared with or without tetracycline was analysed together for all three lines that showed no linkage of the transgene to the sex determining locus. Overall, survival to adult was significantly worse when OX3097 larvae were reared without tetracycline (F= 336, df=1, p<0.001).

No significant difference of survival to adulthood was detected between the two sexes (F=0.32, df=1, p=0.57), nor did the survival to adulthood differ between the different transgenic strains in comparison with their wild-type counterparts. Significantly fewer OX3097A larvae survived to adulthood when no tetracycline was present in the larval water in comparison with the wild-type (t=-4.870, df=3, p<0.001), and none of the OX3097C survived to pupation nor to adulthood (t=-10.080, df=3, p<0.001). However, surprisingly, the analysis revealed a significant increase in survival of OX3097D larvae to adulthood in comparison with wild-type (t= 2.529, df=3, p = 0.0143).

OX3097E line was analysed separately since the progeny exhibited a strong bias towards heterozygous females that statistical analysis confirmed (p<0.001). The absence of tetracycline had no impact on survival to neither pupation nor adulthood (p=0.51 and p=0.60 respectively). However though not significant, heterozygous males experienced approximately 12% reduction of survival to adulthood as opposed to 4% for heterozygous females (F=6.1484, df=1, p=0.06824).

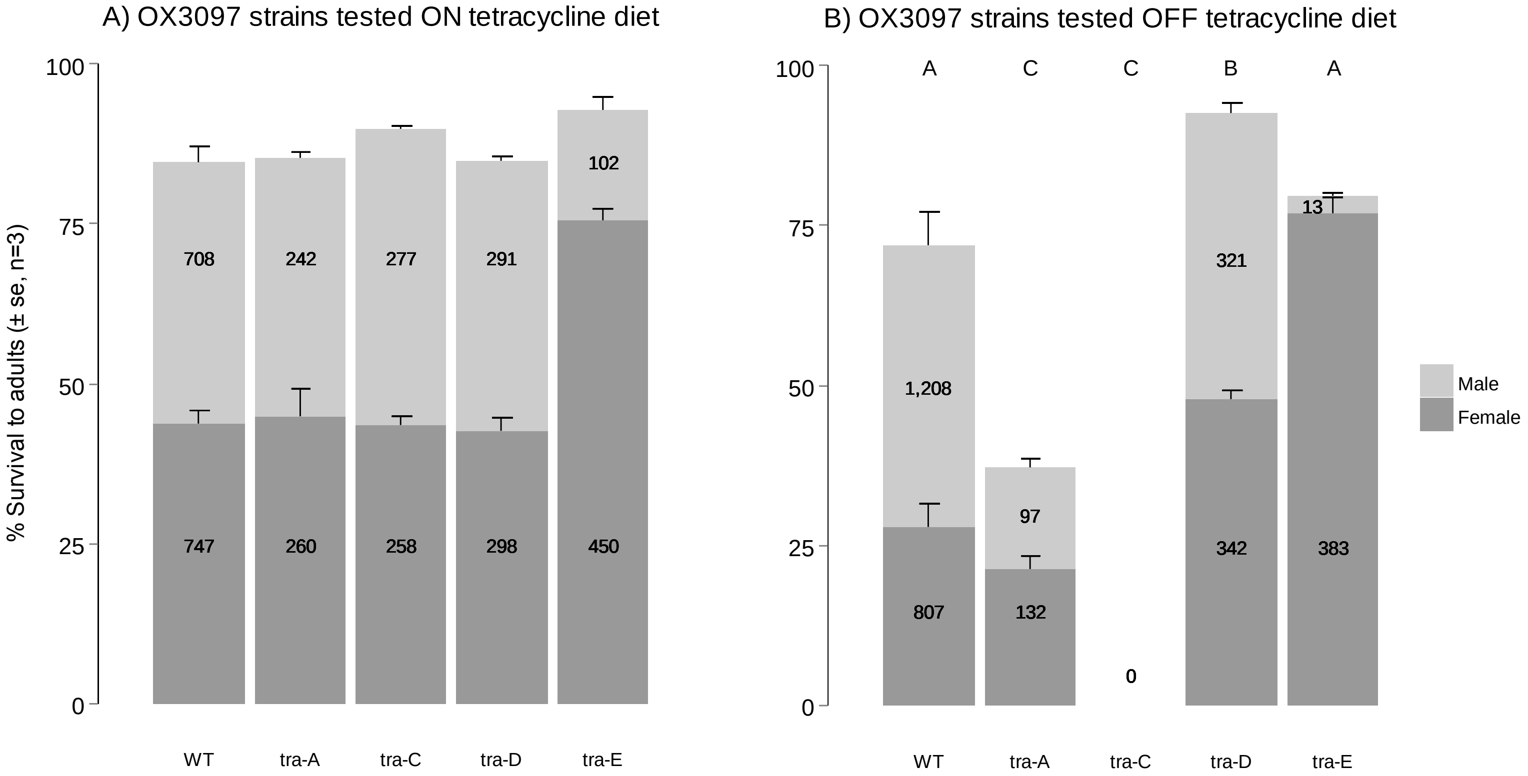


Figure 4.8. Survival of progeny expressing the Hr5*ie1*-DsRed2 (*tra* lines) marker to adult males and females compared with wild-type (WT) progeny modulated by the presence (ON) or absence (OFF) of tetracycline in the larval diet.

A starting number of 300 first instar larvae of both sexes of each heterozygous transgenic OX3097 lines (*tra*-A, C, D, and E) and of 900 first instar larvae for the wild-type strain were tested with 30 µg.ml^{-1} tetracycline diet (**A**) and without (**B**). For each test (ON and OFF tetracycline) and each strain (WT and *tra* lines), the number of live male and female pupae was recorded (numbers shown at the bottom of each bar) and the resulting proportion that survived to adulthood represented on the Y-axis. Note that each strain tested has approximately the same number of male and female pupae (**A**) (*tra*-A, C, D and wild-type) with the exception of line *tra*-E which progeny is skewed towards transgenic female (approximately 80 : 20 GM female to GM male - refer to table 4.2.) Groups denoted by the same letter (Latin capital letter for comparisons between strains comparison) are not significantly different from each other (ANOVA).

Out of the four heterozygous transgenic lines obtained with the OX3097 construct, one (OX3097C) exhibited a fully repressible bi-sex lethal phenotype similar to the RIDL strain OX513A (Phuc et al., 2007) in the absence of the antidote tetracycline (**Figure 4.8.B**).

Reverse-transcription-PCR confirmed the conditional bi-sex lethal phenotype observed in the strain OX309C. Extraction of the two cDNA bands observed on the electrophoretic gel (**Figure 4.9.**) confirmed the presence of the *transformer* expected female transcript in both *tra*-transformed *Ae. aegypti* male and female individuals (the faint band below 600 bp). Sequencing of the cDNA bands matched the F1 *transformer* female transcript (refer to the diagram presented in **Figure 1.8.**). The tra-female splice variant F1 is the only one in *C. capitata* to produce functional tTAV protein and eventually leading to the death of both sexes in OX3097C when expressed in a feedback loop system.

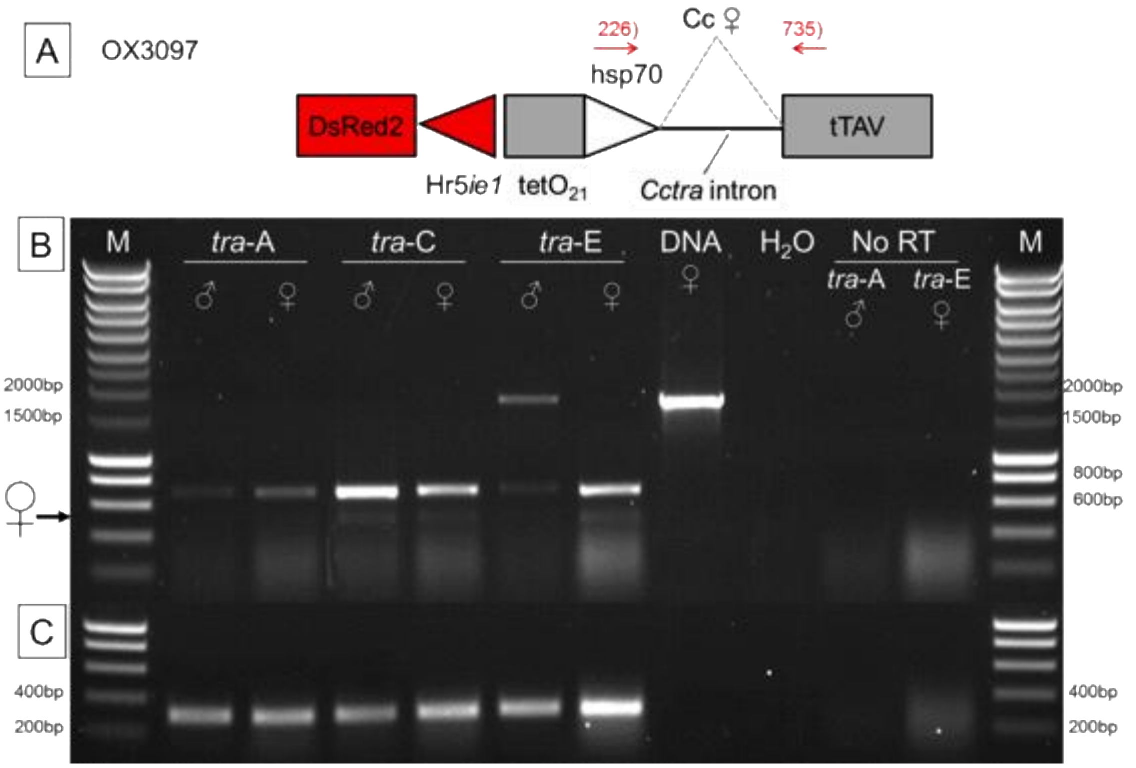


Figure 4.9. RT-PCR on *Ae. aegypti tra*-transformed male and female pupae with construct OX3097 (A).

RT-PCRs were performed on the same samples with primer pairs 226) Hsp70-seq and 735) tTAV+365seq (**B**) as well as primer pairs 1319) AeCytL and 1320) AeCytR (**C**). The samples are tra-A♂, tra-A♀, tra-C♂, tra-C♀, tra-E♂, tra-E♀ which were respectively male and females OX3097 pupae from different lines (A, C and E) from lane 2 to 7; lane 8: DNA of OX3097-A female pupa; lane 9: water only as template for negative control; Lanes 10 and 11 are the same RNA samples than OX3097 A♂ and E♀ (lanes 2 and 7) run without SuperScript III reverse transcriptase as a negative control; lanes 1 and 12 are the DNA Smart ladder.

Female-specific expression using *Aedsx*

Out of the seven heterozygous OX4489 lines tested, three of them (OX4489A, C and F) produced very few heterozygous males (OX4489-C), if any (lines A and F), when tested at generation three after injection (G_0) (**Figure 4.10.A**). The analysis of difference in survival to pupation on and off tetracycline was then carried out on the putative single-inserted, non-sex linked lines (i.e. OX4489B, D and H). When compared to the wild-type laboratory strain, survival to pupation was the same whether larvae were reared with or without tetracycline, for all strains (wild-type, OX4489 heterozygous B, D and H), and regardless of whether larvae were male or female. The interactions between tetracycline treatment and strain showed no significant difference as well as the interactions between tetracycline and sex, or the tetracycline:strain:sex 3 way interaction. However, the ANOVA result indicated that the interaction between strain and sex was slightly significant (F=3.1, df=3, p=0.04036), suggesting that the cross between heterozygous OX4489D males and wild-type females produced more heterozygous males than females (58.75%), irrespective of the tetracycline treatments (**Figure 4.10.A** and **4.10.B**). When the strains are compared to each other, the difference is significant at the 10% but not at the pre-selected 5% confidence interval (t=1.731, df=3, p=0.0932).

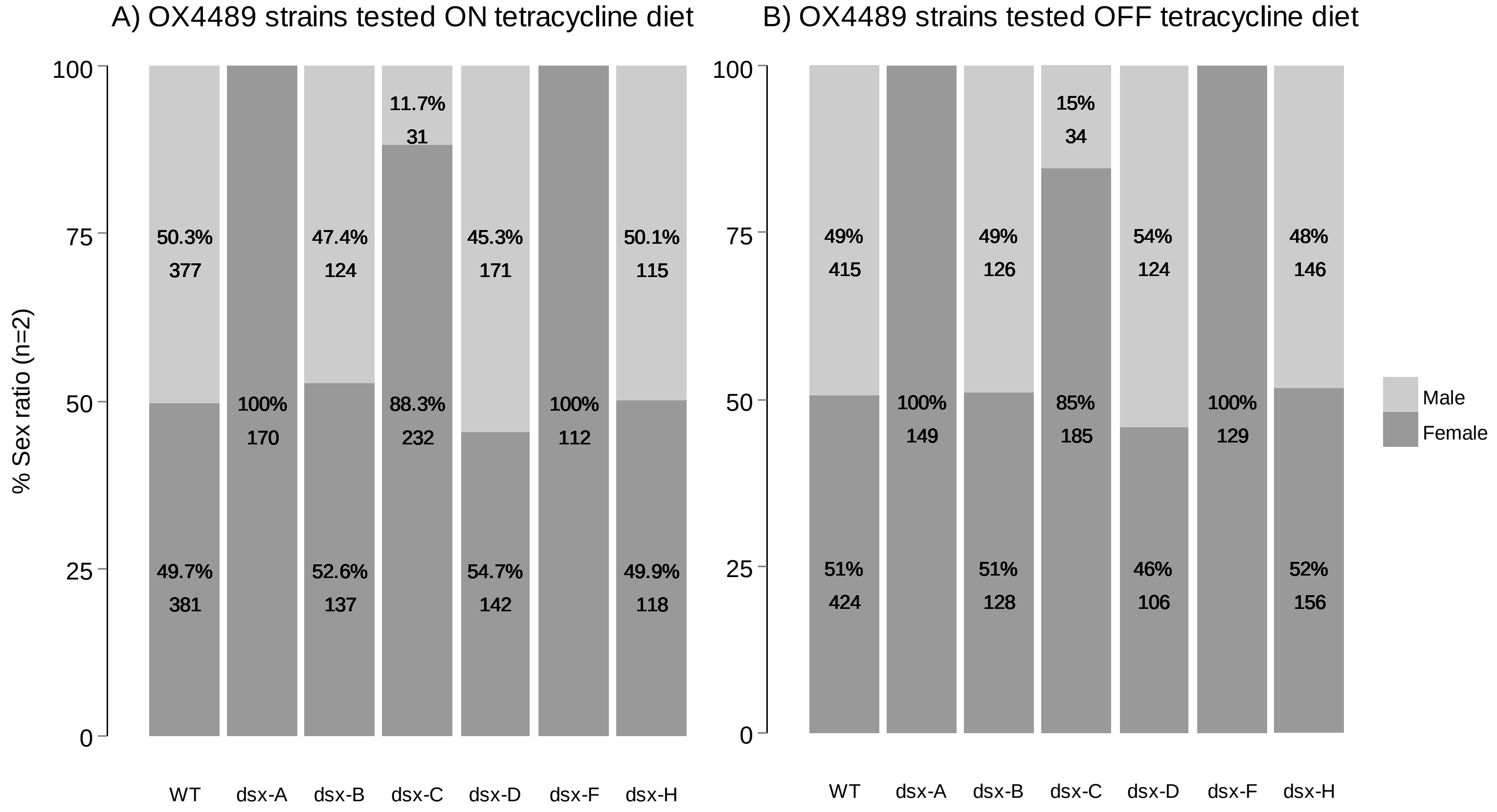


Figure 4.10. Sex ratio of pupae progeny expressing the 3xP3-DsRed2 marker (*dsx* lines) compared with wild-type (WT) progeny, modulated by the presence (ON) or absence (OFF) of 30 µg.ml^{-1} tetracycline in the larval diet.

A starting number of 300 first instar larvae of both sexes of each heterozygous transgenic OX4489 lines (*dsx*-A, B, C, D, F and H) and of 900 first instar larvae for the wild-type strain were tested with 30 µg.ml^{-1} tetracycline diet (**A**) and without (**B**). For each test (ON and OFF tetracycline) and each strain (WT and *dsx* lines), the number of live male and female pupae was recorded (numbers shown at the bottom of each bar) and the resulting sex ratio represented on the Y-axis.

Reverse Transcription-PCR was performed on transgenic OX4489B, D and H male and female pupae, showing the expected sex-specific splicing (**Figure 4.11.**). Sequencing of the RT-PCR fragments indicated that the splicing occurred precisely as in expected (results not shown). In females of lines *dsx*-B, D and H (**Figure 4.11.B**), only the brighter fragment has been sequenced as it corresponded to the expected *dsx*-female transcript but larger fragments can be observed (fainter).

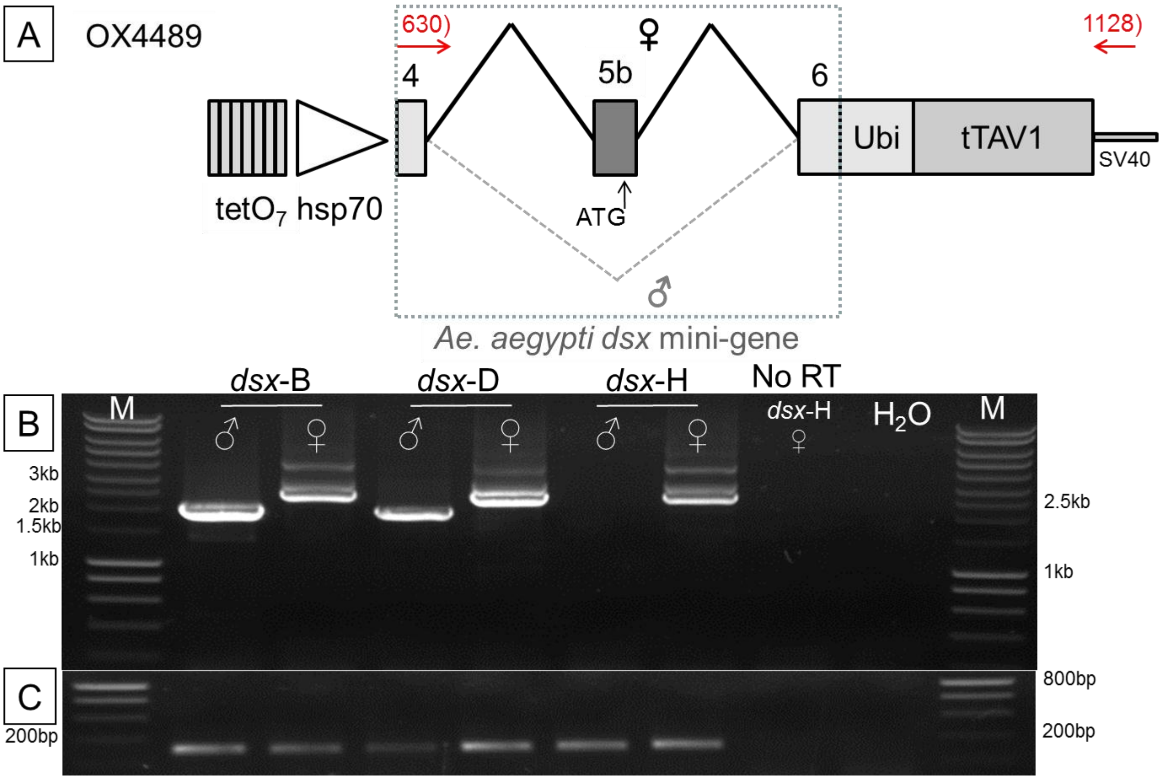


Figure 4.11. RT-PCR on *Ae. aegypti dsx*-transformed male and female pupae (OX4489).
RT-PCRs were performed on the same samples with primer pairs 630) Agdsx-e3-f and 1128) Diag2-SV40 (**A** and **B**) as well as primer pairs 1319) AeCytL and 1320) AeCytR (**C**). The samples are *dsx*-B♂, *dsx*-B♀, *dsx*-D♂, *dsx*-D♀, *dsx*-H♂, *dsx*-H♀ which are respectively male and females OX4489 pupae from different *dsx* lines (B, D and H) from lane 2 to 7; Lane 8 is No RT: same RNA samples OX4489 H ♀ (lane 7) was run without SuperScript III reverse transcriptase as a negative control; Lane 9 is H_2O: water only as template for negative control; Lanes 1 and 10 ‘M’ is the DNA Smart ladder.

Test crosses to tetO-Reaper-KR: the effect of tetracycline treatments

Alternative splicing was tested in both OX3097 and OX4489 *piggyBac* based constructs to selectively kill females in a repressible way using the tetracycline repressible system. Both constructs responded differently to the on and off tetracycline treatments: the former (*Cctra*) exhibiting a total bi-sex lethality ‘off tet’ (line OX3097C) or partial bi-sex lethality in OX3097A and the latter (*Aedsx*) showed no evident lethality (OX4489B, D and H). However, sex-specific fluorescent expression was observed in heterozygous female pupae when tetracycline was absent in the larval rearing water in the *dsx* lines OX4489 B, D and H. Additionally, RT-PCR and sequencing of the *dsx* male and female transcripts confirmed that tTAV is expressed in females only, and the ultimate confirmation was given via test crosses of the three OX4489 (*dsx*) lines to a tetO-effector line carrying the *D. melanogaster* cell death protein, Reaper-KR (White *et al*. 1994; Domingues & Ryoo 2012) (**Figure 4.12.**).

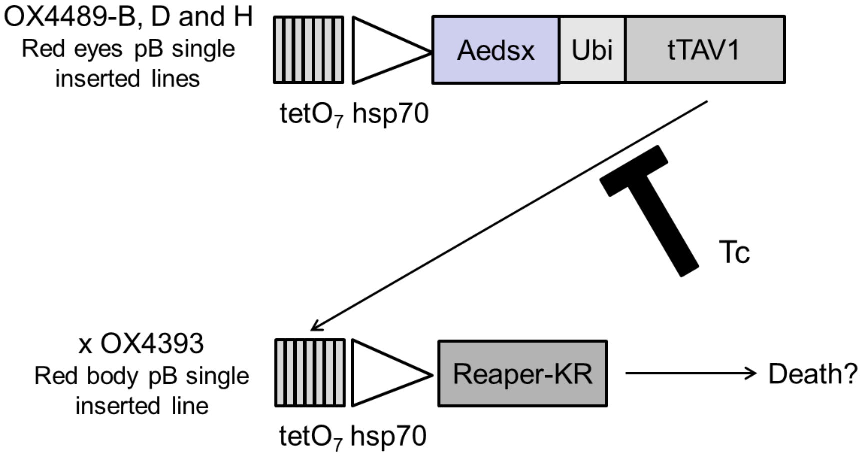


Figure 4.12. Diagram of the test cross experimental design of *dsx* lines OX4489-B, D and H to the tetO-effector line OX4393.

The transactivator tTAV1 was placed under the minimal promoter hsp70 and the alternatively spliced gene *Aedsx* (engineered in a female specific way) in the *piggyBac*-based (pB) single insertion lines (refer to **figure 4.5.** for more details). First on and off tetracycline assays showed no female specific lethality in either OX4489-B, D or H. *Dsx*-lines were then crossed individually to the OX4393 effector line and their resulting progeny was tested for survival on and off tetracycline (Tc).

Independently of tetracycline treatment, sex ratio was biased towards females when the progeny of *dsx*-D crossed to OX4393 effector line was analysed (F=63.332, df=7, p<0.001) in comparison with the wild-types (**Figure 4.13.** and **4.14.**). Analyses revealed that the percentage of female pupae progeny resulting from the cross *dsx*-B / tetO-Reaper was significantly lower when reared *without* tetracycline (F= 2.6261, df=7, p= 0.0203) (**Figure 4.13.**) and even lower when the percentage of female adult progeny was scored (**Figure 4.14.**) (F=62.927, df=7, p<0.001).

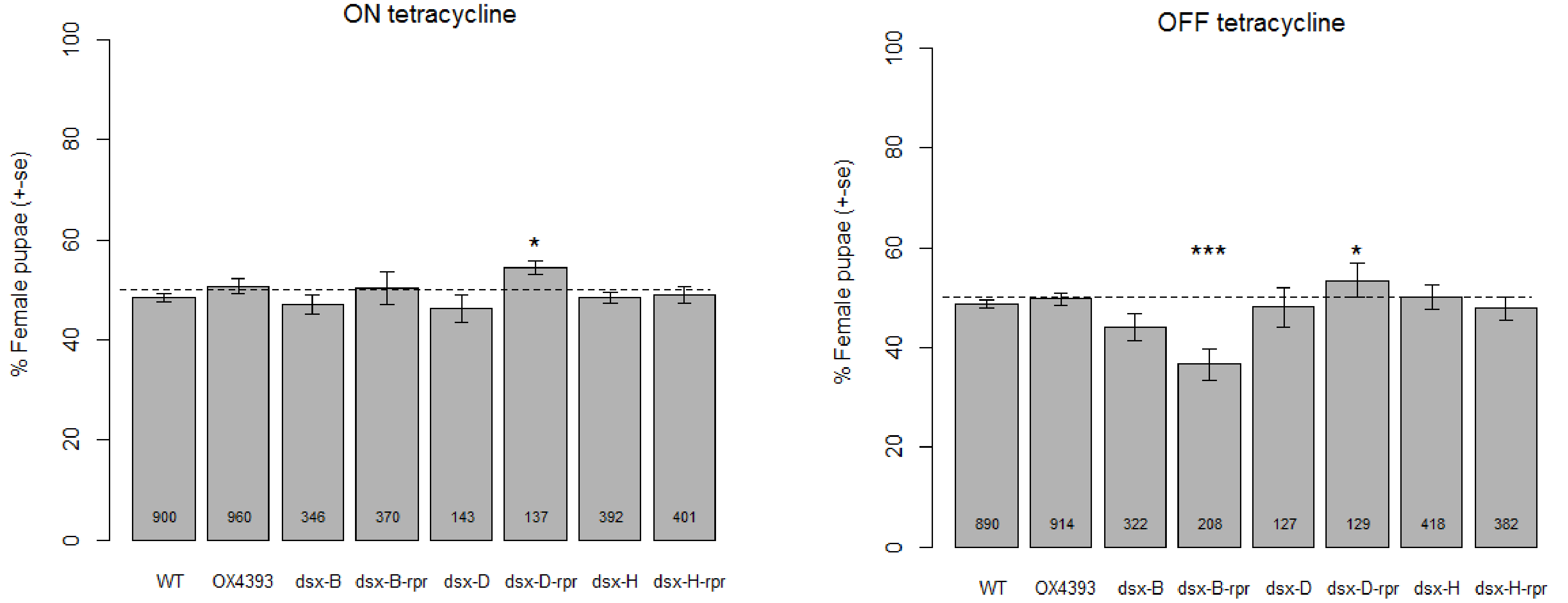


Figure 4.13. Repressibility of female pupae lethality in three *dsx* strains and their crosses to OX4393 effector line in comparison with wild-type. Strains *dsx*-B, D and H are three insertion lines of OX4489 in *Ae. aegypti*. Repressibility of female-specific lethality was assessed by crossing heterozygous females of each strain to OX4393 males (tetO-Reaper) and collecting eggs. The progeny was then reared with either 30 µg.ml^{-1} tetracycline (on tetracycline) or 0 µg.ml^{-1} (off tetracycline). The sex ratio of pupae progeny expressing both DsRed2 profiles (red eyes only, red body only or both) are shown for each strain comprared with the wild-type (WT) progeny. Line *dsx*-B crossed with OX4393 effector line showed a weak female specific lethality when reared in the absence of tetracycline (off tetracycline); that is they produced 69% of female pupae progeny off tetracycline in this assay in comparison with a 98% female pupae survival in the wild-types. The proportion of females was calculated as a proportion of the number of live female pupae collected (numbers shown at the bottom of each bar) over the sum of live pupae, dead larvae and pupae and the rest of larvae left in the tray that did not pupate. Error bars represent the standard error of the mean percentage of females. The star symbols '*' and '***' represent the significance level of female percentage between the different lines tested against the wild-types ($p<0.05$ and $p<0.001$ respectively) (GLM).

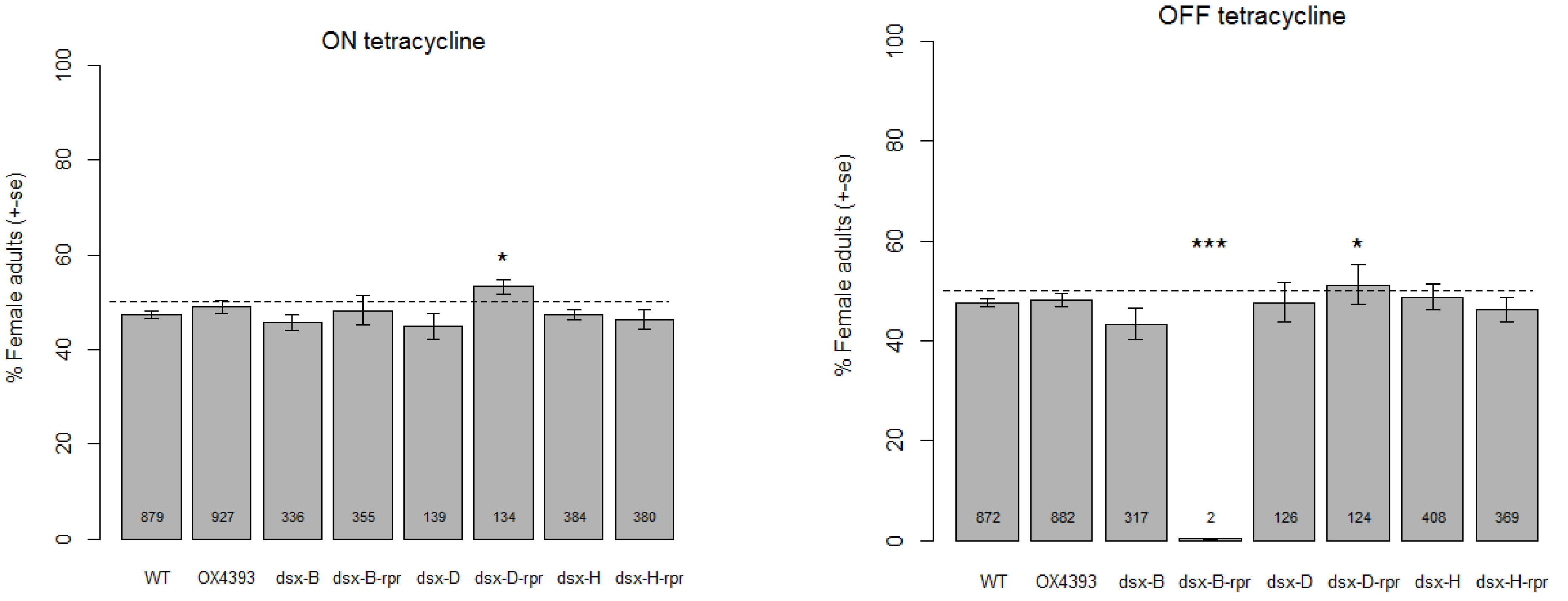


Figure 4.14. Repressibility of female adult lethality in three *dsx* strains and their crosses to OX4393 effector line in comparison with wild-type. Strains *dsx*-B, D and H are three insertion lines of OX4489 in *Ae. aegypti*. Repressibility of female-specific lethality was assessed by crossing heterozygous females of each strain to OX4393 males (tetO-Reaper) and collecting eggs. The progeny was then reared with either 30 µg.ml^{-1} tetracycline (on tetracycline) or 0 µg.ml^{-1} (off tetracycline). The sex ratio of adult progeny expressing both DsRed2 profiles (red eyes only, red body only or both) are shown for each strain comprared with the wild-type (WT) progeny. Line *dsx*-B crossed with OX4393 effector line showed fully penetrant female specific lethality when reared in the absence of tetracycline (off tetracycline); that is they produced only 2 females progeny off tetracycline in this assay. The proportion of females was calculated as a proportion of the number of live female pupae collected that successfully eclosed as adult females (numbers shown at the bottom of each bar). Error bars represent the standard error of the mean percentage of females. The star symbols '*' and '***' represent the significance level of female percentage between the different lines tested against the wild-types ($p<0.05$ and $p<0.001$ respectively) (GLM).

4.4. Discussion

To summarise, this chapter's major finding is the achievement of the first *Ae. aegypti* fsRIDL created using solely alternative splicing of an *Aedsx* mini-gene. However, chronologically, the first attempts, using a *C. capitata* intron, failed not to produce an alternatively spliced tTAV in frame in females but, instead, reproduced the original *Ae. aegypti* RIDL bi-sex lethal phenotype in one line OX3097C out of five transgenic lines obtained with the construct. This was not surprising since mosquitoes are phylogenetically distant from tephritids (Ruiz et al. 2007). An endogenous alternative splicing system presumably has a better chance of being correctly processed, even in the context of an artificial transgene, than does an exogenous one, especially from a species only distantly related. Furthermore, alternative splicing of *Cctra* is mediated by Tra protein; no *tra* homologue has been identified in mosquitoes, so it is possible that the necessary function is absent (or not sex-specific). Lacking an *Aedes* homologue of tra, attention was focused instead on *Aedsx* instead. *doublesex* is another sex determination gene with sex-specific alternative splicing, however it is better conserved and more readily recognisable than *tra*. The *Aedsx* gene was characterised recently (Salvemini et al., 2011) and a mini gene engineered in 2008. However, transformation with OX4489 construct carrying an *Aedsx* mini-gene did not exhibit female specific lethality in the absence of tetracycline in the larval water. Instead, *dsx*-transformed larvae exhibited divergent fluorescent expression of the transformation marker when reared in the absence of tetracycline. Conditional female lethality was achieved only once line OX4489 B was out-crossed to effector lethal gene Reaper-KR.

4.4.1. OX3097C as a RIDL-like strain

Reverse transcription PCRs and sequencing of the male and female dominant transcripts obtained for three OX3097 lines out of the five created confirmed on/off tetracycline results: putative female transcripts are produced in both sexes (**Figure 4.9.**). This indicates that, i) *Cctra* is not differentially spliced in *Ae. aegypti* and ii) that the female *Cctra-tTAV* transcript produces a certain degree of lethality depending of the insertion site of the piggyBac-based construct. Indeed, line OX3097A showed partial lethality when reared without tetracycline, and full lethality was observed in line OX3097C, occurring before pupation (**Annexe 8**, **Figure 8.3.** and **8.4.**). OX3097C could potentially be used as a back-up or alternative to OX513A, since the lethal phenotype is similar, and perhaps more highly penetrant. OX3097C larvae and pupae exhibit a strong red spotted fluorescence all over the body even when immature and reared with 30 $\mu g.ml^{-1}$ tetracycline; this is rather easier to score than the marker in OX513A. The presence of fish food does not compromise the clear distinction between transgenic and wild-type individuals. An extra element (*Cctra* first intron) has been added to the classic tetracycline repressible system (tetO-hsp70-tTAV) in OX513A (Phuc et al., 2007), this may be a negative feature as regulatory authorities are generally leery of non-functional or redundant elements. Also, experiments would need to ensure that the strain fitness is not compromised when made homozygous (i.e., larval survival to pupation and adulthood in presence of 30 $\mu g.ml^{-1}$ tetracycline must be assessed, as well as male longevity and mating competitiveness). The strain can also then be tested for mass rearing (i.e., testing for fecundity and fertility).

4.4.2. Conditional female specific expression using endogenous *Ae. aegypti doublesex* gene

In 2011, Magnusson *et al.* reported the achievement of a sexually divergent fluorescence phenotype using *dsx* in the malaria vector *Anopheles gambiae* (**Figure 4.15.**). Although the construct was designed to give female specific fluorescent expression, the plasmid construct injected in *An. gambiae* resulted in both male and female larvae expressing red fluorescence in the eyes through the expression of *3xP3*-DsRed marker, as well as a diffuse green expression in the abdomen, more pronounced in the male larvae than in the female (Magnusson et al., 2011), allowing consistent discrimination between sexes using flow cytometry (Marois et al., 2012). Explanations of the phenomenon are: DmActin 5C promoter expression is stronger in *An. gambiae* males than in females, hence a stronger green fluorescent expression is observed in the latter; *An. gambiae dsx* splice variants produce functional eGFP proteins in both males and females, however, male transcripts might be more abundant than female transcripts and, finally, the divergent fluorescence could be the result of both DmActin5C and *dsx* actions. The second explanation seems more plausible as DmActin5C drives the fluorescent marker DsRed2 in *Ae. aegypti* RIDL line at Oxitec (Phuc et al., 2007), but no difference of fluorescence has been observed between the sexes. However, expression of DsRed2 is so weak in the OX513A strain that distinction of divergent fluorescence might be difficult.

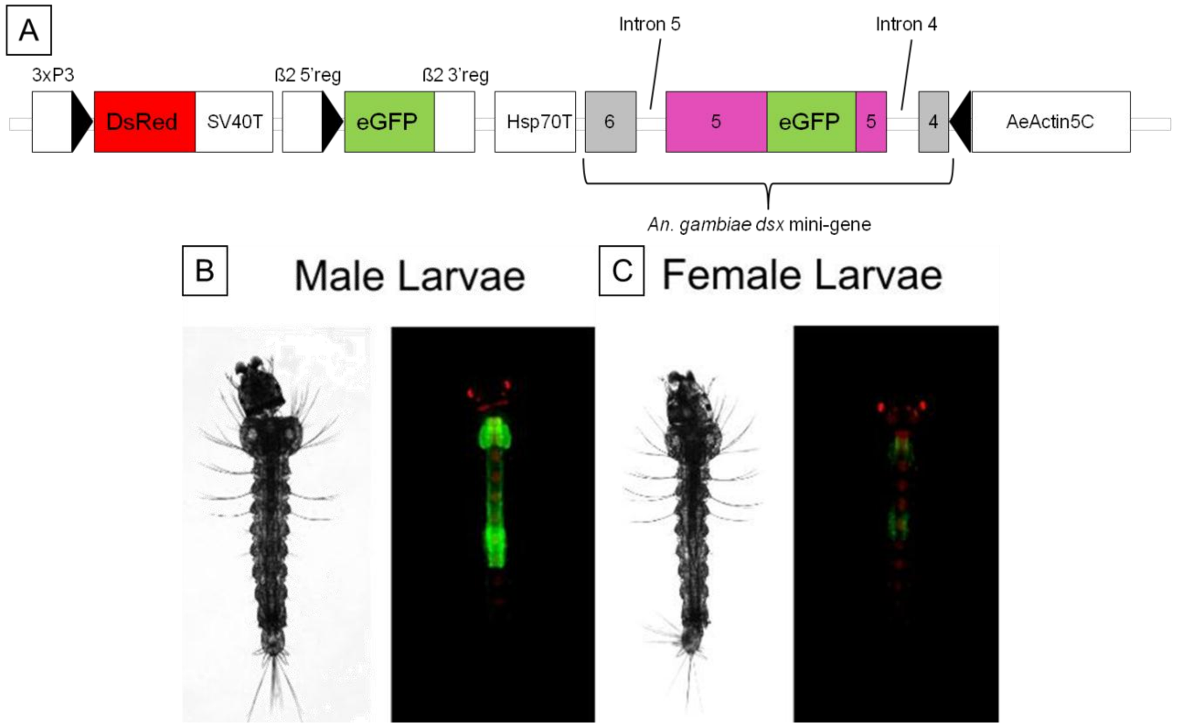


Figure 4.15. Diagram of the *An. gambiae* eGFP-dsx *piggyBac* construct that allowed a sexually divergent fluorescence phenotype (modified from Magnusson *et al.*, 2011)**.**
The *An. gambiae* eGFP-*dsx* construct is a *piggyBac*-based plasmid carrying a 'red eyes' transformation marker through the expression of DsRed gene under the control of 3xP3 promoter. A male-specific promoter, ß2 tubulin, is controlling the eGFP gene so that males express green fluorescent gonads (Catteruccia *et al.* 2005). Finally, another GFP gene is inserted in an engineered *An. gambiae dsx* mini-gene presicely in the female specific exon 5 (**A**). The phenotype of such a construct resulted in a stronger abdomen green fluorescence in male larvae (**B**) in comparison with female larvae (**C**), both sexes carrying the red-eye marker (**A** and **B**).

In *Ae. aegypti*, conditional female specific expression was achieved using a different strategy: instead of placing tTAV elements in the female exon so only females splice out tTAV element as opposed to males (**Figure 4.15.**), tTAV was placed downstream of the alternative splicing and an engineered start codon was added in the female exon (**Figure 4.4.**); tTAV would then be translated in frame in females only. *Aedsx* has been used for the first time in an engineered system, and proven to generate consistent sexually divergent fluorescent expression in three independent *piggyBac* lines (data not shown). The explanation for this phenomenon is due to the bi-directional element tetO driving both ways in the

OX4489 constructs: on one side tTAV is read in frame in females only thanks to the engineered start codon placed in the female exon; on the other side of the construct, enough tTAV is produced in females to bind to tetO generating ubiquitous red fluorescent expression in females when reared in absence of tetracycline.

4.4.3. Engineering a conditional female-specific lethal in *Ae. aegypti*

Female-specific expression was consistently achieved in three independent transgenic lines transformed with *dsx* mini-gene placed between the minimal promoter Dmhsp70 and tTAV. This result probably reflects that enough functional tTAV is being produced in construct OX4489 to produce divergent fluorescent expression. However, not enough is produced to generate lethality as only one OX4489 line out of three exhibited late pupal lethality when out-crossed to tetO-Reaper-KR toxic effector line (**Figure 4.13.** and **4.14.**) and tetO-Nipp1 (another effector toxic line) (data not shown). Moreover, in Chapter 5, the same *Aedsx* cassette was combined with the *DmAdh* inducible lethal system described in chapter 2 failing to induce female specific expression or lethality in presence of long term exposure to ethanol. The reasons for the *AeDsx* working in construct OX4489 remain unclear and are further discussed in the next chapter. From the evidence gathered in this chapter, and to complete the study on engineering the first *Ae. aegypti* fsRIDL using alternative splicing only, one could think of taking advantage of the bi-directional tetO element to combine both toxic elements tTAV and Reaper in an all-in-one Reaper-tetO-dsx-tTAV loop as proposed in **Figure 4.16.**

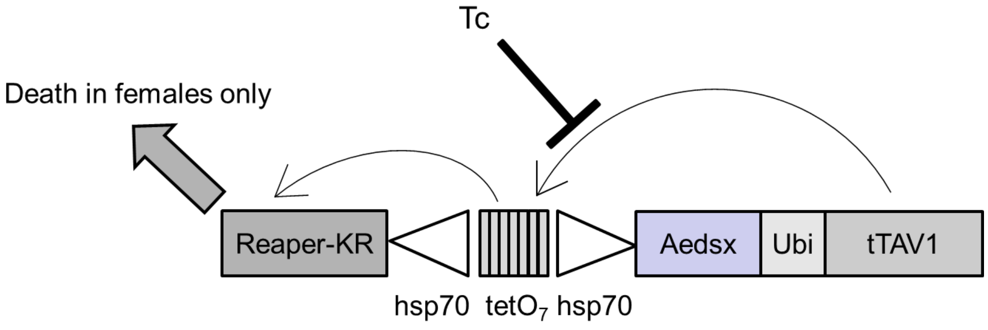


Figure 4.16. Diagram of the all-in-one fsRIDL construct in *Ae. aegypti*.

In this diagram, a ‘all-in-one fsRIDL’ construct is proposed: like in previous construct OX4489 (Figure **4.4.**) minimal promoter hsp70 is driving the *Ae. aegypti* Oxitec mini-gene version (Figure **1.11.**) with the fusion protein Ubiquitin-tTAV1 placed downstream the sex-specific splicing. tTAV1 is in frame in the females transcript only leading to a functional protein and binding to its receptor tetO placed upstream the minimal promoter hsp70. Because the original OX4489 construct did not lead to female killing, we would take advantage of the bi-directionality of tetO and place another hsp70 promoter the other side driving a cytotoxic gene Reaper-KR. The female-killing system would switch off simply by adding tetracycline (Tc) to the larval diet as tetracycline binds to tTAV disabling the binding of tTAV to tetO and induce the feedback loop system.

Chapter 5: Towards a female specific inducible lethal system in *Ae. aegypti*

5.1. Introduction

In SIT-related programmes, only sterile males are released to mate with wild females, in order to reduce the local population and would, in theory, help the transmission of disease by the vector, or prevent infestation of crops in endemic areas. As previously mentioned in Chapter 1, sex separation methods have exploited *Ae. aegypti* pupal sexual dimorphism (the female pupa being generally larger than the male pupa) and sex-sorting devices have been used since 1959 (Ansari et al., 1977b; Fay and Morlan 1959; Focks 1980). Today, the same techniques are used for the release of engineered *Ae. aegypti* males carrying a Repressible Dominant Lethal (RIDL) in Malaysia and Grand Cayman (Harris et al., 2011; Harris et al., 2012b; Lacroix et al., 2012). In the first field release of the strain OX513A in Grand Cayman, Harris et al. (2011) reported a successful mechanical sex separation of female pupae with accuracy up to 99.5% (95% confidence interval of 99.46% and 99.63%); in Malaysia, 33 female pupae were collected out of a total of 10,029 pupae (0.33%) after mechanical sex sorting (Harris et al., 2011; Harris et al., 2012b; Lacroix et al., 2012). Pupae were inspected under a microscope following sorting as a quality-control additional step and to remove any remaining females, due to specific experimental or permit requirements (Harris et al., 2011; Harris et al., 2012b; Lacroix et al., 2012).

While traditional mechanical sex sorting is still in use and is proven to efficiently remove most of female pupae with a maximum of 0.5% failure rate, original studies reported a major loss of male pupae, up to 60% (Ansari et al., 1977b).

Today, transgenesis tools can offer great improvements to sexing methods by creating engineered sexing strains. The flightless strain OX3604C (fsRIDL) (Chapter 4) presents the advantage of carrying a conditional, female-specific flightless trait, and can act as a sexing strain while allowing the release of both males and females: males are healthy and able to fly from the release site, but females, unable to fly (hence to seek for sugar or blood meal) are highly vulnerable to predation. Once the fsRIDL males have mated with the wild type females, 100% of the female progeny will be unable to fly when there is no tetracycline to repress the toxicity of tTAV in their indirect flight muscles, and 100% of the male progeny will carry on, passing on the dominant fsRIDL gene and increasing the efficiency of the suppression programme (Phuc et al., 2007). While such a system presents a significant advantage post-release, several practical issues remain to be addressed pre-release. Because *Ae. aegypti* eggs can be stored for several months, OX3604 eggs could be distributed and hatched in natural or artificial breeding sites, reducing the production costs. An alternative would be an early-acting inducible system expressed in a particular sex (Chapter 1) i.e. inducing lethality of *Ae. aegypti* larvae in the presence of the inducing factor ethanol (see Chapter 3) (**Figure 5.1.B.**). Since a functional bi-sex lethal is available in the strain OX513A (Chapter 6) and that the release of RIDL adults is already in place we can engineer an early-acting, inducible, female-lethal to combine with the RIDL trait. There are two key advantages of this approach, the first is the removal of female larvae early in development will allow the production of containers composed of a RIDL-male-only larval population, halving production cost and space requirements (e.g. staff requirements, sex sorting, materials) as well as improving the efficiency of the release of a RIDL-male-only population

(**Figure 5.1.**). The second advantage is that one would not have to worry about resistance management, as only the release generation is being exposed to the chemical ethanol, and not routinely exposed in the mosquito mass-rearing facility.

Such an inducible lethal system was created (Chapter 3), and showed promising results when the *DmAdh* gene was under the control of *AeHsp83* promoter in *Ae. aegypti,* selectively killing *Adh*-transformed larvae but not wild-type ones under long-term exposure to ethanol (EtOH) in the larval water (between 0.5% and 1% v/v). Combined with the alternatively spliced *Aedsx* mini-gene (Chapter 4), we investigated the possibility of engineering an *Ae. aegypti* GSS using this chemical selection.

This chapter reports the successful transformation of *Ae. aegypti* with the OX4597 construct (**Figure 5.2.**) carrying the following components: *Ae. aegypti* hsp83 promoter previously tested in the *DmAdh* construct OX4346 (Chapter 3), *Aedsx* mini gene previously used in construct OX4489 (Chapter 4) and, finally, the fused elements *Ubiquitin-DmAdh* instead of *Ubiquitin-tTAV* previously tested in construct OX4489 (Chapter 4). Four independent transgenic lines were created and tested with long-term ethanol exposure, as well as the sequencing of both male and female pupal mRNA transcripts produced as products of *Aedsx* alternative splicing.

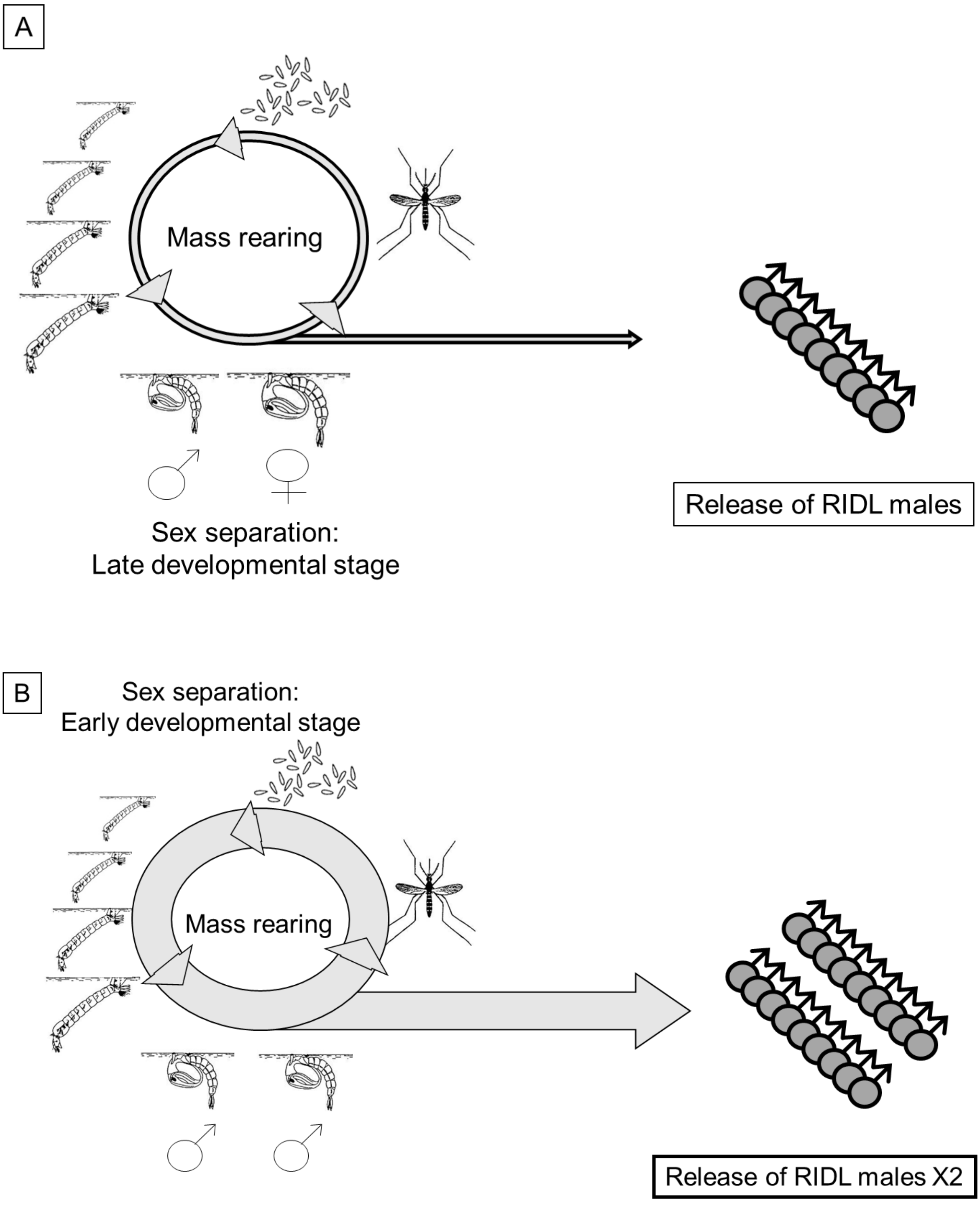


Figure 5.1. Diagram showing the advantages of early sex separation for the release of a male-only RIDL population for local suppression of *Ae. aegypti*.

A: Mass rearing and release process when sex separation occurs at late developmental stage (pupae); **B**: Mass rearing and release process when sex separation occurs at early developmental stage (embryonic or first instar larvae). The weight of the grey line represents the proportion of males produced.

5.2. Material and methods

5.2.1. Construct OX4597 and plasmid construction

Briefly, OX4597[5] plasmid is derived from the OX4489 construct, with tTAV1 element from OX4489 plasmid replaced with *DmAdh* from plasmid OX4345. The tetO$_7$-hsp70 elements have been replaced by the Aehsp83 promoter, and finally, the 'red eyes' *3xP3-DsRed2* replaced by the 'blue body' marker *Hr5IE1-AmCyan* (**Figure 5.2.**).

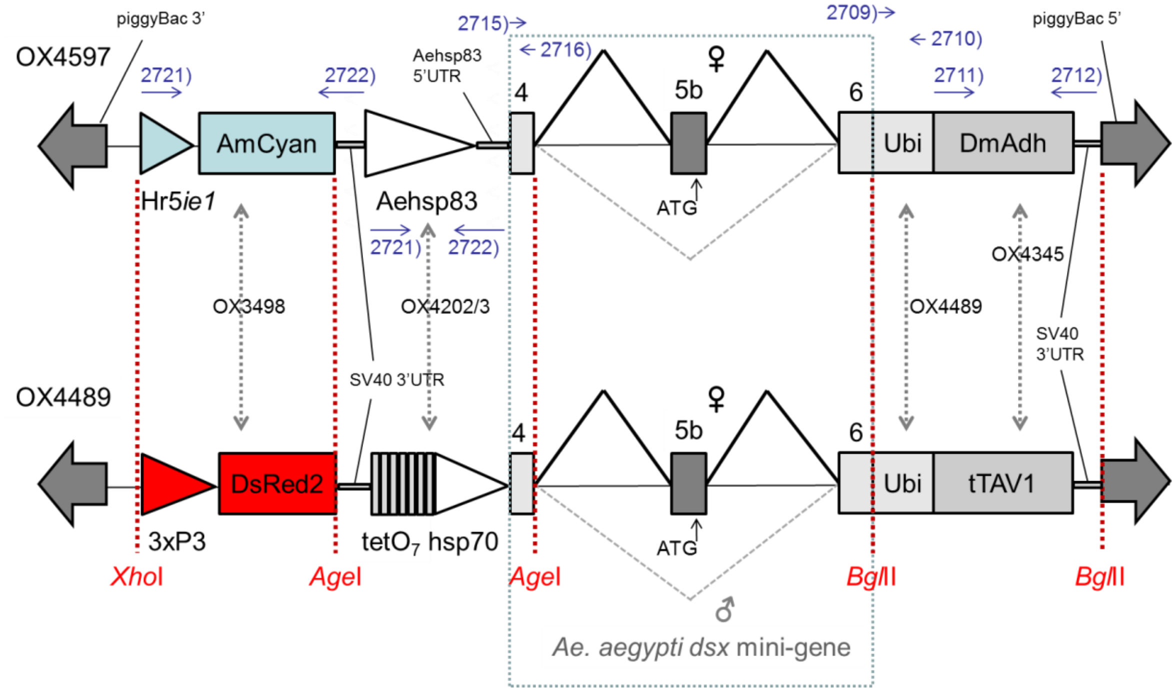


Figure 5.2. Diagram of *Ae. aegypti piggyBac*-based constructs OX4489 and OX4597 and DNA construction process.

OX4489: a tetO-tTAV1 cassette is inserted into *Ae. aegypti doublesex* (*Aedsx*) mini-gene and adjoins a transformation marker (3×P3-DsRed2). OX4597 carries a 'blue body' marker (Hr5-*ie1* promoter driving AmCyan), *Ae. aegypti* Hsp83 promoter driving the alternatively spliced Dsx gene and the fused elements *Ubiquitin-DmAdh*. *Aedsx* engineered mini-gene is composed of two shared exons (4 and 6) surrounded by 2 shortened introns, and a female-specific exon, 5b, with an engineered start codon in frame with the *Ubiquitin-DmAdh* coding sequences. Restriction enzymes (red) as well as the main primer pairs (blue) are indicated in to help understanding the process of DNA construction of plasmid OX4597. Other plasmid constructs from elements were amplified and assembled in the OX4489 construct to create OX4597 end product are also indicated (i.e. OX4345, OX3498, OX4489 and OX4202/3).

---

[5] Plasmid construct designed with the help of Tarig Dafa'alla, Ph.D. at Oxitec Ltd.

Firstly, to build construct OX4597, plasmid OX4489 was digested with enzyme *Bgl*II resulting in a 10,712 bp product, dephosphorylated and purified. The process of DNA construction proceeded in two further steps: 1. The fusion of the OX4489 – Adh elements and 2. The fusion of the first cassette OX4489-Adh to Aehsp83-Hr5ie1-AmCyan elements. The *Ubiquitin* element was amplified from plasmid construct OX4489 (**Figure 5.2.**) with primer pairs 2709) and 2710) (**Table 5.1.**) resulting in a 261 bp product and subsequently digested with *Bgl*II and *Bsa*I, then purified. The elements DmAdh-SV40 were amplified from plasmid construct OX4345 (not shown) with primer pairs 2711) and 2712) (**Table 5.1.**) resulting in an end product of 1,036 bp. The digested elements OX4489 and DmAdh-SV40 were transformed-ligated (OX4489-DmAdh-SV40). The colonies were screened with primer pairs 987) and 2418) then digested with *Bam*HI. The resulting elements were sent for sequencing with primer pairs 987) and 2416). The resulting element OX4489-DmAdh-SV40 were digested with *Age*I and *Xho*I (10,106 bp) and purified. The element Aehsp83 promoter was amplified from the construct OX4202/3 with primer pairs 2713) and 2714) (**Table 5.1.**) resulting in a 4,384 bp product, digested with *Bsa*I and purified. Part of the Aedes dsx mini-gene (188 bp) was amplified from construct OX4489 with primer pairs 2715) and 2716) (**Table 5.1.**) and digested with *Bsa*I and *Age*I and purified. Elements Hr*ie1*-AmCyan-SV40 were amplified from construct OX3498 (not shown) with primer pairs 2721) and 2722) (**Table 5.1.**), digested with BsaI and purified. Elements were ligated-transformed to form OX4489-Adh-Aehsp83-Hr5ie1-AmCyan and colonies were screened with primer pairs 2720) and 1917) resulting in a 395 bp product which was then digested with *Xho*I and *Age*I. The final product was sent for sequencing with primer pairs 222), 980), 584), 107) and 2720). The 3 elements were ligated and transformed into OX4489-*DmAdh*, generating OX4597 *piggyBac* construct pB[*Hr5IE1-AmCyan-AeHsp83-Aedsx-Ubi-DmAdh*-SV40].

Primer name	***Primer nucleotide sequence***
2709) Ubiq-bgl-f	CATATTCTGCACAGCATACATCGGACA
2710) Ubiq-bsa-r4	ACGCGGTCTCTACCACCGCGCAGGCGCAG
2711) DmAdh-bsmb-f2	AGCGCGTCTCATGGTATGTCGTTTACTTTGACCAACAAGAAC
2712) SV40-bsmb-r7	ACGCGTCTCAGATCTTAAGATACATTGATGAGTTTGGACAAAC
987) Diag-aedsx-m	CTGGAAGAGAGAAAGTTTCGGC
2418) Diag3-DmADH-3	ATGCCGGGGTTCACAGTGTAAG
2713) Aehsp83pro-bsa-f2	ACGCGGTCTCACCTAGGTCTACTTCGCAAATCTCACGCTTG
2714) Aehsp83-5utr-bsa-r	ACGCGGTCTCAGGCGCGCCTGGAGCGAGAAATAAGGGACTCG
2715) Aedsx-e4-bsa-f3	ACGCGGTCTCACGCCACGACGAACTTGTCAAACGATCT
2716) Aedsx-int-Age-r	ACCTCGGAAACAGTCCCAACCGGTAAG
2720) Aedsx-seq-6	CACTGTTGGGAATAGACTCCGTC
1917) seq-Aehsp83pro-10	GAAGCAGGTCAGAATGCCCAC
2721) hr5-bsa-xho-f	ACGCGGTCTCATCGAGATAGCGGCCGCATGGTACCCAT
2722) SV40-bsa-r15	ACGCGGTCTCATAGGCTCGCGTTAAGATACATTGATGAGT

Table 5.1. Primer sequences used for OX4597 plasmid construction (see **Figure 5.2.** for binding sites on the plasmid)***.***

5.2.2. Reverse-Transcription PCR and sequencing

In order to study the splice variant forms of *Ae. aegypti doublesex* mini-gene expressed from the *DmAdh* cassette (Chapter 4), RNA was extracted from OX4597 single male and female pupae using RNeasy Qiagen kit (Hilden, Germany) 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. First strand cDNAs were synthesised using RevertAid first-strand synthesis system (Fermentas) with polyT primers, using approximately 0.5 µg extracted RNA per simple. PCR was then performed on cDNA samples using the primers shown in **Table 5.2.** Actin primers were the same used to amplify *Bombyx mori actin 1* in Canning et al. (1996) and were used on the same samples each time a RT-PCR was run as a positive control.

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 sequenced by GATC Biotech (Konstanz, Germany) using pJET primers (**Table 5.2.**). The expected male and female splice sequences were aligned using the Clustal Omega software package (Goujon 2010; Sievers et al., 2011) and explored using jalview version 2.8 (Waterhouse et al., 2009).

Primer name	***Primer nucleotide sequence***
630) Agdsx-e3-f	GAGATGATGCCCCTGATGTACG
1128) Diag2-SV40	GATGAGTTTGGACAAACCACAACTAGA
1319) AeCytL	TTCACCACCACCGC(C/T)GAG
1320) AeCytR	ATACCGGGGTACATGGTGG
1156) pJET-FP2	ATCAACTGCTTTAACACTTGTGC
1157) pJET-RP2	AAAGAAGAACATCGATTTTCCATG

Table 5.2. Primer sequences used for Reverse Transcription PCR on OX4597-transformed male and female pupae.

5.2.3. Phenotype analysis of the OX4597 lines via ethanol bioassays

Long term exposure to ethanol

As mentioned in Chapter 3, OX4597 larvae carried the inducible *DmAdh* lethal system which was triggered by long-term exposure to 0.5% and 1% ethanol in the larval medium. Bioassays were then performed as previously described (Chapter 3; paragraph 3.2.5) with the exception of the initial number of first instar larvae counted per batch (600 instead of 300). Larvae were reared in 600 ml final volume (density of 1 larva.ml^{-1}) for a final ethanol concentration of 0.5% v/v and 1% v/v. As a control, OX4597 larvae were reared in deionised water only and batches were set up in triplicates for each treatment applied (no ethanol, 0.5% and 1% ethanol). All rearing vessels were covered to reduce evaporation of the ethanol during the experiment.

Comparison between the *Adh*-lines (OX4346, chapter 3) and the OX4597 lines (*dsx-Adh*-lines) under standard rearing conditions

As an extra control, both transgenic lines transformed with the *DmAdh* gene were compared under standard rearing with no ethanol added (deionised water only with Tetramin fish food). The *Adh*-lines OX4346-G, O and N (Chapter 3) were compared to the *dsx-Adh*-lines OX4597-A, B, C and D. The starting number of first instar larvae was 300 for the OX4346 lines and 600 for the OX4597 lines reared in, respectively, 300 ml and 600 ml final rearing volumes of deionised water so the larval density of 1 larva per ml was respected. Every assay was performed in triplicates for each strain tested. The larval feeding regime was performed as previously described in the General Material and Methods Chapter (2).

5.2.4. Statistical analysis

Statistical analyses were performed using R software version 2.12.2 (R Development Core Team, 2011). OX4597 larval survival was estimated as the proportion of transgenic individuals to wild-type surviving to adulthood in each pot, data were ArcSin transformed and analysed using ANOVA including ethanol treatment, line and sex as well as their interactions. The proportion of wild-type individuals surviving to pupation in each pot was used as a control.

When both heterozygous transgenic lines OX4597 and OX4346 survival to adulthood were compared in standard rearing conditions, the proportion of adults recorded was ArcSin transformed and analysed using ANOVA including line (OX4597 or OX4346), type (wild-type or transgenic) and sex (male or female).

5.3. Results

5.3.1. The OX4597 *Ae. aegypti* strains

A total of 1,138 *Ae. aegypti* wild-type embryos were injected with the construct OX4597 (**Figure 5.2.**), out of which only 48 survived to pupal stage (4.22%), and even fewer made it to adulthood (N=45, 3.95%), 25 males and 23 females (respectively 52% and 48%). Two transgenic lines resulted from separate adult male G_0 pools, OX4597A and B, and 2 from separate adult female pools, OX4507C and D. Another adult G_0 male pool produced a few transgenic G_1 but only 1 female pupa survived and the line was lost. All 4 transgenic lines expressed a bright blue all-over-the-body expression pattern due to *Hr5*IE1-*AmCyan* marker.

5.3.2. Impact of long-term exposure to ethanol on heterozygous OX4597 larvae

Four heterozygous OX4597 lines were established and reared to generation five (G_5). Each line was then tested for survival of larvae to adulthood relative to that of their wild-type counterparts (**Figure 5.3.**). Overall, long term exposure to 0.5% as well as 1% ethanol had a significant impact on survival from first larval instar to adulthood (F=4.8541, df=1, p=0.02943). Survival was highly significantly different between all the OX4597 strains in comparison with the wild-types (F= 58.4266, df=4, p< 0.001) as well as survival was different between sexes (F= 29.9603, df=1, p<0.001) with a higher proportion of males surviving to adulthood (t=2.227, df=1, p=0.028). The 2-way-interactions Ethanol:Strains was non significant (F= 1.1856, df=4, p=0.32045) and so was the 2-way-interaction Strains:Sex (F=1.1453, df=4, p=0.33844). Out of the four OX4597 heterozygous strains tested, none showed a female-specific-lethal induced with ethanol treatments (interaction between ethanol and Sex, F=0.4972, df=1, p=0.48206) as well as the three-way-interaction EtOH:Strain:Sex (F=0.1109, df=4, p=0.97852) (**Figure 5.3.**).

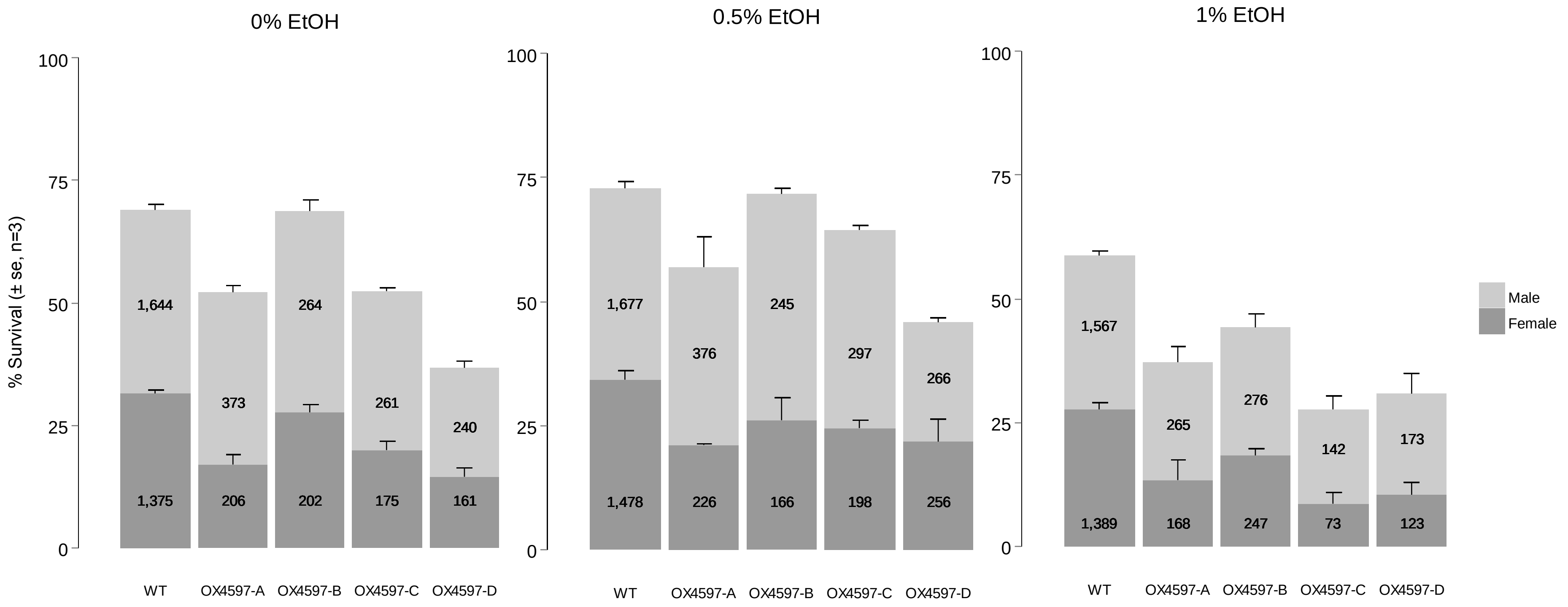


Figure 5.3. Survival of progeny expressing the Hr5*ie1*-AmCyan (0X4597 lines) marker to adult males and females compared with wild-type (WT) progeny when submitted to ethanol treatments.

Ae. aegypti OX4597 transformed lines (OX4597A, B, C and D) were reared in standard conditions (0% EtOH) as well as submitted to long term exposure to 0.5% and 1% ethanol (EtOH) v/v. For each test (0%, 0.5% and 1% ethanol) and each strain (WT and OX4597 lines), the number of live male and female pupae was recorded (numbers shown inside each bar) and the resulting proportion that survived to adulthood represented on the Y-axis. Error bars represent the standard error of the mean survival percentage out of three samples (n=3).

5.3.3. No female inducible lethality with OX4597 construct: Investigating the causes

The desired female specific inducible lethal was not achieved with plasmid construct OX4597 (**Figure 5.2.**): long term exposure to ethanol did not have a significant impact on OX4597 heterozygous larvae in comparison with wild-type ones, even though reverse transcription PCR clearly showed divergent splicing of *Aedsx* in males and females (**Figure 5.4.**).

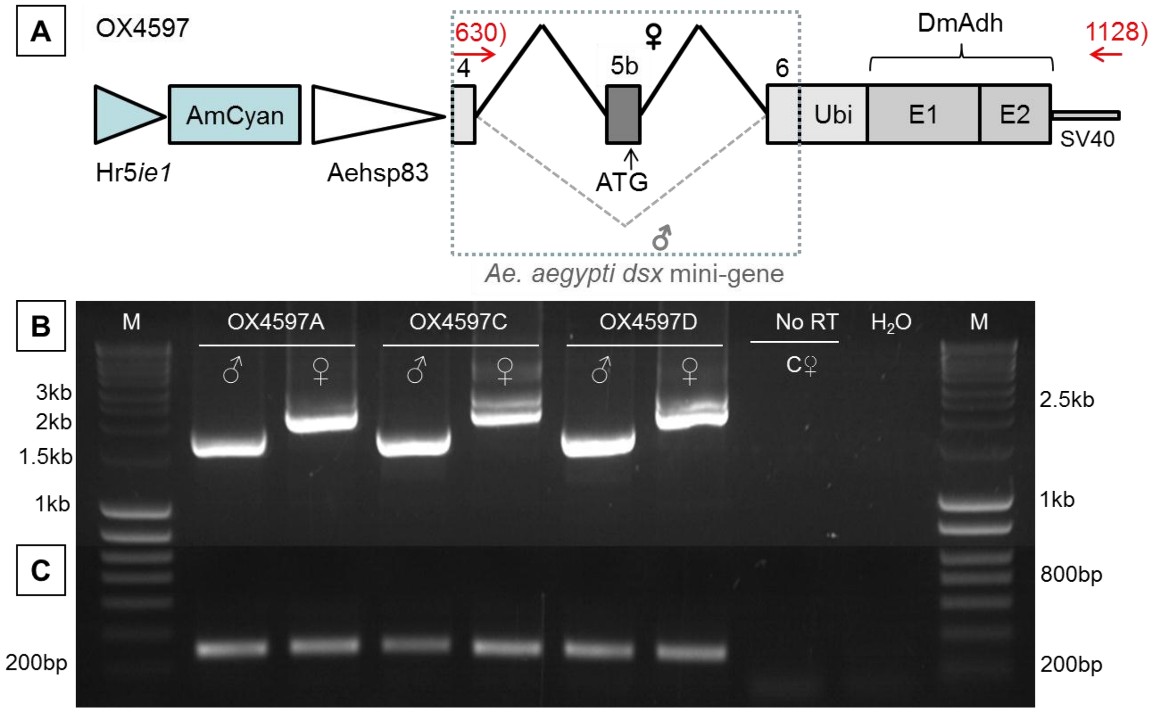


Figure 5.4. RT-PCRs on *Ae. aegypti dsx-DmAdh* male and female pupae with construct OX4597.

RT-PCRs were performed on the same samples with primer pairs 630) Agdsx-e3-f and 1128) Diag2-SV40 (**A** and **B**) as well as primer pairs 1319) AeCytL and 1320) AeCytR (**C**)**.** The samples are alternatively OX4597 males and females from line OX4597-A, C and D (lane 2 to 7). Lane 8 is a No RT sample OX4597 C♀ run without RevertAid reverse transcriptase during cDNA synthesis for negative control. Lane 9 is water only as template for negative control. Lane 1 and 10 of both gels represent Eurogentech Smart ladder (M) from 200 to 10’000 bp.

During the rearing of the OX4597, it was been observed that some of the lines had difficulties surviving to pupation when reared in tap water only (data not shown).

During long term ethanol bioassays, controls (without ethanol) showed a low survival rate of the OX4597 larvae compared to wild-type ones and, for that reason, further experiments were run to analyse this pattern: *Adh*-transformed larvae (OX4346 larvae, Chapter 3) as well as *dsx-Adh*-transformed larvae (OX4597) were both reared under standard rearing conditions (as described in section 4.2.3) in triplicates and survival to adulthood was compared to see whether the production of DmADH alone was causing the extra mortality of *Adh*-transformed heterozygous individuals seen in controls. Also, male and female pupae of both OX4489 (Chapter 4) and OX4597 were extracted for total RNA and the engineered *Aedsx* transcripts sequenced to verify whether they matched the expected male and female transcripts.

Comparing the different lines transformed with *DmAdh* gene (OX4346 and OX4597): survival to adulthood when reared without ethanol

Survival of larvae to adulthood was expressed relative to that of wild-type counterparts for each of four heterozygous OX4597 G_5 and three OX4346 G_3 strain tested (**Figure 5.5.**) when reared under standard conditions (deionised water and fed with fish food). The expected sex ratio for each type of larva (wild-type or transgenic) was 50% as half of the progeny were male or female. All the heterozygous strains tested carried single insertions and exhibited a 1:1 wild-type to transgenic ratio as well as a 1:1 male to female ratio. On average 42.8% OX4346 larvae survived to adults whereas only 41.2% OX4597 survived under standard rearing conditions. Analyses showed a strong significant difference of survival between the strains tested (F=107.5705, df=6, p<0.001) with lower survival of the OX4597 larvae to adults in comparison with the *Adh*-line OX4346-G (42.8%): OX4597-A larvae to adults (40.8%, t=-4.554, df=6, p<0.001), OX4597-B

(41.4%, t= -3.837, df=6, p<0.001), OX4597 C (41.5%, t=-3.65, df=6, p<0.001) and OX4597-D (41%, t=-4.313, df=6, p<0.001).

Survivorship of the transgenics larvae to adults was significantly linked to the OX4597 strains: on average, transgenic OX4597 survived to 39.4% in comparison with an average of 42.8% of the transgenic OX4346. An average of 39.9% OX4507-A larvae survived to adulthood (t=-3.905, df=, p<0.001), 38.8% OX4597-B (t=-4.641, df=, p<0.001), 40.3% OX4597-C (t=-3.641, df=, p<0.001) and 38.4% OX4597-D (t=-4.841, df=, p<0.001).

Finally, three out of the four OX4597 lines had their transgenic males associated with a significantly higher survival to adulthood with 44.9%, 44.8% and 44.6% respectively for the OX4597-A, B and C transgenic males in comparison with a 42.7% average survival of the OX4346 transgenic males.

Survivorship in general was lower for all the OX4597 strains tested (41.2%) in comparison with the OX4346 (42.8%). Both OX4597 and OX4346 were heterozygous strains and wild-type individuals were raised in the same containers as the transformed ones. It was observed that survivorship of the wild-type individuals in pools containing OX4597 lines was lower (32.8%) than the OX4346 (46.8%). When transgenic larvae or pupae died in the containers it may have caused bacterial over-growth and compromised the survival of the wild-type individuals. It is likely that this resulted in the lower survivorship of wild-type individuals observed in OX4597 tests in comparison with OX4346.

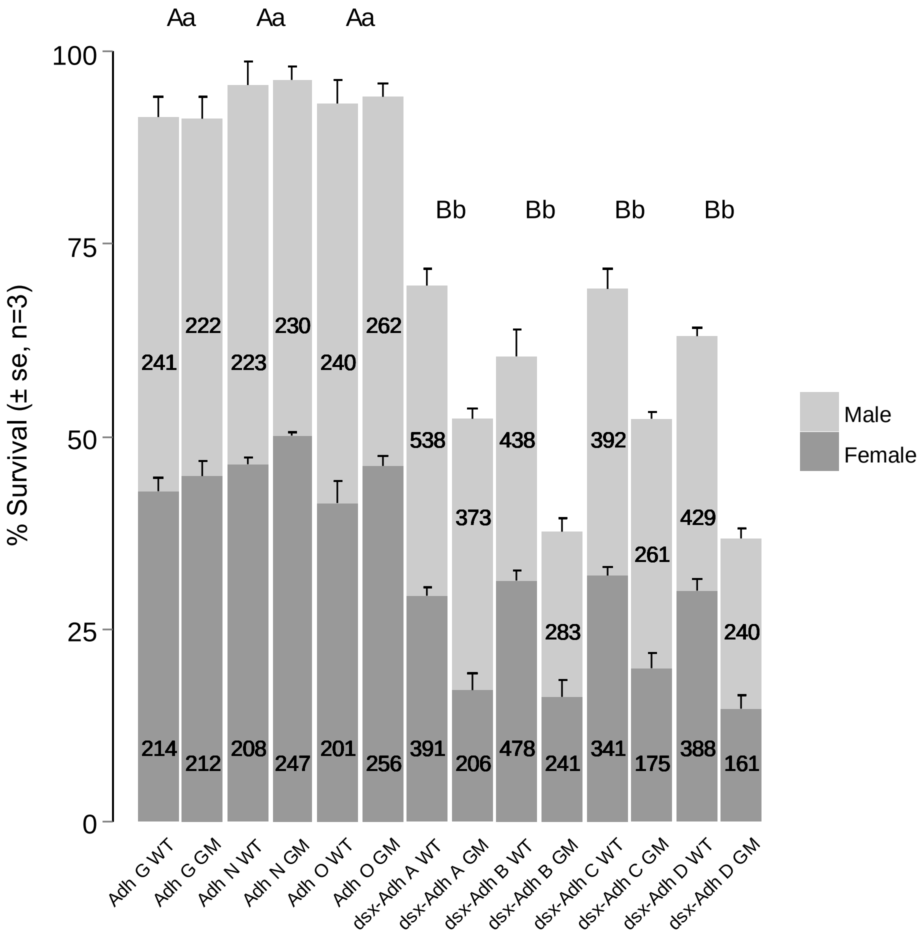


Figure 5.5. Survival of progeny expressing the 3xP3-DsRed2 (*Adh* lines) and Hr5*ie1*-AmCyan (*dsx-Adh* lines) marker to adult males and females compared with wild-type (WT) progeny when reared under standard conditions.

Comparison of survival to adults (%) of heterozygous larvae from *Adh*-transformed *Ae. aegypti* (constructs OX4346, refer to Chapter 3) with *dsx-Adh*-transformed larvae (construct OX4597, see Materials and Methods section of this chapter). the number of live male and female pupae was recorded (numbers shown inside each bar) and the resulting proportion that survived to adulthood represented on the Y-axis. Error bars represent the standard error of the mean survival percentage out of three samples (n=3). Groups denoted by the same letter (Latin capital letter for strains comparison, Latin minuscule letter for type comparison, wild-type or heterozygous) are not significantly different from each other (ANOVA). .

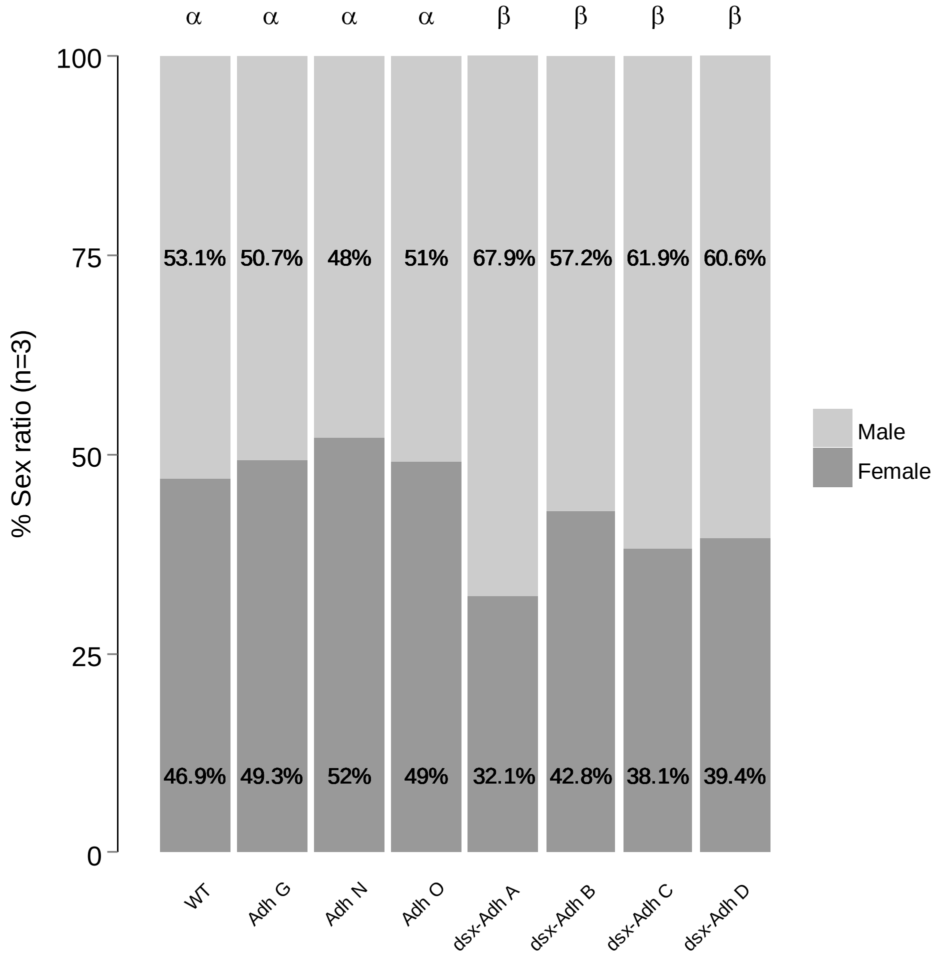


Figure 5.6. Sex ratio of the adult progeny male and female expressing the 3xP3-DsRed2 (*Adh* lines) and Hr5*ie1*-AmCyan (*dsx-Adh* lines) marker compared with wild-type (WT) progeny when reared under standard conditions.

For each strain (WT, *Adh* and *dsx-Adh* lines), the number of live male and female adult was recorded and the resulting ratio plotted (numbers shown inside each bar). Groups denoted by the same letter (Greek letter for sexes comparison) are not significantly different from each other (ANOVA).

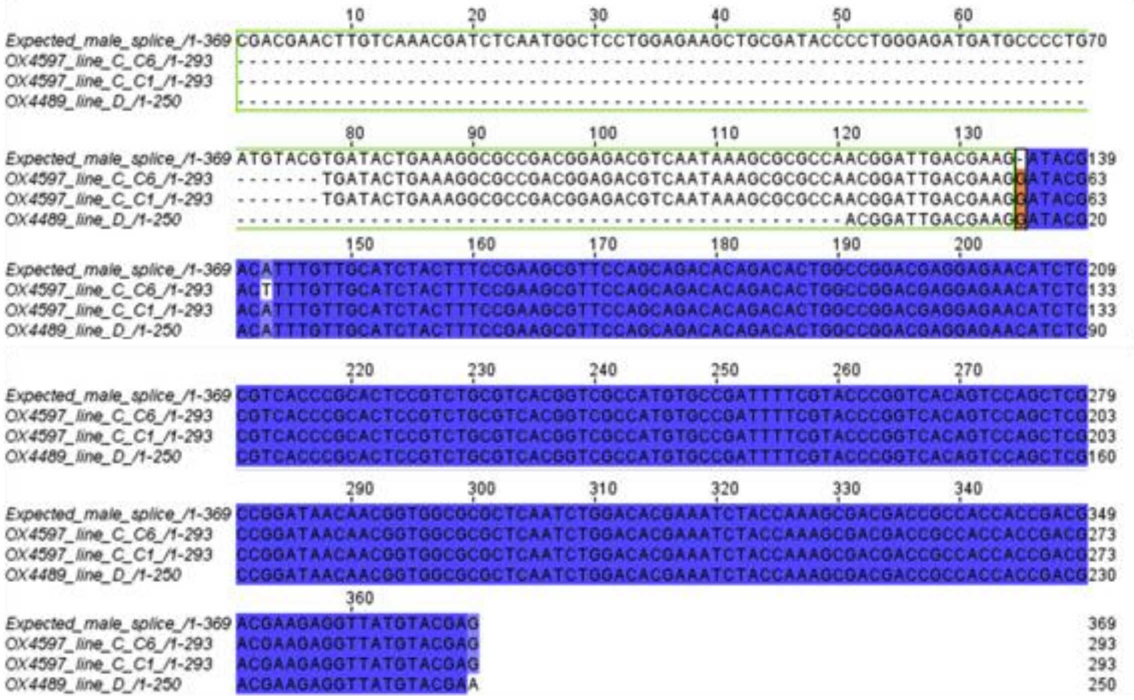
Comparing the different lines transformed with *Aedsx* gene (OX4489 and OX4597): aligning expected *Aedsx* transcripts with sequenced male and female transcripts

In both constructs OX4489 and OX4597 (**Figure 5.2.**), the *dsx* mini gene comprises *dsx* exon 4, the female specific *dsx* exon, 5b, and *dsx* shared exon 6, as well as two shortened introns derived from introns 5 and 7 surrounding the female-specific exons in the native gene. A start codon has been engineered into the female-specific exon, 5b, which is in-frame with the downstream tTAV open reading frame in construct OX4489 and the *DmAdh* exon in OX4597. In males though, both the shortened introns 5 and 7 as well as the female specific exon containing the start codon are expected to be spliced out of the *Aedsx* mRNA. Consequently the processed *Aedsx* mRNA will not code either for the tTAV protein in OX4489 or the DmADH protein in OX4597 in transgenic males.

In order to analyse processing of the *Aedsx* mRNA in OX4489 transgenic individuals, cDNA was prepared as described (4.2.2) and sequences of the transcript of one OX4489 male pupa from line D was aligned against the expected male *Aedsx* transcript, as well as 2 sequences obtained from the same OX4597-C male pupa (clones 1 and 6) (**Figure 5.6.**). Both male transcripts showed a perfect identity match with the expected one with the exception of minor base changes (see positions 142 and 369 in **Figure 5.6.**) and one base addition (a Guanine) right after the shared exon 4 that would probably result in a shift in the reading frame (**Figure 5.6.**, G underlined in orange). This insertion (G) would shift the out-of-frame with tTAV or *DmAdh* *dsx*-male transcripts. However, because this insertion was also present in the

OX4489 sequence this was not responsible of the differences observed between the *dsx-DmAdh* and *dsx*-tTAV constructs (OX4597 and OX4489).

The transcript of one OX4489 female pupa from line B was aligned against the expected engineered female *Aedsx* one [4-5b-6] (as described in the general introduction, **Figure 1.11.**) as well as sequences obtained from the same OX4597C female pupa (clones 9 and 14) (**Figure 5.7.**). Both female transcripts showed a perfect identity match with the expected one with the exception of 4 minor base changes (see positions 45, 80, 118 and 436; **Figure 5.7.**) occurring upstream of the engineered start codon. Transgenic females carrying the OX4489 construct should in theory produce a functional tTAV protein, while those carrying the OX4597 transcript should produce a functional DmADH protein. It has already been proven that OX4489 females produce a functional tTAV since, when crossed to a tetO-Reaper line, the progeny give rise to males only when reared without tetracycline (**Figure 4.14.**).



```
                                    10        20        30        40        50        60
Expected_male_splice_/1-369 CGACGAACTTGTCAAACGATCTCAATGGCTCCTGGAGAAGCTGCGATACCCCTGGGAGATGATGCCCCTG70
OX4597_line_C_C6_/1-293     ----------------------------------------------------------------------
OX4597_line_C_C1_/1-293     ----------------------------------------------------------------------
OX4489_line_D_/1-250        ----------------------------------------------------------------------
                                    80        90       100       110       120       130
Expected_male_splice_/1-369 ATGTACGTGATACTGAAAGGCGCCGACGGAGACGTCAATAAAGCGCGCCAACGGATTGACGAAG-ATACG139
OX4597_line_C_C6_/1-293     -------TGATACTGAAAGGCGCCGACGGAGACGTCAATAAAGCGCGCCAACGGATTGACGAAGGATACG63
OX4597_line_C_C1_/1-293     -------TGATACTGAAAGGCGCCGACGGAGACGTCAATAAAGCGCGCCAACGGATTGACGAAGGATACG63
OX4489_line_D_/1-250        --------------------------------------------------ACGGATTGACGAAGGATACG20
                                   150       160       170       180       190       200
Expected_male_splice_/1-369 ACATTTGTTGCATCTACTTTCCGAAGCGTTCCAGCAGACACAGACACTGGCCGGACGAGGAGAACATCTC209
OX4597_line_C_C6_/1-293     ACTTTTGTTGCATCTACTTTCCGAAGCGTTCCAGCAGACACAGACACTGGCCGGACGAGGAGAACATCTC133
OX4597_line_C_C1_/1-293     ACATTTGTTGCATCTACTTTCCGAAGCGTTCCAGCAGACACAGACACTGGCCGGACGAGGAGAACATCTC133
OX4489_line_D_/1-250        ACATTTGTTGCATCTACTTTCCGAAGCGTTCCAGCAGACACAGACACTGGCCGGACGAGGAGAACATCTC90
                                   220       230       240       250       260       270
Expected_male_splice_/1-369 CGTCACCCGCACTCCGTCTGCGTCACGGTCGCCATGTGCCGATTTTCGTACCCGGTCACAGTCCAGCTCG279
OX4597_line_C_C6_/1-293     CGTCACCCGCACTCCGTCTGCGTCACGGTCGCCATGTGCCGATTTTCGTACCCGGTCACAGTCCAGCTCG203
OX4597_line_C_C1_/1-293     CGTCACCCGCACTCCGTCTGCGTCACGGTCGCCATGTGCCGATTTTCGTACCCGGTCACAGTCCAGCTCG203
OX4489_line_D_/1-250        CGTCACCCGCACTCCGTCTGCGTCACGGTCGCCATGTGCCGATTTTCGTACCCGGTCACAGTCCAGCTCG160
                                   290       300       310       320       330       340
Expected_male_splice_/1-369 CCGGATAACAACGGTGGCGCGCTCAATCTGGACACGAAATCTACCAAAGCGACGACCGCCACCACCGACG349
OX4597_line_C_C6_/1-293     CCGGATAACAACGGTGGCGCGCTCAATCTGGACACGAAATCTACCAAAGCGACGACCGCCACCACCGACG273
OX4597_line_C_C1_/1-293     CCGGATAACAACGGTGGCGCGCTCAATCTGGACACGAAATCTACCAAAGCGACGACCGCCACCACCGACG273
OX4489_line_D_/1-250        CCGGATAACAACGGTGGCGCGCTCAATCTGGACACGAAATCTACCAAAGCGACGACCGCCACCACCGACG230
                                   360
Expected_male_splice_/1-369 ACGAAGAGGTTATGTACGAG                                                  369
OX4597_line_C_C6_/1-293     ACGAAGAGGTTATGTACGAG                                                  293
OX4597_line_C_C1_/1-293     ACGAAGAGGTTATGTACGAG                                                  293
OX4489_line_D_/1-250        ACGAAGAGGTTATGTACGAA                                                  250
```

Figure 5.7. Part of the *dsx* transcript from OX4489 and OX4597 male pupae.

The expected male spliced transcript for both OX4489 and OX4597 is shown (Expected male splice row) aligned with sequenced RT-PCR products from OX4597 male pupa line C, clone 6 (2nd row), another clone from the same individual (clone 1 in 3rd row) and OX4489 male pupa line D (4th row). The sequence bordered in green represent *dsx* shared exon 4 (from 1 to 134; see **Figure 5.2.**) and the rest of the sequence coloured in different shades of blue depending of the degree of identity between the expected sequence and the aligned sequences represents part of *dsx* shared exon 6 (from 136 to 623, here shown up to base 369 only): The first shared exon (4) has a 100% identity between expected and aligned sequences. A base has been added (Guanine in orange) in the males' splicing in both the male pupa sequenced from line OX4597 C (2 clones) as well as the one from OX4489 D; 100% identity is shown with 2 exceptions, base A (position 142) and G (position 369).

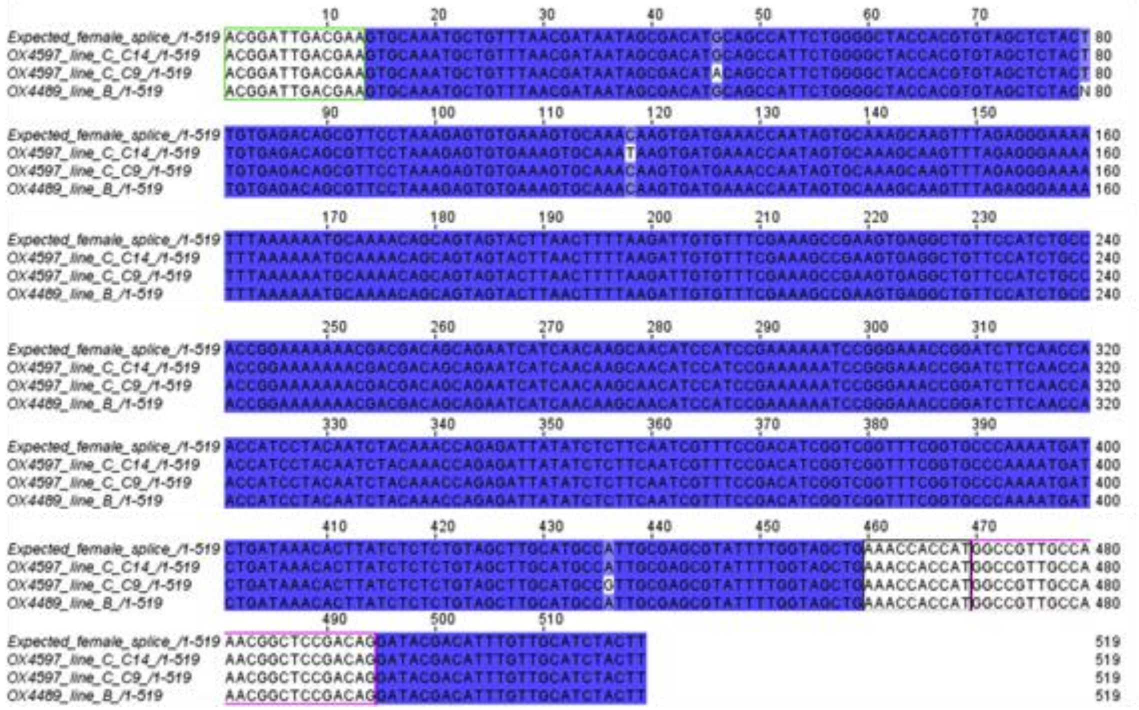


Figure 5.8. Part of *dsx* splicing in OX4489 and OX4597 female pupae.

The expected female spliced transcript for both OX4489 and OX4597 is shown (Expected female splice, top row) aligned with sequenced RT-PCR products from OX4597 female pupa line C, clone 14 (2nd row), another clone from the same individual (clone 9, 3rd row) and OX4489 female pupa line B (4th row). The sequence bordered in green represent part of *dsx* shared exon 4 (from 1 to 13; see **Figure 5.2.**) and the rest of the sequence coloured in different shades of blue depending of the degree of identity between the expected sequence and the aligned sequences represents part of the female *dsx* exon 5b from 14 to 459 and the remaining of the female exon 5b from 470 to 494 (bordered in pink) (**Figure 5.2.**). The added ATG start codon is flanked with the consensus sequence for the eukaryotic translation site (*CCACC*ATG*G*; Kozak, 1984) (bordered in black from position 460 to 469, see ATG on **Figure 5.2.**). The rest of the sequence represent part of the shared exon 6 (**Figure 5.2.**) from position 495 to 519. Four exceptions to a 100% identity between the expected female splice and the aligned sequences can be observed at positions 45, 80, 118 and 436.

5.4. Discussion

The transformation of *Ae. aegypti* with the OX4597 construct did not lead to the desired early-acting female-specific lethal phenotype. Instead, pupal mortality in some of the OX4597 heterozygous lines was observed when the lines were maintained in the insectary (data not shown), and heterozygous larvae reared under standard conditions showed poorer survival to pupation and adulthood in comparison with *Adh*-transformed ones (OX4346) during bioassays (**Figure 4.5.**). Careful analysis of the OX4597 transcript showed that the *DmAdh* ATG start codon had not been removed. This opened up the possibility that transcription initiation could take place at this codon and hence that ADH expression in the males is possible despite correct splicing of the *dsx* transcript and exclusion of the engineered ATG start codon from the male transcript.

This possibility is nonetheless not sufficient to explain the observed phenotype in OX4597 because this ATG is also present in the *DmAdh*-only lines OX4346 (Chapter 2) and non-ethanol treated animals do not have viability issues. Sequencing has shown that the *Aedsx* transcript in both OX4489 (Chapter 3) and OX4597 female pupae exhibit a perfect match with the expected female transcript of *Aedsx*. Even if ADH is produced from the original start codon in males given the observed phenotype in OX4489 individuals, we do not expect it to give fitness effects in the absence of ethanol given the observed phenotype of OX4346 individuals. I did not attempt to assess protein levels directly, for example by Western Blot, due to lack of resources and time.

In summary, two phenomena are observed when *Ae. aegypti* is transformed with plasmid OX4597: splicing of the *Aedsx* transcript appears to occur correctly, in a sex-specific manner; however, some excess mortality in both sexes is observed when OX4597 larvae are reared without ethanol, and lethality fails to be induced with long-term exposure to ethanol in the larval water.

As discussed in Chapter 3 of this thesis, early acting toxicity is not achieved under the tetO-dsx-tTAV system alone (construct OX4489 in **Figure 4.8.** and Chapter 3); instead, lethality is achieved at very late pupal stage in OX4489 individuals from a single line, only when crossed to the tetO-Reaper effector line (Chapter 3). In both OX4489 and OX4597 constructs, *Aedsx* has been designed to produce a single female transcript (based on female-specific exon 5b); however, we cannot exclude the possibility of multiple female transcripts being produced as multiple bands are observed in the RT-PCR analysis (**Figures 3.7.** and **4.3)**. Consequently, one explanation of the difficulties in engineering female-specific lethality with *Aedsx* might lie in the fact that alternatively spliced genes can be inefficient due to the production of several transcripts, some which do not produce functional open reading frames. This can attenuate the lethality in females as seen with the *Ceratitis capitata transformer* intron alternatively spliced in the published fsRIDL in the Medfly (Fu et al., 2010; Fu et al., 2007). This might explain the failure of induction of lethality with long-term exposure to ethanol of *Aedsx*-*DmAdh*-transformed larvae in comparison with *DmAdh*-alone-transformed larvae (**Figure 4.5.** and **4.8.**). In the tetracycline repressible system, over expression of the tTAV transcription factor causes death and placing a lethal effector under its control can enhance the toxicity of the positive feedback loop. We cannot use such tetO-Effector lines to improve induction of lethality in a sex specific manner like we did for OX4489 (Chapter 3). We would need another system, orthogonal to the tetracycline system but based on a transcription factor like tTAV such as an Auxin-based inducible system (Nishimura 2009) rather than a one-component-inducible-system using *DmAdh*.

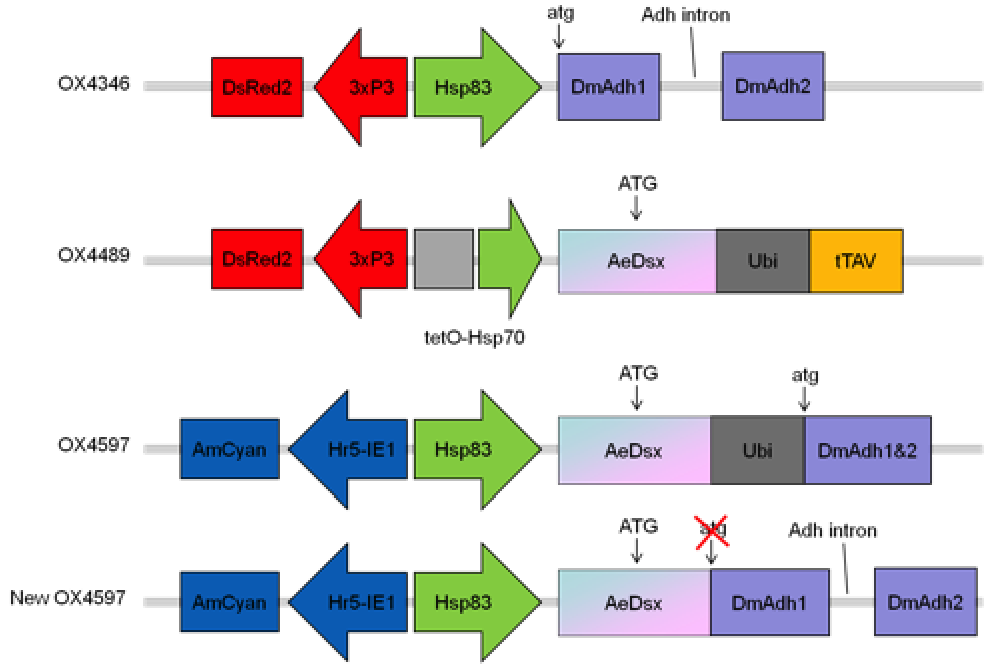


Figure 5.9. Diagram of the three *piggyBac* constructs compared in this Chapter: OX4346, OX4489, OX4597 and finally a suggested construct 'New OX4597' for future work.
OX4346 plasmid construct carries a red eye marker 3xP3-DsRed2 as well as the full *DmAdh* sequence of the gene encoding the Fast isoform of Drosophila ADH (Chapter 3; Christophides et al., 2001) under the control of *AeHsp83* promoter; OX4489 carries the same red eye marker as above with the minimal *DmHsp70* promoter driving the alternatively spliced *Aedsx* gene (refer to Figure 4.2. for more details) with the *Ubiquitin-tTAV* fused elements and finally, OX4597 carries the blue body marker *Hr5-IE1-AmCyan* with *AeHsp83* promoter driving *Aedsx* gene with the *Ubiquitin-DmAdh* fused elements. The engineered start codon in the *Aedsx* female exon is represented by the capital letters ATG. The small letters 'atg' is the start codon at the beginning of the *DmAdh* gene in both constructs OX4346 and OX4597. Construct 'New OX4597' presents suggestions to improve construct OX4597 for future work.

Alternative splicing plays a key role in regulating gene expression in many developmental processes, including sex determination in insects (Gempe and Beye 2011) , but has not yet been used widely in genetic engineering (Fu et al., 2010; Fu et al., 2007; Magnusson et al., 2011). The *Aedsx* gene is alternatively spliced early in development and remains a promising choice for engineering a sex specific early acting lethal in *Ae. aegypti*. High levels of tTAV expression can cause death in *Ae. aegypti* RIDL strains at late larval and pupal stages (Phuc et al., 2007) or flightless-ness when tTAV is under the control of the AeAct4 promoter in *Ae. aegypti* (Fu et al., 2010) as well as in *Ae. albopictus* (Labbé et al., 2012). tTA, a similar protein to tTAV, can be highly deleterious if expressed at very high levels and cause the disruption of transcription in the cell it is expressed (Gossen and Bujard 1992), as well as the disruption of the ubiquitin-dependent protein degradation system (Salghetti et al., 2001). Ubiquitination of the *DmAdh* gene might cause disruptions in the transcription process and might result in a compromised survival of *Aedsx-DmAdh* larvae even when levels of transcription are basal (without ethanol in the larval water).

Based on sequence analysis of transcripts, both transcription and splicing of the *Aedsx* system appears to occur as expected; however, the manner in which these transcripts are translated and the resulting protein products have not been subjected to any analysis at this stage. We can hypothesise that something is going wrong after translation because we observe an unexpected phenotype: it is therefore possible that the ADH protein is not properly expressed, folded or functional in transgenic individuals bearing construct OX4597 as opposed to OX4346 however the reasons remain unclear. The peculiarity of the *piggyBac* transposable element is its random insertion in the genome into TTAA sequence (Fraser et al. 1983), and, with such a short recognition site it is likely to insert randomly into the host genome (Thibault et al. 1999). The phenotype observed in all four OX4597 lines seems unlikely to be due to positional effects of the insertion of the *piggyBac* vector as the four transgenic lines came from separate G_0 individuals (see Material and Methods).

The mapping of the *piggyBac* insertions of all four transformed lines has been considered since it would have given more insight on whether the insertions were simply disrupting key features in the mosquito genome. However it has been judged not essential to do so for diverse reasons: since the original intended fsRIDL OX4489 (Chapter 4) was not efficient in causing death to females only in absence of tetracycline, I hypothethised that the splicing of the engineered *Aedsx* gene needed improving to move on working and injecting new constructs (data not shown); also, the chance to have generated four *piggyBac* strains that inserted into some particular location in the genome compromising each time the survival of the larvae is too small.

Another possible explanation of the unexplained lack of fitness of the OX4597 individuals is that the fused elements *Ubiquitin-DmAdh* is failing to produce a functional ADH as with construct OX4346 (Chapter 3), but, instead, produce a somewhat deleterious product affecting OX4597 larvae under standard rearing conditions. High expression of tTAV can disrupt the ubiquitin-dependent protein degradation system (Salghetti et al., 2001), although a large number of *Ubiquitin-tTAV* constructs have previously been observed to work well and not be especially toxic (Oxitec, unpublished data). The reading frame in the engineered *Aedsx* cassette seems intact; however, the RNA polymerase might misread and convert the genomic region coding for the fused elements *Ubiquitin-tTAV* or *Ubiquitin-DmAdh* into aberrant proteins that are more toxic than *DmAdh* (Hoe et al., 2011). We do not have much reason to suspect there is a problem with the elements *Ubiquitin-tTAV* in construct OX4489 as opposed as *Ubiquitin-DmAdh* in OX4597 though since no toxicity was observed in OX4489 individuals in presence of tetracycline. The hypothesis inferring the production of functional ADH protein in *DmAdh*-alone larvae (OX4346, **Figure 4.8.**) but not in *Aedsx-DmAdh*-larvae (OX4597) might be tested by using Western Blot analysis to examine the engineered ADH proteins in both constructs for comparison.

The OX4597 construct is the first attempt at creating an inducible, female-specific lethal in *Ae. aegypti,* and its failure is not fully understood, given the previous success with a related *Adh* construct. The problem may lie in the efficiency of the alternative splicing, the production of aberrant *Ubiquitin-DmAdh* proteins generating toxicity under standard conditions, a combination of both phenomena, or something else completely. The very first step would be to delete the endogenous start codon at the beginning of the *DmAdh* coding sequence since this might generate ADH proteins independently of the *Aedsx* alternative splicing and compromise the survival of *Aedsx-DmAdh* males as well as females. Among the different issues encountered in this study, the first, i.e. efficiency of the splicing, can be addressed by combining *Aedsx* alternative splicing with a more toxic tTAV variant. This issue is particularly discussed in chapter 3.

The Ubiquitin-fusion protein system provides an efficient tool for studying proteins of interest (Matentzoglu and Scheffner 2009), and in RIDL-like constructs is thought to enhance the efficiency of the production of the protein of interest. However, in that particular case, we might want to add an amino acid linker between *Ubiquitin* and *DmAdh* to provide accessibility to the tag by Ubiquitin-specific proteases and process them into their respective free proteins (Franco et al., 2011) or remove the *Ubiquitin* all together. In the first *DmAdh* construct OX4346 (Chapter 2) no *Ubiquitin* was fused the 5’end of the full

Drosophila *Adh* gene sequence and the system proved to be strong enough to kill at larval stages.

In the *DmAdh*-alone construct (OX4346) the full sequence of Drosophila *Adh* was inserted whereas in *Aedsx-DmAdh* OX4597 the *Adh* intron has been deleted. Perhaps one can suggest going back to the original *DmAdh* architecture used in OX4346 without its endogenous ATG for a final attempt to create a female-specific lethal using *Aedsx* and *DmAdh* (**Figure 4.8.**). Another possibility is to investigate the Ubiquitin-fusion system in OX4597 by replacing the *DmAdh* coding sequence by a fluorescent marker, i.e. *DsRed2*, and analyse the fluorescence patterns in both male and female individuals. If successful, the resulting strains would offer the additional advantage of sex separation using the fluorescence of the DsRed2 protein in females only (because it is spliced in-frame in females only, see **Figure 4.2.**).

Chapter 6: Assessing female fecundity of transgenic and wild type laboratory strains of *Aedes aegypti* and its modulation by tetracycline

6.1. Introduction

Novel approaches to mosquito control modelled on SIT use transgenic mosquitoes that carry repressible lethal genes. By manipulating the conditions which control the expression of these lethal genes, it is possible to selectively kill those insects that carry the transgenic elements. RIDL® (Thomas et al., 2000) is such a strategy, which is being developed at Oxitec Limited. The leading candidate line is OX513A; transgenic OX513A *Aedes aegypti* develop normally throughout their life cycle in the presence of dietary tetracycline, whilst specimens reared in the absence of tetracycline will die.

For the implementation of a control program based on the use of OX513A, mosquitoes would be mass-reared in the laboratory and the larval medium would be supplemented with tetracycline for the mass production of eggs. Following release, OX513A males would compete with wild males for access to wild females, those they mate would produce offspring that would die as late larvae or pupae, due to the absence of tetracycline in the natural environment.

Several research projects are studying various aspects of OX513A male fitness and mating competitiveness, as these are crucial requirements for a successful SIT programme. Indeed, irradiated males in classical SIT programmes can suffer significant reduction in fitness (Benedict and Robinson 2003) and the induction of sterility by bioengineering is therefore an interesting alternative. Success of a control trial may, furthermore, depend on the ease with which sufficient quantities of mosquitoes can be supplied. The actual number required will depend largely on the size of the target population: to cause a severe population decline, the mosquito males released need to reach or exceed a proportion of the total male population that can be estimated from models of *Ae. aegypti* population dynamics (Phuc et al., 2007). Since full equal competitiveness is unlikely to be achieved, it is likely that the OX513A males will need to out-number the wild males by a significant margin (i.e. a sterile: wild male ratio or ‘over-flooding ratio’ considerably greater than one) for a successful SIT-RIDL based programme. The fecundity of females in the mass rearing facility is therefore of importance to the production of sufficient numbers of eggs.

Fecundity is assessed by the number of eggs produced by a female. During mating, the male transfers the sperm, filling successively the 3 spermathecae of the *Ae. aegypti* female which are subsequently used to fertilise the eggs (Clements and Potter 1967). *Ae. aegypti* females can take multiple blood meals during their lifetime, and, after each blood meal, the female lays a batch of eggs (each cycle constituting a gonotrophic cycle). Early work positively correlated *Ae. aegypti* body size (e.g. wing length) or weight (inferred by pupal or adult mass) with the number of oocytes (Christophers 1960; Steinwascher 1984; Harrington & Edman 2001) but the link with actual egg production was missing (Roy in Christophers 1960; Christophers, 1960 and Steinwascher 1984). A better association with *Ae. aegypti* fecundity seems to be blood meal size (Woke et al. 1956; Steinwascher 1982).

Genetic control methods for disease vectors, such as the RIDL or SIT technique inevitably involves deepening the knowledge of male general biology but more particularly its reproductive fitness (Bargielowski et al. 2011; Bargielowski et al. 2012; Helinski et al. 2008; Boyer et al. 2011). Consequently, because these programmes need releasing more ‘sterile’ or RIDL males than the wild ones, another crucial process is the artificial rearing of a productive egg colony to sustain the releases. To summarise, the rebirth of such SIT or RIDL programme allows us to further investigate *Ae. aegypti* reproductive biology (Helinski and Harrington 2011).

The bi-sex lethal strain OX513A proposed as a tool for such approach is typically reared on larval diet of supplemented with tetracycline to a final concentration of 30 $\mu g.ml^{-1}$. Tetracycline, as well as other antibiotics, is routinely used as growth promoters in animal husbandry in some countries (Swann 1969) and, in insects, such antibiotics are commonly used to eliminate the bacterium Wolbachia, a maternally inherited endosymbiotic bacterium found in arthropods and nematodes and used for the Incompatible Insect Technique, a method derived from SIT (Bourtzis 2008). In *Ae. aegypti*, an initial study reported the increase of lipid stores in adult males when tetracycline was added at 30 $\mu g.ml^{-1}$ in the larval diet (Bargielowski et al., 2012). Tetracycline treatments between 0.5 $\mu g.ml^{-1}$ and 100 $\mu g.ml^{-1}$ promoted development rates for some wild-type strains previously unexposed to the antibiotic or increasing life span for tetracycline tolerant wild-type strains, possibly by suppressing deleterious bacteria (E. V. Ridley PhD thesis).

This chapter aims to evaluate the fecundity and fertility (inferred by the hatch rate of the eggs laid per female) of a RIDL line (OX513A) and its wild-type counterpart as well as the impact of 30 µg.ml^{-1} tetracycline on the same wild-type strain's fecundity and fertility. For that purpose, single mating pairs were constituted as follows: OX513A females were mated with OX513A males, wild-type females with wild-type males and finally tetracycline-treated-wild-type females with tetracycline-treated-wild-type males in single mating pairs. Also, for comparison, we crossed OX513A females with wild-type and tetracycline-treated wild-type males, wild-type females with OX513A males and tetracycline-treated wild-type males and finally, tetracycline-treated wild-type females with OX513A and wild-type males. Parameters evaluated included the total number of eggs produced by each cross per female over the course of their lifetime as well as per gonotrophic cycle (fecundity), the relationship with wing length (size) and finally the egg hatch rate per female (fertility) over the course of the fecundity assays.

6.2. Material and Methods

Two main sets of fecundity assays were carried out to assess the number of eggs *Ae. aegypti* females routinely produce in artificial rearing conditions, as well as the fertility of the eggs laid (hatch rate), by comparing the wild-type strain (reared with or without tetracycline) with the transgenic OX513A strains.

6.2.1. Experiment 1: Maximum egg number laid by a RIDL and a wild-type female over the course of their lifetimes; an evaluation of the hatch rate of the most egg-productive females

Single-pair mating crosses were set up individually in tubes as follows: 1 wild-type female with 1 OX513A male, 1 OX513A female with 1 OX513A male, 1 wild-type female with 1 wild-type male, 1 OX513A female with 1 wild-type male (n=105) (**Figure 6.1.**). In total, 420 females were tested for egg production with four different single-mating groups. *Ae. aegypti* adults were 4 days old when tested (four days after emergence of the adult from the pupae case) and fecundity assays were performed in laboratory conditions with temperatures ranging between 26 and 30°C and relative humidity between 50 and 90%. Females were blood fed and eggs collected through each gonotrophic cycle (up to 8 in total) and so on until all of the 420 females of the experiment died. Eggs were counted for each female of each cross as well as for each gonotrophic cycle. Females' time of death and wing length, as a proxy of body size, were recorded. Out of the 105 females that were tested per single-mating group, the 20 that laid the most eggs were tested for their hatch rate (i.e. 80 females in total) 2 months and a half after being laid. Live larvae were counted 24 hours after hatch stimulus.

6.2.2. Experiment 2: Impact of 30 µg.ml^{-1} of tetracycline on fecundity and fertility over the course of 3 gonotrophic cycles

After emergence, females were placed in tubes for four days, as follows: 1 wild-type female with 1 wild-type male both reared without tetracycline (tet-), with a wild-type male reared with 30 µg.ml^{-1} tetracycline (tet+) or a OX513A male (obligatorily reared with 30 µg.ml^{-1}). This was repeated for the wild-type female reared with tetracycline (tet+) as well as the OX513A female (also obligatorily reared with 30 µg.ml^{-1}) (**Figure 6.1.**). This fecundity assay represented a total number of 630 samples so it was staggered through three repeats (n=20, n=30 and n=20) hence undertaken at different dates (December 2010, May 2011 and

August 2011) under laboratory conditions at Oxitec limited. Females were blood fed and eggs collected over the course of the first 3 gonotrophic cycles. Eggs were counted for each female of each cross as well as for each gonotrophic cycle. Female's lifespan as well as wing length were recorded. Finally, the 116,981 eggs collected from all the females tested were hatched 4 to 7 days post oviposition and live larvae counted 24 hours after hatch stimulus. Temperatures recorded in December ranged between 26.2 and 28.8°C and relative humidity between 57 and 78%. In May 2012, temperatures varied between 27.1 and 27.6°C; relative humidity was between 56 and 80%. Finally, in August 2011, mosquitoes were reared at temperatures between 24.5 and 27.5°C and relative humidity between 57 and 90.5%. The experiment was run in an insectary with an outside wall and was more subject to outdoor climatic variations. Three humidifiers were placed across the room and manually filled throughout the day and weekends but could quikly run out of water hence humidity dropping at night.

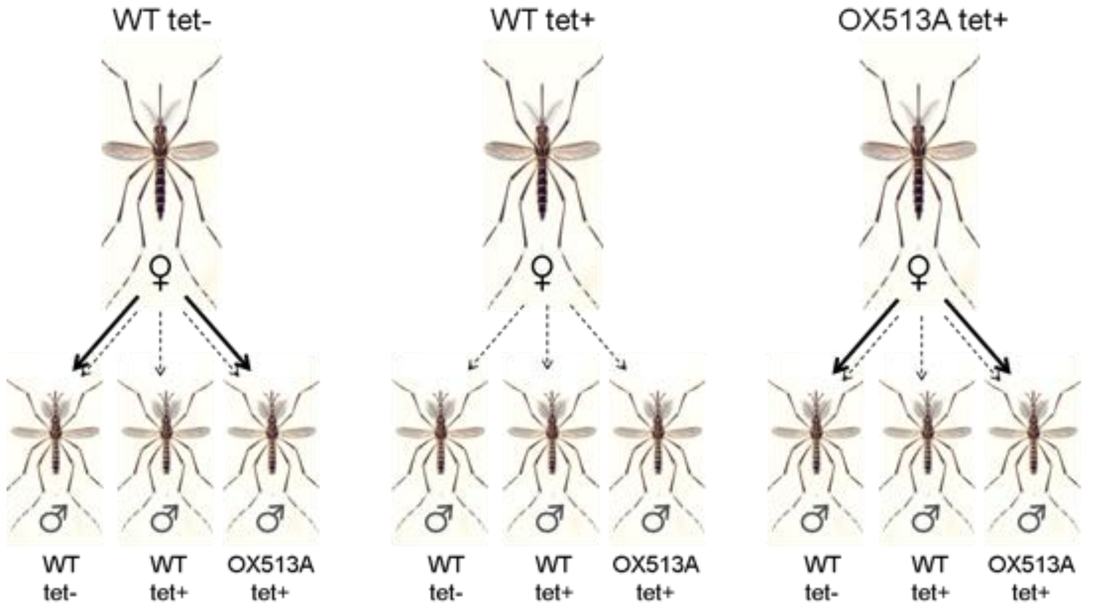


Figure 6.1. Diagram of fecundity experiments 1 and 2 performed with the bi-sex lethal strain OX513A in comparison with the wild-type.

Ae. aegypti single pair mating crosses between wild-type (WT) and OX513A females with wild-type and OX513A males conducted for the two fecundity assays described in this chapter. Experiment 1 (black arrows) focuses on the maximum egg production capacity over the course of *Ae. aegypti* lifetime (8 gonotrophic cycles) while experiment 2 (dashed arrows) analyses the egg production capacity as well as fertility of the strains but focuses on the impact of tetracycline on the wild-type strain when dosed at 30 µg.ml^{-1}.

6.2.3. Fecundity assessment: number of eggs laid per female

After two days, males were removed from the tubes and females were blood fed every week on defibrinated horse blood until their death. Females were checked following each blood meal; those that had not fed were provided repeated blood meals. Eggs were laid on damp cotton wool provided at the bottom of the tube. The cotton wool was replaced daily. Deposited eggs were kept separately according to the number of the corresponding female and her group assignment, as well as the laying date. Eggs were counted under a dissecting microscope and the total number of eggs deposited for each female was recorded as a score/measure of egg production.

6.2.4. Fertility assessment: egg hatch rate

Eggs were between 70 to 87 days old (experiment 1) or 4 to 7 days old (experiment 2) when hatched in tubes adding deionised water and one drop of Liquifry No. 1 (Interpet Ltd., Dorking, Surrey, UK) as food. The number of larvae that hatched was recorded 24h later. Microscopic examination revealed that some eggs had already hatched on the cotton wool prior the experiment, as evidenced by the missing tip of the posterior end of the egg (operculum), and the visible remaining of the dead hatched larva out of the shell (**Figure 6.2.**). Similarly, some eggs were observed as being physically damaged (i.e. egg collapsed because of desiccation or other factors). Consequently, eggs that were physically damaged and did not present an intact shell were discarded from the hatch rate analysis. On the other hand, eggs that previously hatched on the cotton wool and whose larvae dried out, as well as any other egg that presented a full and intact shell were considered as viable and included in the total egg count.

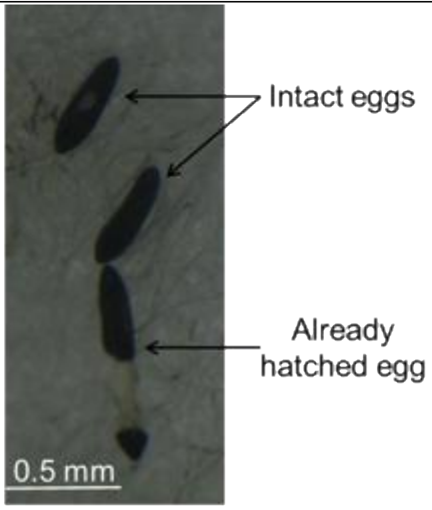


Figure 6.2. Photographs of *Ae. aegypti* eggs observed under microscope prior hatching. Two *Ae. aegypti* eggs can be seen intact (un-hatched eggs) and one already hatched on a cotton wool.

6.2.5. Wing length measurements

Adult wing length measurements are chosen in most studies as a suitable measure of mosquito body size (Nasci 1990). However, due to experimental constraints, females submitted to the fecundity assay and blood fed in Drosophila tubes often exhibited damaged wings (tip of the wing missing) (**Figure 6.3.b**). Therefore, *Ae. aegypti* wing length from alular notch to apex was chosen as the traditional measure of size for mosquitoes (**Figure 6.3.a**) (Nasci 1990) and wing length from the end of the anal vein to the alular notch as an alternative estimator. Females' wings were removed and mounted on microscope slides with 70% ethanol. A photograph of the wings together with a graticule was taken for measurement, using a Canon powershot S5IS camera and a 99 mm adapter (S/N:3754, Martin Microscope Company). Wings were then measured using ImageJ software (http://rsbweb.nih.gov/ij/). Overall a strong correlation was calculated between measurements taken of the traditional wing length (apex) and the alternative method (anal vein) (**Figure 6.4.**).

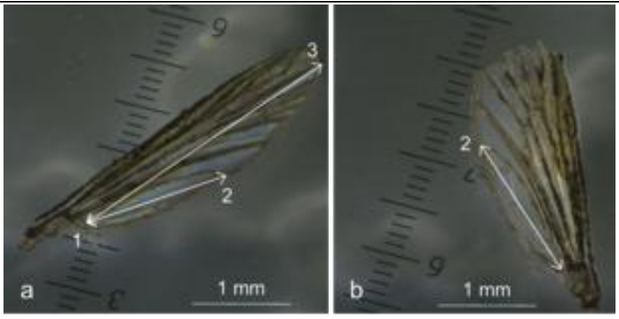


Figure 6.3. Photographs of *Ae. aegypti* intact and damaged wings as well as measurements.

A full-length dissected wing (a) as well as a damaged wing (b) mounted on a microscope slide and photographed with a graticule. Estimates of the full wing length were measured from the end of radial vein R_{4+5} (landmark 3) to the alular notch (1) and the apex of the wing (3) excluding fringes. The alternative measurements were estimated from the end of the anal vein (2) to the alular notch (1).

6.2.6. Statistical analysis

Statistical analyses were performed using R software version 2.12.2 (R Development Core Team 2011). Wing length was analysed with an ANCOVA, a regression between the classical wing length measurements (up to the apex) with the alternative wing length measurements (up to the anal vein) including the strain of the adult mosquito tested, replicates of the experiment run three times (December 2011, May 2012 and August 2013) as well as their interaction. Linear regression analysis was employed to compare the relationship between wing length measurements and the number of eggs produced per female. Female fecundity (number of eggs per gonotrophic cycle) was analysed separately for each gonotrophic cycle with an ANOVA including female or male type (wild-type either bred with 30 µg.ml^{-1} or not and OX513A), experiment replicate and their interaction. Overall reproductive success (number of offspring per female) was analysed with an ANOVA including the female or male type (wild-type either bred with 30 µg.ml^{-1} or not and OX513A), experiment replicate as well as their interaction.

6.3. Results

6.3.1. Wing length measurements as an estimation of *Ae. aegypti* adult’s body size

Wing length measurements from the end of the anal vein to the alular notch enabled us to distinguish size differences among treatments as well as among different mosquito strains*.* In the first experiment, overall, wild-type females exhibited larger wings than OX513A females (**Table 6.1.**), on average 2.98 mm when measured up to the apex (Nasci, 1990) in comparison with and 2.85 mm (t=2.941, df=1, p=0.00455) (**Figure 6.4.a)**.

However, in the course of the second experiment, adult females from both genotypes exhibited on average smaller wings (2.7mm) than during the first experiment (**Table 6.1.**). Females reared with or without tetracycline as well as OX513A female's wings length measured respectively 2.709 ± 0.043, 2.7 ± 0.043 and 2.67 ± 0.031 mm (**Figure 6.4.b)** when measured up to the apex. A similar difference was observed when the wings were measured from the alular notch up to the end of the anal vein (respectively 1.30 ± 0.027, 1.30 ± 0.027 and 1.31 ± 0.019 mm). Female wing length was on average bigger in December (full length: 2.86 ± 0.043 mm or up to anal vein: 1.424 ± 0.027 mm) than when the experiment was conducted in May or August 2010 ($t=4.383$, $df=2$, $p<0.001$; $t=4.219$, $df=2$, $p<0.001$). There was no evidence of interaction of the "strain" (transgenic versus wild-type strain as well as tetracycline treatment) and among the replicates (experiments conducted separately in December, May and August); the effects of both variables were additive to the wing length of the female individuals tested.

OX513A males exhibited similar full wing length when compared with wild-type males, whether reared with or without tetracycline (respectively 2.07 ± 0.019, 2.09 ± 0.027 and 2.11 ± 0.027 mm) ($t=0.840$, $df=2$, $p=0.404$ and $t=1.62$, $df=2$, $p=0.109$). This was also found when the wings were measured from the alular notch up to the end of the anal vein (respectively 0.98 ± 0.017, 0.98 ± 0.017 and 0.97 ± 0.012 mm) ($F=19.2$, $df=2$, $p=0.08$) (**Figure 6.4.c**). Male wing length was on average bigger in December (full length: 2.18 ± 0.027 mm or up to anal vein: 1.03 ± 0.017 mm) than when the experiment was conducted in May or August 2010 ($t=4.246$, $df=2$, $p<0.001$; $t=3.462$, $df=2$, $p<0.001$). There was no evidence of interaction of the "strain" (transgenic versus wild-type strain as well as tetracycline treatment) and the "repeat" (experiments conducted separately in December, May and August) variables; the effects of both variables were additive to the wing length of the male individuals tested (F=0.4293, df=4, p=0.78).

Treatment	***Sex***	***Experiment***	***Apex (mm)***	***N***	***Anal vein (mm)***	***N***
WT	Females	1	2.98 (0.022)	41	1.45 (0.006)	145
OX513A		1	2.85 (0.017)	32	1.43 (0.004)	146
WT	Females	2	2.7 (0.043)	29	1.30 (0.027)	29
WT tet+		2	2.709 (0.043)	30	1.30 (0.027)	30
OX513A		2	2.67 (0.031)	30	1.31 (0.019)	30
WT	Males	2	2.11 (0.027)	30	0.98 (0.017)	30
WT tet+		2	2.09 (0.027)	30	0.98 (0.017)	30
OX513A		2	2.07 (0.019)	30	0.97 (0.012)	30

Table 6.1. Mean wing length measurements of *Ae. aegypti* male and females analysed during the fecundity tests.

Mean wing length measurements are shown for experiment 1 where the focus is on the maximum egg yield during *Ae. aegypti* lifespan under laboratory conditions. Mean wing length measurements are shown for experiment 2 where the wild-type were reared in presence or absence of the antibiotic tetracycline (tet+, tet-) at immature stage and the egg production and fertility compared with the bi-sex lethal strain OX513A. The standard error of the mean is mentioned in parenthesis. Sample size (N) for each parameter is provided.

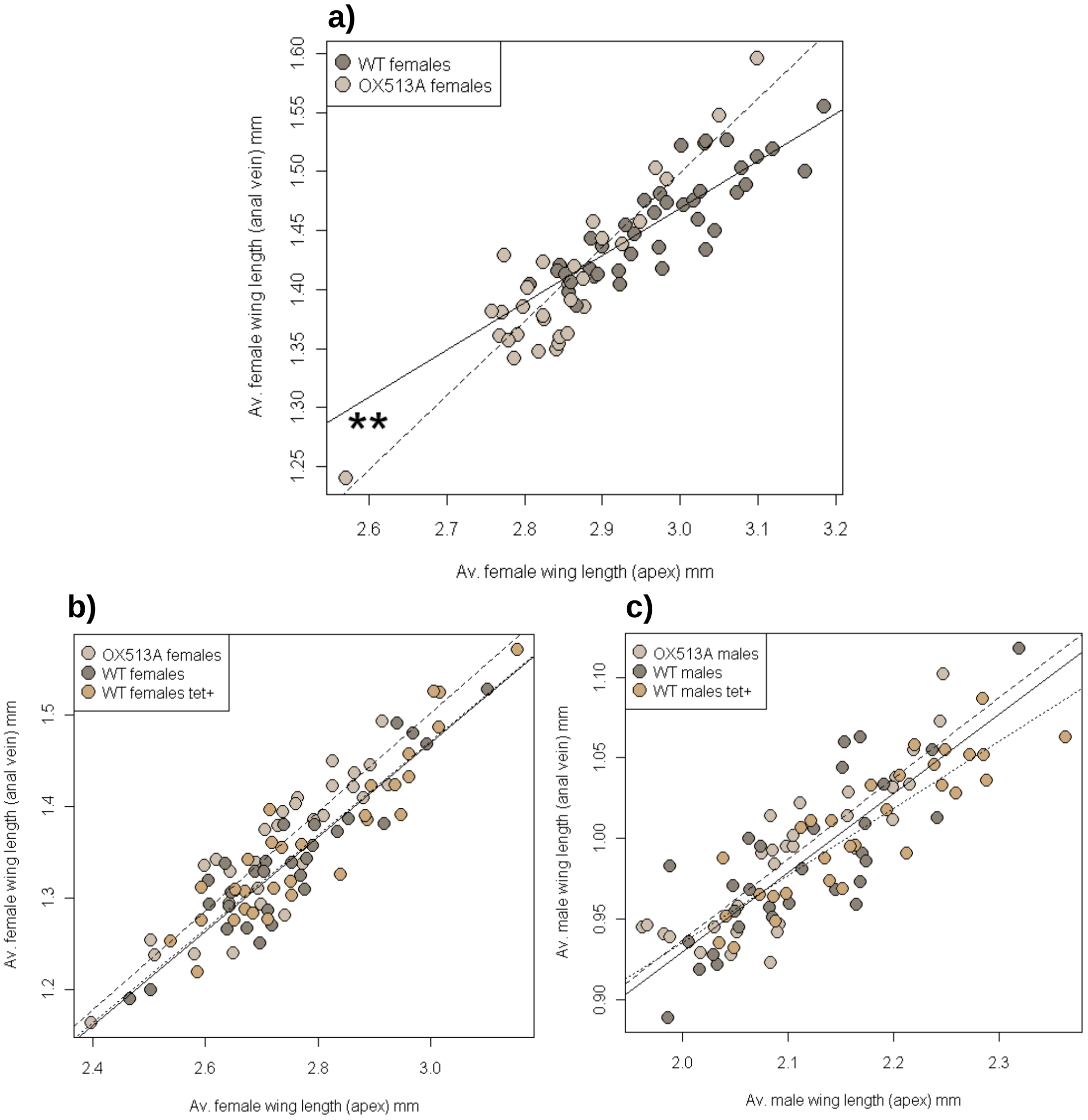


Figure 6.4. A strong positive correlation is observed between two different *Ae. aegypti* wing length measurements (from the alular notch to apex and to anal vein).

Wing length measurements were made with 287 wild-type (WT) females in total (a) as well as OX513A *Ae. aegypti*. Wing length (apex, N=72) was strongly correlated with wing length (anal vein, N=287) (r^2=0.7848). Regression significantly departed from zero (t=12.752, df=1, p-value: < 0.001). Regression lines were significantly different between wild-type and transgenic females (significance level is indicated as follow: ** for p-values < 0.01) confirming that, in the first fecundity assay, wild-type females were recorded larger than OX513As. Wing length measurements were made with 90 females in total (10 females from each treatment) (b) and males (c) wild-type as well as OX513A *Ae. aegypti*. Wing length (apex) was strongly correlated with wing length (anal vein) in females (r^2=0.8135) as well as in males (r^2= 0.7085). Regression significantly departed from zero in females (t=11.036, df=2, p<0.001) and males (t=8.769, df=2, p-value: < 0.001). The black line represents the fitted regression line for wild-type individuals, the dotted line for wild-type reared with tetracycline, and the dashed line for transgenic individuals.

Wing length and total egg production do not correlate

Ae. aegypti female wing lengths varied from 2.5 up to 3.2 mm and between 0 to over 600 eggs were laid over the course of 8 gonotrophic cycles. The total number of eggs produced per female was tested against wing length (**Figure 6.5.a and 6.5.b**), whether measured to the apex or to the end of the anal vein, and no correlation was shown (adjusted r^2: 0.1736 and r^2= 0.06228). In the second experiment, the total number of eggs per female produced was comprised between 0 and 345 and full wing length was between 2.4 and 3.1mm. The total number of eggs tested against full wing length (up to the apex) (**Figure 6.5.c**) again showed no correlation (adjusted r^2=0.4835, t=-0.958, df=8, p=0.3408).

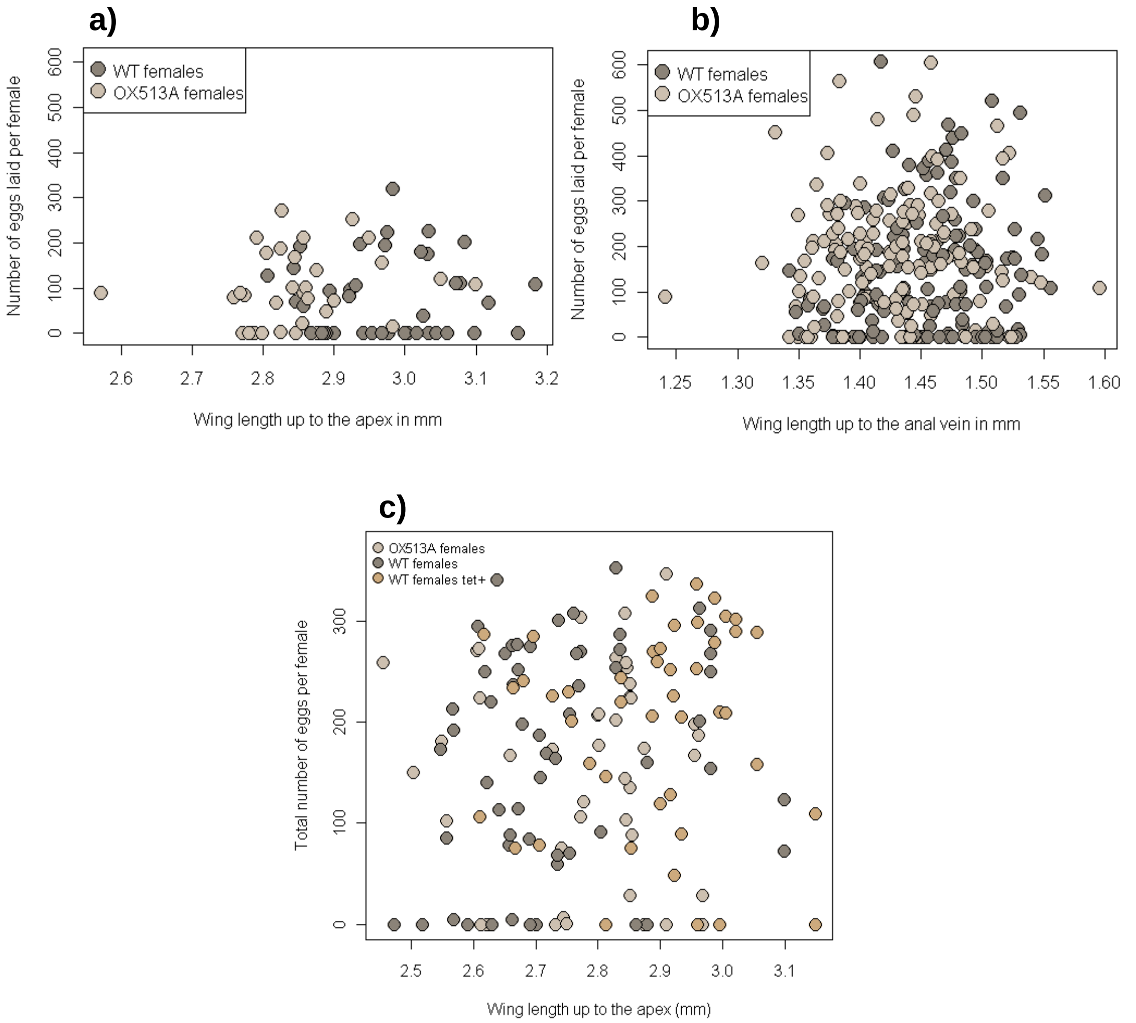


Figure 6.5. No correlation was observed between the total number of eggs laid per *Ae. aegypti* female with wing length measurements during the fecundity experiments.

The total number of eggs laid from the first fecundity experiment by *Ae. aegypti* bi-sex lethal (OX513A in grey) and wild-type females (WT in black) was plotted against full wing length (a) as well as against partial wing length (up to the apex, b) (experiment 1). The total number of eggs laid from the second fecundity experiment by Ae. aegypti bi-sex lethal (OX513A in grey) and wild-type females (WT in black) as well as wild-type from larvae fed with tetracycline (WT tet+ in golden) was plotted against full wing length (c).

6.3.2. An analysis of *Ae. aegypti* fecundity

Comparison of survival and overall longevity between OX513A and wild-type *Ae. aegypti* adult females

Ae. aegypti females were blood fed until full engorgement was observed. The number of live females per gonotrophic cycle was recorded (i.e. females successfully blood fed and susceptible to have laid eggs). The lifespan of the female was reported as the number of days they lived from egg hatching (day 0). Out of the 420 adult females tested, 29 wild-type (6.9%) as well as nineteen OX513A (4.5%) died before producing eggs for the first gonotrophic cycle and were removed from the analysis. OX513A females lived on average 38.3 ± 0.97 days in comparison with wild-type females that lived on average 37.07 ± 1.37 days (**Figure 6.6.**), result not significantly different (t=-0.911, df=1, p=0.363). A survival analysis (Kaplan-Meier) (Kaplan & Meier 1958) gave similar results (X^2=3.4, df=1, p=0.0661). Overall, half of the females tested died between the second and the third (between 29% and 79%) (**Table 6.2.**). However, if we count the females that died before the first gonotrophic cycle, cumulatively, fewer transgenic than wild-type females died before the fourth gonotrophic cycle (75.8 versus 82.8%). In total, 79% percent of the females died after having produced eggs for the third gonotrophic cycle (and 91% after the 4th gonotrophic cycle).

During the second fecundity assay, between 6 and 7% of the females tested died before reaching the first gonotrophic cycle (N=210). Between 9 and 18% of the females died after producing eggs for the first gonotrophic cycle as well as between 24 and 42% died after the second gonotrophic cycle. Finally, between

31 and 59% of the females survived through the experiment and produced eggs for the third gonotrophic cycle (**Table 6.3.**). Survival throughout the successive gonotrophic cycles showed significant differences between mating groups (X^2=23.1, df=8, p=0.0033) and even more strongly significant between female type (X^2=21.9, df=2, p<0.001) with wild-type females surviving better than wild-type reared with 30 µg.ml^{-1} tetracycline and the wild-type females either treated or not with tetracycline surviving better than the OX513A females during experiment 2 (**Figure 6.7.**).

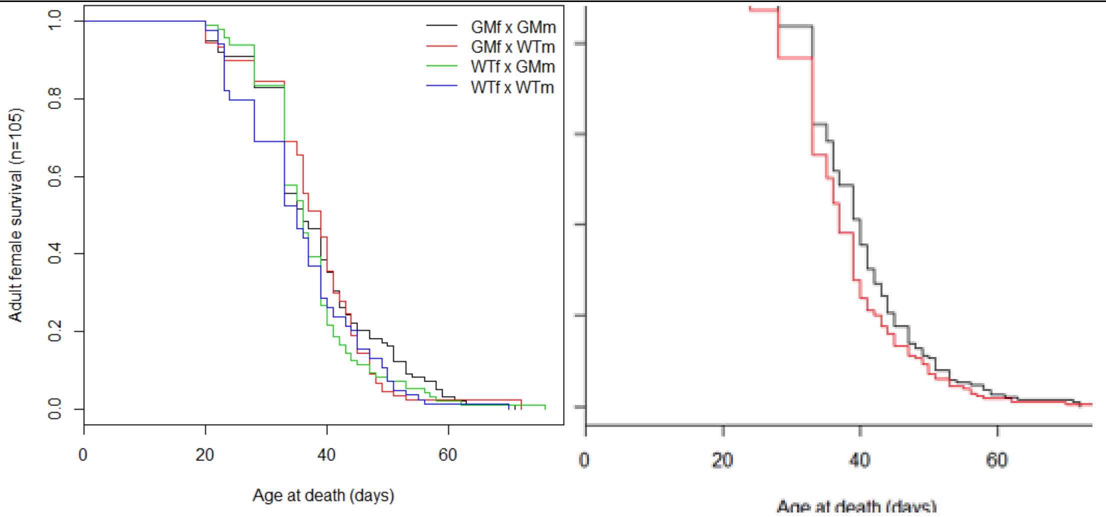


Figure 6.6. Survival curves of *Ae. aegypti* female wild-type (WTf) and OX513A (GMf) from different single-pair mating group (experiment 1).

All treatment started with 105 individual females per mating group: OX513A female mated to a OX513A male (GMf x GMm), OX513A female mated to a wild-type male (GMf x WTm), wild-type female mated to a OX513A male (WTf x GMm) and, finally, wild-type female mated to a wild-type male (WTf x WTm).

Cross *(female x male)*	*Gonotrophic cycle* 0	 1	 2	 3	 4	 5	 6	 7	 8	*N*
A: WT x GM	8 (7.6)	6 (5.7)	10 (9.5)	65 (62)	8 (7.6)	3 (2.9)	3 (2.9)	1 (0.9)	1 (0.9)	105
B: GM x GM	6 (5.7)	9 (8.6)	8 (7.6)	56 (53.3)	9 (8.6)	9 (8.6)	5 (4.8)	2 (1.9)	1 (0.9)	105
C: WT x WT	21 (20)	17 (16.2)	9 (8.6)	38 (36.2)	11 (10.5)	7 (6.7)	1 (0.9)	0 (0)	1 (0.9)	105
D: GM x WT	15 (14.3)	9 (8.6)	5 (4.8)	51 (48.6)	21 (20)	2 (1.9)	0 (0)	0 (0)	2 (1.9)	105
WT female	29 (13.8)	23 (11)	19 (9)	103 (49)	19 (9)	10 (4.8)	4 (1.7)	1 (0.5)	2 (0.95)	210
GM female	21 (10)	18 (8.6)	13 (6.2)	107 (51)	30 (14.3)	11 (9.3)	5 (2.4)	2 (0.95)	3 (1.4)	210
Total **(cumul)**	50 **(12)**	41 **(22)**	32 **(29)**	210 **(79)**	49 **(91)**	21 **(96)**	9 **(98)**	3 **(99)**	5 **(100)**	420

Table 6.2. Number of *Ae. aegypti* wild-type (WT) and OX513A (GM) females that died following each of the 8 gonotrophic cycles from different mating crosses (experiment 1). The table above reports the number of females and the percentage in parenthesis that died following the listed gonotrophic cycles (1 to 8) from different mating crosses between OX513A (GM) and wild-type (WT) males (N=105). The bottom row shows the total number of dead females following each of the gonotrophic cycle listed as well as the cumulative percentage (cumul) in bold. N indicates the sample size.

Female	*Male*	*Dead before testing*	*Gonotrophic cycle* **1**	 **2**	 **3**	*N*
WTf	WTm	4	6	16	42	70
WTf	WTm tet+	8	7	16	37	70
WTf	OX513Am	2	5	18	45	70
WTf tet+	WTm	3	6	22	38	70
WTf tet+	WTm tet+	1	8	22	38	70
WTf tet+	OX513Am	9	8	16	34	70
OX513Af	WTm	4	11	34	18	70
OX513Af	WTm tet+	4	12	29	24	70
OX513Af	OX513Am	4	14	26	24	70
WT females		14 (7%)	18 (9%)	50 (24%)	124 (59%)	210
WT females tet+		13 (6%)	22 (10%)	60 (29%)	110 (52%)	210
OX513A females		12 (6%)	37 (18%)	89 (42%)	66 (31%)	210
Total (cumul)		**39 (6%)**	**77 (18%)**	**199 (50%)**	**300 (98%)**	**630**

Table 6.3. Number of *Ae. aegypti* OX513A and wild-type (WT) females either bred with tetracycline (tet+) or not that died following each of the three gonotrophic cycles (experiment 2).

Number of females that died following the listed gonotrophic cycles for each single pair mating cross between individuals OX513A and wild-type (WT) either bred with tetracycline (tet+) or not. The third column shows the number of females that died before the experiment started (before producing eggs for the first gonotrophic cycle). The bottom row shows the total number of dead females following each of the gonotrophic cycle listed as well as the cumulative percentage (cumul) in bold. N indicates the sample size.

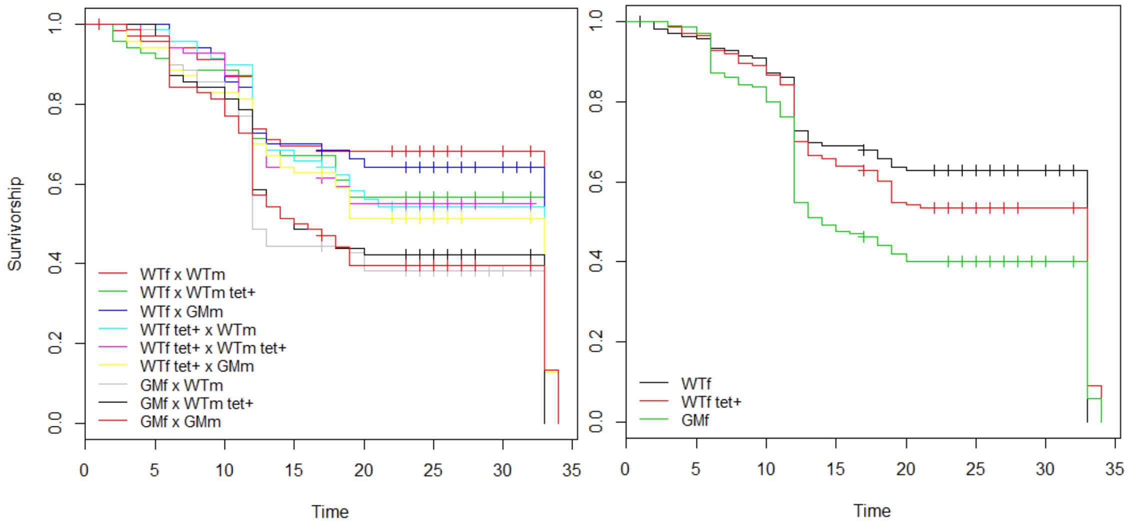


Figure 6.7. Survival curves of *Ae. aegypti* female OX513A (GMf) and wild-type (WTf) reared without (WTf) or with 30 µg.ml^{-1} tetracycline (WTf tet+) from different single-pair mating group (experiment 2).

During the fecundity assay, female mortality was checked daily (Time) and all treatment started with 70 individual females per mating group (graph on the left): wild-type female reared without (WTf) or with 30 µg.ml^{-1} tetracycline (WTf tet+) as well as OX513A females (GMf) were mated to either wild-type males reared without (WTm) or with 30 µg.ml^{-1} tetracycline (WTm tet+) or with OX513A males (GMm). The graph on the right is showing the same survival curves over time (in days) per female type (wild-type, wild-type reared with tetracycline and finally OX513A, N=210). The fecundity experiment was stopped after the third gonotrophic cycle and females that survived through were then censored.

Female	*Male*	*Dead before testing*	*Gonotrophic cycle* **1**	**2**	**3**	*N*
WTf	WTm	4	6	16	42	70
WTf	WTm tet+	8	7	16	37	70
WTf	OX513Am	2	5	18	45	70
WTf tet+	WTm	3	6	22	38	70
WTf tet+	WTm tet+	1	8	22	38	70
WTf tet+	OX513Am	9	8	16	34	70
OX513Af	WTm	4	11	34	18	70
OX513Af	WTm tet+	4	12	29	24	70
OX513Af	OX513Am	4	14	26	24	70
WT females		14 (7%)	18 (9%)	50 (24%)	124 (59%)	210
WT females tet+		13 (6%)	22 (10%)	60 (29%)	110 (52%)	210
OX513A females		12 (6%)	37 (18%)	89 (42%)	66 (31%)	210
Total (cumul)		**39 (6%)**	**77 (18%)**	**199 (50%)**	**300 (98%)**	**630**

Table 6.3. Number of *Ae. aegypti* OX513A and wild-type (WT) females either bred with tetracycline (tet+) or not that died following each of the three gonotrophic cycles (experiment 2).

Number of females that died following the listed gonotrophic cycles for each single pair mating cross between individuals OX513A and wild-type (WT) either bred with tetracycline (tet+) or not. The third column shows the number of females that died before the experiment started (before producing eggs for the first gonotrophic cycle). The bottom row shows the total number of dead females following each of the gonotrophic cycle listed as well as the cumulative percentage (cumul) in bold. N indicates the sample size.

Number of eggs produced per female throughout 8 successive gonotrophic cycles

The number of eggs produced per female per cross was tested separately for the four first gonotrophic cycles (there were too few female survivors from the 5th gonotrophic cycle and onwards). No significant difference was shown when the number of eggs laid per female was tested against the female strain (OX513A or wild-type) as well as the male they mated with (OX513A or wild-type) (**Table 6.4.** and **Figure 6.8.**).

During the second fecundity assay, one sample out of 630 was excluded from the analysis (Female D14, repeat = December), as this was lost during the handling process. On average, *Ae. aegypti* females laid 89.74 eggs during the first gonotrophic cycle, 88.21 during the second and 78.53 during the third. Overall, females seemed to lay more eggs over the course of the first 2 gonotrophic cycles than during the 3rd (**Figure 6.9.**). While wild-type females treated with 30 µg.ml^{-1} tetracycline laid more eggs in total than wild-type non-treated and OX513A females (**Table 6.5.**) the difference between the mean egg numbers laid per cross for each gonotrophic cycle was not significant. However, over the course of the first gonotrophic cycle, the interaction between wild-type male and Repeat December was significant (t=-2.143, df=4, p=0.0345) as was the three way interaction WT-female:WT-male:Repeat December (t=2.310, df=8, p=0.0213). On average, egg production recorded 93.7 ± 3 eggs in December, 84.8 ± 2.8 in May and 87.6 ± 2.2 in August. Most laboratory parameters were similar between the three repeats in December, May and August (i.e. mosquito rearing pots, larval density, tetracycline concentrations, food regime and photoperiod), however temperatures and humidity varied among repeats with higher temperatures recorded in December than in May and August 2011 (see 5.2. Material and Methods, 5.2.2. Experiment 2).

Female	*Male*	*Gonotrophic cycles* 1	 2	 3	 4	 5	*No eggs*
WT	WT	76 (4.1)	82 (5.3)	68 (6.4)	59 (12.2)	70 (10.1)	12,167
WT	OX513A	87 (4.0)	73 (4.5)	62 (5.9)	65 (14.5)	58 (14.2)	15,158
OX513A	WT	84 (3.4)	78 (4.2)	79 (5.6)	60 (9.7)	72 (12.5)	16,227
OX513A	OX513A	83 (3.0)	73 (4.3)	74 (5.3)	70 (8.3)	63 (7.5)	18,435
WT	-	81.8 (2.9)	76.7 (3.4)	64.8 (4.3)	62.5 (9.1)	64.2 (8.4)	27,325
OX513A	-	83.9 (2.2)	75.4 (3.0)	76.3 (3.8)	65.9 (6.2)	65.7 (6.3)	34,662

Table 6.4. Number of eggs produced by *Ae. aegypti* OX513A and wild-type (WT) females following each of the first five gonotrophic cycles (experiment 1).

Total number of eggs laid per female and mean number of eggs laid per gonotrophic cycle and standard error of the mean in parenthesis; Values are calculated per female OX513A and wild-type (WT) as well as per mating cross (N=105).

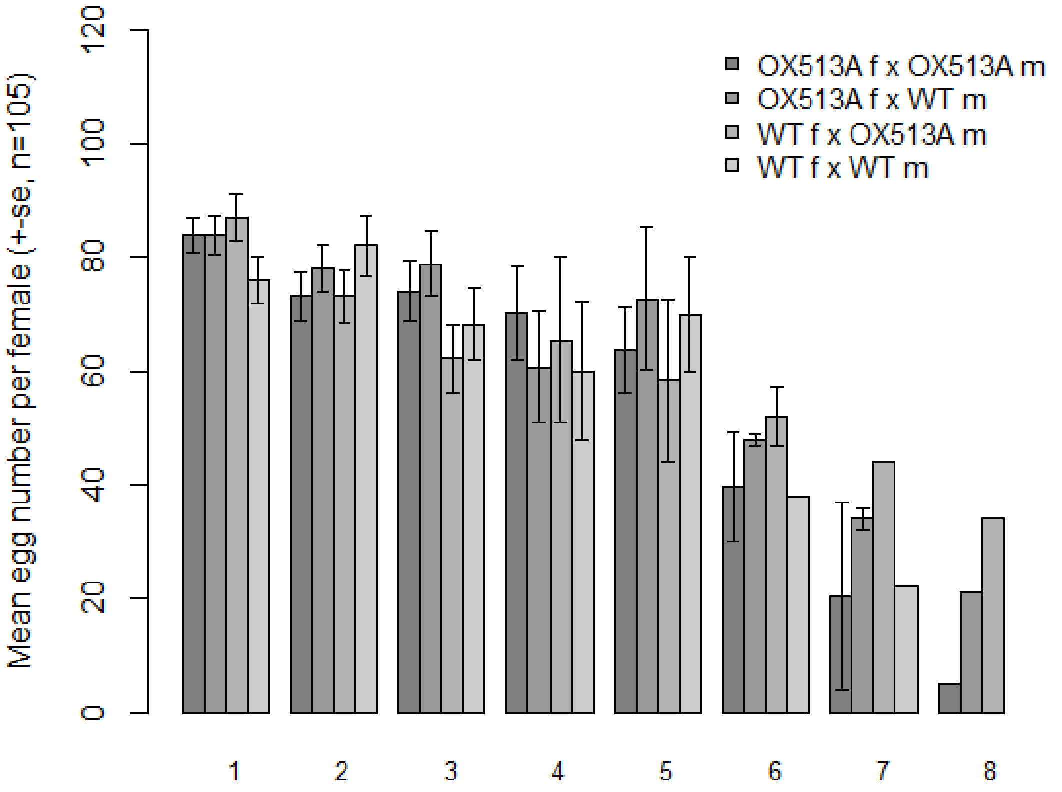


Figure 6.8. Comparison of egg production over 8 gonotrophic cycles when OX513A and wild-type were mated with OX513A or wild-type males (experiment 1).

The mean number of eggs per female per gonotrophic cycle (x axis) per cross is represented on this barchart: wild-type female (WT f) mated with an OX513A male (OX513A m), OX513A female (OX513A f) mated with an OX513A male (OX513A m), wild-type female mated with a wild-type male (WT m) and an OX513A female mated with a wild-type male. Error bars represent standard error of the mean.

Female	Male	Gonotrophic cycle 1	 2	 3	No of eggs
WT tet+	WT tet+	101.4 (2.9)	93.9 (2.8)	84.1 (3.0)	15,536
WT tet+	WT	96.8 (3.5)	95.2 (2.9)	83.4 (3.1)	14,909
WT tet+	OX513A	97.6 (4.4)	95.3 (3.5)	86.4 (4.2)	13,262
WT	WT tet+	84.4 (3.2)	89.2 (2.9)	77.2 (4.0)	12,640
WT	WT	84.5 (2.9)	88.1 (2.7)	83.2 (2.0)	13,931
WT	OX513A	79.0 (3.0)	82.7 (3.4)	71.2 (3.7)	12,910
OX513A	WT tet+	87.4 (3.8)	82.8 (3.9)	74.2 (8.0)	11,214
OX513A	WT	88.6 (3.6)	87.8 (3.2)	76.5 (5.6)	11,856
OX513A	OX513A	88.0 (3.5)	78.7 (4.5)	71.3 (7.0)	10,723
WT tet+	-	98.7 (2.1)	94.8 (1.8)	84.6 (2.0)	43,707
WT	-	82.6 (1.7)	86.6 (1.7)	77.1 (2.0)	39,481
OX513A	-	88.0 (2.1)	83.3 (2.2)	73.9 (3.8)	33,793

Table 6.5. Number of eggs produced by *Ae. aegypti* OX513A and wild-type (WT) females either bred or not with 30 µg.ml^{-1} tetracycline following each of the first three gonotrophic cycles (experiment 2).

Total number of eggs laid per female OX513A, wild-type (WT) and wild-type treated with 30 µg.ml^{-1} (tet+) per mating cross as well as mean number of eggs per gonotrophic cycle and standard error of the mean in parenthesis.

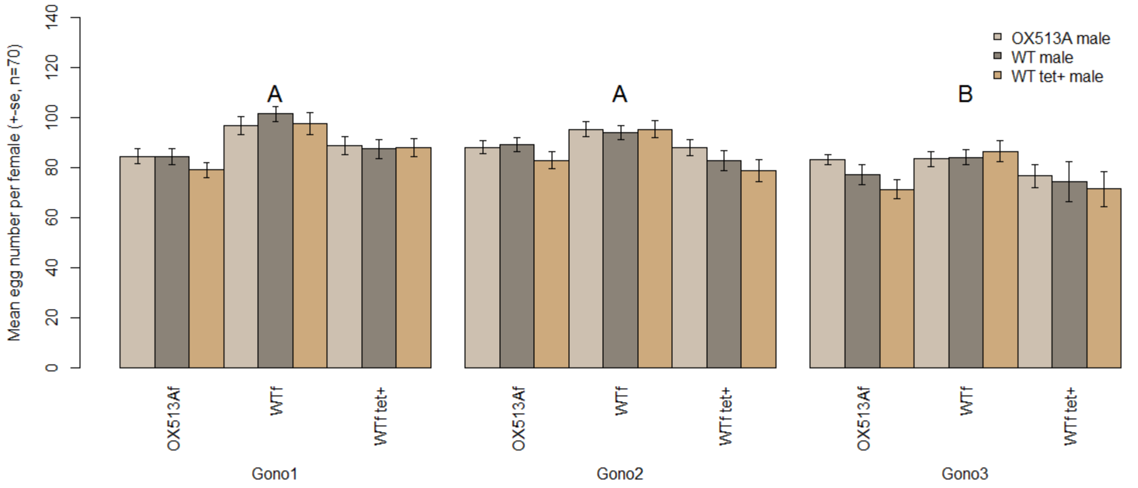


Figure 6.9. Comparison of egg production over three gonotrophic cycles when OX513A and wild-type reared with or without tetracycline were mated with OX513A or wild-type reared with or without tetracycline males (experiment 2).

Mean number of eggs produced per female for each of the 3 gonotrophic cycles among 9 single pair crosses: a wild-type female (WTf) mated with either a wild-type male, a wild-type male reared with 30 µg.ml^{-1} tetracycline or an OX513A male; a wild-type female reared with 30 µg.ml^{-1} tetracycline (WTf tet+) mated with either a wild-type male, a wild-type male reared with 30 µg.ml^{-1} tetracycline or an OX513A male and finally an OX513 female (OX513Af) mated with either a wild-type male, a wild-type male reared with 30 µg.ml^{-1} tetracycline (tet+) or an OX513A male. Error bars represent standard error of the mean egg number per female. Groups denoted by the same capital letter are not significantly different from each other (ANOVA).

Overall egg production per female

Over the course of fecundity assay 1,344 blood meals produced egg batches from 420 females (81.9%); in total 62,074 eggs and, on average, 147.8 eggs per female. Twenty five out of 169 (14.8%) wild-type females did not blood feed during the experiment, showing a similar pattern with the number of OX513A females that did not blood feed (25 out of 175 or 14.3%) (χ^2=0.1161, df=1, p =0.7334). Taking into account the male females mated with, 9.4% of the females that did not blood feed were mated with a OX513A male (19 out of 203) as opposed to 17% of the females with wild-type males (33 out of 193) (χ^2= 4.5382, df =1, p =0.03315). There was no association between the number of females that did not produce any eggs and the type of female (wild-type or OX513A) or the type of male they mated with. Overall, OX513A females did lay significantly more eggs than their wild-type counterpart (200.553 ± 10.964 versus 164.588 ± 12.948) (F=3.92, df=2, p=0.02075) when egg laying females only were included in the analysis, and this result is confirmed when both non egg laying and egg laying females were included in the analysis as well (p=0.00984) (**Figure 6.10.**). In both analyses, the male's genetic background (OX513A or wild-type) did not affect the number of eggs laid by *Ae. aegypti* females (p=0.68 when only egg laying females were included in the analysis and p=0.16 when both non-egg-laying and egg-laying were included in the analysis).

During the second experiment (assessing the impact of tetracycline on *Ae. aegypti* fecundity), 582 out of 630 females tested (92%) blood fed and laid eggs. In total 117,070 eggs were produced (on average 185.7 eggs per female) over the course of three gonotrophic cycles. Fourteen wild-type females did not blood feed during the experiment out of 210 (6.7%) showing a similar pattern with the number of wild-type females reared with tetracycline (13 out of 210, 6.2%) as well as OX513A females (15 out of 210 – 7.1%). Taking into account the strain of male that females were mated with, 6% of the females that did not blood feed were mated with a OX513A male (16 out of 210) as opposed to 6% (12 out of 210) of the females with wild-type males and 7% (14 out of 210) with a wild-type male treated with tetracycline. There was no association between the number of females that did not produce any eggs and the type of female (WT, WT tet+ or OX513A) or the type of male the females were mated with. On average, OX513A females laid significantly fewer eggs than their wild-type counterpart (161 ± 6.4 versus 188 ± 9) as well as wild-type females treated with tetracycline (209 ± 9) (t=5.3, df=2, p<0.001). This result is confirmed when egg-laying-females only are included in the analysis (**Figure 6.11.**). Females produced the same number of eggs whether mated with an OX513A male (175.7 ± 6.5), a wild-type male (194.7 ± 9.2) or a wild-type male treated with tetracycline (187.6 ± 9.2). This result was confirmed when egg-laying females only were included in the analysis.

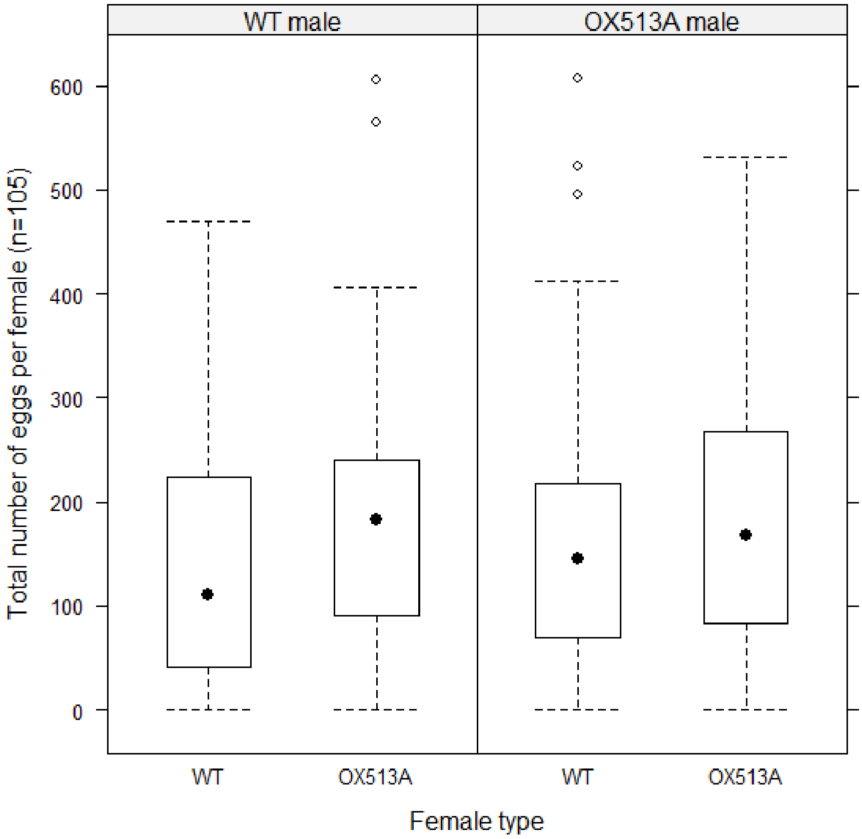


Figure 6.10. Box plot showing the distribution of the total number of eggs produced by *Ae. aegypti* wild-type and OX513A females when mated with wild-type or OX513A males (experiment 1).

Median and 1st (Q1) and 3rd (Q3) quartiles of number of eggs adult female *Ae. aegypti* produced from different single mating crosses: wild-type female (WT) mated with an OX513A male; OX513A female mated with an OX513A male; wild-type female mated with a wild-type male and OX513A female mated with a wild-type male (n=105). The whiskers of the box-plot represent 1.5*Q1 and 1.5*Q3 and circles represent outliers.

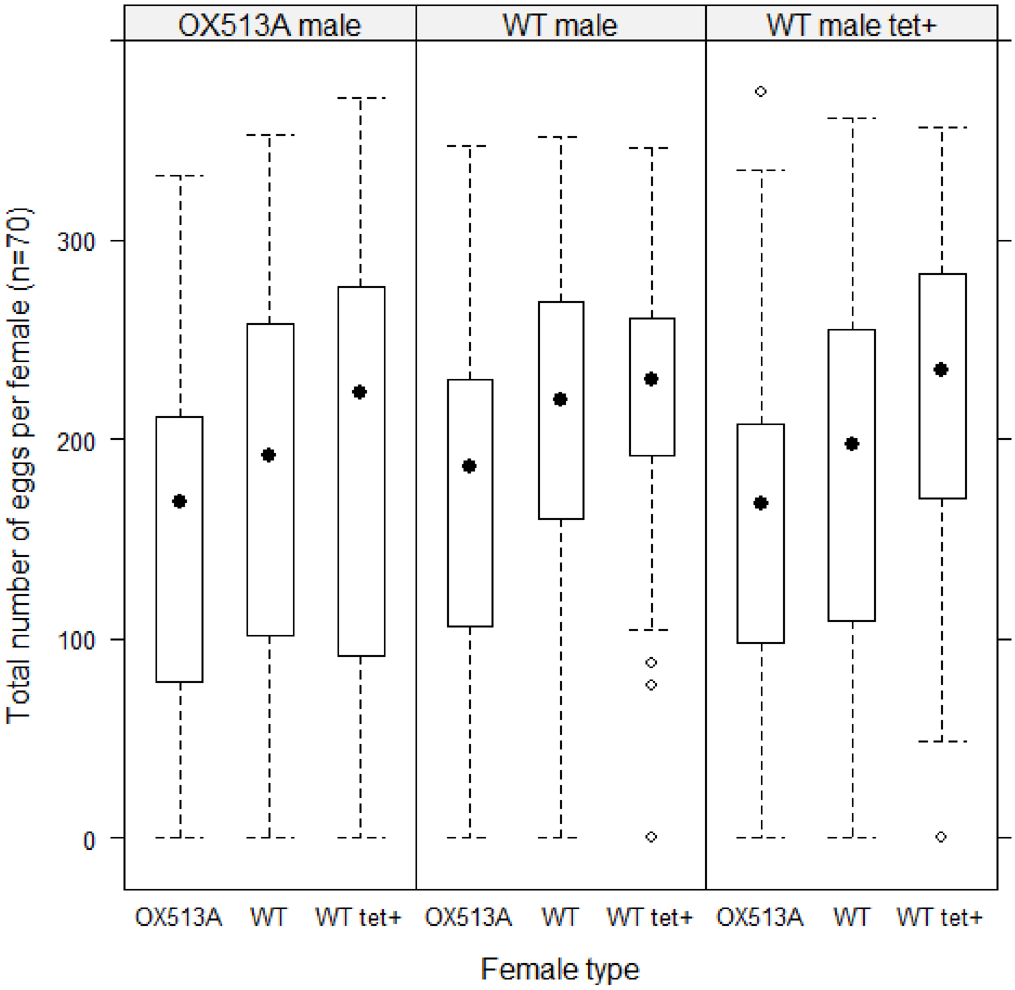


Figure 6.11. Box plot showing the distribution of the number of eggs produced by *Ae. aegypti* OX513A and wild-type females (reared with or without tetracycline) when mated with OX513A or wild-type males (reared with or without tetracycline) (experiment 2). Median and 1st (Q1) and 3rd (Q3) quartiles of number of eggs produced from the 3 different type of female tested (from left to right, OX513A, wild-type and wild-type reared with 30 µg.ml^{-1} tetracycline) mated with either an OX513A male (OX513A m), a wild-type male (WTm) or a wild-type male reared with 30 µg.ml^{-1} tetracycline (WTm tet+) (n=70). The whiskers of the box-plot represent 1.5*Q1 and 1.5*Q3 and circles represent outliers.

6.3.3. Fertility of *Ae. aegypti*: ‘strain’ effect as well as the impact of tetracycline treatment

Hatch rate analysis on “fresh” eggs (4 to 7 days post oviposition)

During the second fecundity experiment, a total of 117,070 eggs were produced over the course of three gonotrophic cycles, 95,298 hatched, 1,190 were found ‘already-hatched’ and finally a total of 3,320 (36 samples) did not hatch at all. No association existed between the number of eggs that did not hatch and the genetic background of the female or the male she mated with. During the first repeat of the fertility assay, 9 samples out of 630 were removed from the original ‘hatch rate’ dataset: a greater number of hatched-live-larvae (first instar) were recorded than the original number of eggs counted to be hatched. This was simply due to the “already-hatched larvae” being mistaken for live ones. Those 9 errors were all committed during the first experiment in December 2010 and attention has been paid to this for the next repeats in May and August 2011. Hatch rate did not differ between the repeats or between the different types of females (from 67% hatch rate for wild-type females to 73% hatch rate for wild-type females treated with tetracycline as well as OX513A females) (**Figure 6.12.**). However, hatch rate was significantly reduced when females were mated with an OX513A male in comparison with both a wild-type male treated with tetracycline (p=0.0167) or a non treated wild-type male (p=0.0317) (**Table 6.6.**).

Female	***Male***	***Already hatched***	***Total egg***	***Total hatched***	***Mean hatch rate***
WTf	WTm	62 (0.51)	12,137	9,850	79.2 ± 3.3
WTf	WTm tet+	34 (0.27)	12,705	9,819	72.0 ± 4.0
WTf	OX513Am	242 (1.89)	12,837	10,030	69.4 ± 3.9
WTf tet+	WTm	173 (1.07)	16,123	13,411	78.8 ± 3.1
WTf tet+	WTm tet+	171 (1.16)	14,690	11,584	80.7 ± 3.0
WTf tet+	OX513Am	107 (0.85)	12,642	10,604	62.3 ± 4.6
OX513Af	WTm	105 (0.77)	13,666	11,668	72.1 ± 4.0
OX513Af	WTm tet+	131 (1)	13,142	11,428	68.9 ± 3.8
OX513Af	OX513Am	165 (1.81)	9,128	6,904	53.5 ± 4.0
OX513f	-	401 (1.12)	35,936	30,000	64.7± 2.3 **a**
WTf tet+	-	451 (1.04)	43,455	35,599	73.8± 2.2 **a**
WTf	-	338 (0.9)	37,679	29,699	73.6± 2.2 **a**
-	WTm	340 (0.81)	41,926	34,929	76.8± 2.0 **a**
-	WTm tet+	336 (0.83)	40,537	32,831	73.7 ± 2.1 **a**
-	OX513Am	514 (1.49)	34,607	27,538	61.7± 2.5 **b**

Table 6.6. Summary table of total eggs analysed for the hatch rate of eggs by *Ae. aegypti* OX513A and wild-type females treated or not with 30 µg.ml^{-1} (experiment 2).

The table is showing the total number of eggs that were found already hatched on the cotton wool prior to hatching (percentage in parentheses), total number of eggs counted for each cross (n=70) as well as the mean hatch rate ± standard error. Wild-type females or males (WTf or WTm) were crossed to OX513A males or females (OX513Am or OX513Af). Wild-type females and males were bred with 30 µg.ml^{-1} tetracycline (tet+) or without tetracycline (tet-). Values denoted with the same letter did not differ significantly (GLM).

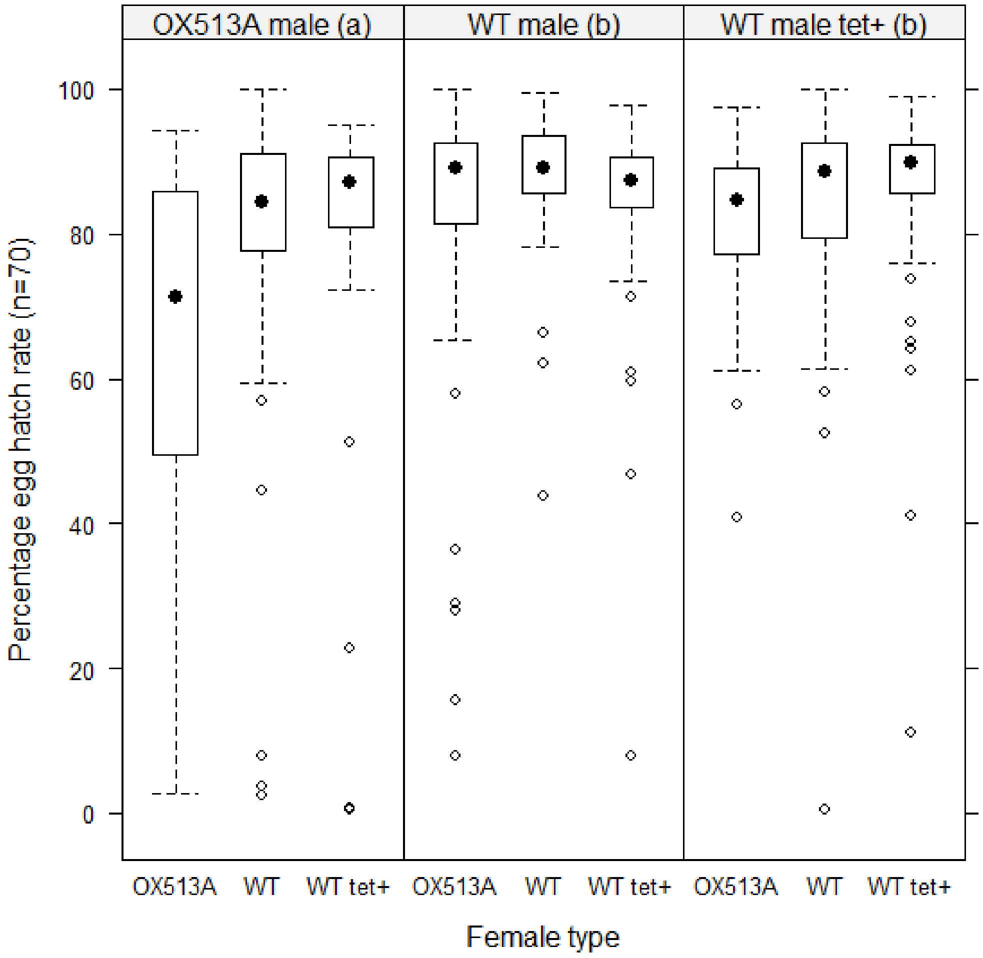


Figure 6.12. Box plot showing the distribution of hatch rate of eggs produced from *Ae. aegypti* OX513A and wild-type females (reared with or without tetracycline) when mated with OX513A or wild-type males (reared with or without tetracycline) (experiment 2). Median and 1st (Q1) and 3rd (Q3) quartiles of egg hatch rates from the 3 different type of female tested (from left to right, OX513A, wild-type and wild-type reared with 30 µg.ml^{-1} tetracycline) mated with either an OX513A male (OX513A m), a wild-type male (WTm) or a wild-type male reared with 30 µg.ml^{-1} tetracycline (WTm tet+) (n=70). Male genotypes in upper panels denoted with the same letter did not differ significantly (GLM). The whiskers of the box-plot represent 1.5*Q1 and 1.5*Q3 and circles represent outliers.

Egg hatching two and a half months after oviposition from the 20 most 'egg-productive females'

During the first fecundity experiment, out of the 20 most egg-productive females per mating group, a total of 27,821 eggs were deposited, but 1.1% had already hatched prior the experiment (299 eggs) and 0.3% were physically damaged (80 eggs) (**Table 6.7.**). A total of 27,741 eggs were considered viable and therefore included in the hatch rate analysis (99.7%). In total, 45.5% of eggs hatched (12,319 out of 27,741). There was no association between the number of eggs that prematurely hatched and the type of female or male (wild-type or OX513A). The eggs collected for one female out of 80 did not hatch at all (an OX513A female mated with a wild-type male). On average, a significantly higher proportion of wild-type female eggs hatched (54.9% ± 1.7) than OX513A female eggs (36.2% ± 2.6) (t=5.293, df=1, p<0.001). Also more eggs hatched when the female was mated with a wild-type male (48.6% ± 2.8) than with an OX513A male (42.4 % ± 2.4) (t=2.47, df=1, p=0.0156) (**Figure 6.13.** and **Table 6.8.**).

Cross (female x male)	*No of eggs intact*	*No of eggs that were already hatched*	*No of eggs physically damaged*	*Total No of eggs*
OX513A x OX513A	7,760 (99.8%)	249 (3.2%)	17 (0.2%)	7,782
OX513A x WT	6,789 (99.6%)	32 (0.47%)	24 (0.35%)	6,755
WT x OX513A	6,789 (99.5%)	16 (0.23%)	32 (0.47%)	6,794
WT x WT	6,430 (99.08%)	2 (0.03%)	7 (0.1%)	6,490
Total	**27,668 (99.5%)**	**299 (1.1%)**	**80 (0.3%)**	**27,821**

Table 6.7. Summary table of total eggs analysed for the hatch rate of eggs by *Ae. aegypti* OX513A and wild-type after long term storage (experiment 1).

Total number of eggs that were counted, recorded as 'physically damaged' or 'already hatched' per mating cross and their percentage in parenthesis: OX513A and wild-type (WT) females were mated with wild-type and OX513A males (N=20).

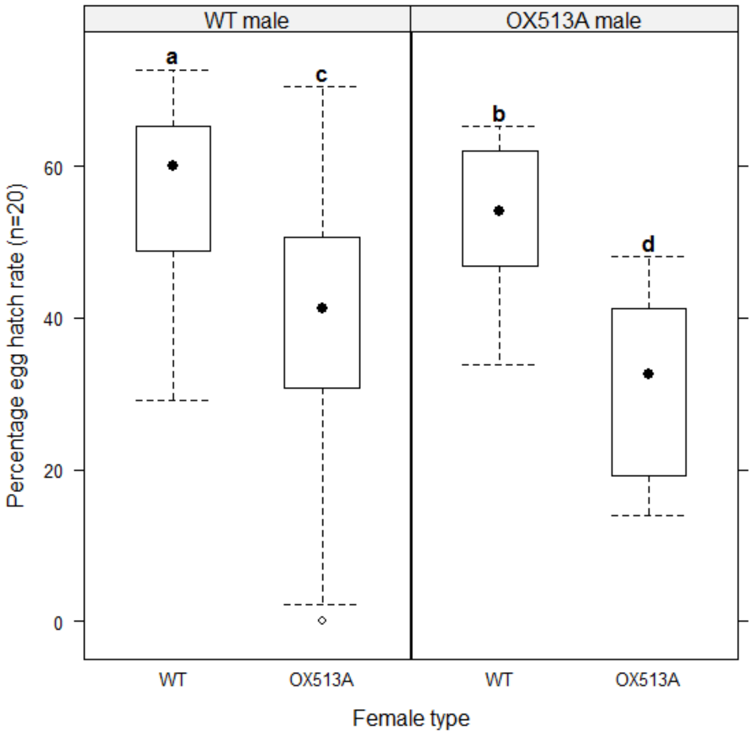


Figure 6.13. Box plot showing the distribution of hatch rate of eggs produced by *Ae. aegypti* OX513A and wild-type females mated with OX513A or wild-type males after 2 and a half month storage (experiment 1).

Median and 1st (Q1) and 3rd (Q3) quartiles of hatch rate of eggs produced by wild-type (WT f) and OX513A females (OX513A f) mated with wild-type (WT m) or OX513A males (OX513A m) (n=20). The whiskers of the box-plot represent 1.5*Q1 and 1.5*Q3 and the circle represent an outlier. Groups represented by a different letter are significantly different (GLM).

Cross (female x male)	*Mean egg hatch rate in % (SE)*	*Total No of eggs counted*	*Total No of eggs hatched*	*No of crosses*
OX513A x OX513A	31.5 (2.6)	7,782	2,413	20
OX513A x WT	40.8 (4.2)	6,755	2,755	20
WT x OX513A	53.4 (2.1)	6,794	3,575	20
WT x WT	56.4 (2.7)	6,490	3,576	20
WT female	54.9 (1.7)	13,284	7,151	40
OX513A female	36.2(2.6)	14,537	5,168	40
WT male	48.6 (2.8)	13,245	6,331	40
OX513A male	42.4 (2.4)	14,576	5,988	40
Total	**45.5 (1.85)**	**27,821**	**12,319**	**80**

Table 6.8. Summary table of total eggs analysed for the hatch rate of eggs by *Ae. aegypti* OX513A and wild-type (experiment 1).

Mean hatch rate and standard error of mean in parenthesis (SE) of OX513A and wild-type (WT) females mated with wild-type (WT) and OX513A males (N=20).

Overall reproductive success: comparing the number of offspring per female

Interestingly, in the first experiment, an average of 178.8 offspring per wild-type female (± 7.6 se) in comparison with 129.2 per OX513A female (± 9.3 se) were obtained after two and half month storage (t=52.8, df=1, p=0.00126). However, during the second experiment, an average of 198 offspring were obtained per wild-type female treated with 30 $\mu g.ml^{-1}$ tetracycline (± 5.3 se) which was significantly different from the number of offspring per non treated wild-type and OX513A females (respectively 177.8 ± 5.5 se and 142.8 ± 5.4 se; t=2.675, df=2, p=0.00771).

6.4. Discussion

6.4.1. OX513A has a good potential for mass production of eggs

In this chapter, fecundity has been assessed by the number of eggs a transgenic female *Ae. aegypti* can produce after a single pair mating in comparison with a wild type. Both experiments resulted in a similar number of eggs produced per female from both genotypes when gonotrophic cycles were analysed separately. If OX513A egg production over the course of 3 gonotrophic cycles was consistent (on average, 162 eggs during the first experiment and 161 eggs during the second), the overall mean egg production per female showed that OX513A females exhibited a greater fecundity than wild-type females in the first experiment producing on average 162 eggs (23 more than a wild-type female producing 139 eggs) out of the three first gonotrophic cycles. When calculated over the course of 8 gonotrophic cycles, OX513A females laid 200 eggs per female in comparison with 164 for wild-type females. During the second experiment however, the tendency is reversed: wild-type females, whether treated with tetracycline or not, laid more eggs than OX513A females over the course of 3 gonotrophic cycles, producing on average 209 eggs (wild-type tetracycline treated females) and 188 eggs (wild-type females) and 161 eggs (OX513A females).

The first gonotrophic cycle produced between 82.5 and 90 eggs per female (first and second fecundity assay), the second between 76.5 and 88 eggs and the third between 70.75 and 78.5 eggs per female. Those results are consistent with previous studies on *Ae. aegypti* females when fully engorged (Christophers 1960). Overall, however, wild-type females non treated to tetracycline did produce more offspring per female (178.8 during the first experiment and 177.8 during the second) than the OX513A (129.2 and 142.8 respectively). Interestingly, wild-type females treated with 30 µg.ml-1 tetracycline obtained more offspring than the wild-type and the OX513A females (198 larvae) during the second experiment.

In a mass production cage system at Oxitec Limited, each week, cages of mosquitoes (dimensions: 70 cm x 70 cm x 30 cm) are blood fed twice and eggs collected once; the cage is kept for 3 to 4 weeks. Over the period of a cage life time, females of variant of the OX513A strain (namely the ‘Latin’ strain) produce approximately 206 eggs. Since the number of females that blood fed each week is not recorded, the weekly egg collection might not exactly reflect the number of eggs per gonotrophic cycle. However, if we speculate that the cage life time corresponds to 3 gonotrophic cycles (the cage is only kept for 3 consecutive weeks), it relates to a production of 70 eggs per OX513A female per gonotrophic cycle. This number has greatly improved since the beginning of OX513A mass production (number of eggs per female was below 10) thanks to several arrangements in the cage system: a better artificial blood feeding apparatus offering a greater surface and homogeneous heat; an optimised adult mosquito density in the cage (6,000 adults in a 150 L capacity) as well as adult male and females ratio (1 male for 3 females) (Neil Naish, personal communication); a greater sugar feeding surface and resting places for adult mosquitoes and, finally, a greater surface offered for oviposition.

6.4.2. OX513A fertility

The present study reveals that *Ae. aegypti* female genetic background (transgenic or wild-type) or exposure to tetracycline during larval development do not impact neither egg production (fecundity) nor hatch rate (fertility). However, RIDL males are associated with a poorer fertility than their wild-type counterparts (whether treated or not with tetracycline). This is the second report of such phenomenon: earlier studies reported an increase of ‘sterile egg batches’ associated with the increase of the number of OX513A males present in a mating competitiveness experiment (I. Bargielowski PhD thesis). The present single-pair mating study did not associate OX513A males with a greater production of sterile egg batches but with a poorer mean hatch rate. This slight fitness deficit of the OX513A strain means that, when a transgenic male will mate with wild-type females, fewer eggs will hatch and, consequently, less transgenic larvae will be present to compete with the wild larvae. These peculiarities of the OX513A strain could reduce the potential benefit that a ‘late-acting lethal’ strain has relative to conventional early-acting lethal systems such as radiation-based SIT (Phuc et al., 2007), implying a somewhat larger numbers of transgenic males may need to be released to achieve suppression of the targeted population than would be required for a hypothetical ‘perfect’ late-acting lethal strain. This novel result can be taken into account in future modelling of RIDL SIT-based control programmes using OX513A.

6.4.3. OX513A long-term egg storage

Ae. aegypti eggs like many other Aedes species, are resistant to desiccation and can be stored up to 8 and a half months if kept moist (Fielding et al. 1919 in Christophers, 1960). Although the egg sample was not optimally selected, our study suggests that RIDL eggs (OX513A) do not store as well as wild-type ones long term (respectively 36% versus 55% hatch rate). Such a phenomenon has previously been observed in a wild-type *Ae. aegypti* strain that was artificially selected for resistances towards the bio-larvicide *Bti*. Recently, a study reported a 68% fitness cost attributed to 4 months storage, with fewer eggs hatching from the Bti-resistant-strain in comparison with the Bti-sensitive one (Paris et al., 2011a). Although such results need confirming, it is possible that OX513A presents tTAV leakiness during egg diapause causing this 34.5% hatch rate reduction over wild-types, OX513A larvae losing more energy reserves than wild-types. This restrictive condition towards long term storage of the RIDL eggs means little when OX513A is in continuous mass production for mass release in a suppression programme. However, it implies more frequent “re-hatches” over the course of the maintenance of the RIDL strain.

Solutions exist to prevent and help OX513A egg long term storage. First, improving the egg storage conditions might help keep them longer e.g. by sterilizing the surface of the eggs with the fungicide benzalkonium chloride

(Lang et al., 1972) or sodium chlorite (Trpiš 1970) to prevent any bacterial or fungus growth. Secondly, and more importantly, RIDL females can be sugar fed with tetracycline added so that the embryos produced by RIDL females might have access to tetracycline so that the positive feedback system is repressed.

6.4.4. No impact of tetracycline on *Ae. aegypti*'s fecundity

Body size and blood meal size have been proven to be positively associated with fecundity (Steinwascher 1984; Harrington and Edman, 2001). In this study, there was no correlation between wing length and the mean number of eggs produced per female. However, body size may be correlated to fecundity and treatments with tetracycline might provoke a change too subtle to be associated with body weight or wing length. Tetracycline-treated larvae give rise to adults exhibiting higher lipid content stores (Bargielowski et al., 2012) and, sometimes, larger wings (E.V. Ridley PhD thesis). The antibiotic treatment between 0.5 and 100 µg.ml^{-1} is thought to remove deleterious bacteria and increase life span for the tetracycline-tolerant wild-type strain. Impacts of tetracycline treatments have been poorly studied and are of major interest regarding insect artificial rearing.

Previously it has been shown that the presence of tetracycline reduced *Drosophila* female's fecundity (number of eggs produced per female) but the removal of bacteria (bacteria depletion) does not (females produced from dechorionated eggs) (E.V. Ridley PhD thesis). In *Ae. aegypti*, different tetracycline concentrations were added to the larval diet (from 0 to 100 µg.ml^{-1}) and energy reserve tests were performed on adult mosquitoes. Those concentrations were not enough to remove all the bacteria found in the adult mosquitoes which could explain a higher lipid content of mosquitoes reared on tetracycline as well as the increase of life span and wing length. To confirm that the presence of tetracycline has such an impact on mosquitoes, rather than bacteria depletion as in Drosophila, mosquitoes could be reared axenically (by dechorionation of the eggs) and tested at different life-stages for lipid content. Egg dechorionation can be obtained by treating *Ae. aegypti* to bleaching treatments (Sodium Hypochlorite or Benzalkonium). Aedes embryos can also be injected with high doses of tetracycline as this is routinely used to clear *Wolbachia* infection and could be applied in that study to produce axenically mosquitoes (control: injected eggs with deionised water only).

Chapter 7: Summary and conclusions

Dengue fever is a serious illness currently affecting over 40% of the world's population (WHO, 2012) for which no vaccine has shown efficacy against all four serotypes of the virus (Sabchareon et al., 2012; Schmitz et al., 2011). Controlling mosquito populations as a means to stop dengue transmission remains the most effective way to limit contacts between the disease vector, *Aedes aegypti,* and the human hosts. SIT-based techniques such as RIDL show promise in combating such a disease: they are environmentally friendly and can efficiently replace pesticide spraying, which has been reported to be less and less efficient against adult *Ae. aegypti* (Gubler 2011), and certain insecticides like DDT kill insects in general and are highly persistent in the environment (Spencer et al., 1996) encouraging the development of resistances in the mosquito populations. The RIDL technology is complementary to existing mosquito control methods such as larval breeding sites removal or management (using fish or other mosquito larvae as predators, or bio-larvicides such as the *Bti* toxins), and therefore can as such be part of an Integrated Pest Management. RIDL mosquitoes can improve classical SIT affecting male fitness (I. Bargielowski PhD thesis). As a result, genetically-engineered-sterile males can challenge their wild counterparts in actively seeking for the wild-type females, producing offspring that die as immature stages and therefore reduce *Ae. aegypti* populations (Harris et al., 2012a).

Past classical SIT programmes in El Salvador and India were interrupted for political reasons, but inefficient mass production and release of a male-only population were major weaknesses of the programmes. Key issues for SIT-related techniques such as RIDL remain the same: an efficient sex separation of males from females prior to release as well as the automation of the mass production of high quality laboratory-bred mosquitoes. Striving to meet the needs for developing and improving the rearing processes, the scientific community in coming up with solutions and implementing new methods: larval rearing systems (Balestrino et al., 2012), artificial blood feeding system, automation of the larval aliquoting and egg counting, separation of larvae from pupae (Balestrino et al., 2011), have all been recently investigated.

There is scope for improving the release of high quality and high numbers of a male-only-RIDL population through the development of an efficient genetic sexing system. Such a system needs being inexpensive and killing females at early stages. Removing the females at early developmental stage is clearly beneficial in term of male production efficiency for the release generation (Caceres 2002). To date, engineered sexing systems were solely achieved through divergent fluorescence pattern in *An. gambiae* males (Catteruccia et al., 2005; Magnusson et al., 2011) (**Figure 1.6.** and **Figure 3.12.**) requiring the acquisition of expensive flow-cytometer equipment for every release site undoubtedly in regions or countries lacking strong financial support for mosquito control. Engineered sexing systems exist also as an “all-in-one” female-specific-RIDL system: the *Ae. aegypti* flightless strain (Fu et al. 2010) (**Figure 1.5.**). Although such a strain has the potential to eradicate more quickly the wild-population and present a real post-release advantage, females are incapacitated very late in developmental stage (they are flightless and cannot mate nor forage for food). An early acting-female-specific lethal system would be the solution to help an efficient male – only release and could be coupled to either an SIT or RIDL programme.

The properties of the fruit fly *D. melanogaster* to tolerate high concentration of environmental alcohol have been exploited since 1981 (Robinson and Heemert 1981) to generate a genetic sexing system. The ADH pathway in *D. melanogaster,* responsible for the metabolism of ethanol in the diet – with the fast *Adh* allele (*Adh*-f) showing greater catalytic activity), is one of the most studied by population and evolutionary biologists (Cohn & Moore 1988; McDonald & Kreitman 1991; Betrán & Ashburner 2000). Also, the *D. melanogaster Adh*-f allele was used to generate the first GSS using the ADH system in the same insect: the *Adh*-f allele, linked to the Y-male chromosome by translocation into an *Adh*-null strain conferred resistance to the males when larvae were exposed to 4% v/v ethanol. The females, homozygous for the null allele were sensitive to ethanol and died. Using such a system in *Ae. aegypti* would be cheaper than sorting every individual through fluorescence using a flow-cytometer (Marois et al. 2012) and ethanol is an inexpensive and widely available product.

In this thesis, the use the *DmAdh* gene as an inducible, selectable marker in *Ae. aegypti* to discriminate between transgenic and wild-type individuals was investigated in Chapter 2. While my initial intent was, logically, to engineer a transgenic *Ae. aegypti* strain resistant to ethanol using *DmAdh* gene encoding for the fast ADH allozyme, results yielded the opposite: I developed transgenic strains highly susceptible to low dosage of ethanol (up to 1% v/v) in comparison to wild-types when dosed throughout immature stages. The work conducted in this thesis did not investigate further the mechanism of why this result has been observed. One can speculate that, unlike *D. melanogaster*, *Ae. aegypti* cannot metabolise the acetaldehyde produced as a by-product from the first reaction between *Dm*ADH and ethanol, and that would imply that acetaldehyde is not detoxified by *Drosophila* ADH as it has originally been speculated or that acetaldehyde is more toxic to *Ae. aegypti*.

While I did not investigate the mechanism of this toxicity further, one could take advantage of the engineered *Ae. aegypti Adh*-lines (OX4346) to test the impacts of secondary alcohols on the survival of larvae in comparison with wild-types to help better understand the ADH pathway in this species. It would also be interesting to detect and quantify the accumulation of acetaldehyde in the *Adh*-transformed larvae in comparison with wild-types to confirm our speculations.

Incidentally, the *DmAdh* gene presented all the characteristics of a suitable marker for a genetic sexing straing (GSS) when transformed in *Ae. aegypti*: i) low ethanol doses are needed to consistently discriminate *Adh*-transformed larvae from wild-types (between 0.5% and 1% v/v) and ii) this dose does not impose obvious fitness effects on *Ae. aegypti* male longevity and fertility. Because the *Adh*-transformed larvae are not sex-specifically targeted (as this was only the first step in creating a GSS), fitness impact have not further been investigated. The combination of a female-specific promoter or alternatively-spliced gene with *DmAdh* would, no doubt, lead to a different phenotype than the OX4346 alone. Once successfully engineered, more experiments could be carried out to evaluate male mating competitiveness of any *Adh*-female-specific strains.

Genetic engineering in insects is a promising tool for Pest Management with the development of novel genetic sexing systems for example. Also, *per se*, genetic engineering in insects provides us great opportunities to explore insect metabolic pathways outside their usual conditions and learn more out of them.

The use of alternatively-spliced genes such as *C. capitata transformer* (*tra*) and *Ae. aegypti doublesex* (*dsx*) to generate female-specific expression has been investigated in Chapter 3. Unsurprisingly, divergent conditional female specific expression was obtained with *Aedsx,* but not *Cctra* since the *transformer* gene, despite a great deal of effort, has not been identified in the dengue fever mosquito. The work described here allowed identification of elements from an alternatively spliced gene that can be used in a tetracycline repressible cassette to generate the female-specific lethal phenotype. When the tTAV element was placed downstream of the full *dsx* mini gene sequence (introns and exons) such that tTAV would be spliced in frame in females but not in males, female specific expression was observed. An additional start codon was inserted in the female specific exon to allow opening the reading frame at that precise location. Fused with tTAV, at the 3' end of the mini-gene, is an ubiquitin gene, thought to help in generating functional tTAV protein based on previous work at Oxitec Ltd (data not shown). Expression of tTAV from the the tetO-dsx-tTAV positive feedback loop alone was not enough to kill at any life stage, but female-specific lethality was observed at very late pupal stages when the tetO-dsx-tTAV elements were crossed to tetO-Reaper effector elements.

This finding is a major step in the genetic engineering of *Ae. aegypti* since the *doublesex* mini-gene cassette configuration can be used in combination with the lethal effector gene Reaper-KR (White et al., 1994) to create an all-in-one fsRIDL construct as proposed in **Figure 3.13**. I have also generated female specific fluorescence expression that can be used as a sexing strain, in combination with classical SIT. A fluorescence sorter able to distinguish fluorescence patterns in mosquito larvae has been developed, but sorting the fluorescent female larvae from the male larvae on site would mean providing a flow-cytometry sorter for each release site which would be costly (Marois et al., 2012). Also, using classical sterilization methods via irradiation on genetically modified males might seem paradoxical since one does not avoid the political difficulties associated with either the GM or radiation. Perhaps most interestingly, sex-specificity can also be combined with novel inducible or repressible systems such as ADH or the Auxin based system (Nishimura et al., 2009).

In Chapter 4, attempts to combine the *DmAdh* gene together with the *Aedsx* elements failed to generate female-specific lethalily induced by larval exposure to ethanol. As previously discussed, we believed the repressible lethal system configuration based on *doublesex* alternative splicing should be successful when tTAV is replaced by the *DmAdh* gene. Causes of the failure of the system may have been multiple: whether the efficiency of the alternative splicing is to blame, the production of aberrant *Ubiquitin-DmAdh* proteins generating toxicity under standard conditions, the combination of either phenomena, or something else completely is unknown.

Despite this failure, another configuration can be tried to create a GSS using the ADH system: instead of fusing the elements *Ubiquitin* and *DmAdh*, we could use the full intron and exon sequence of the *DmAdh* gene as in the original *Adh*-only construct, OX4346, after removing its endogenous start codon

ATG, and place it downstream the *AeDsx* mini gene cassette. If unsuccessful, rather than working on a protein effector system such as the ADH one, we might be more successful with genetic systems based on transcription factors (e.g. tetracycline or auxin-based systems). When induction is successful but lethality fails, such systems can be improved by crossing them to effector constructs.

Releasing RIDL male mosquitoes in the field is a reality (Harris et al., 2011, Harris et al., 2012, and Lacroix et al., 2012), however the treated areas were small, and the question remains: will scaling up the production of RIDL mosquitoes be possible? The fecundity assessment of the transgenic strain OX513A, the candidate strain for suppression trials in Chapter 5 is reassuring: OX513A female and male size is similar to that of the wild-type laboratory strain, and so is the egg production per female. Current mass rearing estimations of a variant of the OX513A strain (namely the ‘Latin’ strain) are about 70 eggs per female, per week. Moreover, the production capacity in Brazil rose from 0.5 to 4 million males per week and need targeting 20 million eggs per week in the near future for suppression programmes (Andrew McKemey, personal communication). While the size of our transgenic mosquitoes is similar, and tetracycline appeared not to affect female fecundity when supplemented at 30 µg.ml^{-1}, one particular result in Chapter 5 raised new questions: the loss of 34.5% viability of the OX513A eggs in comparison to the wild-type strain after two and half months of storage in the insectary. Is this trend common to other RIDL-like strains? If yes, it might suggest a leak of tTAV, impacting the survival of RIDL eggs in storage, and this needs special attention: it can lead to selection of resistances to the tTAV system, and could be prevented by providing tetracycline throughout the life cycles (adding tetracycline in the sugar water as well as in the larval water, for a start). Also, improving the egg storage conditions might help keep them longer (e.g. sterilizing the surface of the eggs). Rearing quality insects remains central for the success of SIT-related programmes, and gains can be made by improving general survival throughout the mosquito life cycle by developing and improving the larval diet (Damiens et al., 2012), adjusting tetracycline dosage (E. V. Ridley PhD thesis), improving adult maintenance by sugar solution supplemented with antibiotics and/or fungicides, cage systems integrating resting spaces, controlling the sex ratio and density in cages, and optimizing blood and sugar meal availability. All of these variables can be explored in the laboratory, but field studies assessing impacts they have on release cohorts may be necessary to make real gains in efficiency and cost effectiveness of a release. Developing *Ae. aegypti* mass rearing procedures and technology together with insect quality remains an on-going challenge calling for further researches.

8. Annexe

8.1. Summary tables of survival to pupation of *Adh*-transformed larvae (OX4346) when reared under standard conditions and long-term exposure to ethanol (Chapter 3)

Transgenic line tested	Phenotype	No. of male pupae	Prop.	No. of female pupae	Prop.	χ^2	P-value
OX3599	Total	1000	77%	307	23%	1.51	0.219
	GM	509	51%	491	49%	0.29	0.591
	WT	155	50%	152	50%	0.01	0.909
Adh-F males	Total	703	52%	645	48%	2.41	0.121
	GM	480	70%	204	30%	110.60	**0.000**
	WT	223	34%	441	66%	70.90	**0.000**
Adh-F females	Total	696	51%	668	49%	0.53	0.465
	GM	344	49%	355	51%	0.14	0.705
	WT	352	53%	313	47%	2.17	0.141
Adh-D males	Total	542	51%	511	49%	0.86	0.355
	GM	258	53%	226	47%	1.99	0.159
	WT	284	50%	285	50%	0.00	1.000
Adh-B males	Total	702	50%	696	50%	0.02	0.894
	GM	625	88%	82	12%	415.50	**0.000**
	WT	77	11%	614	89%	415.80	**0.000**
Adh-B females	Total	770	54%	661	46%	8.15	**0.004**
	GM	377	54%	316	46%	5.20	0.023
	WT	393	53%	345	47%	2.99	0.084
Adh-A	Total	525	58%	381	42%	22.60	**0.000**
	GM	245	54%	207	46%	3.03	0.082
	WT	280	62%	174	38%	24.30	**0.000**
Adh-G	Total	432	59%	298	41%	24.23	**0.000**
	GM	223	61%	142	39%	17.53	**0.000**
	WT	209	57%	156	43%	7.41	**0.007**
Adh-H	Total	463	52%	426	48%	1.46	0.227
	GM	222	51%	212	49%	0.19	0.666
	WT	241	53%	214	47%	1.49	0.223
Adh-N	Total	453	50%	455	50%	0.05	0.817
	GM	230	48%	247	52%	0.54	0.464
	WT	223	52%	208	48%	0.46	0.500
Adh-O	Total	502	52%	457	48%	2.02	0.155
	GM	201	43%	262	57%	7.78	**0.005**
	WT	240	48%	256	52%	0.45	0.501

Table 8.1. Summary table of Adh-transformed and wild-type proportions as well as sex-ratios for each strain obtained with the OX4346 construct.

The number of male and female as well as *Adh*-transformed (GM) and wild-type (WT) pupae are obtained from crosses between OX4346 heterozygote and wild-type individuals; Male and female ratio deviates from the expected 1:1 as evaluated by χ^2 when P-values < 0.05 (Prop.); Number of replicates = 5 with 300 of starting first instar larvae in each replicate (except for line *Adh*-A: 200 of starting first instar larvae per replicate).

Strain	**EtOH**	**Pupae (%)**		No of pupae		Mean No of pupae		SE mean		χ²	P-value
	(% v/v)	**GM**	**WT**	GM	WT	GM	WT	GM	WT		
Adh-F	0	**98%**	**95%**	735	716	147	143.2	0.3	0.3	0.2	0.637
1st assay	1	**0%**	**60%**	0	451	0	90.2	0	0.4	448	0
Adh-F	0	**107%**	**90%**	800	677	160	135.4	0.5	0.5	10.1	0.002
2nd assay	0.5	**14%**	**53%**	106	399	21.2	79.8	0.8	0.4	169	0
	1	**0%**	**38%**	0	287	0	57.4	0	0.7	285	0
	1.5	**0%**	**7%**	0	55	0	11	0	1.9	53	0
	2	**0%**	**0%**	0	2	0	0.4	0	0.6	0.5	0.48
Adh-F males	0	**91%**	**89%**	684	664	136.8	132.8	0.4	0.2	0.3	0.605
	0.5	**1%**	**36%**	5	273	1	54.6	1	2	256	0
	1	**0%**	**37%**	0	279	0	55.8	0	1.2	277	0
Adh-D	0	**85%**	**88%**	639	660	127.8	132	0.5	0.3	0.3	0.579
	0.5	**19%**	**85%**	144	638	28.8	127.6	1.4	0.1	311	0
	1	**0%**	**74%**	0	553	0	110.6	0	0.5	551	0
	1.5	**0%**	**2%**	0	17	0	3.4	0	0.8	15.1	0
	2	**0%**	**2%**	0	14	0	2.8	0	0.6	12.1	0.001
Adh-A	0	**90%**	**91%**	452	454	90.4	90.8	0.2	0.1	0	0.973
	0.5	**73%**	**85%**	364	425	72.8	85	1.1	1.2	4.6	0.033
	1	**0%**	**89%**	2	444	0.4	88.8	0.4	0.3	436	0
Adh-B	0	**77%**	**88%**	579	659	115.8	131.8	0.5	0.4	5	0.025
	0.5	**4%**	**84%**	29	629	5.8	125.8	0.6	0.5	545	0
	1	**0%**	**77%**	0	577	0	115.4	0	0.3	575	0
	1.5	**0%**	**33%**	1	249	0.2	49.8	0.4	1.2	244	0
	2	**0%**	**0%**	0	3	0	0.6	0	0.5	1.3	0.248
Adh-G	0	**66%**	**66%**	359	357	119.7	119	10.5	11.5	0.001	0.97
	0.5	**69%**	**78%**	302	463	100.7	154.3	8.4	3.5	33.5	0
	1	**0%**	**52%**	0	468	0	156	0	0.6	466	0
Adh-H	0	**95%**	**95%**	430	449	143.3	149.7	5.8	9.2	0.37	0.544
	0.5	**33%**	**63%**	141	473	47	157.7	10.7	3.2	178	0
	1	**5%**	**48%**	22	424	7.3	141.3	6.8	5.8	360	0
Adh-N	0	**97%**	**96%**	473	425	157	142	5.36	11	2.46	0.117
	0.5	**48%**	**63%**	224	427	75	142	0.88	7.3	63	0
	1	**19%**	**56%**	83	457	28	152	5.66	3.3	257	0
Adh-O	0	**100%**	**100%**	503	432	168	144	5.33	1.5	5.24	0.022
	0.5	**12%**	**48%**	68	399	23	133	21.7	15.4	233	0
	1	**0%**	**46%**	0	415	0	138	0	8.7	413	0

Table 8.2. Number of pupae survival to long term exposure to ethanol for *Adh*-transformed lines.

Transgenic (GM) and wild-type (WT) proportions deviates from the expected 1:1 as evaluated by χ ², df 1 when P-values < 0.05 (values in bold); Number of replicates = 5 with 300 of starting first instar larvae in each replicate (except for line *Adh*-A: 200 of starting first instar larvae per replicate).

Strain	**EtOH**	**Pupae (%)**		No of pupae		Mean No of pupae		SE mean		χ^2	P-value
	(% v/v)	**GM**	**WT**	GM	WT	GM	WT	GM	WT		
OX3599	0	**89%**	**82%**	1000	307	200	61.4	0.17	0.31	1.5121	0.2188
	0.5	**89%**	**92%**	1002	344	200.4	68.8	0.44	0.46	0.1941	0.6594
	1	**63%**	**61%**	712	230	142.4	46	2.22	1.42	0.1415	0.7067
WT colony 1st assay	0	**-**	**84%**	0	1260	0	252	0	0.1	-	-
	0.5	**-**	**81%**	0	1214	0	242.8	0	0.7	-	-
	1	**-**	**89%**	0	1329	0	265.8	0	0.3	-	-
WT colony 2nd assay	0	**-**	**98%**	0	1465	0	293	0	0.2	-	-
	0.5	**-**	**90%**	0	1355	0	271	0	0.6	-	-
	1	**-**	**80%**	0	1197	0	239.4	0	1.5	-	-

Table 8.3. Pupal survival following long term exposure to ethanol for the original wild-type (WT) laboratory strain.

Transgenic (GM) and wild-type (WT) proportions deviates from the expected 0.75:0.25 enriched OX3599 line (see Material and Methods) as evaluated by χ^2, df 1 when P-values < 0.05 (values in bold); Number of replicates = 5 with 300 first instar larvae in each replicate.

8.2. Comparison of survival to pupation and adulthood of heterozygous larvae transformed with OX3097 construct (Chapter 4)

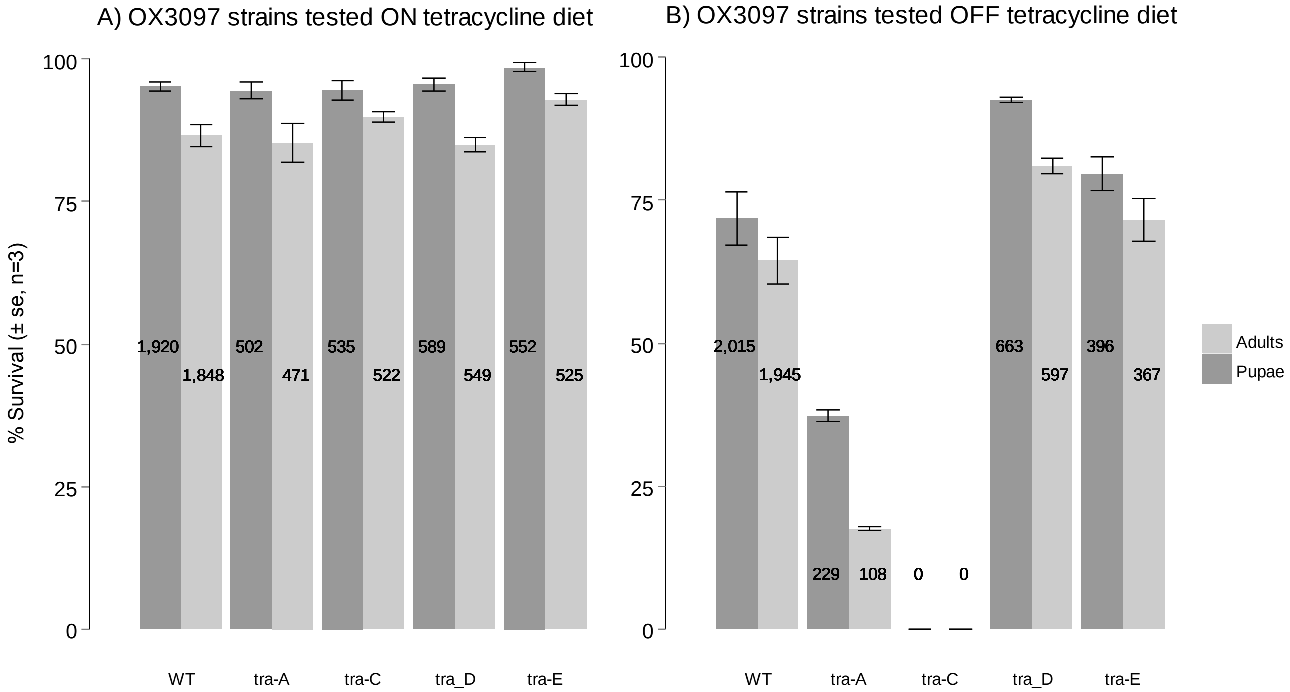


Figure 8.1. Survival of progeny expressing the Hr5*ie1*-DsRed2 (*tra* lines) marker to pupae and adults compared with wild-type (WT) progeny modulated by the presence (A) or absence (B) of tetracycline in the larval diet.

Mean percentage survival to pupae and adults for the heterozygous transgenic strains transformed with the *tra*-transformed larvae from OX3097 construct (tra-A, C, D and E) as well as *Ae. aegypti* wild-type (WT) as a control. Mean percentage is calculated out of the three assays (n=3) repeated in presence of 30 µg.ml^{-1} tetracycline (A) as well as in absence of tetracycline (B) in the larval rearing water. Error bars represent the relative standard error of the mean percentage survival to pupation as well as the mean percentage survival to adulthood.

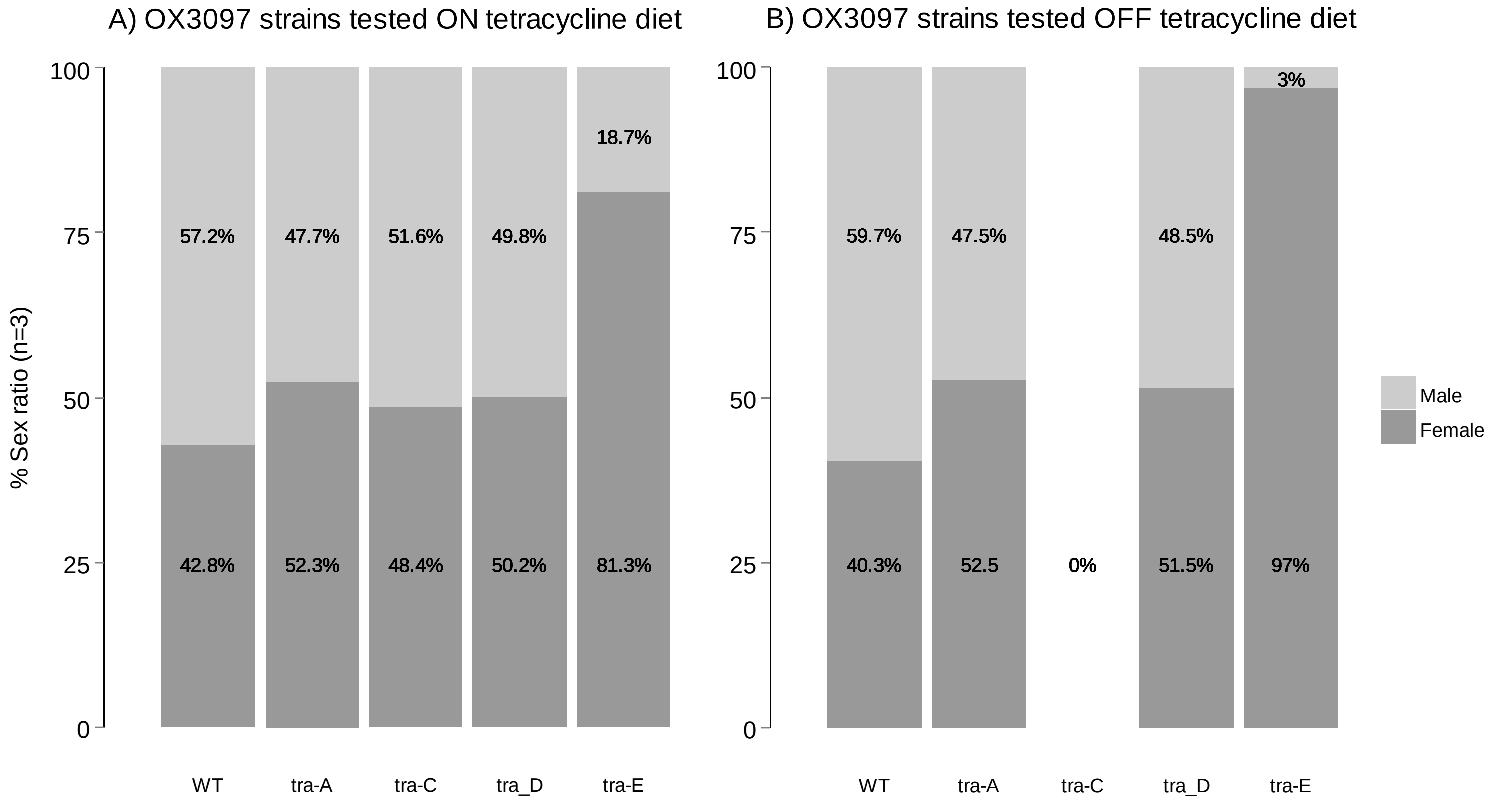


Figure 8.2 Sex ratio of the adult progeny male and female expressing the Hr5*ie1*-DsRed2 (*tra*-lines) marker compared with wild-type (WT) progeny modulated by the presence (A) or absence (B) of tetracycline in the larval diet.

For each strain (WT and *tra* lines), the number of live male and female adult was recorded and the resulting ratio of sexes plotted (numbers shown inside each bar).

Glossary of Terms

3xP3 – Three times *Pax-6* artificial promoter

Act5C – Cytosqueletal Actin gene from *Drosophila melanogaster*

Adh – Drosophila melanogaster Alcohol dehydrogenase gene

ADH-F – Alcohol Dehydrogenase Fast allozyme

ADH-S – Alcohol Dehydrogenase Slow allozyme

AeAct4 – Aedes aegypti Actin 4 gene

Aedsx – Aedes aegypti Doublesex gene

AeMuAc – Aedes aegypti Muscle Actin gene

ALDH – Aldehyde Dehydrogenase

Amcyan - *Anemonia majano* cyan fluorescent protein variant

ANCOVA - Analysis of covariance

ANOVA - Analysis of variance

Bora-Bora strain – *Aedes aegypti Bti* susceptible laboratory-cultured World Health Organization (WHO) reference

Bti – Bacillus thurigiensis var. *israeliensis*

Bs – Bacillus sphaericus

Cc – Ceratitis capitata

cM - Centimorgan

DDT – Dichlorodiphenyltrichloroethane

DF – Dengue Fever

df – degree of freedom

DHF – Dengue Hemorragic Fever

Dm – Drosophila melanogaster

DsRed – Discosoma Red fluorescent protein eGFP – Enhanced Green Fluorescent Protein

EtOH - Ethanol

fsRIDL – Female Specific Release of Insects carrying a Dominant Lethal

G_0 – generation zero of individuals surviving microinjections for genetic transformation

G_1 – next generation of individuals from microinjected parents

Gal4 – Galactose induced gene

GLM – General Linear Model

GM – Genetically modified

Gono – Gonotrophic cycle

GSSs – Genetic Sexing Strains

Hr5 / *ie1* – Enhancer and immediate early promoter from baculovirus

hsp – Heat Shock Promoter

IFM – Indirect flight muscle

K10 – Keratine 10 gene from *Mus musculus* species

KW – Kruskall Wallis test

L1, L2, L3 and L4 – Mosquito first, second, third and fourth larval instar

LiTOX strain – *Aedes aegypti* resistant strain to toxic leaf litter containing *Bti*

MSSP a2 – Male Specific Serum Polypeptide alpha 2

MWU – Mann Whitney U test

pB – *piggyBac*

PCR – polymerase chain reaction

Pg – Pectinophora gossypiella

Px – Plutella xylostella

RH – Relative Humidity

RIDL – Release of Insects carrying a Dominant Lethal

Rockefeller strain – *Aedes aegypti* laboratory-cultured international reference for experimental research established in Rockefeller Institute of Medical Research at Princeton, New York, USA, around 1935 (Kuno 2010)

RT-PCR – Reverse transcriptase PCR

SDR – Short-chain Dehydrogenases Reductases

SE – Standard Error (statistics)

SIT – Sterile Insect Technique

SV40 - *Simian vacuolating* virus 40

Tet – Chlortetracycline hydrochloride

tetO – Tetracycline Operator

tra – *Transformer* gene

tRE – Tetracycline Response Element

TSL – Temperature Sensitive Lethal strain

tTAV – Tetracycline controlled TransActiVator protein

UAS – Upstream Activation Sequence

Ubi - Ubiquitin

ULV – Ultra Low Volumes

VVD – fungal light sensor protein Vivid

VP16 – herpex simplex Virus Protein

WHO – World Health Organisation

WT – Wild-type

Bibliography

Abbott W. 1925. A method of computing the effectiveness of an insecticide. *J Econ Entomol,* 18(2):265-7.

Ansari MA, Singh KRP, Brooks GD, Malhotra PR. 1977a. A device for separation of pupae from larvae of aedes aegypti (diptera: Culicidae). *J Med Entomol,* 14(2):241-3.

Ansari MA, Singh KR, Brooks GD, Malhotra PR, Vaidyanathan V. 1977b. The development of procedures and techniques for mass rearing of aedes aegypti. *Indian J Med Res,* 65 Suppl:91-9.

Ant T, Koukidou M, Rempoulakis P, Gong HF, Economopoulos A, Vontas J, Alphey L. 2012. Control of the olive fruit fly using genetics-enhanced sterile insect technique. *BMC Biology,* 10(1):51.

Ashburner M. 1998. Speculations on the subject of alcohol dehydrogenase and its properties in drosophila and other flies. *Bioessays,* 20(11):949-54.

Atkinson MP, Su Z, Alphey N, Alphey L. 2007. Analyzing the control of mosquito-borne diseases by a dominant lethal genetic system. *Proceedings of the National Academy of Sciences*, 104(22), pp.9540–9545.

Atkinson PW. 2009. Vector biology, ecology and control. Springer Verlag.

Bailey DL, Lowe RE, Dame DA, Seawright JA. 1980. Mass rearing the genetically altered MACHO strain of anopheles albimanus wiedemann. *Am J Trop Med Hyg,* 29(1):141-9.

Balestrino F, Benedict M, Gilles J. 2012. A new larval tray and rack system for improved mosquito mass rearing. *J Med Entomol,* 49(3):595-605.

Balestrino F, Gilles JRL, Soliban SM, Nirschl A, Benedict QE, Benedict MQ. 2011. Mosquito mass rearing technology: A cold-water vortex device for continuous unattended separation of anopheles arabiensis pupae from larvae. *J Am Mosq Control Assoc,* 27(3):227-35.

Barbosa P and Peters TM. 1973. Some effects of overcrowding on the respiration of larval aedes aegypti. *Entomol Exp Appl,* 16(2):146-56.

Bargielowski, I. 2010. PhD Thesis: Sexual ecology of transgenic mosquitoes *Stegomyia (Aedes) aegypti.* Imperial College London.

Bargielowski I, Nimmo D, Alphey L, Koella JC. 2011a. Comparison of life history characteristics of the genetically modified OX513A line and a wild type strain of aedes aegypti. *PloS One,* 6(6):e20699.

Bargielowski I, Alphey L, Koella JC. 2011b. Cost of mating and insemination capacity of a genetically modified mosquito aedes aegypti OX513A compared to its wild type counterpart. *PloS One,* 6(10):e26086.

Bargielowski I, Kaufmann C, Alphey L, Reiter P, Koella J. 2012. Flight Performance and Teneral Energy Reserves of Two Genetically-Modified and One Wild-Type Strain of the Yellow Fever Mosquito Aedes aegypti. *Vector-Borne and Zoonotic Diseases*, 12(12):1053-8.

Becker N, Petric D, Zgomba M, Boase C, Madon M, Dahl C, Kaiser A. 2010. Mosquitoes and their control. Springer.

Bellini R, Calvitti M, Medici A, Carrieri M, Celli G, Maini S. 2007. Use of the sterile insect technique against Aedes albopictus in Italy: first results of a pilot trial. In M. J. B. Vreysen, A. S. Robinson, & H. J. Dordrecht, eds. Springer, pp. 505–516.

Benedict MQ and Robinson AS. 2003. The first releases of transgenic mosquitoes: An argument for the sterile insect technique. *Trends Parasitol,* 19(8):349-55.

Berghammer AJ, Klingler M, Wimmer EA. 1999. A universal marker for transgenic insects. *Nature,* 402(6760), pp.370–371.

Betrán E and Ashburner M. 2000. Duplication, dicistronic transcription, and subsequent evolution of the Alcohol dehydrogenase and Alcohol dehydrogenase-related genes in Drosophila. *Molecular Biology and Evolution*, 17(9), pp.1344–1352.

Boyer S, Gilles J, Merancienne D, . 2011. Sexual performance of male mosquito Aedes albopictus. *Medical and Veterinary Entomology*, 25(4), pp.454–459.

Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, Moyes CL, Farlow AW, Scott TW, Hay SI. 2012. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. R. Reithinger, ed. *PLoS neglected tropical diseases*, 6(8), p.e1760.

Caceres C. 2002. Mass rearing of temperature sensitive genetic sexing strains in the mediterranean fruit fly (ceratitis capitata). *Genetica*, 116(1):107-16.

Canning E, Penrose M, Barker I, Coates D. 1996. Improved detection of barley yellow dwarf virus in single aphids using RT-PCR. *J Virol Methods*, 56(2):191-7.

Canyon DV and Muller R. 1999. Effect of diet on biting, oviposition, and survival of aedes aegypti (diptera: Culicidae). *J Med Entomol*, 36(3):301.

Carvalho E, Solferini VN, Matioli SR. 2009. Alcohol dehydrogenase activities and ethanol tolerance in anastrepha (diptera, tephritidae) fruit-fly species and their hybrids. *Genetics and Molecular Biology*, 32(1):177-85.

Catteruccia F, Benton JP, Crisanti A. 2005. An anopheles transgenic sexing strain for vector control. *Nat Biotechnol*, 23(11):1414-7.

Chamberlin M, McGrath J, Waskell L. 1970. New RNA polymerase from Escherichia coli infected with bacteriophage T7. *Nature*, 228(5268), pp.227–231.

Christophers SR. 1960. *Aëdes aegypti (L.), the yellow fever mosquito: its life history, bionomics, and structure*, Cambridge University Press.

Christophides GK, Savakis CA, Mintzas C, Komitopoulou K. 2001. Expression and function of the drosophila melanogaster ADH in male ceratitis capitata adults: A potential strategy for medfly genetic sexing based on gene-transfer technology. *Insect Mol Biol,* 10(3):249-54.

Christophides GK, Livadaras I, Savakis C, Komitopoulou K. 2000. Two medfly promoters that have originated by recent gene duplication drive distinct sex, tissue and temporal expression patterns. *Genetics*, 156(1):173-82.

Clements AN. 1963. The physiology of mosquitoes. Pergamon Press ed. Oxford: .

Clements AN and Potter SA. 1967. The fine structure of the spermathecae and their ducts in the mosquito aedes aegypti. *J Insect Physiol,* 13(12):1825-36.

Clements AN. 1992. The biology of mosquitoes. volume 1. development, nutrition and reproduction. Chapman & Hall, London.

Cohn VH and Moore GP. 1988. Organization and evolution of the alcohol dehydrogenase gene in Drosophila. *Molecular Biology and Evolution*, 5(2), pp.154–166.

Curtis, C.F., Akiyama J, Davidson G. 1976. A genetic sexing system in Anopheles gambiae species A. *Mosquito News*, 36(4), pp.492–498.

Dafa'alla T, Fu G, Alphey L. 2010. Use of a regulatory mechanism of sex determination in pest insect control. *Journal of Genetics*, 89(3):301-5.

Dame DA, Lowe RE, Williamson DL. 1981. Assessment of released sterile anopheles albimanus and glossina morsitans morsitans. In: Cytogenetics and genetic of vectors: Proceedings of a symposium of the XVIth international congress of entomology. . 231 p.

Damiens D, Benedict M, Wille M, Gilles J. 2012. An inexpensive and effective larval diet for anopheles arabiensis (diptera: Culicidae): Eat like a horse, a bird, or a fish? *J Med Entomol,* 49(5):1001-11.

David JR, Van Herrewege J, De Scheemaeker-Louis M, Pla E. 1981. Drosophila alcohol dehydrogenase: Detoxification of isopropanol and acetone, substances not used in energy metabolism. *Heredity*, 47(2):263-8.

Domingues C and Ryoo HD. 2012. Drosophila BRUCE inhibits apoptosis through non-lysine ubiquitination of the IAP-antagonist REAPER. *Cell Death and Differentiation*, 19(3), pp.470–477.

Dyck VA, Hendrichs J, Robinson AS. 2005. Sterile insect technique: Principles and practice in area-wide integrated pest management. Dordrecht, The Netherlands: Springer.

Erickson JW and Quintero JJ. 2007. Indirect effects of ploidy suggest X chromosome dose, not the X: A ratio, signals sex in drosophila. *PLoS Biology*, 5(12):e332.

Fay RW and Morlan HB. 1959. A mechanical device for separating the developmental stages, sexes and species of mosquitoes. *Mosq News,* 19:144-7.

Fay RW, McCray Jr EM, Kilpatrick JW. 1963. Mass production of sterilized male aedes aegypti. *Mosq News,* 23:210-4.

Fletcher TS, Ayala FJ, Thatcher DR, Chambers GK. 1978. Structural analysis of the ADHS electromorph of drosophila melanogaster. *Proceedings of the National Academy of Sciences,* 75(11):5609-12.

Focks DA. 1980. An improved separator for the developmental stages, sexes, and species of mosquitoes (diptera: Culicidae). *J Med Entomol*, 17(6).

Franco M, Seyfried NT, Brand AH, Peng J, Mayor U. 2011. A novel strategy to isolate ubiquitin conjugates reveals wide role for ubiquitination during neural development. *Molecular & Cellular Proteomics*, 10(5).

Franz G. 2005. Genetic sexing strains in mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique. Springer. 427 p.

Fraser MJ, Smith GE, Summers MD. 1983. Acquisition of Host Cell DNA Sequences by Baculoviruses: Relationship Between Host DNA Insertions and FP Mutants of Autographa californica and Galleria mellonella Nuclear Polyhedrosis Viruses. *J Virol*, 47(2), pp.287–300.

Fu G, Lees RS, Nimmo D, Aw D, Jin L, Gray P, Berendonk TU, White-Cooper H, Scaife S, Phuc HK, et al. 2010. Female-specific flightless phenotype for mosquito control. *Proc Natl Acad Sci USA*, 107(10):4550-4.

Fu G, Condon KC, Epton MJ, Gong P, Jin L, Condon GC, Morrison NI, Dafa'alla TH, Alphey L. 2007. Female-specific insect lethality engineered using alternative splicing. *Nature Biotechnology*, 25(3):353-7.

Gempe T and Beye M. 2011. Function and evolution of sex determination mechanisms, genes and pathways in insects. *Bioessays,* 33(1):52-60.

Gibbons RV and Vaughn DW. 2002. Dengue: An escalating problem. *Br Med J,* 324(7353):1563-6.

Gibson JB, May TW, Wilks AV. 1981. Genetic variation at the alcohol dehydrogenase locus in drosophila melanogaster in relation to environmental variation: Ethanol levels in breeding sites and allozyme frequencies. *Oecol*, 51(2):191-8.

Glare TR and O'Callaghan M. 1998. Environmental and health impacts of bacillus thuringiensis israelensis. Wellington, NZ: Report to the Ministry of Health.

Gong P, Epton MJ, Fu G, Scaife S, Hiscox A, Condon KC, Condon GC, Morrison NI, Kelly DW, Dafa'alla T, et al. 2005. A dominant lethal genetic system for autocidal control of the mediterranean fruitfly. *Nat Biotechnol,* 23(4):453-6.

Gossen M and Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA*, 89(12):5547.

Goujon MM. 2010. A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res,* 38.

Gratz NG. 1991. Emergency control of aedes aegypti as a disease vector in urban areas. *J Am Mosq Control Assoc*, 7(3):353-65.

Gubler D. 2005. The emergence of epidemic dengue fever and dengue hemorrhagic fever in the americas: A case of failed public health policy. *Pan Am J Public Health*, 17(4):221-4.

Gubler DJ. 1989. Aedes aegypti and aedes aegypti-borne disease control in the 1990s: Top down or bottom up. *Am J Trop Med Hyg,* 40(6):571-8.

Gubler DJ. 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol,* 10(2):100-3.

Gubler DJ. 2012. The economic burden of dengue. *Am J Trop Med Hyg*, 86(5):743-4.

Gubler DJ. 2011. Emerging vector-borne flavivirus diseases: Are vaccines the solution? *Expert Rev Vaccines*, 10(5):563-5.

Guo R and Ren J. 2010. Alcohol and acetaldehyde in public health: From marvel to menace. *International Journal of Environmental Research and Public Health*, 7(4):1285-301.

Harrington LC and Edman JD. 2001. Indirect evidence against delayed “skip-oviposition” behavior by aedes aegypti (diptera: Culicidae) in thailand. *J Med Entomol*, 38(5):641-5.

Harris AF, Nimmo D, McKemey AR, Kelly N, Scaife S, Donnelly CA, Beech C, Petrie WD, Alphey L. 2011. Field performance of engineered male mosquitoes. *Nat Biotechnol,* 29(11):1034-7.

Harris AF, McKemey AR, Nimmo D, Curtis Z, Black I, Morgan SA, Oviedo MN, Lacroix R, Naish N, Morrison NI. 2012a. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nat Biotechnol*, 30(9):828-30.

Harris AF, McKemey AR, Nimmo D, Curtis Z, Black I, Morgan SA, Oviedo MN, Lacroix R, Naish N, Morrison NI, et al. 2012b. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nat Biotechnol*, 30(9):828-30.

Hay B, Jan LY, Jan YN. 1988. A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. *Cell*, 55(4), pp.577–587.

Heinrich JC and Scott MJ. 2000. A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proc Natl Acad Sci USA*, 97(15):8229-32.

Heinrich JC, Li X, Henry RA, Haack N, Stringfellow L, Heath AC, Scott MJ. 2002. Germ-line transformation of the australian sheep blowfly lucilia cuprina. *Insect Mol Biol*, 11(1):1-10.

Heinstra PWH, Eisses KT, Schoonen W, Aben W, Winter AJ, Horst DJ, Marrewijk WJA, Beenakkers AMT, Scharloo W, Thörig GEW. 1983. A dual function of alcohol dehydrogenase in drosophila. *Genetica*, 60(2):129-37.

Helinski MEH, Hassan MM, El-Motasim WM, Malcolm CA, Knols BG, El-Sayed B. 2008. Towards a sterile insect technique field release of Anopheles arabiensis mosquitoes in Sudan: Irradiation, transportation, and field cage experimentation. *Malaria Journal*, 7.

Helinski MEH and Harrington LC. 2011. Male mating history and body size influence female fecundity and longevity of the dengue vector aedes aegypti. *J Med Entomol*, 48(2):202-11.

Hemingway J and Ranson H. 2000. Insecticide resistance in insect vectors of human disease. *Annu Rev Entomol*, 45(1):371-91.

Hickey WA and Craig Jr GB. 1966. Genetic distortion of sex ratio in a mosquito, aedes aegypti. *Genetics*, 53(6):1177.

Hoe N, Huang CM, Landis G, Verhage M, Ford D, Yang J, van Leeuwen FW, Tower J. 2011. Ubiquitin over-expression phenotypes and ubiquitin gene molecular misreading during aging in drosophila melanogaster. *Aging* (Albany NY) 3(3):237.

Ito J, Ghosh A, Moreira LA, Wimmer EA, Jacobs-Lorena M. 2002. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature*, 417(6887):452-5.

Jin L, Walker AS, Fu G, Harvey-Samuel T, . 2013. Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology*, 2(3), pp.160–166.

Jörnvall H, Persson B, Krook M, Atrian S, Gonzalez-Duarte R, Jeffery J, Ghosh D. 1995. Short-chain dehydrogenases/reductases (SDR). *Biochemistry* (N Y) 34(18):6003-13.

Kaplan EL, and Meier P. 1958. Nonparametric estimation from incomplete observations. *Journal of the American statistical association*, 53(282), pp.457–481.

Kim KY, Kwon SY, Lee HS, Hur Y, Bang JW, Kwak SS. 2003. A novel oxidative stress-inducible peroxidase promoter from sweetpotato: Molecular cloning and characterization in transgenic tobacco plants and cultured cells. *Plant Mol Biol*, 51(6):831-8.

Klassen W and Curtis C. 2005. History of the sterile insect technique. In: Sterile insect technique. principles and practice in area-wide integrated pest management. Dyck, V.A.; Hendrichs, J.; Robinson, A.S. ed. Dordrecht, The Netherland: Springer. 3 p.

Knipling EF. 1959. Sterile-male method of population control. *Science*, 130:902-4.

Koureas M, Tsakalof A, Tsatsakis A, Hadjichristodoulou C. 2012. Systematic review of biomonitoring studies to determine the association between exposure to organophosphorus and pyrethroid insecticides and human health outcomes. *Toxicology letters*, 210(2), pp.155–168.

Kuno G. 2010. Early history of laboratory breeding of Aedes aegypti (Diptera: Culicidae) focusing on the origins and use of selected strains. *Journal of medical entomology,* 47, pp. 957–971.

Labbé GMC, Scaife S, Morgan SA, Curtis ZH, Alphey L. 2012. Female-specific flightless (fsRIDL) phenotype for control of aedes albopictus. *PLoS Neglected Tropical Diseases*, 6(7):e1724.

Lacey LA and Orr BK. 1994. The role of biological control of mosquitoes in integrated vector control. *Am J Trop Med Hyg,* 50(6 Suppl):97.

Lacroix R. 2012. Open field release of genetically engineered sterile male aedes aegypti in malaysia. *PLoS One*, 7(8):e42771.

Lang CA, Basch KJ, Storey RS. 1972. Growth, composition and longevity of the axenic mosquito. *J Nutr,* 102(8):1057-66.

Lees R. 2008. PhD Thesis: Developing transgenic *Aedes aegypti* for a Release of Insects with a Dominant Lethal (RIDL) programme. University of Oxford.

Lis JT, Simon JA, Sutton CA. 1983. New heat shock puffs and β-galactosidase activity resulting from transformation of drosophila with an hsp70-lacZ hybrid gene. *Cell*, 35(2 I):403-10.

Lofgren CS, Dame DA, Breeland SG, Weidhaas DE, Jeffery G, Kaiser R, Ford HR, Boston MD, Baldwin KF. 1974. Release of chemosterilized males for the control of anopheles albimanus in el salvador. III. field methods and population control. *Am J Trop Med Hyg*, 23(2):288-97.

Lowe RE, Fowler JEF, Bailey DL, Dame DA, Savage KE. 1981. Separation of sexes of adult anopheles albimanus by feeding of insecticide-laden blood. *Mosq News*, 41(4):634-8.

Magnusson K, Mendes AM, Windbichler N, Papathanos PA, Nolan T, Dottorini T, Rizzi E, Christophides GK, Crisanti A. 2011. Transcription regulation of sex-biased genes during ontogeny in the malaria vector anopheles gambiae. *PloS One*, 6(6):e21572.

Marois E, Scali C, Soichot J, Kappler C, Levashina EA, Catteruccia F. 2012. High-throughput sorting of mosquito larvae for laboratory studies and for future vector control interventions. *Malaria Journal*, 11(1):302.

Matentzoglu K and Scheffner M. 2009. Ubiquitin-fusion protein system: A powerful tool for ectopic protein expression in mammalian cells. *BioTechniques*, 46(1):21-8.

McCray RMJ. 1961. A mechanical device for the rapid sexing of *aedes aegypti* pupae. *J Econ Entomol*, 54(4):819.

McDonald JH and Kreitman M. 1991. Adaptive protein evolution at the Adh locus in Drosophila. *Nature*, 351(6328), pp.652–654.

McKechnie SW and Morgan P. 1982. Alcohol dehydrogenase polymorphism of drosophila melanogaster: Aspects of alcohol and temperature variation in the larval environment. *Aust J Biol Sci*, 35(1):85-94.

Morris AC, Eggleston P, Crampton JM. 1989. Genetic transformation of the mosquito aedes aegypti by micro-injection of DNA. *Med Vet Entomol*, 3(1):1-7.

Nasci RS. 1990. Relationship of wing length to adult dry weight in several mosquito species (diptera: Culicidae). *J Med Entomol*, 27(4):716-9.

Nishimura KK. 2009. An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nature Methods*, 6(12).

Olson KE, Higgs S, Gaines PJ, Powers AM, Davis BS, Kamrud KI, Carlson JO, Blair CD, Beaty BJ. 1996. Genetically engineered resistance to dengue-2 virus transmission in mosquitoes. *Science*, 272(5263):884-6.

Papathanos PA, Bossin HC, Benedict MQ, Catteruccia F, Malcolm CA, Alphey L, Crisanti A. 2009. Sex separation strategies: Past experience and new approaches. *Malar J*, 8(Suppl 2):S5.

Paris M, Boyer S, Bonin A, Collado A, David JP, Despres L. 2010. Genome scan in the mosquito Aedes rusticus: Population structure and detection of positive selection after insecticide treatment. *Molecular ecology*, 19(2), pp.325–337.

Paris M, David JP, Despres L. 2011a. Fitness costs of resistance to bti toxins in the dengue vector aedes aegypti. *Ecotoxicology*, 20(6):1184-94.

Paris M, Tetreau G, Laurent F, Lelu M, Despres L, David JP. 2011b. Persistence of bacillus thuringiensis israelensis (Bti) in the environment induces resistance to multiple Bti toxins in mosquitoes. *Pest Manag Sci*, 67(1):122-8.

Park K and Kwak IS. 2009. Alcohol dehydrogenase gene expression in Chironomus riparius exposed to di (2-ethylhexyl) phthalate. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 150(3), pp.361–367.

Patterson RS, Weidhaas DE, Ford HR, Lofgren CS. 1970. Suppression and elimination of an island population of culex pipiens quinquefasciatus with sterile males. *Science*, 168(3937):1368-9.

Perry MJ, Venners SA, Barr DB, Xu X. 2007. Environmental pyrethroid and organophosphorus insecticide exposures and sperm concentration. *Reproductive toxicology*, 23(1), pp.113–118.

Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ, Pape G, Fu G, Condon KC, Scaife S, Donnelly CA, et al. 2007. Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology*, 5:11.

Pialoux G, Gauzere BA, Jaureguiberry S, Strobel M. 2007. Chikungunya, an epidemic arbovirosis. *Lancet Infect Dis*, 7(5):319-27.

Poupardin R, Riaz MA, Jones CM, Chandor-Proust A, Reynaud S, David JP. 2012. Do pollutants affect insecticide-driven gene selection in mosquitoes? experimental evidence from transcriptomics. *Aquatic Toxicology*, 114:49-57.

Prasad H, Prasad RN, Haq S. 1993. Control of mosquito breeding through gambusia affinis in rice fields. *Indian J Malariol*, 30(2):57-65.

R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rendon P, McInnis D, Lance D, Steward J. 2000. Comparison of medfly male-only and bisexual releases in large scale field trials. In: Area-wide control of fruit flies and other insect pests. TanK-H, editor. Pulau Pinang, Penerbit Universiti Sains Malaysia. pp. 517–525.

Rendon P, McInnis D, Lance D, Stewart J. 2004. Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *J Econ Entomol*, 97(5), pp.1547–1553.

Rezza G. 2009. Chikungunya and west nile virus outbreaks: What is happening in north-eastern italy. *Eur J Public Health*, 19(3):236-7.

Ridley EV. 2011. PhD Thesis: The impact of chlortetracycline on *Drosophila melanogaster* and *Aedes aegypti*. University of York.

Robinson AS and Van Heemert C. 1981. Genetic sexing in drosophila melanogaster using the alcohol dehydrogenase locus and a Y-linked translocation. *Theor Appl Genet*, 59(1):23-4.

Robinson AS and Van Heemert C. 1982. Ceratitis capitata—a suitable case for genetic sexing. *Genetica*, *58*(3), pp. 229-237.

Robinson AS, Riva ME, Zapater M. 1986. Genetic sexing in the mediterranean fruit fly, ceratitis capitata, using the alcohol dehydrogenase locus. *Theor Appl Genet*, 72(4):455-7.

Robinson A and Heemert C. 1981. Genetic sexing in drosophila melanogaster using the alcohol dehydrogenase locus and a Y-linked translocation. *TAG Theoretical and Applied Genetics*, 59(1):23-4.

Robinson A, Riva M, Zapater M. 1986. Genetic sexing in the mediterranean fruit fly, ceratitis capitata, using the alcohol dehydrogenase locus. *TAG Theoretical and Applied Genetics*, 72(4):455-7.

Robinson AS. 2002. Genetic sexing strains in medfly, ceratitis capitata, sterile insect technique programmes. *Genetica*, 116(1):5-13.

Rodems SM and Friesen PD. 1993. The hr5 transcriptional enhancer stimulates early expression from the autographa californica nuclear polyhedrosis virus genome but is not required for virus replication. *Journal of Virology,* 67(10):5776-85.

Ruiz MF, Milano A, Salvemini M, Eirin-Lopez JM, Perondini ALP, Selivon D, Polito C, Saccone G, Sanchez L. 2007. The Gene *Transformer* of *Anastrepha* Fruit Flies (Diptera, Tephritidae) and Its Evolution in Insects. *PLoS ONE*, 2(11), p.e1239.

Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, Suvannadabba S, Jiwariyavej V, Dulyachai W, Pengsaa K, Wartel TA, et al. 2012. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in thai schoolchildren: A randomised, controlled phase 2b trial. *Lancet*, 380(9853):1559-67.

Saccone, G., Salvemini, M. & Polito, L.C., 2011. The transformer gene of Ceratitis capitata: A paradigm for a conserved epigenetic master regulator of sex determination in insects. *Genetica*, 139(1), pp.99–111.

Salghetti SE, Caudy AA, Chenoweth JG, Tansey WP. 2001. Regulation of transcriptional activation domain function by ubiquitin. *Science Signalling*, 293(5535):1651.

Salvemini M, Mauro U, Lombardo F, Milano A, Zazzaro V, Arcà B, Polito LC, Saccone G. 2011. Genomic organization and splicing evolution of the doublesex gene, a drosophila regulator of sexual differentiation, in the dengue and yellow fever mosquito aedes aegypti. *BMC Evolutionary Biology*, 11(1):41.

Schmitz J, Roehrig J, Barrett A, Hombach J. 2011. Next generation dengue vaccines: A review of candidates in preclinical development. *Vaccine*, 29(42):7276-84.

Seawright JA, Kaiser PE, Dame DA, Lofgren CS. 1978. Genetic method for the preferential elimination of females of anopheles albimanus. *Science*, 200(4347):1303-4.

Sharma VP, Patterson RS, Ford HR. 1972. A device for the rapid separation of male and female mosquito pupae. *Bull World Health Organ*, 47(3):429.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, et al. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using clustal omega. *Molecular Systems Biology*, 7:539.

Sinkins SP and Hastings IM. 2004. Male-specific insecticide resistance and mosquito transgene dispersal. *Trends Parasitol*, 20(9):413-6.

Somwang P, Yanola J, Suwan W, Walton C, Lumjuan N, Prapanthadara L, Somboon P. 2011. Enzymes-based resistant mechanism in pyrethroid resistant and susceptible aedes aegypti strains from northern thailand. *Parasitol Res*, 109(3):531-7.

Spencer WF, Singh G, Taylor CD, LeMert RA, Cliath MM, Farmer WJ. 1996. DDT persistence and volatility as affected by management practices after 23 years. *J Environ Qual*, 25(4):815-21.

Steinwascher K. 1982. Relationship between pupal mass and adult survivorship and fecundity for aedes aegypti. *Environ Entomol*, 11(1):150-3.

Steinwascher K. 1984. Egg size variation in aedes aegypti : Relationship to body size and other variables. *Am Midl Nat*, 112(1).

Swann Report. Report of the Joint Commitee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine. 1969. London.

Tan A, Fu G, Jin L, Guo Q, Li Z, Niu B, Meng Z, Morrison NI, Alphey L. 2013. Transgene-based, female-specific lethality system for genetic sexing of the silkworm, Bombyx mori. *Proceedings of the National Academy of Sciences*, 110(17), pp.6766–6770.

Teixeira, C.F., Augusto, L.G. da S. & Morata, T.C., 2003. Saúde auditiva de trabalhadores expostos a ruído e inseticidas. *Rev Saúde Pública*, 37(4), pp.417–423.

Tetreau G, Bayyareddy K, Jones CM, Stalinski R, Riaz MA, Paris M, David JP, Adang MJ, Després L. 2012a. Larval midgut modifications associated with bti resistance in the yellow fever mosquito using proteomic and transcriptomic approaches. *BMC Genomics*, 13(1):248.

Tetreau G, Alessi M, Veyrenc S, Périgon S, David JP, Reynaud S, Després L. 2012b. Fate of bacillus thuringiensis subsp. israelensis in the field: Evidence for spore recycling and differential persistence of toxins in leaf litter. *Appl Environ Microbiol*, 78(23):8362-7.

Tetreau G, Bayyareddy K, Jones CM, Stalinski R, Riaz MA, Paris M, David J-, Adang MJ, Després L. 2012c. Larval midgut modifications associated with bti resistance in the yellow fever mosquito using proteomic and transcriptomic approaches. *BMC Genomics*, 13(1).

Thibault ST, Luu HT, Vann N and Miller TA. 1999. Precise excision and transposition of piggyBac in pink bollworm embryos. *Insect Molecular Biology*, 8(1), pp.119–123.

Thomas DD, Donnelly CA, Wood RJ, Alphey LS. 2000. Insect population control using a dominant, repressible, lethal genetic system. *Science*, 287(5462):2474-6.

Trpiš M. 1970. A new bleaching and decalcifying method for general use in zoology. *Can J Zool*, 48(4):892-3.

Wang X, Chen X, Yang Y. 2012. Spatiotemporal control of gene expression by a light-switchable transgene system. *Nature Methods*, 9(3):266-9.

Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, 25(9):1189-91.

Weaver R.F. 2011. *Molecular Biology*, fifth ed. McGraw-Hill International Companies, Inc. NY, p. 892.

Webster DP, Farrar J, Rowland-Jones S. 2009. Progress towards a dengue vaccine. *Lancet Infect Dis*, 9(11):678-87.

White K, Grether ME, Abrams JM, Young L, Farrell K, Steller H. 1994. Genetic control of programmed cell death in drosophila. *Science*, 264(5159):677.

WHO. 2011. Yellow fever. Fact sheet N°100. [Online]. Available from http://www.who.int/mediacentre/factsheets/fs100/en/ [Accessed December 2012]

WHO. 2012. Dengue and severe dengue. *Fact sheet N°117*. [Online]. Available from http://www.who.int/mediacentre/factsheets/fs117/en/ [Accessed December 2012]

Wilke ABB, Nimmo DD, St John O, Kojin BB, Capurro ML, Alphey L. and Marrelli, MT. (2009). Mini-review: Genetic enhancements to the sterile insect technique to control mosquito populations. *Asian-Pacific Journal of Molecular Biology and Biotechnology*, 17(3): 65-74.

Woke PA, Ally MS, Rosenberger. 1956. The numbers of eggs developed related to the quantities of human blood ingested in aedes aegypti (L.)(diptera: Culicidae). *Ann Entomol Soc Am*, 49(5):435-41.

Xiao H and Lis JT. 1989. Heat shock and developmental regulation of the drosophila melanogaster hsp83 gene. *Mol Cell Biol*, 9(4):1746-53.

Yamada H, Benedict MQ, Malcolm CA, Oliva CF, Soliban SM, Gilles JRL. 2012. Genetic sex separation of the malaria vector, Anopheles arabiensis, by exposing eggs to dieldrin. *Malaria Journal*, 11:208.

Zhao L, Becnel JJ, Clark GG, Linthicum KJ. 2010. Expression of AeaHsp26 and AeaHsp83 in aedes aegypti (diptera: Culicidae) larvae and pupae in response to heat shock stress. *J Med Entomol*, 47(3):367-75.