Sexual ecology of transgenic mosquitoes
*Stegomyia (Aedes) aegypti*

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Abstract

*Aedes aegypti* is the main vector of the virus that causes dengue fever and its more severe form dengue hemorrhagic fever. As traditional control methods have been unable to prevent its global re-emergence as a significant threat to human health, the development of new control methods is gaining importance. One possibility is a genetics-based control strategy modelled on the traditional sterile insect technique (SIT). The RIDL system (Release of Insects carrying a Dominant Lethal) is such an approach, and has been engineered in *Ae. aegypti* with tetracycline-dependent repression of a dominant lethal gene construct.

This thesis examines some of the aspects of *Aedes aegypti* mating ecology and behaviour that are relevant for the implementation of SIT-based control programmes, focusing on the competitive fitness of the genetically modified males. The transformed mosquitoes differed from unmodified mosquitoes with a similar genetic background with regard to several life history traits. Though the modified mosquitoes pupated earlier - which may be useful in the mass-rearing of such insects - most of the differences suggest reduced competitive performance of the modified males. These included reduced larval survival, adult longevity, insemination capacity and flight ability. In caged mating trials the modified males were less competitive than their wild type counterparts in direct competition for females. Genetically modified mosquitoes were generally smaller than unmodified mosquitoes reared at high larval densities, highlighting the value of optimising rearing conditions as females preferentially selected larger males for mating. Females exhibited no propensity to re-mate over several gonotrophic cycles, unless they had been mated to sperm-depleted males, in which case secondary insemination was common.

In conclusion, genetic manipulation reduced the performance of mosquitoes. However, their competitive disadvantage could be compensated for by high over-flooding ratios upon release. Therefore, accurate estimates of competitive ability, as presented in this thesis, are essential if this control strategy is to prove successful.
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Author’s Declaration

I declare that all the work presented in this thesis is my own original research with the following acknowledgements. The energetic reserves presented in Chapter 4 were measured by Dr Christian Kauffmann (Vector Entomology, Institute of Parasitology, University of Zürich, Zürich, Switzerland.)

Supervisor
Professor Jacob Koella
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Chapter 1

1 Introduction

1.1 Aedes aegypti – necessity for control and review of traditional control methods

*Aedes aegypti* (or *Stegomyia aegypti*) is a predominantly urban, anthropophilic, day-biting mosquito species and is one of the most common vectors of various tropical and subtropical diseases, thus posing a substantial threat to human health in infected areas. In terms of its devastating social and economic effects, some of the most damaging diseases transmitted by *Ae. aegypti* are dengue fever and its more severe form dengue hemorrhagic fever (mortality as high as 30% if not properly managed (described by Nimmannitya 1997)), which are now endemic to most tropical countries as well as some sub-tropical countries and consequently present a major public health problem (Halstead 1980; Rosen 1982; Gubler 1994). Current estimates suggest dengue fever is endemic in at least 100 countries. In addition, imported cases have been reported in several non-endemic countries (Domingo, Palacios et al. 2004), thus placing approximately 40% of the world population (2.5 billion people) at risk (WHO 2009). Dengue fever and dengue hemorrhagic fever are recognised as re-emerging diseases with their incidence increasing dramatically throughout the world in recent decades in line with the expansion in their geographic distribution (WHO 2009).

To date, no specific vaccine for the treatment of dengue fever has been developed. Other major diseases transmitted by *Ae. aegypti* include yellow fever, chikungunya fever and Rift Valley fever. The majority of yellow fever infections result in symptoms ranging from high fevers, nausea, vomiting, loss of appetite, muscle pain, headaches and shivers. Although most patients recover within a matter of days, 15% of patients enter a ‘toxic phase’ of infection resulting in death in 50% of these cases (WHO 2009). The yellow fever virus is responsible for 200,000 recorded cases and 30,000 deaths every year, almost all of which occur in Africa (WHO 2009). The mosquitoes are an active reservoir for the disease as vertical transmission is common and infected, desiccation-resistant eggs can harbour the disease throughout the dry season until hatching when the rains
begin, consequently ensuring transmission from one year to the next. The most effective protection against yellow fever is the live virus 17-D yellow fever vaccine which was developed in 1937 by Max Theiler and confers immunity for a period of up to ten years. Though successful population-wide immunisation has been conducted in some instances (e.g. in Trinidad and Tobago, Gambia; (WHO 2009)) very few countries in Africa have achieved the 80% vaccination rate required to prevent outbreaks from spreading.

Chikungunya fever virus was first isolated from a patient in Tanzania in 1953, but is now present in most of Africa, parts of Southeast Asia and India. The first incidence of the disease spreading to Europe was reported from north-eastern Italy in August 2007 (Enserink 2007a; Enserink 2007b). The word ‘chikungunya’ translates as ‘that which bends up’ describing the contorted posture of sufferers experiencing severe joint pain. As yet, no vaccine or cure is available and treatment usually comprises fluids and analgesics (WHO 2008).

Finally, even though Rift Valley fever primarily affects livestock and brings about significant economic losses due to the death and extremely high abortion rates among infected animals, it can also be transmitted to humans and cause severe illness, vision loss, neurological impairment or death. The majority of human infections result from direct contact with the blood of infected animals (in abattoirs) as well as through inhalation of aerosols produced during slaughter. Yet, infections have also been reported following the bites of infected mosquitoes (mainly *Aedes* species) as well as other hematophagous flies (WHO 2008).

As yet there is no licensed, commercially available vaccine against Rift Valley fever.

Due to the absence of effective vaccines or treatments for many of the diseases transmitted by *Ae. aegypti*, as with the examples listed above, the only suitable method of disease control is population reduction or vector elimination.

Effective vector reduction and eradication programmes have proven this principle in the past. Examples include the highly successful campaign directed by the Pan American Sanitary Board from 1946 to 1970 (Schliessmann and Calheiros, 1974) and vector control operations in Cuba in 1981 (Kouri et al., 1981). These programmes employed a rigorous, top-down approach of intensive insecticidal treatment coupled with reduction of available habitats (source reduction). A similar programme, with a very strong source reduction component, successfully kept dengue fever at very low incidence for 15 years in Singapore (Chan, 1985), though the disease has re-emerged in the last decade.
With few exceptions, commonly used, traditional methods of control (often employed in response to epidemic outbreaks (‘fire-fighting’), rather than as preventative measures) are not effective at curbing the widespread re-emergence of dengue fever.

The most widespread approach is the use of insecticides. These can be applied either to breeding sites as larvicides or they can target adult mosquitoes as part of thermal fogging programmes. The use of larvicides for controlling *Ae. aegypti* is problematic due to the multiplicity and inaccessibility of their breeding sites. The main environments for breeding sites are created in areas with inadequate infrastructure and potable water supply, thus making rainwater harvesting necessary (Barrera and Gonzales-Tellez 1993; Barrera, Mora et al. 1995). In combination with inadequate storage techniques, the constant replenishment of ideal breeding conditions is inevitable and comprehensive larvicidal treatment unrealistic.

Thermal fogging outdoors or space-spraying indoors are further possible insecticidal strategies. In cases of reported outbreaks and high densities of adult mosquitoes they can be an effective tool for dramatically reducing adult numbers, particularly in urban areas. Yet, they can also be costly measures with only transient and limited impact on adult mosquito populations and disease transmission. Used as a general method of control, i.e. routine spraying of a specific area, they have been shown to be relatively ineffective, as the majority of adults, particularly the females, will rest indoors and consequently escape insecticide fogging (Mani 2005; Reiter 2007). Even when indoor spraying is conducted concurrently, not all houses and buildings will be sprayed, leaving refuge areas for mosquitoes to survive in. This is particularly problematic as even very small numbers (0.5-1.5 per person) of mosquitoes have been shown to support the transmission of disease (Focks, Brenner et al. 2000).

In addition to the practical difficulties affecting these methods, increased insecticide resistance is hampering any sustained and effective use of insecticides for vector control. A further common approach to mosquito control is water – or oviposition site management. Any receptacle containing water is a possible oviposition site for gravid females. This means an effective drive to eliminate such sites can be a valuable instrument in reducing mosquito numbers. Such control programmes require a coordinated community effort, usually backed by an education campaign. If conducted properly such programmes can be highly successful (Yasuoka 2006), especially in urban areas where most breeding sites are artificial. However, poverty stricken, rural areas with unreliable supplies of potable water may struggle to deliver the close control of breeding
sites necessary for this method to be successful in reducing the numbers of disease-transmitting adult mosquitoes (Barrera and Gonzales-Tellez 1993; Barrera, Mora et al. 1995).

As opposed to their usage in the fight against other, mostly nocturnally transmitted vector-borne diseases such as malaria, treated bed nets have not been used extensively as a control strategy for *Ae. aegypti*, as this species is diurnally active. A recent study conducted in Haiti (Lenhart, Orelus et al. 2008), however, showed bed nets to have a negative impact on diurnally active dengue vector populations, thus raising the possibility of integrating treated bed nets in systematic dengue control programmes. Even though these findings are encouraging, issues concerning insecticide resistance and adequate area-wide coverage will remain intrinsic problems of bed net use as a control strategy.

Finally, biological control methods, the use of larvivorous fish for instance, such as *Claris fuscus*, *Tilapia nilotica*, and *Macropodus spp.* have successfully been used to manage *Aedes* larvae (Neng, Shusen et al. 1987). Besides fish, various insects are adapted as predators of mosquito larvae. In Myanmar, dragonfly larvae, for example, have been used successfully to target *Ae. aegypti* larvae (Sebastian, Sein et al. 1990). Copepods of the genus *Mesocyclops* have also shown potential as successful biological control agents of *Aedes* larvae (Riviere, Kay et al. 1987; Vu, Nguyen et al. 2005) and have proven successful as part of a large scale community-based approach to *Aedes* control in Vietnam (Marten, 2000).

However, attempts at larvicidal control in general are subject to specific drawbacks. For instance, the destruction of only a portion of the larvae at any one site will not always lead to a reduction in the number of adults emerging from this site, as this stage of development is strongly governed by density-factors. This means the removal of a proportion of the larvae will release resources for the remaining individuals, leading to the emergence of larger, more productive adults (Agudelo-Silva and Spielman 1984; Washburn, Mercer et al. 1991).

Though still in the trial-phase of development, the recent discovery of a strain of *Wolbachia* that inhibits the ability of a range of pathogens (including the dengue virus) to infect *Ae. aegypti* (Moreira et al. 2009) and significantly shortens the life span of adult mosquitoes (McMeniman et al. 2009), may provide a promising bio-control tool for vector management.

Finally, the production of ‘biological’ larvicides that utilise the toxins produced by bacteria, such as *Bacillus thuringiensis israelensis*, or plant extracts (Spielman and...
Lemma 1973; Minjas and Sarda 1986; Green, Singer et al. 1991) can be used as species-specific, environmentally friendly control methods (Lacey and Undeen 1986). Nevertheless, as with chemical larvicides, the emergence of resistance is a problem (Boyer, Tilquin et al. 2007).

1.2 Development of new methods (RIDL – Release of Insects carrying a Dominant Lethal)

Traditional control methods as described above have shown only limited success in their application and are still plagued by a variety of drawbacks thus the need for new approaches in this field is evident.

In 1982 Rubin and Spradling achieved the first transformation of *Drosophila melanogaster* using transposable element vectors (Rubin and Spradling 1982). This new technology, referred to as transposon-mediated germ line transformation, enabled insertional integration of a predetermined DNA fragment, flanked by the ends of a transposable element, into the genome of the chosen host insect (Alphey and Andreasen 2002). By the mid 90s non-*Drosophila* species such as the medfly had been successfully transformed utilising this technology (Loukeris, Livadaras et al. 1995). This was shortly followed by insects of medical importance such as *Aedes aegypti* in 1998 (Coates, Jasinskiene et al. 1998; Jasinskiene, Coates et al. 1998), *Anopheles stephensi* in 2000 (Catteruccia, Nolan et al. 2000) and *Culex quinquefasciatus* in 2001 (Allen, O’Brochta et al. 2001).

The development of this technology in vectors of disease such as the mosquitoes mentioned above paves the way for new approaches to their control and population suppression.

Two genetic-based strategies can be envisaged for use in vector control programmes. The first, known as population replacement, aims to introduce gene sequences into the natural population, rendering it refractory to disease transmission. The second, known as population suppression or elimination, is modelled on the traditional sterile insect technique (SIT) and involves the release of engineered insects that produce non-viable progeny, thus suppressing, or if enough insects are released over a sufficient amount of time, eliminating the target population (Thomas, Donnelly et al. 2000). The mosquitoes
used and studied in this thesis are engineered following the principles of this latter approach.

The RIDL system (release of insects carrying a dominant lethal) is such a strategy (Thomas, Donnelly et al. 2000; Alphey 2002; Alphey, Nimmo et al. 2008; Alphey, Benedict et al. 2010). Strains of *Ae. aegypti* have been engineered using tetracycline-dependent repression of a dominant lethal gene (Phuc, Andreasen et al. 2007; Fu, Lees et al. 2010). Tetracycline can be introduced as a dietary supplement for mosquitoes reared in the laboratory, but is not readily available in the wild; hence the lethal system is repressed in the laboratory and activated upon release. Upon their release, transformed males, which are homozygous for this lethal construct, would pass one copy of the dominant lethal to their offspring by normal Mendelian inheritance. These would subsequently die as larvae or pupae in the wild due to the absence of tetracycline. The method takes advantage of the insects’ intrinsic mate-seeking behaviour, thus making it an effective tool in the control of species difficult to target with more conventional methods.

This system offers various improvements to the SIT strategy it is based on.

Firstly, irradiation is no longer needed to sterilise males. Although some field trials showed chemically or genetically sterilised males could successfully compete with males in the wild (measured by egg batch sterility) (Lofgren, Dame et al. 1974; Grover, Suguna et al. 1976), many field trials were unsuccessful due to the fitness effects of sterilisation (reviewed in Benedict and Robinson 2003).

Secondly, late-acting lethality, as described above, in a species limited by density dependent effects, can be significantly more effective than conventional SIT (Phuc, Andreasen et al. 2007). As the larval stages of *Ae. aegypti* are under strong density dependent pressure any reduction in their numbers will benefit the surviving larvae by releasing valuable, limited resources. Therefore, late-acting lethality is preferable so that the RIDL larvae will compete for food and space with the wild population.

Thirdly, transgenic mosquitoes can be engineered to carry fluorescent markers, allowing easy identification upon recapture and reliable monitoring of a release programme, while making it possible to dispense with the use of fluorescent dust which, apart from being expensive and possibly detrimental to the performance of released males, can affect the health of workers in the mass-rearing facility (Robinson, Franz et al. 2004).
Finally, a refinement of the RIDL system, female-specificity of the lethal system, would eliminate the need for manual pupal sorting. Sex-separation can be achieved by mechanical sorting on the basis of pupal size or time of eclosion but is rarely 100% effective and can be time consuming. Several sexing systems that used classical genetics relied on the translocation of a dominant selectable marker (e.g. temperature sensitivity) onto the Y-chromosome (Seawright, Kaiser et al. 1978; Hendrichs, Franz et al. 1995; Robinson 2002), however, these chromosomal aberrations impacted the fitness of the carrier and thus proved successful only within limits. A genetics-based sexing system is therefore desirable. In this case, the promoter used to control expression of the lethal gene would need to be female-specific. This would function in the same way as described above, with females surviving under permissible conditions, i.e. with tetracycline in their diet, and dying under restrictive conditions, i.e. the absence of tetracycline. In this way a colony could be kept in the mass rearing facility under permissible conditions and once ready for release, tetracycline would be omitted from the diet of the final generation. The females would die and again genetically sterile males, homozygous for the lethal construct, could be released into the field. The daughters of these males will die in the wild, while the sons, heterozygous for the lethal gene, will survive to mate again. In the following generation half the daughters will die as they will inherit the lethal gene and half the sons again will inherit the lethal system and so forth. In this way, the controlling effect of RIDL is exerted over several generations in the wild population leading to more efficient population suppression or eradication.

1.3 Description of mosquito lines used in this thesis

1.3.1 Wild type line (WT)

The WT line originates from field-caught *Aedes aegypti* from Jinjang, Selangor, Malaysia. It was colonised in 1975, and has been held in the laboratory for many generations. It can therefore be considered a highly lab-adapted strain and is unlikely to be representative of field bred males; however it was chosen because of its genetic similarity to the modified RIDL lines (see below).
1.3.2  **OX513A – bi-sex lethal line**

OX513A is a homozygous RIDL line of *Ae. aegypti*, transformed with a tetracycline repressible, lethal positive feedback system (Phuc, Andreasen et al. 2007). A tetracycline-repressible transcriptional transactivator (tTAV) (Gossen and Bujard 1992; Gong, Epton et al. 2005) under the control of its own binding site (tetO) creates a positive feedback loop. Consequently, the absence of tetracycline leads to high levels of tTAV production and possibly, due to the interaction of the VP16 domain with key transcription factors (Phuc, Andreasen et al. 2007), to increased cell toxicity (transcriptional squelching) (Lin, McGrath et al. 2007) in the genetically modified mosquitoes, leading to their death. The addition of tetracycline, on the other hand, leads tTAV to bind tetracycline, in which form tTAV can no longer bind to tetO and the cycle is interrupted (Phuc, Andreasen et al. 2007).

Mosquitoes of this line are identifiable by red fluorescence due to the expression of DSRed2 under the control of an Act5C promoter (Phuc, Andreasen et al. 2007). The OX513A line was originally created in the Rockefeller strain and subsequently out-crossed into a Mexican line of *Ae. aegypti*. It has since been crossed to the WT line described above in such a fashion that at least 97-99% of their genome should correspond.

1.3.3  **OX3604 – female-specific flightless line**

OX3604 is a homozygous line of *Ae. aegypti*, transformed with a tetracycline repressible, sex-specific system that produces a ‘flightless’ phenotype in females (Fu, Lees et al. 2010). In this line the production of tTAV is controlled by the AeACT-4 promoter in combination with a sex-specifically modified alternative splicing region, targeting gene expression only in the indirect flight muscles (IFMs) of females. Consequently males of this line can survive without the addition of tetracycline to their larval diet.

Mosquitoes of this line are identifiable by all-over-body red fluorescence due to the expression of DSRed2 and blue eyes due to the expression of 3xP3-AmCyan (Fu, Lees et al. 2010).

This line was created in the WT line and thus has a similar genetic background.
1.3.4 OX3878 – sperm marker line

OX3878 is a sperm marker line created in *Aedes aegypti*. In this line, a promoter of the male germline, beta2-tubulin, a testis specific tubulin, drives the production of a GFP (green fluorescent protein) marker. Additionally the construct carries a promoter-marker set conferring red fluorescence in the eyes, P3DsRed2. Both males and females of this line can therefore be distinguished by selecting for ‘red eyes’, while the males produce distinctively florescent (green) sperm.

OX3878 was created in a ‘site-specific’ line. This ‘site-specific’ line contains a docking site, attB, into which foreign gene constructs can be inserted (for details see Nimmo, Alphey et al. 2006) and was created in an Asian wild type (Bangkok) genetic background.

1.4 Need for assessing the performance of RIDL mosquitoes and importance of species specific knowledge of mating behaviour for SIT-based strategies

Although highly successful SIT programmes have been carried out against a number of pest species, such as the eradication of the New World screw worm *Cochliomyia hominovorax* in the Americas (Wyss 2000), the control of an outbreak of screw worm in Libya in 1989 (Lindquist, Abusowa et al. 1992), the Mediterranean fruit fly (medfly) *Ceratitis capitata* in Latin America (Hendrichs, Franz et al. 1995) and the tsetse fly (*Glossina* spp.) in parts of Africa (e.g. in Zanzibar (1997) (Msangi, Saleh et al. 2000), successes of SIT programmes applied to mosquito control have been sparse in comparison.

Releases of mosquitoes, even sterile ones, would preferably be restricted to males, as the repeated release of large numbers of females might increase biting nuisance as well as the transmission of disease. Furthermore, the simultaneous release of sterile females may reduce the efficiency of the release programme as sterile males may be distracted from seeking out wild females. Consequently, it is the performance of male mosquitoes that is of paramount importance to SIT-based control strategies. Mating opportunities will only present themselves to males that are able to disperse from the release sight seek out females, live long enough to do so and successfully court females once they are
encountered. Furthermore, the ability to induce refractory behaviour in mated females may play a role in *Ae. aegypti*.

The importance of establishing a basic understanding of the mating ecology and behaviour of a species that is to be targeted by SIT-based control programmes is made clear in a review of past SIT trials by Benedict and Robinson (2003). This review examined 27 trials in total, 15 of which had the aim of reducing or eliminating local mosquito populations (Benedict and Robinson 2003). Only three of these trials could be classed as successful (Laven 1967; Laven, Cousserans et al. 1972; Curtis, Brooks et al. 1982), with the most common reason for failure being identified as behavioural incompatibilities and inferior mating competitiveness of sterilised males upon release (Ferguson, John et al. 2005). Other reasons included the immigration of mated females into the control area as well as the production and release of insufficient numbers of sterile males.

Despite their obvious importance for the success and design of release programmes these aspects of mosquito quality have not yet been assessed for RIDL lines (Alphey, Benedict et al. 2010).

It is worthwhile to note that it is not necessary for RIDL males to be equally as competitive with wild type males as a deficiency in competitive ability may be overcome by releasing more sterile males. However, knowledge of exactly how competitive RIDL males are compared with their wild type counterparts will allow a more precise estimate of the numbers needed for a successful control programme. Control programmes in the past have failed due to the release of too few males (Benedict and Robinson 2003). However, a threshold value for the necessary competitive ability of released *Ae. aegypti* males has not yet been estimated.

Finally, knowledge of the mating ecology of a species may also offer possibilities of improvement to an SIT-based control programme. In the case of the Mediterranean fruit fly *Ceratitis capitata* for example, mass-reared sterile males generally have poorer mating competitiveness than do the wild type (Barry, McInnis et al. 2003), but providing males with a protein-rich diet before release (Yuval 2002) as well as exposing males to ginger root oil (Shelly and McInnes 2001), greatly enhances mating success.
1.5 Possible fitness costs affecting RIDL mosquitoes

Even though insects that are genetically modified for sterility with dominant lethal systems such as RIDL are predicted to suffer less fitness costs than their irradiated counterparts, certain fitness burdens may remain. Three main sources can be identified: (i) costs associated with the transgene, (ii) costs associated with inbreeding (line creation) and (iii) costs associated with mass rearing.

1.5.1 Costs associated with the transgene

The act of inserting a foreign gene into a genome may, depending on its insertion site, disrupt native gene function (Spradling 1995; Spradling 1999; Thibault 2004). Severe forms of this insertional mutagenesis are likely to be fatal. Other cases may however only become apparent in the homozygous state, as heterozygosity will offer a buffer, masking the detrimental effect of the insertion event. Encouragingly, observations show most non-fatal insertions to have little to no effect on fitness due to insertional mutagenesis, possibly because they either integrate into regions that do not encode genes or do not substantially disrupt gene function (Lyman 1996; Marrelli, Moreira et al. 2006).

A more significant factor may be the expression of alien proteins by the integrated foreign genes, a build up of which may be toxic to the host cells in which they are expressed. Transgenic insects typically express multiple foreign genes and the fitness burden created by alien protein expression will depend to some extent on the nature of the protein produced (e.g. Moreira, Wang et al. 2004) and its expression pattern, with ubiquitous expression of alien proteins possibly being more damaging than tissue specific expression or life stage-specific expression.

These toxological effects are an obvious concern with the RIDL system, as one of the main functions of the transgene, at least in the OX513A line, is to kill the individual carrying it under specific conditions. The tight control of such a system is therefore important. Any basal leakiness or off-target production of tTA may have a negative impact on the genetically modified insects by increasing cell toxicity (transcriptional squelching) (Lin, McGrath et al. 2007).

Different promoter regions are used in the creation of the two RIDL lines used in this thesis; this may affect the two lines differently. The Act5C promoter used in OX513A results in ‘all over body’ expression of the gene. Furthermore the production of tTAV is driven by a non-sex-specific positive feedback system in this line. In the OX3604 line, on
the other hand, the AeACT-4 promoter is used in combination with a sex-specifically modified alternative splicing region to control tTAV production, targeting gene expression only in the IFMs of females. Consequently males of this line can survive without the addition of tetracycline to their larval diet and are far less likely to suffer excess effector production.

1.5.2 Fitness costs associated with inbreeding / genetic bottlenecks

The creation of a transgenic strain involves the initial transformation of a single individual upon which the entire line is based, whereas the production of a line homozygous for the inserted construct, needed for release, entails many generations of inbreeding. Both these processes will inevitably limit the genetic variability of the strain. To date, studies testing the fitness of transgenic mosquitoes compared to wild type counterparts in the laboratory using homozygous strains have shown a significant fitness load relative to non-transgenic lines (Catteruccia, Godfray et al. 2003; Irvin, Hoddle et al. 2004). This decline in fitness may be due to the effects of the transgene itself, as discussed above, or it could be attributable to the unwanted side effects of inbreeding to achieve homozygosity. Most genomes contain numerous recessive mutations that are capable of reducing the fitness of the carrier in a homozygous state (Simmons and Crow 1977; Halligan and Keightley 2003). The insertion of a transgene in the vicinity of such a negative recessive mutation and subsequent breeding efforts to make the strain homozygous for the inserted construct will at the same time make it homozygous for the recessive allele; this is known as the hitchhiking effect (Marrelli, Moreira et al. 2006) and can lead to the fixation of such alleles causing severe inbreeding depression. This may indeed be one of the most influential factors on the fitness of transgenics as a study by Moreira et al. (2004) comparing heterozygous transgenics to non-transgenic mosquitoes concluded no reduction of fitness for mosquitoes carrying the SM1 construct, showing that a foreign gene in itself need not negatively impact the fitness of the carrier. Heterozygous lines may therefore be useful for testing the fitness implications of the transgene, yet any mosquito destined for field release in a control programme would have to be homozygous. It is therefore vital to choose the fittest strain possible. If a hitchhiking event is suspected, out-crossing the line to wild type counterparts for multiple generations may genetically remove the deleterious allele (Marrelli, Moreira et al. 2006), yet the line would subsequently have to be made homozygous again for release.
1.5.3 **Fitness costs associated with mass rearing and distribution**

For a RIDL programme to be a sustainable success, an extremely large number of sterile mosquitoes would have to be produced continuously. If this is to be done in a cost effective manner, the rearing density of larvae would have to be as high as possible while still producing competitive, fit males. Previous studies have shown that increasing larval density has precisely the opposite effect, resulting in increased larval mortality, delayed pupation and the emergence of smaller, shorter lived, less fecund adults (Bar-Zeev 1957; Wada 1965; Gilpin and McClelland 1979; Dye 1982; Dye 1984; Agnew, Hide et al. 2002; Bedhomme, Agnew et al. 2003). Furthermore, the rearing densities examined in all previous studies have been lower than the densities considered for use in future *Aedes aegypti* rearing facilities.

Apart from the more obvious measures of fitness such as life span and body size, as mentioned above, mass rearing conditions may affect the suitability of mosquitoes for RIDL programmes in other ways, such as inducing behavioural changes. The intense crowding in mass rearing facilities may promote shorter courting and faster mating behaviour, as proposed by Rull and Mendez (2005), resulting in inconsistencies with behaviour in the wild, consequently giving rise to assortative mating upon release. Such behavioural changes have been observed to appear in a time span as short as three generation (Reisen 2003) and have been documented in previous SIT programmes such as in the control efforts of the melon fly in Japan. This example additionally illustrates the need for constant monitoring of control programmes and male fitness as the processes involved are dynamic with, in this case, assortative mating appearing 20 years after commencement of the control programme (Hibino and Iwahashi 1989; Hibino and Iwahashi 1991). An immediate and dramatic increase in the number of sterile males released, led to a disruption in the assortative mating preferences of wild females to select wild males over sterilised counterparts, thus avoiding the spread of a potential ‘sterile insect-resistant’ genotype through the wild population and thereby achieving the successful eradication of the melon fly in 1993 (Koyama and al. 2004).

A further reason constant monitoring of the release strain is necessary is the possibility of rare genetic or molecular events occurring and becoming fixed in the population. In view of the large numbers of insects reared in selective conditions, only a small selective advantage is needed for a mutation to sweep through the entire population, making the
strain unsuitable for release, or in the most extreme case, resulting in the loss of traits, i.e. sterility, for which the strain was bred (Robinson, Franz et al. 2004).

Finally, the transport and dispersal methods employed in the field may further reduce male fitness. Limiting time in transit by producing the insects close to the release site is obviously desirable, as is a minimisation of crowding and physical interference.

1.6 Current state of knowledge of *Aedes aegypti* mating ecology and behaviour

1.6.1 General description and distribution

*Aedes aegypti* is a small, dark mosquito, easily identified by its conspicuous white markings and banded legs. The scutum has a dorsal pattern of white scales in the form of a ‘lyre’ with two central stripes, thus clearly distinguishing it from *Aedes albopictus*, which is generally very similar in appearance but has only one central stripe. *Ae. aegypti* is a highly domesticated species found largely in and around human habitation, widely, but sporadically distributed throughout the tropics and subtropics between latitudes of 40ºN and 40ºS, roughly following the 20ºC isotherm. It is considered a low-density species and does not form large, station-keeping swarms, unlike many other mosquito species (Clements 1999).

1.6.2 Reproductive cycle

The developmental cycle of *Aedes aegypti* (from egg to adult) can be completed in less than 10 days under optimal conditions but can take up to three weeks in less favourable environments. Males generally emerge 1-2 days before the females (of a synchronously hatched batch) and are able to inseminate females only after their genitalia have undergone a 180° rotation, which takes 15-24 hours to complete (Roth 1948). Females are also refractory to insemination immediately after emergence and only become sexually receptive after 48-72 hours (Gwadz and Craig 1968). This lag in sexual maturity possibly serves the purpose of allowing adequate time for dissemination from the breeding site, thus minimising the risk of inbreeding (Hartberg 1971). Mating, or successful insemination at any rate, therefore does most likely not occur at the breeding site itself (Hartberg 1971), but rather at another location, most likely close to the host. This assumption is supported by the findings of numerous early studies on the feeding
and mating behaviour of *Ae. aegypti* (e.g. Teesdale 1955; Hartberg 1971). Although exceptions occurred, generally mating was recorded after the female had fed, often while the female was flying away from the host, suggesting mating takes place at or near the host. Further evidence backing this notion can be found in studies using biting catches designed to trap females (Yasuno and Tonn 1970; Hartberg 1971). Unusually, compared to biting catches of other mosquito species, a small percentage of male mosquitoes were also trapped. Furthermore, an early study undertaken by McClelland (1959) in Uganda describes witnessing several mating attempts in close proximity to hosts. Males have been observed to fly a figure of eight pattern close to the host and usually initiate mating in flight after identifying the female by sound (Roth 1948). It is possible that other attractants include visual (Sippell and Brown 1953; Fay 1968) and chemical cues (Grant 1969; Cabrera and Jaffe 2007). For this reason, the mating system more closely resembles a 3-D lekking system, as described by (Cabrera and Jaffe 2007), than a stationary swarm. Once the female has fed and mated, it takes 2-3 days for her to digest her blood meal and produce eggs; the gravid female will oviposite her eggs in or around the edges of natural pools of standing water such as puddles or, as is often the case, in water collected in containers, discarded tyres or debris. The eggs can withstand up to six months of desiccation and can therefore survive dry seasons, ready to emerge when conditions are more favourable. Once submerged, the eggs hatch and subsequently metamorphose through four morphologically similar larval instars. The larval stages are entirely aquatic with the larvae feeding on organic matter and microorganisms present in the water. As soon as the larvae have acquired enough energetic reserves they pupate and within a day or two, depending on environmental conditions, the adult mosquitoes emerge.

### 1.6.3 Mating system: female monogamy and male polygamy

Early studies on the mating behaviour of *Aedes aegypti* suggest females are monandrous (Leahy and Craig 1965; Craig 1967), thus inseminated only once over the course of a lifetime. The transferred complement of sperm is stored and used to fertilise egg batches over a number of gonotrophic cycles, the female being refractory to any secondary insemination attempts. Polygamous behaviour (acceptance of insemination by a second male) was documented under certain conditions, including forced mating and following
insemination by sperm depleted males or interruption during insemination (Christophers 1960; Gwadz and Craig 1970). Unlike many other mosquito species *Ae. aegypti* males do not form an insemination plug after mating a female, rather the refractory behaviour seen in mated females is thought to be controlled by an accessory gland substance transferred during mating known as ‘matrone’. Components of mosquito semen in general appear to be essential in regulating post mating behaviour (as is the case in *Drosophila* (Kalb, di Benedetto et al. 1993; Xue and Noll 2000)). Female behaviour, such as flight (Jones 1981; Chiba, Shinkawa et al. 1992), response to host cues (Lavoipierre 1958; Judson 1967; Hartberg 1971), oviposition (Leahy and Craig 1965; Hiss and Fuchs 1972), fertility and ovarian development (Klowden and Chambers 1991; Klowden 1993), blood digestion (Edman 1970; Downe 1975) and sexual refractoriness (Craig 1967; Fuchs, Craig et al. 1969; Sirot, Poulson et al. 2008) are all influenced to some extent by the transfer of seminal products in mosquitoes.

Conversely, more recent studies conducted in the early 80s throw this presumption of strict lifelong monogamy into question (Williams and Berger 1980; Young and Downe 1982), indicating a willingness of females to re-mate once they have completed one or more gonotrophic cycles.

The propensity of females to re-mate is relevant to an SIT-based control programme, in particular if there are differences in the ability of sterile and wild type males to induce refractory behaviour in females or in the willingness of females to re-mate preferentially if mated to a sterile male.

Males of the species *Ae. aegypti* are thought to be polygamous (Clements 1999), yet the number of females a male can inseminate in a lifetime is thought to be limited; with estimates suggesting on average 3-5.8 females in a day (Gwadz and Craig 1970; Foster and Lea 1975) and 8-9 over the course of a lifetime (Youngson, Welch et al. 1981). Little research has been carried out to identify differences in insemination capacity of different *Ae. aegypti* strains. As with the question of female monogamy, the insemination capacity of males, and any differences between the potential of sterile and wild type males is of interest in designing SIT / RIDL releases.
1.6.4 Mating cues: flight sound and semiochemical function of epicuticular hydrocarbons

Though, it is likely that the host itself is a meeting point for male and female *Aedes aegypti*, mate recognition at the host requires further stimuli, including sound (Roth 1948) and chemical cues (Horne and Priestman 2002). Mate recognition through flight sound has received particular interest recently with the publication of several journal articles (Gibson and Russell 2006; Cator, Arthur et al. 2009). The article by Cator, Arthur et al. (2009) is of particular interest as it describes the acoustic behaviour involved in mate recognition and courtship of *Ae. aegypti*. It suggests the existence of active modulation of wing beat frequencies by both sexes, creating a duet, at frequencies of 1200Hz, higher than the previously assumed limit of hearing in mosquitoes. Furthermore Cator, Arthur et al. (2009) present evidence indicating that previously inseminated females are less likely to respond to the auditory signalling of males, revealing a possible mechanism rendering females refractory to multiple inseminations.

Little work on the chemical signalling between the two sexes has been carried out, yet the characterisation of cuticular hydrocarbons of *Ae. aegypti* thought to be involved in sexual signalling (Horne and Priestman 2002) has to some extent improved current knowledge of this field. Furthermore, the discovery of quantitative differences in cuticular hydrocarbons between males and females (Horne and Priestman 2002) as well as the finding that mating indeed alters the cuticular hydrocarbons in both *Anopheles gambiae* as well as in *Aedes aegypti* females (Polerstock, Eigenbrode et al. 2002), suggests they posses semiochemical functions in signalling between the sexes.

1.6.5 Role of body size and longevity in mating success

The effect of body size on reproductive success of female *Aedes aegypti* has been the subject of a number of early studies. Although a direct relationship between body size and oocyte number (Christophers 1960) was recorded the link with actual egg production is less clear (Roy 1936 in Christophers, 1960; Christophers 1960). It is more likely that the size of the ingested blood meal is more closely correlated to the number of eggs produced than is the size of the female (Woke, Ally et al. 1956). Then again, Okanda, Dao et al. (2002) showed that male *Anopheles* preferentially selected larger females for mating, presenting evidence for male mate choice based on female size in this species.
Some research has been carried out on the effect of male size on mating success in *Anopheles gambiae* (Ng'habi, John et al. 2005; Ng'habi, Huho et al. 2008), concluding that size indeed plays an important role in male competitiveness, with mid to larger sized males being more successful than smaller ones.

Conversely, very few studies have attempted to show any link between male size and reproductive success for *Aedes aegypti*. Dickinson and Klowden (1997) measured the entire protein content of small vs. large adult males before and after mating to assess protein transfer. Small males were shown to have transferred significantly less protein than large males. Ponlawat and Harrington (2007) also found an increased sperm capacity in larger males. Yet, how these findings translate into actual fertilisation success has not yet been assessed.

The question of how longevity contributes to reproductive success in the two sexes is also worth considering. For female *Ae. aegypti* any increase in longevity may allow for the completion of further gonotrophic cycles and will therefore enhance reproductive output. For males, on the other hand, this correlation is not as apparent. If, for example, as is the case with *Anopheles* (Ng'habi, Huho et al. 2008), medium sized, rather than larger males have the highest mating success and longevity is assumed to be correlated with body size, i.e. larger males surviving longer than smaller males, then mating success itself will not necessarily be linearly correlated with longevity. On the other hand, should large males turn out to be more competitive, they may make substantially higher investment in mating, through ejaculate production for instance, than smaller males that do not get the chance to mate, possibly resulting in a trade off with overall life expectancy. For these reasons, unravelling the relationship between male reproductive success and longevity will be partly dependent on assessing other parameters of male ecology.

1.6.6 Flight / dispersal ability

Another aspect of *Ae. aegypti* behaviour that has recently been reinvestigated is their dispersal ability. It was generally the view that females cover only short distances of up to 100 metres during their lifetime (Russel 2004; Harrington, Scott et al. 2005; Russel 2005) yet rapid colonisation has been observed upon introduction into previously vector-free areas. Liew (2004) released rubidium-marked insects in both rural as well as urban settings in Singapore and recorded dispersal distances of several hundred metres in
addition to vertical dispersal throughout a tower block. The females were shown to disperse in a random rather than progressive manner, ovipositing eggs between multiple sites. This form of skip-oviposition has also been described in a recent study by Reiter (2007), showing females usually lay 1-5 eggs before moving on to the next oviposition site.

Furthermore, experiments examining the flight potential of female *Ae. aegypti*, concluded that larger, sugar fed females have greater flight potential than smaller or starved individuals (Briegel, Knuesel et al. 2001).

In contrast, the dispersal ability and flight potential of males, as with other aspects of *Aedes aegypti* biology, has lacked attention, possibly due to the difficulty of recapturing released males in the field. Yet, with renewed interest in SIT-based control programmes, male capacity for dispersal warrants further investigation.

### 1.7 Laboratory vs. field studies

An important point that needs to be taken into consideration when assessing the suitability and fitness of a transgenic line destined for release is the common disparity of results gleaned from laboratory-based and field release trials. In past SIT programmes, strains that were deemed competitive in laboratory trials very often failed to compete successfully under conditions in the wild. Chemo-sterilised *Aedes aegypti*, for example, found to be highly competitive in laboratory trials (Seawright 1975; Curtis 1976; Seawright 1976) where approximately ten times less likely to mate upon release into the wild than their endogenous counterparts in Kenya (Asman and al. 1981). Comparable results were observed for *Culex* and *Anopheles* species as discussed by Reisen (1981; 2003), again showing a significant decline in fitness in the field compared to laboratory trials. Thus, it is essential that any newly developed RIDL strains be trialled extensively both in the laboratory and especially in the field to allow exact assessment of its effectiveness at asserting a controlling effect on natural populations. Yet, the importance of adequate field trials by no means eliminates the necessity for laboratory analysis; in fact, the determination of successful predictors of field success in the laboratory would be extremely beneficial.
1.8 Aims of this thesis

In conclusion, RIDL technology represents a promising new tool in the fight against disease vectors. *Aedes aegypti* is particularly suited to such control programmes, as it is readily reared and manipulated under laboratory conditions and, as such, is suitable for mass rearing (Ansari, Singh et al. 1977). Furthermore, its relatively low density in the wild (Liew 2004) as well as its genetic similarity over large areas in the field (Gubler 1998; Gorrochotegui-Escalante, Gomez-Machorro et al. 2002; Kuwayama, Yamashita et al. 2006) renders it susceptible to eradication through RIDL. Yet, if this new means of mosquito control is to be implemented successfully and not fall into the same traps as its SIT predecessors, it is vital that thus far understudied aspects of mosquito biology pertaining to mating success, mate selection and overall fitness of transgenic mosquitoes be addressed, both in the laboratory as in the field.

The aim of this thesis is twofold. On the one hand, it aims to investigate areas of *Aedes aegypti* mating ecology and behaviour, particularly of males, which are of direct relevance to the implementation of a SIT-based control programme and have as yet received little attention; on the other hand, it aims to consistently examine the differences between OX513A, a RIDL mosquito strain destined for release in just such control programmes, and its wild type counterparts with respect to the questions of mating ecology addressed above. Throughout this thesis OX513A will be considered “a finished product”, i.e. experiments to determine its possible fitness deficits will be carried out on mosquitoes homozygous for the transgenic insertion, as this is how they would be released into the field. Although discussed in the context of this thesis, specifically identifying the proportion of fitness costs incurred due to the process of genetic manipulation itself or the creation (inbreeding) of this specific RIDL line will not be the focus of this research. Rather, the main emphasis will be on ecological and behavioural aspects of how these males may compete upon release.

1.9 Overview of chapter contents / experiments

Chapter 2 compares the life history characteristics (larval mortality, time to pupation, adult size and longevity) of the OX513A line with its wild type counterpart and their respective responses to increasing larval rearing density. The results show that in a controlled laboratory situation the transgenic sterile OX513A line may have somewhat
reduced performance compared to its WT counterpart and that high rearing densities may further reduce performance. Furthermore, it highlights the potential value of optimising mass-rearing systems to alleviate performance issues associated with specific lines or with lab-adapted lines in general.

Chapter 3 examines the insemination capacity and the cost of courtship and mating to males of the OX513A and WT line. Genetically modified males inseminated just over half as many females as the WT males during their lifetime. Providing days of rest from mating had no significant effect on the total number of females inseminated by males of either line, yet it did increase their longevity. Results further showed that sperm production itself is low cost in terms of energy investment, whereas the process of transferring this sperm to a receptive female is costly. Continued mating attempts with refractory females, therefore offer evidence that males may not be able to identify refractory females before investing a substantial amount of energy in courtship. Though, over a lifetime OX513A males inseminated fewer females, the number of females inseminated over the first three days, was similar between males of both lines, suggesting the benefit of a strategy of frequent releases for control programmes.

Chapter 4 compares the flight potential and energetic reserves of males of two genetically modified lines, OX513A and OX3604, with those of the WT line. OX3604 males reared without tetracycline spent 35% less time in flight, while OX513A males covered 42.25% less distance than WT males reared without tetracycline. Such differences in flight may be worth considering in the design of release programmes. Release sites, for example, may need to be sufficiently close together to achieve adequate cover. Additionally this chapter shows that the addition of tetracycline to the larval diet of mosquitoes produces adults with higher average lipid contents.

Chapter 5 investigates the propensity of females to re-mate. Results suggest female Ae. aegypti are generally monogamous over the course of their lifetimes. Of particular interest to SIT-based control programmes is the fact that females showed no preference or ability to selectively re-mate if inseminated by a genetically modified male. Exceptions to this strict monogamy existed when females had been mated to sperm-depleted males; though it remains to be established with what frequency this occurs in the field.
Chapter 6 deals with female mate-selection in the *Ae. aegypti* mating system. It shows that selecting the first pair of mosquitoes to form a couple is not a valid way to assess the mating success of males as females often reject males even at this late stage in courtship. Smaller males were more likely to approach females before larger males, yet larger males fathered more offspring. Males of the OX513A line were as likely as WT males to approach a female, yet WT males fathered more offspring, which may have been due to their generally larger size. These experiments therefore present the possible benefit of adjusting rearing conditions of sterile males destined for release, to achieve larger individuals.

Chapter 7 describes cage trials assessing the performance of OX513A males in direct competition for females. When competing at a ratio of one to one, WT males were almost double as likely to father offspring as the genetically modified mosquitoes under controlled laboratory conditions. WT males were increasingly likely to inseminate more females than their OX513A counterparts with both increasing frequency of WT males as well as increasing overall density of males. Females actively selected to mate with WT males when these were present at low frequency in the population. The performance of OX513A males in cage trials suggest high over-flooding ratios will be necessary for successful implementation of control programmes in the field.
Chapter 2

2 Comparison of life history characteristics of the genetically modified OX513A line and a wild type strain of Aedes aegypti

2.1 Introduction

The development of techniques to transform mosquito species that are vectors of disease, e.g. Aedes aegypti (Coates, Jasinskiene et al. 1998; Jasinskiene, Coates et al. 1998) and Anopheles gambiae (Grossman, Rafferty et al. 2001), has paved the way for new approaches to disease control. One possibility is a genetics-based control strategy modelled on the traditional sterile insect technique (SIT), which uses repressible lethal genes that kill the insect, but which can be repressed to allow rearing of the strain under artificial conditions, i.e. in the laboratory (Thomas, Donnelly et al. 2000).

The RIDL system (release of insects carrying a dominant lethal) is such a strategy (Thomas, Donnelly et al. 2000; Alphey 2002; Alphey, Nimmo et al. 2008; Alphey, Benedict et al. 2010). Strains embodying the concept have been engineered for Ae. aegypti, using tetracycline-dependent repression of a dominant lethal gene (Phuc, Andreasen et al. 2007; Fu, Lees et al. 2010). Tetracycline can be introduced as a dietary supplement for mosquitoes reared in the laboratory, but is not readily available in the wild; hence the lethal system is repressed in the laboratory and activated upon release. Upon their release, transformed males, which are homozygous for this lethal construct, would pass one copy of the dominant lethal to their offspring by normal Mendelian inheritance. These would subsequently die as larvae or pupae in the wild due to the absence of tetracycline. This late-acting lethality, in a species limited by density dependent effects, can be significantly more effective than conventional SIT (Atkinson, Su et al. 2007; Phuc, Andreasen et al. 2007). Over time, releases of sterile males are expected to reduce and perhaps, under appropriate circumstances, eventually eliminate the targeted mosquito population.
Releases of mosquitoes, even sterile ones, would preferably be restricted to males, as only female mosquitoes bite – the repeated release of large numbers of females might increase biting nuisance and/or the transmission of disease. Mating opportunities will only present themselves to males that are fit enough and live long enough to successfully compete for habitat, energetic resources and, of course, females. Thus, the performance of male mosquitoes is of paramount importance to sterile-male-release strategies such as RIDL.

Although RIDL insects are expected to suffer less fitness costs than their irradiated counterparts, certain fitness burdens may remain. Apart from fitness costs associated with the process of transposon-mediated transformation itself and the subsequent genetic pressures of inbreeding to create a homozygous line (Marrelli, Moreira et al. 2006); there may also be costs associated with the mass-rearing required to release the large numbers of males that are necessary to make a RIDL (or SIT) programme effective and sustainable. Previous studies have shown that increasing larval density in various mosquito species increases larval mortality, delays pupation and results in smaller, shorter-lived, less fecund adults (Bar-Zeev 1957; Wada 1965; Southwood, Murdie et al. 1972; Gilpin and McClelland 1979; Dye 1982; Dye 1984; Agnew, Hide et al. 2002; Bedhomme, Agnew et al. 2003).

In this paper we investigate the role of rearing density on the life-history of a RIDL line, OX513A, carrying a tetracycline repressible, lethal positive feedback system (Phuc, Andreasen et al. 2007) in comparison with its wild type counterpart.

The parameters examined in this study were larval mortality, developmental rate (i.e. time to pupation), adult size and longevity.

2.2 Methods

2.2.1 Experimental design and larval rearing

All experiments were conducted in a temperature-controlled insectary at 27 (+/- 2) °C and a relative humidity of 65 (+/- 10) % with a 12h:12h light/dark cycle.

Eggs of the WT Aedes aegypti line and the OX513A line were submerged in water supplemented with tetracycline to a final concentration of 30µg/ml and placed under low
pressure for one hour to ensure synchronous hatching. The following day, larvae were counted out into 100 ml pots (surface area of the water: 79 cm\(^2\), water depth 1.5 cm) at densities of 100, 400 and 800 larvae per pot, thus giving rearing conditions of 1, 4 and 8 larvae/ml.

The larvae were fed the following feeding regime of finely ground TetraMin fish food per larva: day 1 – 0.03 mg, day 2 – no food, day 3 – 0.04 mg, day 4 - 0.08 mg, day 5 – 0.16 mg, day 6 – 0.16 mg, day 7 onwards – 0.32 mg. Rearing was carried out in four consecutive blocks staggered by three days. This blocked design was repeated three times giving a total of 30 pots per treatment.

Pupae were removed from pots by pipette on the day of pupation and their numbers and sex recorded.

One male and one female pupae of each rearing pot (so 30 mosquitoes per treatment) were moved into individual pots to eclose. Emerged adult mosquitoes were supplied with a piece of cotton wool saturated with a 10% sucrose solution, which was refreshed every other day to prevent desiccation. Survival was recorded daily.

The other mosquitoes were frozen and their wing length was measured. Wings were removed in a 70 % ethanol solution under a dissection microscope and mounted on microscope slides. Digital images of the wings alongside a graticule for purposes of scale were taken using a Canon PowerShot S5IS camera and a 99 mm adapter (S/N:3754, Martin Microscope Company). Wings were measured with ImageJ (http://rsbweb.nih.gov/ij/) from the auxiliary incision to the apical margin excluding the fringe (Figure 2.1).
2.2.2 Statistical analysis

Statistical analyses were performed with JMP version 7.0 (http://www.jmpdiscovery.com). Age at pupation and wing length were analyzed with mixed effect ANOVAs (Fisher, 1925) including density, line, sex and up to 2-way interactions (the 3-way interaction was not significant) as fixed factors and pot as a random factor nested within density and line. Larval survival was estimated as the proportion of individuals surviving to pupation in each pot, was Box-Cox (Box and Cox, 1964) transformed, and was analysed as an ANOVA including line, density and their interaction. Adult longevity was analysed as an ANOVA including density, line, sex and their interactions. An ANOVA was used instead of a survival analysis, as no mosquitoes were censored and the distribution of longevity was close to normal. However, a survival analysis (Kaplan-Meier) (Kaplan and Meier, 1958) gave similar results (not shown). As density must be a nominal factor in the nested analysis, to ensure consistency, it was considered nominal in all analyses.
2.3 Results

2.3.1 Larval survival to pupation
On average, 95% (between 66% and 100%) of the larvae in each pot survived to pupation. Density had no effect on survival ($F=1.32$, df=2, $p=0.28$) but wild type mosquitoes survived on average about 5% better than the transformed OX513A line ($F=8.01$, df=1, $p=0.007$) (Table 1).
Table 2.1: Life history characteristics

Comparison of average (n ≥30 (± s.e.m.)) larval survival, age at pupation, wing length and longevity. Values denoted with the same letter do not differ significantly (95%CI). Differences between treatments (rearing density) denoted by lower case letters and differences between lines (WT and OX513A), but within treatments, by capital letters.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th></th>
<th></th>
<th>OX513A</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>1 larvae/ml</td>
<td>4 larvae/ml</td>
<td>8 larvae/ml</td>
<td>1 larvae/ml</td>
<td>4 larvae/ml</td>
<td>8 larvae/ml</td>
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<tr>
<td>Average larval survival</td>
<td>94.30%</td>
<td>99.05%</td>
<td>98.74%</td>
<td>92.30%</td>
<td>94.40%</td>
<td>89.71%</td>
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<tr>
<td>Males</td>
<td></td>
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<tr>
<td>Average age at pupation</td>
<td>10.67 (±0.07)</td>
<td>11.19 (±0.04)</td>
<td>10.98 (±0.02)</td>
<td>9.39 (±0.05)</td>
<td>10.45 (±0.03)</td>
<td>10.51 (±0.03)</td>
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<td>a, B</td>
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</tr>
<tr>
<td>Average wing length (mm)</td>
<td>2.01 (±0.01)</td>
<td>2.03 (±0.01)</td>
<td>1.99 (±0.02)</td>
<td>2.04 (±0.01)</td>
<td>1.94 (±0.01)</td>
<td>1.90 (±0.01)</td>
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<td>b, B</td>
<td>b, B</td>
</tr>
<tr>
<td>Average longevity</td>
<td>31.60 (±1.43)</td>
<td>24.90 (±1.32)</td>
<td>21.27 (±1.26)</td>
<td>29.3 (±1.47)</td>
<td>19.67 (±1.52)</td>
<td>16.63 (±1.32)</td>
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<tr>
<td>Females</td>
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<tr>
<td>Average age at pupation</td>
<td>11.66 (±0.06)</td>
<td>12.47 (±0.04)</td>
<td>12.27 (±0.03)</td>
<td>9.96 (±0.05)</td>
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<td>11.35 (±0.03)</td>
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<td>c, A</td>
<td>a, B</td>
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<td>b, B</td>
</tr>
<tr>
<td>Average wing length (mm)</td>
<td>2.60 (±0.01)</td>
<td>2.62 (±0.02)</td>
<td>2.31 (±0.01)</td>
<td>2.54 (±0.01)</td>
<td>2.53 (±0.02)</td>
<td>2.28 (±0.01)</td>
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<td>a, B</td>
<td>a, B</td>
<td>b, A</td>
</tr>
<tr>
<td>Average longevity</td>
<td>31.10 (±1.16)</td>
<td>24.90 (±1.11)</td>
<td>21.27 (±0.75)</td>
<td>28.03 (±1.22)</td>
<td>20.60 (±1.05)</td>
<td>16.87 (±1.37)</td>
</tr>
<tr>
<td></td>
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</table>
2.3.2 Age at pupation

Males pupated on average after 10.7 days; females after 11.7 days, a significant difference (F=1267, df=1, p<0.001); for both, age at pupation ranged from 7 to 18 days. Age at pupation increased by about 1 day from the lowest to the intermediate density, but was similar at the intermediate and the highest density (F=10.8, df=2, p<0.001). WT larvae pupated on average about 1 day later than OX513A larvae (F=37.5, df=1, p<0.001), and this difference was similar across rearing densities (interaction F=1.58, df=2, p=0.22). The difference between the two lines was less pronounced for males (difference 0.9 days) than for females (difference 1.4 days) (F=154, df=1, p<0.001) (Table 2.1, Figure 2.2).

![Figure 2.2: Age at pupation](image)

Comparisons of age at pupation of WT and OX513A mosquitoes reared at different densities in 100 ml pots; error bars showing 95% CI.

2.3.3 Wing length

Females of both lines were generally larger than the males (F=3976, df=1, p<0.001) and showed a greater decrease in wing length with increasing rearing density than the males (F=83.59, df=2, p<0.001). Both male and female WT mosquitoes were larger than their OX513A counterparts reared at the same density (F=46.8, df=1, p<0.001). Increased larval rearing density decreased adult wing length for both lines (F=208.22, df=2, p<0.001), but the OX513A line showed a greater response to increasing rearing density (difference 0.204 mm)
than the WT line (difference 0.155 mm) ($F=8.05$, $df=2$, $p<0.001$), producing increasingly smaller adults. This effect is mainly due to the stronger reaction of OX513A males compared to their WT counterparts rather than the females ($F=6.79$, $df=2$ $p=0.0013$) (Table 2.1, Figure 2.3).

![Figure 2.3: Wing length](image)

Comparisons of average wing length of WT and OX513A mosquitoes reared at different densities in 100 ml pots; error bars showing 95% CI.

### 2.3.4 Longevity

Adult mosquitoes lived an average of 24 days (1 to 48) irrespective of sex ($F=0.23$, $df=1$, $p=0.63$). As density increased from 1 to 8 larvae/ml, longevity decreased from 30 days to 19 days ($F=80.1$, $df=1$, $p<0.001$). Wild types lived about 4 days longer than OX513A transgenics ($F=26.3$, $df=1$, $p<0.001$) (Table 2.1, Figure 2.4). None of the interactions were significant ($p>0.54$).
Survival curves of male WT (a) and OX513A (b) mosquitoes at different rearing densities. All treatments started with 30 individuals. Solid black line representing rearing density of 1 larva/ml; dashed black line representing rearing density of 4 larvae/ml; gray line representing rearing density of 8 larvae/ml.

2.4 Discussion

Our results reveal statistically significant differences between the life history traits of the genetically modified OX513A line and the wild type line with a similar genetic background. Overall larval survival to pupation was reduced by around 5% in the OX513A line and adult longevity was reduced by about four days. Mosquitoes of the OX513A line pupated on average one day earlier than their wild type counterparts, with this difference being more pronounced for females (1.4 days) than for males (0.9 day). Perhaps as a consequence, adults of the OX513A line were generally smaller than the wild type mosquitoes, this difference was again more pronounced for females.

Increasing larval rearing density delayed pupation by approximately one day from the lowest to the intermediate density, but was similar at the intermediate and the highest density in both lines. Moreover, the decrease in adult longevity followed a similar pattern in the two lines with averages decreasing from 30 to 19 days from low rearing density to high rearing density. The average reduction in longevity in response to increased rearing density in either line is larger than the difference between the two lines. Therefore identifying changes in rearing conditions that reduce this negative effect on lifespan are desirable and have the potential to significantly improve male quality for either line. In contrast, the decrease in adult size was
significantly different between the two lines with increasing larval rearing density with the OX513A line showing a greater reduction in wing length, especially in males, than the wild type line.
The reduced time to pupation of OX513A would be advantageous in mass-rearing. However, OX513A adults have a smaller mean size which may be associated with shorter time to pupation. Previous studies show adult body size may play a role in reproductive success. For females a relationship between body size and oocyte number has been established (Christophers 1960). Furthermore, Okanda et al. (2002), showed that male *Anopheles gambiae* preferentially selected larger females for mating. For males, too, size may play a role. Dickinson and Klowden (1997), for example, measured the entire protein content of small vs. large adult male *Aedes aegypti* before and after mating to assess protein transfer. Small males transferred significantly less protein than large males. Ponlawat and Harrington (2007) also found an increased sperm capacity in larger males. Yet, how these findings translate into actual fertilization success has not yet been assessed. Research on the effect of male size on mating success in *Anopheles gambiae* – which has different mating habits – (Ng’habi, John et al. 2005; Ng’habi, Huho et al. 2008) concluded that size indeed plays an important role in male competitiveness in this species, with mid to larger sized males being more successful than smaller ones. Although not as clear as the argument for survival and longevity, the smaller size of OX513A mosquitoes may additionally contribute towards a fitness cost compared to wild type males and should be further investigated. With this in mind, an assessment of the average size of males in any target population is therefore advisable before designing a release programme.
The differences observed in the two lines may be attributable to several possible, non-exclusive factors. The transgenic construct itself may be deleterious by either of two mechanisms. First, the transgene products may have negative effects, i.e. the build-up of alien gene products resulting from the integrated foreign genes may be deleterious to the host cells in which they are expressed. Second, transposition may be associated with insertional mutagenesis, for example a transgene may insert itself in a transcriptionally active region of the genome where it may disrupt native gene function (Spradling 1995; Spradling 1999; Thibault 2004).
In the case of OX513A in particular the possibility of non-zero expression of the lethal system (incomplete repression) could play a role. Even low basal ‘leakiness’ of the system could potentially weaken the mosquito.
Furthermore, in these experiments (and after release) adult males of this strain no longer have access to dietary tetracycline. This is likely to derepress the lethal system (Gong, Epton et al. 2005). Though this clearly does not rapidly kill the males, it is possible that it contributes to their somewhat reduced lifespan relative to wild type males. These effects may be minimised by suitable design of the construct, but may not be completely eliminated.

Additionally it is likely that fitness is reduced by the inevitable genetic bottleneck associated with starting a new transgenic line from a single transformed individual and the further genetic pressures of inbreeding to make this line homozygous for the lethal gene construct. Most genomes contain numerous recessive mutations that are capable of reducing the fitness of the carrier in a homozygous state (Simmons and Crow 1977; Halligan and Keightley 2003). The insertion of a transgene in the vicinity of such a negative recessive mutation and subsequent breeding efforts to make the line homozygous for the inserted construct will at the same time make it homozygous for the recessive allele; this is known as the hitchhiking effect (Marrelli, Moreira et al. 2006) and can lead to the fixation of such alleles causing severe inbreeding depression. This may indeed be one of the most influential factors on the fitness of transgenics, as studies by Amenya et al. (2010) and Moreira et al. (2004) show that a foreign gene in itself need not negatively impact the fitness of the carrier.

Finally, the two lines should in theory have close to the same (WT) background; however the OX513A line was out-crossed into this (WT) genetic background. At least 97%-99% of their genome should correspond; nevertheless, it is conceivable that the small amount of the Rockefeller and/or the Mexican genetic background that may remain, especially in the region of the insertion site, is contributing to the differences seen between the lines.

Besides the fitness effects associated with the creation and breeding of the line discussed above, environmental factors may also play a role in the fitness of mass-reared mosquitoes. Environmental stress, such as crowding, during the larval stages of development could, for example, impact the adult mosquito’s fitness by reducing its teneral reserves.

Mass rearing environments will contrast with the mosquitoes natural breeding sites in two main respects. Firstly, the larval density in rearing trays will likely be higher than that encountered in natural habitats. Secondly, in addition to being available in high quantities and in constant supply, the food offered may be of a different composition/quality than that found in natural breeding pools. Therefore, in this study we focus specifically on the effect of larval crowding (i.e. rearing density) on life history traits of genetically modified and wild type mosquitoes, and not on food limitation, as space, not food supply, is considered the most
important limiting factor in mass-rearing. Providing each larva with an equal, and sufficient, amount of food daily should eliminate competition for food as a main factor. Nevertheless, as the food was increased in accordance with larval growth, any individuals growing at a slightly faster rate may have eaten proportionately more of the food, to some extent creating competition for food over time.

Moreover, the high larval density will result in large quantities of waste materials such as dead and decomposing larvae, discarded exoskeletons, excretory products and surplus food entering the system. Consequently, as the larvae age the water quality will degrade unless it is changed regularly. Growing in polluted water can have a negative impact on larval development (Bedhomme, Agnew et al. 2005). The possible contaminants in high-density rearing water include allelopathic substances, such as a growth retardant, synthesised in reaction to competitive stress, excretory products such as nitrogenous waste as well as food waste or larval debris, or bacterial growth. These may have a direct effect on larval growth, or indirect effects, for example via effects on microbial composition of the habitat.

Early work on larval crowding (Moore and Fischer 1969; Ikeshoji and Mueller 1970; Moore and Whithacre 1972) suggested the existence of a growth retardant factor excreted by mosquito larvae under intraspecific competition, especially when food became limiting. However, other studies found no such growth retardant factor (Dye 1982). Therefore, the existence of such products is still questionable and the contribution they may make to intraspecific competition undefined, indeed no such product has been identified for any other animal species (Bedhomme, Agnew et al. 2005).

Recent results for various mosquito species, Aedes albopictus, Tripteroides bambusa (Sunahara and Mogi 2002) and Aedes aegypti (Bedhomme, Agnew et al. 2005), demonstrate a negative effect of rearing mosquitoes in water that has already been occupied by a previous batch of larvae. The latter experiment in particular is of interest as mosquitoes were reared individually, thus eliminating the element of competition and therefore any need to produce growth retardants. Furthermore, studies on Aedes triseriatus (Walker, Lawson et al. 1991) showed the accumulation of ammonia in tree-holes occupied by larvae, while Carpenter (1982) showed that the addition of ammonia to microcosms containing Ae. triseriatus had a negative effect on survival and development as well as adult mass.

Finally, it is possible that bacterial or fungal growth could affect life history parameters in mass rearing trays. Depending on the microbial community present in the rearing water this can either benefit larval development as some bacteria acts as an additional food source (Kaufman, Walker et al. 1999), while some fungal species can negatively impact larval
development as shown by Mokany and Shine (2003). Furthermore, the micro-biota could indirectly affect the larvae by contaminating their food supply, rendering it less nutritious or even inedible.

As all the factors described above will increase with increasing number of larvae per millilitre the negative impact water pollution may have will increase accordingly, in line with the results presented here, potentially leading to later pupation and smaller, shorter lived adults. Assessing life-history traits is only one part of fitness. As a complementary study the fitness of the males in competition for females and, in particular, their mating success is discussed in Chapter 7.

Nevertheless, despite possible complications, this study shows that in a controlled laboratory situation the OX513A line may have somewhat reduced performance compared to its wild type counterpart and that high rearing densities necessarily associated with mass-rearing may further reduce performance. Such potential reduction in performance must however be confirmed in the field as laboratory-based and field-based trials do not always show similar effects (Seawright 1975; Curtis 1976; Seawright 1976). Simulation models may be useful to explore the impact of line performance on the effectiveness of any future control programme using such lines. It is likely the modest performance reduction indicated here for OX513A relative to a wild type strain could be compensated by releasing more males. However, there may be some scope for improvement in the construction of future strains. Furthermore, this chapter highlights the potential value of optimisation of mass-rearing systems as optimised rearing methods may be able to alleviate performance issues associated with specific lines or with lab-adapted lines in general to a certain extent. Unfortunately, advances in mosquito mass-rearing have in recent years lagged far behind advances in mosquito genetics.
Chapter 3

3 Cost of mating and insemination capacity of a genetically modified mosquito *Aedes aegypti* OX513A compared to its wild type counterpart

3.1 Introduction

Strategies based on genetic manipulation are becoming more popular in the search for effective techniques for vector control. Advances in the technology for genetic transformation have made such methods feasible for the control of *Aedes aegypti*, the most important vector of dengue fever, yellow fever and other arboviruses (Gubler 2002). One possible approach is the release of insects carrying a dominant lethal (RIDL) (Thomas, Donnelly et al. 2000; Alphey 2002; Alphey, Nimmo et al. 2008; Alphey, Benedict et al. 2010), a control strategy modelled on the traditional sterile insect technique (SIT). That this strategy is feasible has been demonstrated with lines of *Ae. aegypti* transformed with a dominant lethal gene that can be repressed with tetracycline (Phuc, Andreasen et al. 2007; Fu, Lees et al. 2010). These lines can be reared in the laboratory by adding tetracycline to the larval diet (Thomas, Donnelly et al. 2000); however, in nature tetracycline is not readily available, so the lethal system is activated. Transformed males, homozygous for the lethal construct, would then pass one copy of the dominant lethal gene to their offspring by normal Mendelian inheritance and these would consequently die as larvae or pupae. Mathematical models indicate that the continued releases of such ‘sterile’ males would in time lead to the suppression, or elimination, of the targeted mosquito population. As releasing large numbers of females would increase biting nuisance and the transmission of disease, deliberate releases of mosquitoes, even sterile ones, should be restricted to males.

The success of such a control programme depends on the likelihood that the released males can inseminate females and father offspring, which depends on the one hand on the outcome of competition between wild type and RIDL males for access to females and on the other hand on the relative longevities of wild type and RIDL males.
Female *Aedes aegypti* are monogamous (Craig 1967; Spielman, Leahy et al. 1967), i.e. mate only once and are generally refractory to a second insemination. Males, in contrast, are polygamous (e.g. Gwadz and Craig 1970; Foster and Lea 1975; Youngson, Welch et al. 1981; Clements 1999) and can inseminate several females over the course of their lifetimes. The number of females is limited, with estimates of, on average, 3 to 5.8 females in a day (Gwadz and Craig 1970; Foster and Lea 1975) and 8 to 9 over the course of a lifetime (Youngson, Welch et al. 1981). That males can inseminate more females in their lifetimes than they can in a single day suggests that their sperm reserves are depleted by successive matings and must be replenished before they can inseminate further females. The production of sperm, the replenishment of sperm reserves and the effort involved in courting or competing for access to females undoubtedly require energy and thus can be considered costly to males (Sakaluk 1985; Clutton-Brock and Parker 1992; Hyashi 1993; Cordts and Partridge 1996; Mappes, Atalo et al. 1996; South, Steiner et al. 2009). Investment in activities relating to mating success may therefore trade off against other fitness-determining traits, such as longevity. This has been demonstrated in other species, including *Drosophila* (Cordts and Partridge 1996) and the mosquito *Sabethes cyaneus* (South, Steiner et al. 2009).

In this paper we compare the insemination capacity of males (i.e. the number of females a male is capable of inseminating over the course of his lifetime) and the cost of investing in courtship and mating on longevity for two mosquito colonies: a wild type strain of Malaysian origin (‘WT’) and an engineered version of this strain which is homozygous at a single locus for repressible-lethal construct (‘OX513A’).

### 3.2 Methods

#### 3.2.1 Larval rearing
Eggs of the WT line and the genetically altered OX513A line were submerged in water and placed under low pressure for one hour to ensure synchronous hatching. The following day the larvae were placed in individual wells of 12-well plates with 3 ml of water per well and reared on the following regime of finely crushed TetraMin fish food; day 1: 0.06 mg, day 2: 0.08 mg, day 3: 0.16 mg, day 4: 0.32 mg, day 5: 0.64 mg, day 6 and thereafter: 0.32 mg/larva. Fish food was prepared in a tap water solution, mixed to a uniform suspension with a magnetic stirrer and aliquoted into the wells (150μl per well) daily. The water in wells
containing OX513A larvae was supplemented with 30 µg/ml tetracycline. This rearing method was chosen to produce adults (in particular males) of equivalent size for the two strains, while enabling a large number of independent repeats to be reared within a relatively small space and limited time period.

3.2.2 Insemination capacity / longevity

One hundred mating arenas were set up, each a cage of 15 x 15 x 15 cm. One day after emergence, a WT or an OX513A male was placed into a cage. The fifty cages of each strain were treated in two ways. (i) Five virgin females were placed in the cage for ninety minutes every day until the male had died. They were then removed, dissected and their spermathecae assayed for the presence of sperm. (ii) The second treatment differed from the previous one in that on the fourth and fifth day within consecutive 5-day periods, no females were placed into the cage, so that the males had 2 days of ‘rest’.

Mating trials were carried out at the same time daily (9:30 am) to ensure possible circadian rhythms would not bias the results. As only a limited number of dissections could be carried out in a day, the experiment was divided into five consecutive blocks of twenty cages, ten containing wild type males and ten containing OX513A males, half of each with rest days and half without.

3.2.3 Cost of mating / longevity

To compare the effect on male longevity of increasing the number of available females the following cages were set up: 220 cages, half containing one WT male and half one OX513A male. 50 males of each line were kept in isolation, without the addition of females. 30 males of either line were presented either two or four virgin females daily. Dead mosquitoes were removed from their pots and stored for wing length measurements.

As males may be more active when they encounter virgins than previously mated (and therefore refractory) females, the effect of courting receptive (virgin) and refractory (previously inseminated) females on the longevity of WT and OX513A males was compared by setting up an additional 120 cages. Again, 30 males of either line were held with two or four refractory females. Females that had been observed to copulate with other males were selected. In six cages, some of the females died before the male, and were replaced with females from the standard colony, which had probably mated.
The adult mosquitoes were supplied with a piece of cotton wool saturated with a 10% sucrose solution, which was refreshed every other day to prevent desiccation. Mosquitoes were checked daily for survival.

In order to supply enough virgin females to make the daily replacements, three large trays (1 litre) of WT and OX513A larvae were reared in succession at low density (approx. 0.3 larva/ml) on the same food regime as above. The pupae were sexed and transferred to female stock cages.

3.2.4 Wing length measurement

Mosquitoes used for wing length measurements were put into 1.5ml Eppendorf tubes and frozen. The wings were removed in a 70% ethanol solution under a dissection microscope and mounted on microscope slides. Digital images of the wings were taken with a Canon PowerShot S5IS camera and a 99 mm adapter (S/N:3754, Martin Microscope Company). Wings were measured with ImageJ (http://rsbweb.nih.gov/ij/) from the auxiliary incision to the apical margin excluding the fringe.

3.2.5 Statistical analysis

Statistical analyses were performed with JMP version 7.0 (http://www.jmpdiscovery.com). The number of females inseminated and longevity were analysed with an ANOVA (Fisher, 1925) including line (WT, OX513A), treatment (with or without rest days) and their 2-way interactions as factors. The longevity of males caged with increasing numbers of females was analysed with an ANOVA including line (WT, OX513A), number of females (0, 2, and 4) and their 2-way interactions as factors. The difference in longevity between males of either line caged with virgin or refractory females was analysed as a three-way ANOVA including line (WT, OX513A), number of females (2, 4), kind of female (virgin, refractory) and their interactions as factors. Each analysis included block as a nominal factor. The residuals of all analyses were close to Gaussian distributed, justifying the use of the ANOVAs.
3.3 Results

The longer a male of either line lived the more females he inseminated over the course of his lifetime (F=30.4, df=1, p<0.001). There was no difference among blocks (F=1.3, df=1, p=0.263). WT males inseminated more (11.5 ± 0.53 s.e.m.) females than OX513A males (6.6 ± 0.31 s.e.m) (F=61.6, df=1, p<0.001). Rest days, i.e. days on which no females were introduced, had no effect on the total number of females inseminated by males of either line (F=0.05, df=1, p=0.823), and the difference between the two lines was not affected by the availability of rest days (interaction: F=0.03, df=1, p=0.873). Blocking had no effect (F=0.0005, df=1, p=0.982). WT males, regardless of whether rest days were offered, outlived OX513A males by approximately four days (F=19.8, df=1, p<0.001); introducing ‘rest days’ increased the average lifespan for both lines by approximately four days (F=32.8, df=1, p<0.001), and the difference between the lines was not affected by the availability of rest days (F=0.005, df=1, p=0.943). Again, blocking showed no significant effect (F=1.269, df=1, p=0.263).
Figure 3.1: Number of females inseminated by, and longevity of males with and without rest days.

WT males (solid line) inseminate more females than OX513A males (dashed line) both without (panel A)) and with (panel C)) rest days. WT males outlive their OX513A counterparts whether given rest days (panel D)) or not (panel B)). Error bars represent the standard error.
3.3.1 Longevity of males

Number of females

Providing males (WT and OX513A) with virgin females reduced their average lifespan by 43% from 34.35 (±0.84 s.e.m) to 14.62 (±0.74 s.e.m) days (F=313.6, df=1, p<0.001).

The longevity of males decreased with the number of virgin females added from 34.35 (±0.95 s.e.m) days with 0 females to 11.99 (±0.59 s.e.m) days with 4 females (Figure 3.2) (F=17.0, df=2, p<0.001). The number of females affected males of the two lines similarly (F=1.45, df=2, p=0.237) (Figure 3.2).

Figure 3.2: Average longevity of WT and OX513A males caged with virgin females.

The longevity of WT (solid line, solid squares) and OX513A (dashed line, open circles) males decreased similarly with increasing numbers of virgin females (provided daily). Error bars represent the standard error.

Mating status of females

As above, the male’s longevity was higher if he was caged with 2 rather than with 4 females (F=20.95, df=1, p<0.001), and higher for WT than for OX513A males (F=25.12, df=1, p<0.001). Whether the females he was caged with were virgin or refractory had only a slight effect (F=1.314, df=1, p=0.253), but the interaction of these factors was significant (F=4.843,
df=1, p=0.029): adding virgin or refractory females has a similar effect on OX513A males, but only adding virgin females substantially reduced the longevity of WT males (Figure 3.3). None of the other two-way interactions were significant.

Figure 3.3: Effect of the three-way interaction on the longevity of males.

Adding virgin (closed symbols) or refractory females (open symbols) has a similar effect on OX513A males (dashed lines, circles), but only adding virgin females substantially reduced the longevity of WT males (solid lines, squares). Error bars represent the standard error.

3.3.2 Effect of body size on longevity

There was no difference between the average wing lengths of WT males (2.096 ± 0.012 mm) and OX513A males (2.096 ± 0.12 mm) (df = 1, F = 0.0001, p = 0.992) or between the average wing lengths of WT females (2.63 ± 0.011 mm) and OX513A females (2.62 ± 0.011 mm) (df = 1, F = 1.2097, p = 0.274). Body size had no effect on longevity.
3.4 Discussion

This chapter compares the insemination capacity of males of a genetically modified and a wild type line of *Aedes aegypti*, as well as assessing the contributions of mating attempts and successful insemination to the overall cost of mating. Female *Ae. aegypti* are considered to be monogamous (Craig 1967; Spielman, Leahy et al. 1967), i.e. mate only once in their lifetime, after which they become refractory to further insemination. Keeping males with previously inseminated females will therefore principally measure the cost of futile attempts at courtship and mating. Conversely, presenting males with virgin females daily will give an indication of the cost of both coupling and successful insemination, therefore, allowing us to assess the contribution of both factors to the overall cost of mating. The experimental design does not include male contest competition although this may play a role in a more natural setting (reviewed in Gaskin, Futerman et al. 2002). The estimates of the cost of mating and reproduction to male *Ae. aegypti* are therefore conservative and may well increase in a field situation.

We have examined an engineered line intended for use in a population suppression strategy (Alphey 2002; Alphey and Andreasen 2002; Phuc, Andreasen et al. 2007; Alphey, Benedict et al. 2010). However, the same issues of fitness arise with other proposed uses of modified mosquitoes, such as attempts to make wild populations less able to transmit specific pathogens ('refractory insects', Alphey, Beard et al. 2002; Alphey 2009). Indeed, since such insects are intended to establish and persist in the wild, whereas sterile males are merely expected to mate and die, male mating ability may be just one of a much wider range of relevant fitness traits affecting the performance and effectiveness of refractory insects.

The results show distinct differences in the insemination capacity and the cost of mating in males of the genetically modified OX513A and the WT line. Genetically modified males inseminated just over half as many females (on average 6.6) as the WT males (on average 11.5) during their lifetime. Providing days of rest from mating had no significant effect on the total number of females inseminated by males of either line, yet it did increase their longevity. In line with previous studies (Liles and Delong 1960), keeping males confined with females significantly reduced their lifespan, while increasing the number of females a male was caged with further decreased his longevity. The reduction in longevity was similar between males housed with previously inseminated females and males presented with virgin females daily, indicating that sperm production itself is low cost in terms of energy investment, whereas the process of transferring this sperm to a receptive female is costly.
Taken together with the observation that males attempted mating throughout their lives, even when confined with refractory females, it follows that an unsuccessful attempt is as energetically costly as successfully inseminating a female and suggests that males may not be able to identify refractory females before investing a substantial amount of energy. OX513A males, caged with refractory females, showed a greater reduction in longevity with increasing numbers of females than the WT males. Attempting to mate therefore appears more costly in terms of energy investment to the genetically modified males. On the other hand, it was the longevity of WT males that decreased to a greater extent when males were kept with increasing numbers of virgin females. This may seem counterintuitive, but as stated above the WT males are capable of inseminating almost double the number of females than the genetically modified males. Furthermore, Jones (1972) noted that sperm depleted males no longer attempted mating to the extent of ‘fresh’ males. It is therefore conceivable that the OX513A males which ran out of sperm more quickly reduced their mating efforts sooner than the WT males.

The reduced insemination capacity and the higher cost of mating to OX513A males established in this paper is evidence of possible fitness deficits in this line. However, the males of the two lines inseminated a similar number of females over the first three days, following which the performance of the genetically modified line declined. Therefore, the time after release in which OX513A males are effective may be somewhat shorter, yet this in itself does not exclude their potential use in a control programme, as long as a strategy of frequent releases was adopted. Furthermore, in a sterile-male release programme, there will be a large excess of males relative to females, e.g 10:1 ratio (Dyck, Hendrichs et al. 2005; Alphey, Benedict et al. 2010). Consequently, if females mate only once the average life-time number of successful copulations is likely to be low, perhaps 0.1. While there may be considerable variation around this mean, very few males may be affected by sperm depletion. One question that must still be considered with regard to their suitability for release though, is the relative competitive ability of OX513A males in direct competition with wild type males. Fitness deficits observed in the laboratory assays described above may be more pronounced when (i) in direct competition for females and (ii) in competition with field-bred mosquitoes. Further analysis of this genetically modified line’s potential effectiveness through cage and field trials is desirable.
4 Flight potential and energy reserves of two genetically modified and a wild type strain of *Aedes aegypti*

4.1 Introduction

*Aedes aegypti* is one of the most important vectors of dengue fever and is therefore the target of various vector control programmes. Advances in genetic transformation technology have enabled the development of new approaches to its control. One of these strategies is the release of insects carrying a dominant lethal (RIDL) (Thomas, Donnelly et al. 2000; Alphey and Andreasen 2002; Alphey, Nimmo et al. 2008; Alphey, Benedict et al. 2010), a genetics-based control system modelled on the traditional sterile insect technique (SIT). RIDL mosquitoes are engineered with tetracycline-dependent repression (Phuc, Andreasen et al. 2007; Fu, Lees et al. 2010), as tetracycline can be introduced as a dietary supplement for mosquitoes reared in the laboratory, but is not readily available in the wild. Thus the lethal system can be repressed in the laboratory and activated upon release. The transformed males, which are homozygous for the engineered construct, pass one copy of the dominant lethal to their offspring - these subsequently die as larvae or pupae in the wild due to the absence of tetracycline. Thus, releases of sterile males are expected to reduce and, under appropriate circumstances, eventually eliminate the targeted mosquito population. As the control strategy can work only if the engineered males can pass on their genes, the ability of released males to successfully compete for females in the field is a critical aspect in its success.

The flight potential of *Ae. Aegypti* is an important epidemiological factor as well as a possible indicator of reproductive success, as mating takes place at or near the human host (Teesdale 1955; Hartberg 1971) and therefore sometimes far from the breeding site. Indeed, *Ae. aegypti* only become sexually receptive 15-24 h (for males, Roth 1948) or 48-72 h (for females, Leahy and Craig 1965; Gwadz and Craig 1970; Gwadz, Craig et al. 1971) after emergence. This delay in sexual maturity allows adequate time for dispersal from the breeding site, thus minimising the risk of inbreeding (Hartberg 1971). Males must consequently search for hosts and females. Once the males are at the host or mating site, their flight plays an important role...
in the mating behaviour. They fly a characteristic figure-of-eight pattern close to the host and usually initiate mating in flight after identifying the female by sound (Roth 1948), at which point the synchronisation of wing beat frequencies may play a key role in their mating success (Gibson and Russell 2006; Cator, Arthur et al. 2009).

Furthermore, maximal flight potential is probably linked to the available energetic reserves of a mosquito (Briegel, Knuesel et al. 2001; Briegel, Waltet al. 2001; Kaufmann and Briegel 2004) and therefore can be considered an indicator of overall health and fitness. This chapter therefore compares the flight potentials and the teneral energy reserves of males of two RIDL lines (OX513A and OX3604) and their wild type counterpart.

OX513A is a bi-sex lethal line (described by Phuc, Andreasen et al. 2007), in which the absence of tetracycline in the larval diet causes male and female mosquitoes to die at their late larval or pupal stage. Originally created in the Rockefeller line, it has since been out-crossed to an Asian wild type (WT) line, so that 97-99% of their genome should match the wild type. OX3604 is a female specific flightless line (described by Fu, Lees et al. 2010), in which the absence of tetracycline has little phenotypic effect in males, yet in females results in the inability to fly. This line was created directly in the WT line and thus, other than the insert, should have the WT genetic background.

4.2 Methods

Experiments were conducted in a temperature-controlled insectary at 28 ± 2 °C and a relative humidity of 65 ± 10 % with a 12 h:12 h light/dark cycle.

4.2.1 Larval rearing

Mosquito eggs (WT, OX513A and OX3604) were submerged in water and subjected to low pressure for one hour to ensure synchronous hatching. The following day, larvae were placed individually into the wells of 12-well plates containing 3 ml of water and fed the following feeding regime of finely ground TetraMin fish food per larva; day 1: 0.06 mg, day 2: 0.12 mg, day 3: 0.24 mg, day 4: 0.36 mg, day 5: 0.48 mg, days 6 and later: 0.6 mg/individual. WT and OX3604 larvae were reared with or without tetracycline (tet) added to their rearing water (to a final concentration of 30 µg/ml), whereas OX513A (which requires tetracycline for survival) was reared solely on tet.
To ensure uniformity, only mosquitoes that pupated 7 days and emerged 9 days after hatching were used. Mosquitoes used in the flight mill trials and for wing length measurements were kept in large holding cages for three days before being used; the mosquitoes used in the biochemical analyses were killed (briefly frozen) shortly after eclosing (cages were checked every 2 hours). For the biochemical analyses males were fixed in absolute EtOH, which was evaporated over a water bath at 90 °C, and were subsequently stored for less than 2 weeks before analysis (Van Handel, 1985a, b).

4.2.2 Wing length measurements
Wings were removed in a 70 % ethanol solution under a dissection microscope and mounted on microscope slides. Digital images of the wings were taken with a Canon PowerShot S5IS camera and a 99 mm adapter (S/N:3754, Martin Microscope Company). Wings were measured with ImageJ (http://rsbweb.nih.gov/ij/) from the auxiliary incision to the apical margin excluding the fringe.

4.2.3 Flight mill system
The flight-mills were constructed according to the design described by Rowley, Wayne et al. (1968) (flight path circumference 32.7 cm). Three day old male mosquitoes were mounted on one arm of the flight mills with heated wax and their flight was recorded by registering the number of revolutions at 30 second intervals (see Briegel, Knuesel et al. 2001; Briegel, Waltert et al. 2001).

Because males will not actively mate in the first 24 hours following eclosion (Roth 1948) and sucrose-fed females demonstrated peek flight activity from 3 days post eclosion (Briegel, Knuesel et al. 2001), 3 day old males were used. Flight trials were set up daily at approximately 10 am and ran for 20 hours, providing information on the total flight distance covered during this period, the temporal pattern of flight activities, i.e. bursts of continuous flights, or erratic flight pulses, and resting periods for each male tested.

269 flight-trials were carried out (55 WT off tet, 47 WT on tet, 61 OX3604 off tet, 48 OX3604 on tet, 58 OX513A on tet), of which 99 trials yielded useful data, i.e. males had flown more than 500 metres (21 WT off tet, 14 WT on tet, 26 OX3604 off tet, 14 OX3604 on tet, 24 OX513A on tet). Flight trials that comprised less than 500 metres of recorded flight activity were rejected as the most likely explanation for such a lack of activity was injury or inhibition of the wings during attachment.
4.2.4 Biochemical Analyses

To quantify teneral nutrient reserves (lipid, glycogen, and sugar), 25 males of each line and rearing treatment were analysed individually. Total lipid, glycogen, and sugar contents were measured with methods described by Van Handle and Day (Van Handel 1985; Van Handel 1985). Lipid content was quantified with a vanillin-phosphoric acid reaction (3 ml vanillin (Merck 818718)/tube) with 0.1% soybean oil (Sigma S-7381) in chloroform as a standard. Glycogen in the precipitate and sugar in the aqueous fraction were measured with a hot anthrone reaction (3 ml anthrone/tube), with glucose standards (Merck 8337) 0.1% in EtOH (25%). Absorbance values for 100 μl/well of processed experimental males and standard samples were measured in 96 well plates by a microplate reader at λ = 630 or 490 nm for carbohydrate and lipid, respectively, and further converted to microgram per male with a regression line derived from the standard sample values. To compare energy content the metabolites were converted to Joules.

4.2.5 Statistical analysis

Statistical analyses were performed with JMP version 7.0 (http://www.jmpdiscovery.com). As OX3604 can be reared without tetracycline, but OX513A cannot, two separate analyses were used. First, a two-way ANOVA (Fisher, 1925) comparing line (WT and OX3604), rearing treatment (with or without tetracycline) and their interaction was used to compare males of the WT and the OX3604 line as well as the effect of tetracycline on their flight potential and energy reserves. Second, a one-way ANOVA was used to compare males of the WT line (on and off tet) with males of the OX513A line (on tet). Flight distance, time and speed were log-transformed and gave normally distributed residuals. Carbohydrate contents were transformed using a square-root-transformation. In cases where the ANOVA revealed significant differences between lines or treatments post-hoc testing was performed using Tukey’s HSD (Tukey, 1953; Kramer, 1956).
4.3 Results

4.3.1 Flight potential

The two-way ANOVAs comparing males of the WT line (on and off tet) and the OX3604 line (on and off tet) showed no significant effect of line on either the average distance or the average flight speed of males (Table 4.1). However, line did have a significant effect on their average flight time ($t=2.09$, df=3, $p=0.04$) with males of the OX3604 line (off tet) (2.68 ($\pm$ 0.38 s.e.m.) h) spending 35% less time in flight than WT males (off tet) (4.13 ($\pm$ 0.42 s.e.m.) h) (Table 4.1). Treatment (i.e. on or off tet) showed no significant effect, therefore, the presence of tetracycline in the larval diet had no significant impact on the flight potential of males of either the WT or the OX3604 line. Further, the interaction between line and treatment showed no significant effect.

The one-way ANOVA comparing males of the WT line (on and off tet) with males of the OX513A line (on tet) showed them to spend a similar amount of time in flight and have similar flight speeds. However, males of the OX513A line (on tet) flew significantly shorter average distances than their WT counterparts (on and off tet) ($F=5.909$, df=2, $p<0.01$) (Table 4.1).

4.3.2 Biochemical analysis

As glycogen and sugar contents were similar across lines and rearing treatments they were combined as total carbohydrate contents in the analysis.

The two-way ANOVA showed no significant effect of line, treatment or their interaction when comparing the total carbohydrate contents of males of the WT line (on and off tet) and males of the OX3604 line (on and off tet). Furthermore, the one-way ANOVA showed the carbohydrate contents of males of the WT line (on and off tet) and males of the OX513A line to be similar.

In contrast, analysis of the lipid levels (two-way ANOVA) showed line ($t=3.75$, df=3, $p<0.01$), treatment ($t=-7.55$, df=3, $p<0.001$) and their interaction ($t=4.08$, df=3, $p<0.01$) to significantly affect the lipid contents of males of the WT line and the OX3604 line. In general, males of the OX3604 line had higher concentrations of lipids (9.20 ($\pm$0.23 s.e.m.) J) than their WT counterparts (8.23 ($\pm$0.28 s.e.m) J). Mosquitoes reared with the addition of tetracycline to their larval diet had higher (21.52%) levels of lipids (9.76 ($\pm$0.22 s.e.m.) J) than those without (7.66 ($\pm$0.22 s.e.m.) J), and this trend was stronger in the WT males (Table 4.1).
Furthermore, the one-way ANOVA comparing WT (on and off tet) and OX513A (on tet) males showed a significant difference in the average lipid content between males of the different lines \( (F=37.18, \text{df}=2, \ p<0.01) \). Post-hoc analysis revealed males of the WT line (on tet) to have a higher lipid content than WT males (off tet) and OX513A males (on tet) (Tukey’s HSP, \( p<0.01 \)) (Table 4.1).

### 4.3.3 Wing length

The two-way ANOVA showed no significant effect of line, treatment or their interaction on the wing lengths of WT males (on and off tet) and OX3604 males (on and off tet) (Table 4.1). Furthermore, the one-way ANOVA showed WT (on and off tet) and OX513A males (on tet) to have similar wing lengths (Table 4.1).
Table 4.1: Flight parameters, energetic reserves and wing lengths.

This table shows the distance flown, the time spent in flight and the flight speed of males of the three lines (WT, OX513A and OX3604) reared with (on tet) and without (off tet) the addition of tetracycline to their larval diet. Averages are calculated from the performance of 3 day old males that covered more than a minimum of 500m in a twenty hour period. Average lipid and carbohydrate contents were calculated from newly eclosed males (age <2h). Wing lengths were measured on day old males. All averages given ± standard error. Values denoted by the same letter are not significantly different (95%CI). Comparisons between WT (on and off tet) and OX3604 (on and off tet) denoted by capital letters, comparisons between WT (on and off tet) and OX513A denoted by lower case letters.

<table>
<thead>
<tr>
<th>Line</th>
<th>Average distance flown (km)</th>
<th>Average time spent flying (h)</th>
<th>Average flight speed (km/h)</th>
<th>Average lipid contents (J)</th>
<th>Average carbohydrate contents (J)</th>
<th>Average wing length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT off tet</td>
<td>2.58 (± 0.24)</td>
<td>4.13 (± 0.42)</td>
<td>0.65 (± 0.04)</td>
<td>6.66 (± 0.17)</td>
<td>2.92 (± 0.16)</td>
<td>2.05 (±0.02)</td>
</tr>
<tr>
<td></td>
<td>A a</td>
<td>A a</td>
<td>A a, b</td>
<td>A a</td>
<td>A a</td>
<td>A a</td>
</tr>
<tr>
<td>WT on tet</td>
<td>2.20 (± 0.24)</td>
<td>3.22 (± 0.42)</td>
<td>0.72 (± 0.04)</td>
<td>9.80 (± 0.30)</td>
<td>2.89 (± 0.15)</td>
<td>2.07 (±0.02)</td>
</tr>
<tr>
<td></td>
<td>A a, b</td>
<td>A, B a</td>
<td>A a</td>
<td>B b</td>
<td>A a</td>
<td>A a</td>
</tr>
<tr>
<td>OX3604 on tet</td>
<td>2.21 (± 0.23)</td>
<td>3.17 (± 0.41)</td>
<td>0.74 (± 0.04)</td>
<td>9.73 (± 0.33)</td>
<td>3.15 (± 0.13)</td>
<td>2.05 (±0.02)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>A, B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>OX3604 off tet</td>
<td>1.74 (± 0.21)</td>
<td>2.68 (± 0.38)</td>
<td>0.71 (± 0.04)</td>
<td>8.66 (± 0.30)</td>
<td>3.01 (± 0.13)</td>
<td>2.05 (±0.02)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>OX513A on tet</td>
<td>1.49 (± 0.22)</td>
<td>2.99 (± 0.39)</td>
<td>0.57 (± 0.04)</td>
<td>7.40 (± 0.31)</td>
<td>3.11 (± 0.16)</td>
<td>2.03 (±0.02)</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>a</td>
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</tbody>
</table>
4.4 Discussion

The results show that rearing conditions necessary for RIDL mosquitoes, i.e. the addition of tetracycline to the larval diet, does not affect any parameters of flight that were tested (time spent in flight, flight speed and distance flown), at least in the two lines, WT and OX3604, were these comparisons were possible. However, the experiments revealed moderate differences in the flight potential between the RIDL lines and their wild type counterparts.

In the case of a release programme OX3604 mosquitoes would be reared without tetracycline in the final release generation to eliminate the need for sex-sorting in the factory, while OX513A mosquitoes would be reared with tetracycline, as it is necessary for their survival. Pupae would have to be sorted in the factory to ensure an all-male release. In the field males of both RIDL lines would compete with wild males that had no access to tetracycline, therefore the comparison of OX3604 males (off tet) and OX513A males (on tet) is of particular practical interest.

OX3604 males (off tet) showed similar flight speed and covered a similar average distance compared to WT males (off tet), yet spent 35% less time in flight, while OX513A males showed similar flight speed and time spent in flight during a 20h period, yet covered 42.25% less distance. This is a fairly significant difference and might indicate a certain fitness deficit in this line. Furthermore, it may be worth considering in the design of a release programme. Release sites, for example, may need to be closer together than anticipated to achieve adequate cover.

It is worth mentioning that OX3604 males reared on tetracycline performed similarly to WT males (off tet) in all respects, and should a suitable pupal-sexing mechanism be in place in the factory, they may indeed be considered the most suitable insects for release.

A possible explanation for the somewhat diminished flight capacity in the genetically modified mosquitoes is the potentially negative effect of basal or off target production of the effector tTAV in both OX513A and OX3604. Any ‘leakiness’ in this system would lead to higher levels of tTAV production and therefore increase cell toxicity (transcriptional squelching) (Lin, McGrath et al. 2007) in the genetically modified mosquitoes. Different promoter regions are used in the creation of the two lines; this may affect the two lines differently. The Act5C promoter used in OX513A results in ‘all over body’ expression of the gene. Furthermore the production of tTAV is driven by a non-sex-specific positive feedback
system in this line. This is the reason males carrying this construct cannot survive without the addition of tetracycline to their larval diet. In the OX3604 line, on the other hand, the AeACT-4 promoter is used in combination with a sex-specifically modified alternative splicing region to control tTA production, targeting gene expression only in the indirect flight muscles of females. Consequently males of this line can survive without the addition of tetracycline to their larval diet and are far less likely to suffer excess effector production. This fits with our findings, as on the one hand, OX3604 males generally compared more favourably with the WT males than did the OX513A males, and on the other hand, OX3604 males (on tet) to some extent outperformed OX3604 males (off tet), suggesting possible leakiness in the tet-control element of the system.

It should be noted, that there was no significant difference in wing length of the males among the three lines, reared either with or without the addition of tetracycline, so that the differences between the flight potential and energy content of males could not be due to differences in size.

Besides flight capacity, the amount of energetic reserves available to males of the different lines upon eclosure differed among the three lines and rearing treatments. The differences in the energetic reserves were mainly attributable to variation in the lipid content of mosquitoes, rather than variation in their carbohydrate stores. This difference was particularly pronounced when examining mosquitoes reared with and without the addition of tetracycline to their larval diet, the addition of tetracycline leading to a significant increase in lipid stores, producing ‘obese’ (Van Handel 1965) mosquitoes upon emergence. Tetracycline and tetracycline-based compounds have in the past been added to animal feeds, e.g. to chicken and pig feed, as growth promoters (Swan Report 1969). The study suggests that adding tetracycline to the food of insects has a similar effect.

With the unexpected exception of WT males reared off tetracycline, the energetic reserves of males upon eclosure correlated roughly with their flight potential as adults. The WT males reared without tetracycline, however, had the lowest amount of energetic reserves upon eclosure. As the flight mill trials were conducted on three day old mosquitoes supplied unlimited access to 10% sucrose solution during their maturation it was expected for energy levels to change from their state at eclosure, though a complete reversal of ranking, i.e. from least amount of energetic reserves to strongest flyers, is possibly suggestive of a more efficient metabolic use of available energy sources after eclosure in the WT males.

In conclusion, although comparisons with field-bred mosquitoes remain desirable and the transgenic lines examined in general showed somewhat reduced flight potential compared to
their un-manipulated counterparts, the results suggest that the sex-specific ‘flightless’ line, OX3604, in particular, shows promise for use in control trials. Furthermore, this controlled and relatively quick approach to evaluating flight potential shows promise as a quality testing tool in transgenic line development.
5 Re-mating of female *Aedes aegypti*

5.1 Introduction

*Aedes aegypti* is one of the most significant dengue vectors worldwide and its control therefore of major concern and importance in affected countries. With the advancement of genetic transformation technology new approaches to the control of this species have been developed. One of these newly developed systems is RIDL (release of insects carrying a dominant lethal) (Thomas, Donnelly et al. 2000; Alphey 2002; Alphey, Nimmo et al. 2008; Alphey, Benedict et al. 2010), a genetics based control strategy modelled on the traditional sterile insect technique (SIT), engineered for *Ae. aegypti*, using tetracycline-dependent repression of a dominant lethal gene (Phuc, Andreasen et al. 2007; Fu, Lees et al. 2010).

The question of whether female *Aedes aegypti* are monogamous over the course of their lifetime or whether they re-mate is important with regard to SIT-based population control programmes. Early studies show monandrous behaviour of females and female refractory behaviour to any secondary insemination attempt (Craig 1967; Spielman, Leahy et al. 1967), thus demonstrating that females are inseminated only once, using only one complement of sperm to fertilise all future offspring. Yet, more recent studies conducted in the early 80s throw this presumption of strict lifelong monogamy into question (Williams and Berger 1980; Young and Downe 1982), indicating a willingness of females to re-mate once they have completed one or more gonotrophic cycles.

This is particularly crucial should females preferentially re-mate wild type males following insemination by a sterile male, or be more likely to reject a sterile male once inseminated by a wild type male.

The advances in genetic engineering mentioned above, which have created the need for a more detailed investigation into the mating ecology of *Ae. aegypti*, also supply us with a powerful tool kit to undertake investigations into the nature of mosquito reproduction, as most of the constraints and difficulties in distinguishing paternity and even sperm allocation have been alleviated. In this study we used the OX3879 strain which carries a GFP marker.
triggered during sperm production (Figure 5.1), resulting in males with fluorescent sperm in experiments designed to examine the transfer and storage of sperm. Furthermore, we used the OX513A line (Phuc, Andreasen et al. 2007), which carries a red-body marker as well as the RIDL system, and the OX3604C line (Fu, Lees et al. 2010), which carries a blue eye, and red body marker and a female-specific flightless system, in experiments to accurately assess paternity of offspring and any possible propensity of females to re-mate preferentially with wild type versus genetically altered males.

Figure 5.1: Dissection showing fluorescent sperm in the testes of a male *Aedes aegypti* (OX3878).

(Photograph: Derric Nimmo)
5.2 Methods

Two main sets of experiments were carried out to determine the mating behaviour of female *Ae. aegypti*.

The first focused on the likelihood of females to re-mate over the course of their lifetimes. Due to the high degree of handling involved (moving females between small containers for mating crosses and egg laying) mortality was increased, therefore the experiment was conducted in two parts; the first considering the first three gonotrophic cycles, the second considering later gonotrophic cycles.

As no incidence of re-mating was detected in the first set of experiments, i.e. in females that had in all likelihood received a full complement of sperm, a second set of experiments was undertaken to identify possible exceptions to monogamous behaviour in females, in particular following mating with sperm depleted males.

All experiments were conducted in a temperature-controlled insectary at a temperature of 28 (+/-2) °C and a relative humidity of 65 (+/-10) % with a 12h:12h light/dark cycle.

5.2.1 Experiment 1: Re-mating over several gonotrophic cycles

**Part 1**

Mosquitoes used in this experiment were reared at low density in one litre trays (200 larvae per tray) and fed finely ground TetraMin fish food; day 1: 0.06mg per larva, day 2: 0.12mg, day 3: 0.24mg, day 4: 0.36mg, day 5: 0.48mg, days 6 and later: 0.6mg. WT larvae were reared in one-day old tap water OX513A and OX3604C larvae were reared in the same water containing tetracycline (30µg/ml). For each type 15 trays were set up. Mosquitoes were sexed upon pupation and placed in single sex cages to emerge. WT females were moved to 12 cages, 100 in each and crossed with 100 males; two times 3 cages with WT males, 3 cages with OX513A males and 3 cages with OX3604 males. At the beginning of each cycle, mosquitoes were given three days to mate, following which males were removed from the cages and females were presented with a blood meal for three consecutive days. Subsequently females were transferred to individual vials containing wet cotton pads to encourage egg-laying for five days. All recovered eggs were dried for 4 days and counted before being submersed in water and placed under a vacuum to induce hatching. The larvae were then screened to determine paternity. Once paternity had been established, the females (mothers) were once again returned to the large cages to commence a further cycle of mating crosses.
For each gonotrophic cycle, females were combined with different type of males, according to the combinations (A, B, C and D) shown in Table 5.1. Mosquitoes were provided constant access to a 10% sucrose solution throughout the experiment.

**Table 5.1: Experimental design**

Sequence of crosses carried out for experiment 1, part 1. Each combination A, B, C and D was repeated three times.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT Females</strong></td>
<td><strong>WT Females</strong></td>
<td><strong>WT Females</strong></td>
<td><strong>WT Females</strong></td>
</tr>
<tr>
<td>+ WT Males</td>
<td>+ WT Males</td>
<td>+ OX513A Males</td>
<td>+ OX3604 Males</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>First gonotrophic cycle</strong></td>
<td></td>
</tr>
<tr>
<td>+ OX3604 Males</td>
<td>+ OX513A Males</td>
<td>+ WT Males</td>
<td>+ WT Males</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Second gonotrophic cycle</strong></td>
<td></td>
</tr>
<tr>
<td>+ OX513A Males</td>
<td>+ OX3604 Males</td>
<td>+ OX3604 Males</td>
<td>+ OX513A Males</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Third gonotrophic cycle</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Part 2**

As only a few females survived past the third gonotrophic cycle, a second set of experiments, with only WT and OX513A mosquitoes, was carried out to investigate female behaviour in later gonotrophic cycles. Rearing was conducted as in part one and again, cages were set up containing 100 WT females and 100 males (6 cages with WT males and 6 with OX513A males). Females were placed in individual tubes for laying over the first gonotrophic cycle and any females that did not lay viable eggs were discarded to ensure that all females used in the continuation of this experiment had indeed mated successfully. The females were again placed into large cages and progressed through three more gonotrophic cycles. Eggs were collected per cage not per individual. Over gonotrophic cycles 5 to 8 (the main focus of this experiment), females were fed in the large cages, but isolated for egg-laying. Furthermore, the strain of males offered was changed in this part of the experiment, so females that had
initially been presented WT males were now offered OX513A males and vice versa. Eggs were counted and screened to determine paternity.

5.2.2 Experiment 2: Exceptions to monogamous behaviour – mating with sperm depleted males
Mosquitoes used in this set of experiments were reared individually in the wells of 12-well plates (in 3ml of water) and fed the same food regime as described in section 1 above.

Part 1
To assess the degree of sperm depletion in repeatedly mated males, each of 20 WT males was offered five WT females in succession. This was accomplished by placing a male in a large cage and introducing a female, following which the couple was observed until a successful mating had taken place. This female was then removed and a virgin female added. Females were subsequently dissected and the contents of their spermathecae examined.

Part 2
Once sperm depletion in repeatedly mated males had been established, a further set of experiments was designed in order to gauge the readiness of females to re-mate following a mating with a sperm depleted male.
Each of 60 WT males were presented five WT females in succession; this time however half the females were offered an OX3878 (sperm-marked) male or an OX513A male either immediately following the first mating or 24 hours later. The females that had been offered an OX3878 male were dissected and the contents of their spermathecae examined. The females that had been offered an OX513A male were held in individual containers following the mating crosses and offered a blood meal on 3 consecutive days. Two days later, they were given wet cotton pads for egg-laying. The eggs were collected, dried and subsequently hatched at low atmospheric pressure. Pupae were counted and screened to determine paternity.
5.3 Results

5.3.1 Experiment 1: Re-mating over several gonotrophic cycles

Part 1
No re-mating was detected over three gonotrophic cycles. All offspring screened were parented by the first male the female mated in each case, regardless of whether that male was genetically modified or not. The number of eggs produced per gonotrophic cycle did not vary significantly between cycles or between paternity of eggs.

Part 2
As in part one, no re-mating was detected, this time across eight gonotrophic cycles. Again, the average number of eggs laid per female, did not differ between females mated to a wild type or a genetically modified male. The number of eggs laid per gonotrophic cycle on the other hand did vary significantly (F=6.6273, p<0.001) (Figure 5.2), decreasing in later gonotrophic cycles.

Figure 5.2: Number of eggs laid per gonotrophic cycle

Error bars represent the standard error. Cycles 2-4 (shaded area) do not have error bars as eggs were collected per cage, not per individual.
5.3.2 Experiment 2: Exceptions to monogamous behaviour – mating with sperm depleted males

Part 1
Mating females in succession depleted the sperm reserves available for subsequent mating (Figure 5.3). On average a ‘fresh’ male transferred enough sperm to fill at least 2 spermathecae (average 2.05 ± SE 0.088), while a male that had already inseminated four females previously had only enough sperm reserves to fill less than half a spermatheca (average 0.4 ± SE 0.124).

![Figure 5.3: Depletion of sperm reserves of repeatedly mated males](image)

Error bars represent the standard error (N=20).

Part 2
Females offered a second male immediately after insemination by a WT male were more likely to re-mate. This tendency to re-mate becomes more pronounced depending on how many females the WT male had previously inseminated. Specifically, as the sperm reserves of the first male became depleted females were more likely to accept a second mating, if this was offered immediately. In contrast, if the second male was offered 24 hours after the initial exposure to a WT male, females were less prepared to re-mate. The first three females to be mated in succession by the WT male did not re-mate when offered a second male 24 hours later. The fourth and fifth female in the sequence (once the WT male was increasingly sperm depleted), on the other hand, contained fluorescent sperm upon dissection, yet their numbers
were significantly less than corresponding females that had been offered a second mating immediately (Figure 5.4). The paternity results support the results obtained from the spermathecal dissections. In cases where the second mating was offered immediately a substantial amount of females produced mixed batches of offspring (i.e. WT and OX513A) from approximately the second female in sequence onward, whereas if the second mating opportunity was presented 24 hours later, mixed batches of offspring were only common with the fourth and fifth female in sequence (Figure 5.5).

**Figure 5.4: Results of spermathecal dissections**

Spermathecae of females offered an OX3878 male immediately following the first mating (solid line) are more likely to contain fluorescent sperm than those of females offered a second male 24 hours later (dashed line). Error bars represent the standard error.
Figure 5.5: Paternity results

Females offered an OX513A male immediately following the first mating [a]) generally produced more batches of mixed offspring (WT/OX513A - blue) than females offered a second mating 24 hours later [b]). Proportion of WT offspring - white, OX513A - grey, no eggs - black.
5.4 Discussion

The results corroborate Gwadz (1970) and Craig’s (1967) early work and confirm that *Aedes aegypti* females are generally monogamous over the course of a lifetime. As all offspring screened were progeny of the first insemination, we saw no renewal of sexual receptivity among mated females. This contradicts the findings by Williams et al. (1980) and Young et al. (1982) who showed female polygamy in *Aedes aegypti* following completion of the first gonotrophic cycle. One possible explanation for this disparity in results is that differences exist between colonies. However, it should be noted that neither of the more recent papers examined offspring to assess paternity but rather determined insemination success either by dissecting the female to measure the distension of the bursa (Williams and Berger 1980) or with radio-labelled sperm (Young and Downe 1982). Therefore, despite the presence of sperm from a second insemination, the sperm transferred from the secondary mating might not have been used to fertilise eggs, rendering the females ‘monogamous’ in the context of paternity. Spielman, Leahy et al. (1967), for example, describes the expulsion of sperm by previously inseminated females, providing a possible mechanism underlying this form of sperm selection/loyalty.

Yet, as seen in the second set of experiments carried out and in line with previous research (Christophers 1960; Gwadz and Craig 1970), exceptions to the strict monogamy witnessed over a female’s lifetime do exist under certain circumstances, i.e. if a second male attempts mating shortly after the first or if the first male was sperm depleted. Furthermore, forced copulations have been shown to result in secondary insemination (Spielman, Leahy et al. 1967). The question of how often these situations occur in nature is pivotal in assessing the importance of such exceptions. It is conceivable that sterile males or less fit males in general are chased away during a mating attempt or transfer only a small amount of sperm, thus leaving the female receptive to a secondary mating attempt. Alternatively, highly desirable males with mating opportunities may eventually become sperm depleted, again leaving females receptive to consequent mating attempts.

Previous experiments conducted on exceptions to female monogamy in *Ae. aegypti* generally concluded that secondary insemination was possible only before the monogamising effect of *matrone*, a male accessory gland substance that renders the female refractory to further insemination, had set in; i.e. between four to six hours following insemination (Craig 1967; Spielman, Leahy et al. 1967). The results of secondary mating 24 hours after the first
suggests that, if a male is sufficiently sperm-depleted, either it transfers no matrone, or its matrone cannot render the female refractory.

These considerations are particularly interesting in the context of mosquito control with genetically sterile males. OX513A males have a lower insemination capacity than WT males (Chapter 3), i.e. they are able to inseminate fewer females over the course of their lifetime. This may mean they are more likely to become sperm depleted sooner and thus may be more likely to transfer an insufficient amount of sperm to prevent a female re-mating than their wild type counterparts, opening the possibility for secondary insemination by wild-type males and rendering the control strategy less effective.

Although limited by the fact that they were conducted under laboratory conditions and comparison with wild mosquitoes in a more natural field setting remain desirable, our results suggest female *Ae. aegypti* are generally monogamous. Experiments to determine the frequency with which females may copulate in the field are needed to determine the incidence of re-mating due to insufficient sperm transfer, yet we predict the occurrence of multiple inseminations to be the exception rather than the rule. In particular, the absence of any ability or preference of females to selectively re-mate if inseminated by a genetically modified male may be of interest to SIT-based control efforts.
Chapter 6

6 Evidence for post-mating female choice in the dengue fever vector, *Aedes aegypti*

6.1 Introduction

Females of many mosquito species are considered to be monogamous, inseminated only once over the course of a lifetime (Craig 1967; Spielman, Leahy et al. 1967). Thus mated females store the sperm and use it to fertilise egg batches over several gonotrophic cycles, and they are refractory to any secondary insemination attempts. As, therefore, the quality of the single mate has a large impact on the female's reproductive success, evolution is expected to have led to a strong female preference for the mate leading to the greatest reproductive success. Yet, in swarming anophelines scramble competition among males is generally considered to be the predominant factor in mate choice. In *Ae. aegypti*, however, there may be more opportunity for female choice. Their mating behaviour resembles a lekking system (Cabrera and Jaffe 2007), with males aggregating close to a potential host, flying a distinctive figure of eight pattern and approaching the female after she has taken a blood meal. Although female choice has not been documented for *Ae. aegypti*, it is common in species mating in leks (Bateson 1983). Assessing the prevalence of female mate choice of *Ae. aegypti* has practical implications for control programmes, in particular strategies based on the sterile insect technique (SIT), in which sterile males are released into the field to compete for access to females. This chapter therefore compares males of the RIDL line OX513A (Phuc, Andreasen et al. 2007) designed for use in such a programme with their wild type (WT) counterparts. The RIDL system has been engineered for *Ae. aegypti*, using tetracycline-dependent repression of a dominant lethal gene (Heinrich and Scott 2000; Thomas, Donnelly et al. 2000; Phuc, Andreasen et al. 2007). Tetracycline can be introduced as a dietary component to mosquitoes reared in the laboratory but is not freely available in the wild. Therefore, the lethal system is repressed in the laboratory and activated upon release. Upon their release, transformed males, homozygous for this lethal construct, would pass one copy of the dominant lethal to their offspring, which would subsequently die as larvae or pupae in the
wild due to the absence of tetracycline. Thus, over time, releases of genetically modified males are hoped to reduce and, eventually, eliminate the targeted mosquito population. In such a programme it is therefore essential that released males mate with wild type females and fertilize their eggs.

The preference of wild type females for the wild type male or the OX513A male was measured at two stages: pre-mating preference or coupling (whether the female forms a couple with the wild type or with the OX513A male, in which the male may strive for a copulatory position but not achieve it (see Gwadz, Craig et al. 1971)) and post-mating preference or insemination. Whether pre-mating preference, which is often used in mating studies (Ng’habi, John et al. 2005; Ng’habi, Huho et al. 2008), relates to post-mating preference is of particular interest.

6.2 Methods

6.2.1 Experimental design and larval rearing

All experiments were conducted in a temperature-controlled insectary at a temperature of 28 (±2) °C and a relative humidity of 65 (±10)% with a 12h:12h light/dark cycle.

Eggs of the wild type Ae. aegypti line and the genetically modified OX513A line were submerged in water and placed under low pressure for one hour to ensure synchronous hatching. To rear adults of different sizes, the following day, larvae were counted out into 100 ml pots of water supplemented with tetracycline to a final concentration of 30µg/ml at densities of 100, 400 and 800 larvae per pot, thus giving rearing conditions of 1, 4 and 8 larvae/ml. Thirty pots were set up for each treatment. The larvae were fed the following feeding regime of finely ground TetraMin fish food per larva: day 1 – 0.03 mg, day 2 – no food, day 3 – 0.04 mg, day 4 – 0.08 mg, day 5 – 0.16 mg, day 6 – 0.16 mg, day 7 onwards – 0.32 mg.

Mosquitoes used in this experiment all pupated on day 8 and emerged on day 10. One female and two males were picked at random from each pot. One male was used in the ‘first to mate’ experiment while the other was used in the competition crosses described below. The females used in all experiments were never from the same pot as either of the males competing for her.
6.2.2 First male to mate

Five days after emergence, a WT male and an OX513A male were aspirated into a cage (dimensions: 28 x 28 x 28 cm). The order in which the males were aspirated into the cage was alternated. Several minutes later, a five-day old WT female was added. The mosquitoes were observed until the first mating attempt took place. The couple was aspirated out of the cage and placed in a falcon tube. The male who had not mated was placed in a second falcon tube and the three mosquitoes were frozen.

Three sets of ‘first to mate’ competition experiments were carried out (30 repeats of each; 90 in total). In the first set the males (WT and OX513A) were from the same rearing density of 4 larvae/ml. In the second set the WT males were reared at low density (1 larva/ml) while their OX513A competitors were reared at high larval density (8 larvae/ml). In the final set, the OX513A males were reared at low density (1 larva/ml) while their WT competitors were reared at high density (8 larvae/ml). All the females used in these competition crosses were reared at 4 larvae/ml in order to maintain a standard size female for which the males competed.

6.2.3 Paternity

An additional 90 (30 repeats of each treatment) competition crosses were set up. The mosquitoes were left in smaller cages (20 x 20 x 20 cm) for 3 days with unlimited access to sugar solution. On days 4 and 5 the females were offered a blood meal and, once they had fed, were transferred to individual laying tubes. After they had laid eggs, they were frozen. The eggs were counted and hatched. After 3-4 days (so handling was more practical) the larvae were screened under the fluorescence microscope for red body expression to determine paternity. Mosquitoes of the OX513A line carry a red marker gene that is expressed over the whole body (see Chapter 1).

6.2.4 Measurements

In both sets of experiments, the wings were removed under a dissection microscope and mounted on microscope slides. Digital images of the wings alongside a graticule for purposes of scale were taken using a Canon PowerShot SS1S camera and a 99 mm adapter (S/N:3754, Martin Microscope Company). Wings were measured with ImageJ (http://rsbweb.nih.gov/ij/) from the auxiliary incision to the apical margin excluding the fringe.

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PCR analysis was used to determine paternity in these experiments as the fluorescent markers in OX513A, useful in larval or pupal-stage screening, are no longer visible in the adult mosquito. The heads of the male mosquitoes were removed so that the pigments found in the eyes would not interfere with the PCR analysis. The mosquitoes where subsequently stored individually in 1.5 ml Eppendorf tubes. Genomic DNA was extracted using the Nucleospin DNA kit (Macherey-Nagel) and 4 μl was used as a template for the PCR reaction (Table 6.1) under amplification conditions in Table 6.2.

**Table 6.1: PCR template**

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>4 μl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.75 μl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.75 μl</td>
</tr>
<tr>
<td>dNTPmix</td>
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</tr>
<tr>
<td>10xBuffer</td>
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</tr>
<tr>
<td>BSA</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>MiliQ water</td>
<td>15.75 μl</td>
</tr>
</tbody>
</table>

**Table 6.2: PCR amplification**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturing</td>
<td>94°C</td>
<td>2’</td>
<td>x 1</td>
</tr>
<tr>
<td><strong>Cycle 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturing</td>
<td>95°C</td>
<td>10’’</td>
<td></td>
</tr>
<tr>
<td>Annealling</td>
<td>56°C</td>
<td>1’</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>2’</td>
<td>x 3</td>
</tr>
<tr>
<td><strong>Cycle 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturing</td>
<td>95°C</td>
<td>10’’</td>
<td></td>
</tr>
<tr>
<td>Annealling</td>
<td>56°C</td>
<td>30’’</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>55’’</td>
<td>x 34</td>
</tr>
<tr>
<td><strong>Final Extension</strong></td>
<td>72°C</td>
<td>5’</td>
<td>x 1</td>
</tr>
</tbody>
</table>
Two sets of primers were used, one as a control to detect the presence of *Ae. aegypti* DNA, the other to distinguish between OX513A and WT males.

The control primer sequences were:
Forward primer: 5’-GGTAGGAGACTTGAAAGACGTCAG-3’
Reverse primer: 5’-GAAGGAGGACGTCATAAGATGAAG-3’
The expected band length was 450 bps.

The OX513A-detection primer sequences were:
Forward primer: 5’-GGACGAGCTCCACTTAGACGG-3’
Reverse primer: 5’-CAACTCTTCTCGTTTTGAAGTCAGC-3’
The expected band length was 400 bps.

A 1% agarose gel with 1.5 μl of ethidium bromide (10 mg/ml, Invitrogen) was prepared and transferred to a gel cassette to set. 5 μl of the PCR product mixed with 6 μl of bromophenol blue loading dye (0.25% bromophenol blue, 30% glycerol in water) was loaded into each well in the gel. 5 μl of SMART ladder (Eurogentec) was loaded into the first well of each row. The gel was then run at 120 V for 30 minutes. The gel was visualized using a UV light source and video camera system (UVIttech).

### 6.2.5 Statistical analysis
Statistical analyses were performed with JMP version 7.0 (http://www.jmpdiscovery.com). Logistic models (Berkson, 1944) were used to assess the contribution of wing length and rearing density to the likelihood that a WT or an OX513A male would be first to attempt mating or would father offspring. The models were adjusted for the blocking of the experiments by adding ‘day of hatching’ as a fixed effect. Exact confidence intervals are given for all percentages, while Fisher’s exact tests (Fisher, 1922) were used to assess variation between treatments.
6.3 Results

6.3.1 Wing measurements
Overall, WT males had longer wings (2 ± 0.01 mm) than OX513A males (1.97 ± 0.01 mm) (F=17.03, df=1, p<0.001). Increasing the larval rearing density decreased the average wing length (F=37.12, df=2, p<0.001) from 2.03 (±0.01) mm at 1 larva/ml to 1.94 (±0.01) mm at 8 larvae/ml. This effect was stronger for OX513A males (0.14 mm difference from low to high rearing density) than for WT males (0.04 mm difference) (interaction between line and density: F=11.56, df=2, p<0.001).

6.3.2 First male to mate
Overall, WT and OX513A males were equally likely to mate with the female first (Fisher’s exact test, p = 0.877), with WT mating first in 51.19% (95% CI: 40.04% to 62.26%) of the trials. Rearing density had little direct effect on this conclusion (Table 6.3). When WT males reared at 1 larva/ml competed against OX513A males reared at 8 larvae/ml they each mated first in 50% (95% CI: 29.93% to 70.07%) of the trials (13 out of 26), when the two competing males where reared at 4 larvae/ml WT males mated first in 48% (95% CI: 29.45% to 67.47%) of the trials (14 out of 29) and when competing WT males were reared at 8 larvae/ml and OX513A males at 1 larva/ml, WT males mated first in 55% (95% CI: 35.69% to 73.55%) of the trials (16 out of 29).

Wing length, however, was a strong predictor of the likelihood a male would make the first mating attempt (Table 6.3). Smaller males were more likely to approach the female first. The smaller the OX513A male and the larger the WT male, the more likely the OX513A male would be to approach first (Table 6.3). Thus, the smaller of the two competing males (WT and OX513A) was considerably more likely to mate than the larger of the pair (Figure 6.1).

6.3.3 Paternity
In the experiment where multiple matings were allowed, WT males were more successful at fathering offspring than their OX513A counterparts, fathering 65% of the offspring (ChiSquare = 9.77, df = 1, p = 0.002). Rearing density strongly influenced paternity (Table 6.3). When WT males were reared at 1 larva/ml and competing OX513A males at 8 larvae/ml, WT males fathered the offspring of 67% (95% CI: 40.99% to 86.66%) of the females (12 out of 18); when WT and OX513A males were reared at 4 larvae/ml, WT males fathered the offspring of 90% (95% CI: 68.30% to 98.76%) of the females (18 out of 20).
females) and finally when WT males were reared at 8 larvae/ml and OX513A males at 1 larva/ml, WT males fathered the offspring of 38% (95% CI: 18.11% to 61.56%) of the females (8 out of 21 females) (showing no significant difference between WT males and OX513A males).

Wing length influenced paternity in the opposite direction to first mating: Larger males were more likely to father offspring (Figure 6.2, Table 6.3) than smaller ones. Furthermore, the interaction between the sizes of the two males shows that a male's size relative to that of his competitor is a deciding factor in paternal success (Table 6.3).

Table 6.3: Predictors of mating success

Model results showing the effects of rearing density and adult size on the likelihood an OX513A male would either be the first male to mate or actually father offspring. Statistically significant predictors are marked with an asterisk.

<table>
<thead>
<tr>
<th></th>
<th>First male to mate</th>
<th>Paternity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>Chi-Square</td>
</tr>
<tr>
<td>Block (Day of hatching)</td>
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<td>9.74</td>
</tr>
<tr>
<td>Rearing density</td>
<td>2</td>
<td>4.99</td>
</tr>
<tr>
<td>Wing length OX513A</td>
<td>1</td>
<td>7.04</td>
</tr>
<tr>
<td>Wing length WT</td>
<td>1</td>
<td>3.13</td>
</tr>
<tr>
<td>Wing length OX513A x Wing length WT</td>
<td>1</td>
<td>1.04</td>
</tr>
</tbody>
</table>
Figure 6.1: Effect of body size on mating success

Comparison of the relative size of competing males that either made the first mating attempt or actually fathered offspring (all data combined). Error bars represent the 95% CI.

Figure 6.2: Effect of ‘line’ (WT or OX513A) on mating success

Comparison of males that either approached the female first or actually fathered offspring; WT or OX513A (all data combined). Error bars represent the 95% CI.
6.4 Discussion

Due to the lek-like nature of the mating system of *Aedes aegypti* (Cabrera and Jaffe 2007) both forms of sexual selection are likely represented; intra-sexual selection in the form of male-male competition as well as inter-sexual selection in the form of female mate choice. Competition exists between males as they jostle for position at the host and for access to females. Because females are monandrous and rendered refractory to secondary insemination by the transfer of an accessory gland substance (‘matrone’) during mating (Fuchs, Craig et al. 1968; Gwadz 1972), the pressure to be the first male to inseminate a female is high. Conversely, because she will generally use sperm from only one male, mating with the fittest male is a desirable strategy for females to ensure the highest reproductive success. Therefore inter-sexual selection is most likely also an important factor in this mating system. The results of these experiments confirm the existence of female mate choice in *Ae. aegypti*, as the relative size and strain (WT or OX513A) differed between males that made the first mating attempt and males that actually fathered offspring (Figures 6.1 and 6.2). It is conventionally thought that if a male is the first to successfully couple with or seize a sexually receptive virgin female and establish genital contact he will also successfully inseminate that female, however, Gwadz, Craig et al. (1971) showed female sexual behaviour to be a key factor in rendering females refractory to insemination. Young females and females that had previously been inseminated turned their terminalia away from the males’ during coupling, thus preventing complete copulation and insemination (see Figure 6.3). These subtle differences in coupling could not be seen without the aid of a stereoscope and thus are indistinguishable to the naked eye (Gwadz, Craig et al. 1971). Even though this behaviour has so far only been established in refractory females, its existence offers the potential for receptive females to exert mate choice until very late in the courtship ritual. Though they do not verify the exact mechanism involved, the results presented here indicate that female mate choice indeed takes place and that for *Ae. aegypti*, using the first male to attempt mating as a gauge of overall insemination success is not a suitable approach.
Figure 6.3: Differences in the mating behaviour of receptive and refractory female Ae. aegypti

Taken from Gwadz, Craig et al. (1971); Pictures 1 and 2 show the positions of male and female genitalia in successful copulation; Pictures 3 and 4 show the positions of male and female genitalia in an unsuccessful coupling.

The existence of female mate choice has practical implications for SIT-based control, such as the RIDL system. Male intra-sexual selection will be intense at a mating site or host, should large differences exist in the quality of males competing, many males may obtain few mates, while few males may obtain many mates (Arita and Kaneshiro 1985). If females actively select and discriminate in favour of fitter males (Heath, Epsky et al. 1994; Eberhard 2002), they may favour the courtship of a wild male even if he is in the minority. Increasing the proportion of sterile males or over-flooding ratio will in this case be less effective in overcoming reduced sterile male performance. Furthermore, the existence of female mate choice increases the possibility of ‘resistance’ developing in the natural population (Robinson, Knols et al. 2009). An example of this
principle can be found in the appearance of assortative mating among female melon flies during the SIT control programme conducted on Okinawa (Hibino and Iwahashi 1991). As stated in Robinson, Knols et al. (2009) certain requisites must be met for female selection to favour wild type males, in particular the existence of a recognisable trait in the sterile males (and heritability of the recognition of the trait in the field females). For this reason selectively breeding for traits correlated to male mating success, such as for example larger size, should be treated with caution as they may lead to the promotion of such a trait.

A further aim of these experiments was to assess the importance of male size in determining mating success. Little information is available on the importance of male size in mosquito mating systems, though some examples can be found. Ponlawat and Harrington (2007) and Dickinson and Klowden (1997), for example, showed an increase in sperm production and protein transfer in large males, while Ng’habi et al. (2005; 2008) investigated the role of size in swarming anopheline species. As relative body size was the most significant factor in determining which male made the first mating attempt and, to a certain extent, the likelihood of a male fathering offspring, it is evidently of importance in this mating system. Interestingly, smaller males attempted to mate first, while larger males were more likely to successfully father offspring. While it is less surprising that the larger males secured more successful mating opportunities, as this would fit with the current knowledge of the structure of *Ae. aegypti* mating systems, it is more significant that the small males made the first attempt at mating with the female. This may indicate an alternative strategy for small males. As females are more likely to mate with larger males given the choice, small males are at a disadvantage; their only chance at reproduction may be to reach a female before she has time to assess other males. Such alternative strategies, often adopted by smaller or weaker males, can be seen in a number of animal species (Ra’an an and Sagi 1985; McLachlan and Neems 1988; Himuro, Hosokawa et al. 2006) and are thought to contribute to the maintenance of existing variation in male body size.

While more detailed studies are necessary to identify the exact contributions body size and rearing density make to the competitive ability of genetically modified versus wild type males, these results clearly indicate the potential of optimising rearing methods for sterile males as well as underlining the necessity for high quality control during production.
7 Cage trials assessing the competitive ability of males of the RIDL line OX513A compared to an unmodified line (WT) of *Aedes aegypti*

7.1 Introduction

The development of genetic transformation methods in disease vectors such as *Aedes aegypti* (Coates, Jasinskiene et al. 1998; Jasinskiene, Coates et al. 1998) has paved the way for new approaches to disease control. One such approach is RIDL (release of insects carrying a dominant lethal), a genetics-based control strategy modelled on the traditional sterile insect technique (SIT) (Thomas, Donnelly et al. 2000; Alphey 2002; Alphey, Nimmo et al. 2008; Alphey, Benedict et al. 2010). This strategy uses repressible genes that kill the insect when they are expressed (Heinrich and Scott 2000; Thomas, Donnelly et al. 2000) and has been engineered for *Ae. aegypti*, using tetracycline-dependent repression of a dominant lethal gene (Phuc, Andreasen et al. 2007; Fu, Lees et al. 2010). Tetracycline, a compound not readily available in the wild, can be added as a dietary supplement to mosquitoes reared in the laboratory; hence the lethal system is repressed in the laboratory and activated upon release. In the field, transformed males, which are homozygous for this lethal construct, would pass one copy of the dominant lethal to their offspring by normal Mendelian inheritance. As tetracycline is absent, these would subsequently die as larvae or pupae. Thus releases of sterile males are expected to reduce and perhaps, under appropriate circumstances, eventually eliminate the targeted mosquito population.

This chapter compares the competitive ability of a RIDL line, OX513A (Phuc, Andreasen et al. 2007) with that of its wild type (WT) counterpart (a laboratory line with similar genetic background, yet without the transgene insertion). Releases of mosquitoes must necessarily be restricted to males as the repeated release of females might increase biting nuisance or the incidence of disease. Therefore, it is the competitive ability of males, in particular, that is of interest. A lack of competitive ability of males upon release contributed to the failure of previous SIT trials against mosquitoes (reviewed in Benedict and Robinson 2003). Although
insects that are engineered with dominant lethal systems such as RIDL are expected to suffer less fitness costs than their irradiated counterparts used in early SIT programmes, certain fitness burdens may remain: on the one hand from the process of transposon-mediated transformation and the subsequent inbreeding necessary to create a homozygous line (Marrelli, Moreira et al. 2006), on the other hand from the mass-rearing that produces the large numbers of males necessary to make a RIDL programme effective and sustainable (Bar-Zeev 1957; Wada 1965; Southwood, Murdie et al. 1972; Gilpin and McClelland 1979; Dye 1982; Dye 1984; Agnew, Hide et al. 2002; Bedhomme, Agnew et al. 2003).

The previous chapters show that several life history parameters differ between the OX513A and the WT line and are affected by mass-rearing conditions. This chapter expands on these results by comparing the competitive ability, measured as ability to mate, of males of the two lines in controlled cage trials.

### 7.2 Methods

Mosquitoes used in this experiment were reared at low density in one litre trays (200 larvae per tray) and fed the following feeding regime of finely ground TetraMin fish food per larva; day 1: 0.06mg, day 2: 0.12mg, day 3: 0.24mg, day 4: 0.36mg, day 5: 0.48mg, days 6 and later: 0.6mg/individual. WT larvae were reared in day old tap water, while OX513A larvae were reared in water containing tetracycline (30µg/ml). Mosquitoes were sexed upon pupation and placed in single sex cages to emerge and mature for three days. Subsequently, 1000 cages were set up, each containing 10 virgin females (WT) and varying ratios and numbers of WT and OX513A males (50 repeats of each treatment). Five ratios of WT vs OX513A males were examined (1:9, 3:7, 5:5, 7:3 and 9:1), and the total number of males per cage ranged from 10 to 40 with increments of 10.

Mosquitoes remained in these cages for 3 days. Females were offered a blood meal daily. Subsequently females were moved to individual tubes to lay eggs (on moist cotton wool). The eggs were stored for a minimum of 5 days before being hatched and the larvae screened for red fluorescence to assess paternity.

Adult mosquitoes were provided access to a 10% sucrose solution throughout the experiment. The wing lengths of a sample of males (50 of each line), reared according to the protocol described above were measured to check for any differences in size. Wings were removed
and mounted on microscope slides. Digital images of the wings were taken using a Canon PowerShot S5IS camera and a 99 mm adapter (S/N:3754, Martin Microscope Company). Wings were measured with ImageJ (http://rsbweb.nih.gov/ij/) from the auxiliary incision to the apical margin excluding the fringe.

7.3 Results

WT males had a distinct advantage over OX513A males in controlled cage trials (Figure 7.1). Overall, out of 1000 trials (10000 females), WT males inseminated 62.34 % of the females, while OX513A males inseminated 37.66 %. In 1.78% of the trials, both males inseminated the same female and the female produced a mixed batch of offspring. When reviewing only the trials in which males were under actual competition for females (i.e. excluding the trials 10 males caged with 10 females) WT males actually fathered 69% of the offspring when competing at a ratio of one to one with OX513A males.

If there were no competitive advantage of either line, the percentage of females inseminated by WT or OX513A males would increase proportionally to the frequency of the two lines. Indeed, the percentage inseminated by WT increased with its frequency (F=2996.45, df=1, p<0.001). To estimate a competitive advantage, we analysed the difference between the percentage of females inseminated by WT males and the percentage of WT males in a cage; with no competitive advantage or disadvantage, this would be 0, while with an advantage it would be positive.

This measure of competitive advantage increased with increasing male density (number of competing males) (F=48.57, df=3, p<0.001) with WT males inseminating 5% more females than expected at densities of 1 male per female up to 20% more at 4 males per female. The advantage of WT males decreased with increasing frequency of WT males (F=45.6, df=1, p<0.001), showing females to actively select for WT males when they were at low frequencies in the population (Figure 7.1).

The number of females that produced eggs that did not hatch increased significantly with increasing proportion of OX513A males (F=11.83, df=1, p<0.001) from 0.65% (1 OX513A : 9 WT) to 1.6% (9 OX513A : 1 WT).

There was no difference between the average wing lengths of WT males (2.073 ± 0.013 mm) and OX513A males (2.081 ± 0.13 mm) (df = 1, F = 0.002, p = 0.921) reared following the protocol outlined above.
Figure 7.1: Competitive ability of WT males

Comparison of the competitive ability of WT males over several ratios of WT vs OX513A (1 - 9, 3 - 7, 5 - 5, 7 - 3, 9 - 1) and total number of males competing (10 – black line, 20 – red line, 30 – green line, 40 – blue line).

7.4 Discussion

The males of the WT line inseminated a greater proportion of females than their genetically modified counterparts. This corroborates the previous work on the life history parameters (Chapter 2), insemination capacity (Chapter 3) and flight potential (Chapter 4) of these two lines. All of these studies suggest that OX513A are less successful than the WT mosquitoes. When competing at a ratio of one to one (over all densities), males of the WT line inseminated 65% of the females that produce offspring, while OX513A males fathered only
35 %, making WT males 1.86 times as likely to father offspring than the genetically modified mosquitoes.

The relative fitness of the two strains varied with reference to the conditions under which they competed, changing with both the ratio of WT to OX513A males as well as with the total number of males per cage. WT males were increasingly likely to inseminate more females than their OX513A counterparts with both increasing frequency (ratio) of WT males as well as increasing overall density of males (total number). Furthermore, females selectively mated with WT males when they were present at low frequencies. This is relevant with respect to the implementation of control trials. It highlights the importance of accurate estimates of the structure and size of the target field population and influences decisions about suitable release numbers.

Furthermore, the results show a correlation between the number of OX513A males in a trial and the number of females that produced sterile egg batches. This could, in itself, indicate a fitness deficit for this line and might have a slight impact when rearing the line for production. However, in the field, it has little influence, as the males still fulfil their function as ‘sterile’, i.e. inseminating females but producing no surviving offspring. Nevertheless, as demonstrated by Phuc et al. (2007), the production of sterile eggs is less desirable than the production of offspring doomed to death in late larval development or as pupae, as early larval development is strongly density dependent. Removing eggs from breeding sites rather than pupae may thin the population, leaving more resources free for the remaining larvae, causing an increase in survival and thus an increase in population growth rate instead of the desired reduction (Rogers and Randolph 1984).

These trials are the first to assess the competitive ability of a RIDL line in cage trials under controlled laboratory conditions and therefore provide insight into the possible weaknesses of this line. Nonetheless, they cannot be considered a substitute for thorough field testing. On the one hand, confining mosquitoes to caged mating arenas may influence their natural mating behaviour. On the other hand, the ‘wild type’ line used in these trials, chosen because of its similar genetic background to the RIDL line, is a lab-adapted strain, colonized over twenty years ago and therefore will vary considerably to mosquitoes in the field. The estimates of competitive ability for the RIDL line are therefore conservative estimates. Nevertheless, these trials do show the ability of OX513A males to mate at least a considerable proportion of the females. Therefore, given the right conditions and an appropriate release strategy, the OX513A line may be a useful tool in the control of *Ae. aegypti*. 
Chapter 8

8 Summary and Conclusions

8.1 Introduction

In view of the significant re-emergence of dengue fever in large parts of the world (WHO 2009) and the fact that previously used methods of control (e.g. the widespread use of DDT) have become less popular and have therefore failed to suppress the disease (see Chapter 1), it has been necessary to develop new strategies aimed at vector-control. *Aedes aegypti*, genetically engineered for use in SIT-based control programmes, have been developed in response to this need (Thomas, Donnelly et al. 2000; Alphey 2002; Alphey and Andreasen 2002; Fu, Condon et al. 2007; Phuc, Andreasen et al. 2007; Alphey, Benedict et al. 2010).

When I embarked on this field of study the dominant lethal line OX513A of *Ae. aegypti* was at a stage were ‘quality-testing’ of the mosquitoes was important in order to assess their potential use. By necessity, the first trials of any genetically modified organism destined for field-release have to be laboratory-based (largely due to regulatory constraints), with testing of the RIDL line OX513A, the main focus of this thesis, being no exception. I therefore focused on comparing the competitive ability and behavioural aspects of the RIDL line OX513A to the wild type (WT) line it was genetically most similar to under controlled laboratory conditions, thus building a picture of the line’s strengths and weaknesses.

As the WT line used in the experiments for this thesis is highly lab-adapted and inbred, it will itself differ substantially from any target field population and therefore cannot be considered a substitute for testing against field-bred lines. Furthermore, as the WT and the OX513A were maintained under identical laboratory conditions, not only are they genetically similar but they are also likely to be behaviourally more similar compared to a field-bred line.

Consequently, it can be expected that the modified males will face ‘tougher’ competition upon release than these laboratory trials can simulate. The differences detected between the lines here should therefore be treated as conservative estimates. Out-crossing the genetically modified line destined for field release with field-bred, wild mosquitoes may ameliorate these
differences and make the line more competitive. Although, the out-crossed line itself would again have to be inbred to a certain extent to ensure homozygosis of the RIDL insert before release. Field-cage trials using wild mosquitoes should be the next step in quality assessment. For the time being though, the results of laboratory-based research give a good indication of the expected competitive ability of this line.

8.2 Summary of results

The experiments conducted as part of this thesis show significant differences between males of the genetically modified OX513A line and the WT line that share an otherwise similar genetic background. Apart from their faster pupation rate, which may be beneficial in mass-rearing, most of these differences point to reduced performance of males of the OX513A line. Mosquitoes of the OX513A line showed reduced larval survival, produced smaller, shorter-lived adults under similar larval crowding conditions (Chapter 2) and reacted more strongly (greater decrease in wing length) to increased larval density than their WT counterparts (Chapter 2). Males inseminated fewer females; on average 6.6 during their lifetime opposed to 11.5 for WT males (Chapter 3) while more of the females they mated produced sterile eggs (Chapter 7). Furthermore, they were only able to cover 57.75% of the distance an average WT male could fly in a 20h period (Chapter 4). Finally, cage trials showed that in direct competition for females they were consistently outperformed by the WT males (Chapter 7). Their performance decreased with both increasing frequency of WT males as well as with increasing total number of males (competitive pressure).

8.3 Relevance to RIDL application

However, though desirable, males of the genetically modified line need not be equally competitive to wild males for an SIT-based control strategy to succeed. As long as mating between released males and wild females takes place to a certain extent their competitive disadvantage can be compensated for by releasing sufficiently large numbers of males. Some Medfly programmes, for example, operate at ratios of sterile to wild insects as high as 100:1 (InSecta 2007). SIT / RIDL programmes are generally considered most effective as part of an integrated pest management programme, i.e. in combination with other control methods. Lowering the number of mosquitoes in the target population with traditional control methods
can aid in achieving the necessary over-flooding ratios for the SIT / RIDL component of the programme to be effective.

Consequently, possessing an accurate estimate of competitive ability aids programme design insofar as it allows an approximation of the minimum number of sterile males needed to suppress the natural population. However, the final effectiveness of the released males comprises a number of factors, of which competitive ability is just one.

An accurate assessment of the size and distribution of the wild target population is of obvious importance, as well as the evaluation of immigration into the population (in particular of mated females). Life history parameters of sterile and wild males may also play a role. The longevity of OX513A males (as seen in Chapter 2) is shorter than that of WT mosquitoes and most likely will be significantly shorter than that of mosquitoes in the field. The frequency of releases may need to reflect this difference. Furthermore, the dispersal ability of genetically modified males may not be as great as that of their wild counterparts (see Chapter 4). The distribution of release sites may therefore be of particular importance. Finally, a certain proportion of released males may be lost to predation in the wild.

Consequently, the actual number of sterile males needed to inseminate one wild female will be significantly higher than presented by their competitive ability alone. A clear example of this principle can be found in Patterson et al. (1975). Here a line of *Culex quinquefasciatus*, proven highly competitive in laboratory trials, is shown to be far less effective than expected upon release. In fact, 3-4 times more mosquitoes than estimated were needed for release to achieve the desired effect in the field.

The mating behaviour of mosquitoes may also influence the effectiveness of sterile males in the field. *Aedes aegypti* mate at the host (Hartberg 1971), where males fly a distinct figure-of-eight pattern and intercept females on their way to or from a blood meal. Male intra-sexual competition is high as they jostle for position. In the caged mating trials discussed in Chapter 6 no difference was observed between WT and OX513A males’ propensity to approach the female first, suggesting the modified males may be competitive at this stage in courtship (at least with similarly lab-adapted mosquitoes). However, the same set of experiments reveal a degree of female mate choice in the mating system and at this stage in courtship the WT males performed significantly better, fathering more offspring. A possible explanation for female preference of WT males in this set of experiments was their generally larger size; a characteristic that could be selected during mass-rearing of sterile males.
If indeed female mate choice plays a significant role in the wild, the differential mating success of males could be large, with only a small number of males achieving the majority of matings. Under these circumstances, the importance of releasing high quality males gains in importance compared with merely increasing the over-flooding ratio as females may preferentially mate with the wild males even if these represent a minority (see Chapter 7).

Another aspect of mating behaviour that is relevant to the implementation of an SIT-based programme was examined in Chapter 5 that deals with monogamy of females. Although monogamous behaviour is not a prerequisite for the success of such a programme, it is important to establish whether there is any preferential re-mating of females with wild males after insemination by a sterile male. No re-mating was detected over up to eight gonotrophic cycles and therefore no preferential re-mating. Exceptions exist when females are mated by males that are sperm-depleted, yet it remains to be established how common this may be in the wild. OX513A males can mate fewer females over the course of a lifetime than their WT counterparts and thus may become sperm depleted sooner, potentially leaving a greater percentage of females likely to re-mate. Yet, this effect (should it occur) could again be counteracted by increasing the over-flooding ratio. Furthermore, in a sterile-male release programme, there will be a large excess of males relative to females, e.g 10:1 ratio (Dyck, Hendrichs et al. 2005; Alphey, Benedict et al. 2010). Consequently, if females mate only once the average life-time number of successful copulations for males is likely to be low, perhaps 0.1. While there may be considerable variation around this mean, very few males may be affected by sperm depletion and sterile males may not need to inseminate more than one female for the programme to succeed.

8.4 Recommendations for future work

Though, as described above, overall the genetically modified OX513A strain shows reduced performance compared to its unmodified counterpart, the differences between the strains do not exclude its potentially successful use in vector control programmes. In fact, this research highlights areas of future study that may be able to ameliorate these differences. One promising field of work is the improvement of mass-rearing systems for *Ae. aegypti*. The results described in Chapter 2 reveal larval rearing conditions to be a greater predictor of certain life history traits than the genetic background (WT or OX513A) of a strain (e.g.
differences in adult survival were larger between rearing treatments than they were between strains). Furthermore, crowded rearing conditions adversely affected larval survival, pupation rate and adult body size. There is therefore large scope for improvement in this area.

Apart from the possibility of improving mass-rearing methods, gaps in knowledge of behavioural aspects of mosquito ecology offer areas of future research that may increase the sterile males’ chance of success upon release.

The following table of recommendations provides a series of suggestions to close the gaps in knowledge identified by the research conducted for this thesis as well as suggesting steps necessary in view of potential field releases.

**Table 8.1 Table of recommendations for future research**

<table>
<thead>
<tr>
<th>Recommendation</th>
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<tbody>
<tr>
<td><strong>Mass rearing</strong></td>
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<tr>
<td>Feeding regime</td>
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<tr>
<td>Research should focus on optimising the quantity as well as the quality/composition of food provided. Supplying sterile fruit flies with a high protein diet before release, for example, enhanced their competitive performance (Yuval 2002).</td>
</tr>
<tr>
<td>Quality of rearing water</td>
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<tr>
<td>Water quality/pollution has been shown to influence larval development (e.g. Bedhomme, Agnew et al. 2005). Benefits of keeping rearing water fresh, either through replacement or filtration should be investigated.</td>
</tr>
<tr>
<td>Effect of tetracycline</td>
</tr>
<tr>
<td>The addition of tetracycline to the rearing water of <em>Ae. aegypti</em> produced adults with a higher lipid content. The effect of tetracycline on overall performance therefore merits further attention, possibly with particular respect to the influence on the natural gut-flora of mosquitoes.</td>
</tr>
<tr>
<td>Rearing system design</td>
</tr>
<tr>
<td>Development of rearing systems (ranging from rearing container design to the possible application of high intensity closed-circuit systems) offers one of the largest areas for possible improvements in production quality and quantity and should therefore be considered a priority for future research.</td>
</tr>
</tbody>
</table>
Distribution and release
To date, sterile mosquito releases have been predominantly ground releases, comprising relatively simple packaging and transport protocols. The expansion of release methodology to include aerial distribution may provide a more effective means of cover as well as reducing pre-release damage.

Behavioural ecology
Mate recognition and mating cues
Studies focusing on aspects of mating behaviour can make valuable contributions to the application of SIT-based strategies. Of particular interest are questions regarding mate recognition and mating cues: What cues attract males and females to mating sites? At a mating site, which cues initiate mating behaviour? How precisely does mate recognition function in this species? What role do semio-chemical compounds such as cuticular hydrocarbons play in mate recognition?

Mate selection
Additional research is needed to identify correlates of mating success in Ae. aegypti. Results presented in this thesis show size to be a likely predictor of mating success, but the precise link warrants further study, particularly under field conditions. Furthermore, other possible correlates such as for example, age and wing symmetry, require consideration. The degree to which male competition for access to females and female mate choice influence the mating success of individuals also calls for more study.

Steps towards field release
Field-bred stock
Comparing males of the OX513A line with field-bred mosquitoes is key in more accurately assessing their ability to compete with wild males as well as detecting any propensity for assortative mating.

Environmental conditions
Exposing males of the OX513A line to more realistic environmental conditions (i.e. field cages) will allow for more reliable measures of life history traits (e.g. adult longevity) as well as mating behaviour under natural conditions.

Resistance
It is important to examine the possibility of resistance developing in the RIDL system. The impact of resistance through mutation events could be minimised by the development of lines with multiple effector genes, while behavioural studies will be necessary to establish the likelihood of behavioural resistance developing among wild females.
8.5 Conclusions

This thesis demonstrates in particular the value of behavioural / mating studies of SIT target species as well as the potential of optimising rearing conditions for improving sterile male quality. To date there have been no large-scale SIT programmes in operation against mosquito species. Perhaps because of this, knowledge of mating behaviour relevant to SIT-based approaches is not as developed as for some other pest species such as the Medfly for example, a popular SIT target. If SIT for mosquito control is to be as successful as this technique has proven in other species, this deficit in knowledge needs to be addressed and the findings translated into improvements in technology application. Encouragingly, as seen in this thesis, RIDL technology makes an effective contribution to tackling some of these issues.
9 References


Alphey, L. (2002). Re-engineering the sterile insect technique. Insect Biochemical and Molecular Biology, 32, 1243-1247.


Minjas, J. N., & Sarda, R. K. (1986). Laboratory observations on the toxicity of Swartzia madagascariensis (Leguminosae) to mosquito larvae. Transcripts of the Royal Society of Tropical Medicine and Hygiene, 80, 460-461.


