Sex-specific genetic control of *Ae. aegypti* and *Ae. albopictus*

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For my daughter, Ivy.

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Declaration of Authorship

I declare that this thesis was composed by myself and that the work contained herein is my own except where explicitly stated otherwise in the text. Of particular note is Dr Tarig Dafa'alla, who built the genetic constructs used throughout this thesis. The work presented here has not been submitted for any other degree or professional qualification.

Abstract

"One of the deadliest animals in the world", is the description given to the mosquito by the World Health Organisation. Collectively mosquitoes are a vector for a range of pathogens to humans. Recent developments in genetic engineering have demonstrated that the transgenic self-limiting technology developed by Oxitec Ltd can be deployed to reduce populations of *Aedes aegypti*. The technology uses a transgene that, when inherited, causes lethality if the mosquito does not have access to tetracycline during development. Recent advancements at Oxitec Ltd involve targeting the self-limiting system specifically to females. This allows for males (which are required for a release) to be readily separated from females without the need for costly mechanical separation.

The work presented in this thesis aims primarily to further development of the Oxitec Ltd technology in *Ae. aegypti* and *Aedes albopictus.* In *Ae. aegypti* this is achieved through the development of paternal effect, a male specific construct which render the sperm inviable if reared in the absence of tetracycline. This paternal effect is subsequently combined with a genetic sexing strain in an 'all in one' construct, such that when reared in the absence of tetracycline only sterile males are produced. Development in *Ae. albopictus* comes through characterisation of certain life history traits of the wild type strains currently maintained at Oxitec Ltd. This is necessary as regulatory approval is unlikely to be granted to a product developed in a background strain with "negative" traits. One WT strain was determined unfit for use due to its low productivity under mass rearing conditions, and another for potential resistance to permethrin.

In conclusion the work presented here represents a major advancement of Oxitec Ltd's self-limiting technology in *Ae. aegypti*, through production of a genetic sexing strain capable of producing sterile males. In *Ae. albopictus* wild type characterisation has laid the foundations for future transgenic development in a suitable background strain.

Acronyms

1G – 1st generation technology

2G – 2nd generation technology

3G – 3rd generation technology

AWT – Asian Wild Type (*Ae. aegypti*)

CAA - Centro Agricoltura Ambiente "G. Nicoli"

CD – Cleavage domain

CDC – Centers for disease control and prevention

CI - Cytoplasmic Incompatibility

DENV – Dengue Virus

dsx - Doublesex

ED50 – Estimated dose 50%

G_0 – Generation number post injection

GC – Gonotrophic Cycle

GSS – Genetic Sexing Strain

HAW – Hawaii WT (*Ae. albopictus*)

HEG - Homing Endonuclease Gene

IRS – Indoor residual spraying

ITA – Italian WT (*Ae. albopictus*)

KLP – Kuala Lumpur WT (*Ae. albopictus*)

L 1/2/3/4 – Larval instar 1-4

LL.3 – Log logistic (3 parameters)

LLIN – Long lasting insecticide treated bed net

LSHTM – London School of Hygiene and Tropical Medicine

LWT – Latin Wild Type (*Ae. aegypti*)

MAL – Malaysian WT (*Ae. albopictus*)

PCR – Polymerase Chain Reaction

PE – Paternal Effect

qRT-PCR – Quantitative Real Time Polymerase Chain Reaction

REU – Reunion WT (*Ae. albopictus*)

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

SIT – Sterile Insect Technique

tetO – Tetracycline operator

TG – Transgenic

topi - matotopetli

tra - transformer

tTAV – Transcriptional transactivator

WHO – World Health Organization

WHO DD – World Health Organization Diagnostic Dose

WT – Wild Type

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Chapter 1: Introduction

Of all haematophagous vectors of disease, the mosquito is often labelled as the most significant. The WHO refers to mosquitoes as “one of the deadliest animals in the world”, and with good reason. Collectively mosquitoes are vectors for a wide range of pathogens to humans including protozoa, bacteria, filarial worms and viruses. Malaria alone was responsible for half a million deaths worldwide in 2015 [1].

1.1. Mosquito Vectors *Aedes aegypti* & *Aedes albopictus*

Two of the most significant mosquito vectors of human disease are *Aedes aegypti* and *Aedes albopictus* as they are both responsible for the transmission of a number of medically important arthropod borne viruses (arbovirus) such as dengue, chikungunya, Zika and yellow fever.

Originating from Africa, *Ae. aegypti* is now distributed throughout the tropics (Figure 1). It is believed to have arrived in the New World between the 15th and 17th centuries, carried aboard slave ships, and into tropical Asia in the late 19th Century [2]. The more recent spread of *Ae. albopictus* is similar to that of *Ae. aegypti* (Figure 2), believed to have been facilitated by transportation of eggs through global trade, particularly in used tyres [3]. *Ae. albopictus* is also rapidly spreading into more temperate areas including Europe, the Middle East and North America (Figure 3), putting potentially immunologically naïve populations at risk of new diseases.

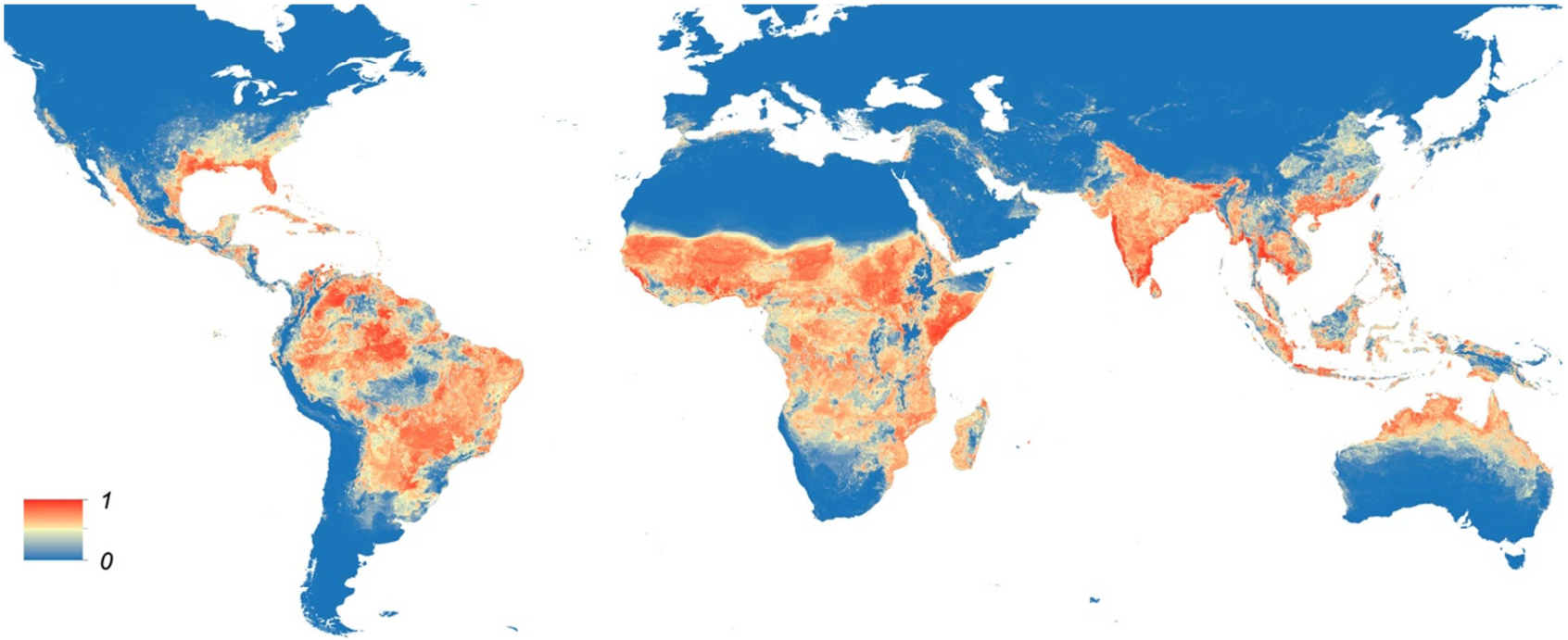

Figure 1: Predicted geographic distribution of *Ae. aegypti.* The map depicts the probability of occurrence (from 0 blue to 1 red) at a spatial resolution of 5 km × 5 km. [4]

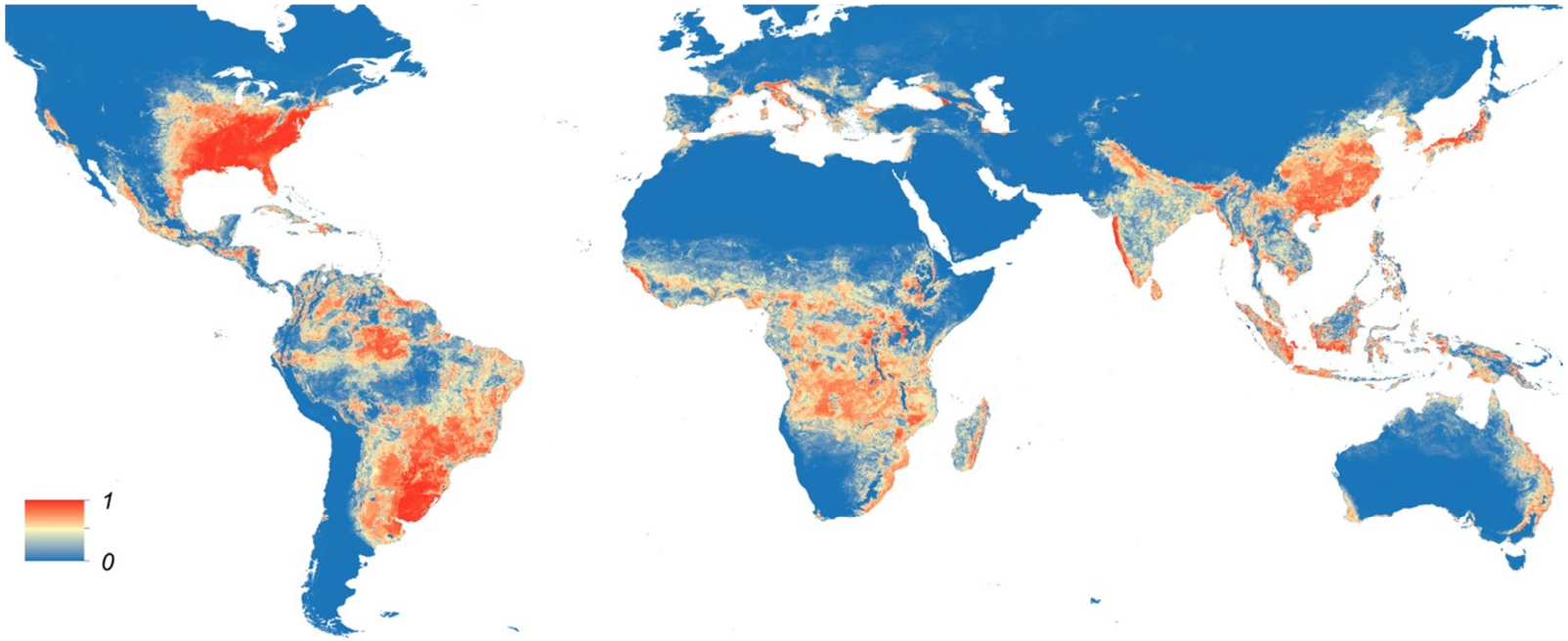

Figure 2: Predicted geographic distribution of *Ae. albopictus.* The map depicts the probability of occurrence (from 0 blue to 1 red) at a spatial resolution of 5 km × 5 km. [4]

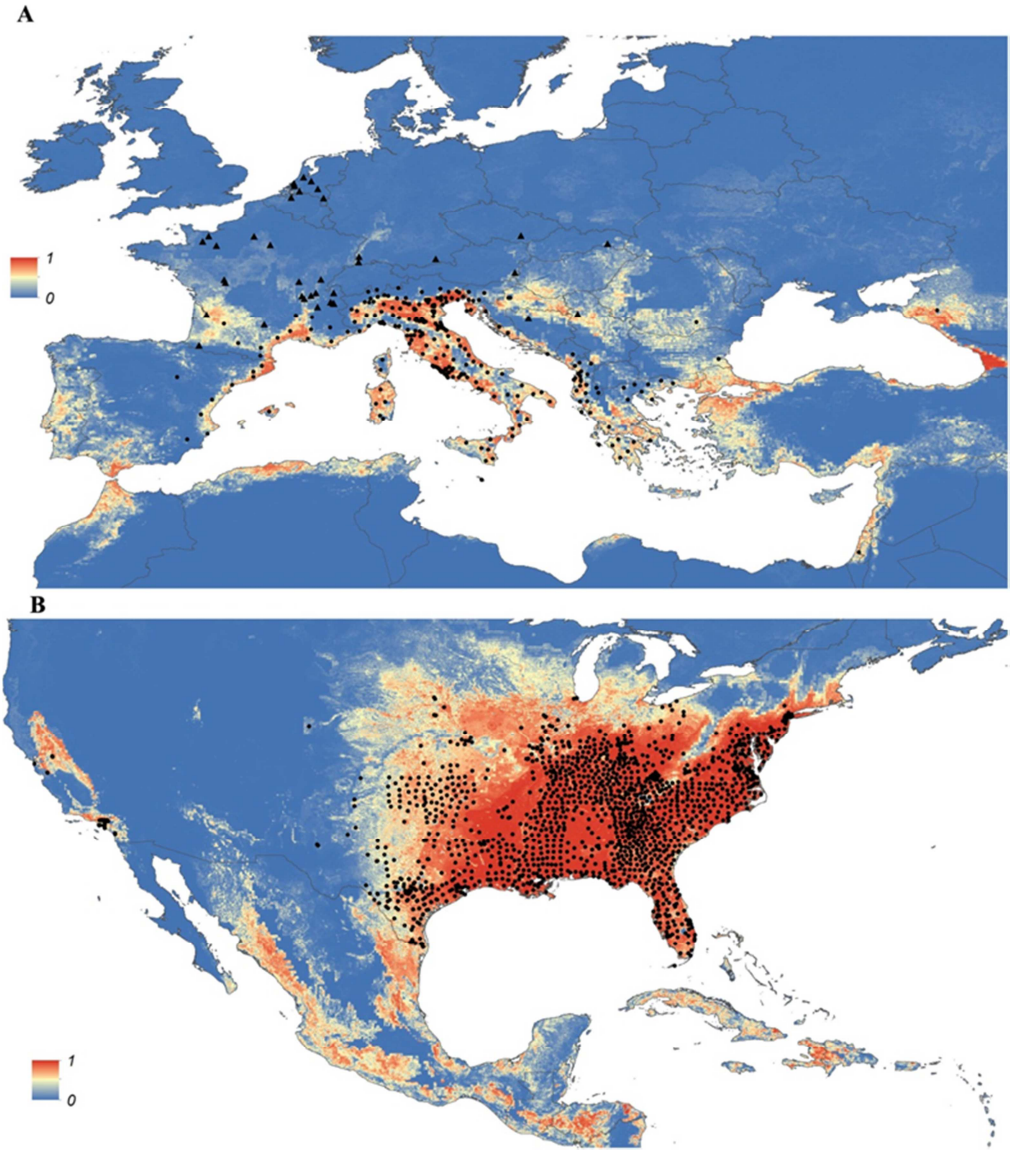

Figure 3: Predicted distribution of *Ae. albopictus* in: **A)** Europe, **B)** United States. Points represent known occurrences (transient [triangles] or established [circles]) until the end of 2013. [4]

The *Ae. aegypti* and *Ae. albopictus* mosquitoes have an aquatic (growth) and terrestrial (reproductive) life cycle (Figure 4). The eggs, laid in small water containers, are able to withstand desiccation for several months. This trait has allowed for their colonisation of every inhabited continent, and is one of the barriers to successful control.

Disease transmission begins during the adult terrestrial stage when a female takes an infected blood-meal. The disease pathogen develops over time (the extrinsic incubation period) before being transmitted when the female takes another blood-meal. It is only the females that take a blood-meal, as they require the protein for egg production; the males subsist on a diet of nectar and are therefore not agents of disease transmission. Disease transmission is therefore closely associated with adult female longevity.

Both *Ae. aegypti* and *Ae. albopictus* are found predominantly in urban and semi-urban settings, with peak activity during the mornings and early evenings [5][6]. *Ae. aegypti* is highly anthropophilic, and an efficient vector of disease in humans; in contrast *Ae. albopictus* is less specific, feeding on a variety of animals, but is a very aggressive biter. Whilst *Ae. aegypti* is considered to be the primary vector of dengue virus, it is important to recognise the threat of *Ae. albopictus*, not only also as a major vector of dengue virus in certain areas of the world [7], but also with its association with other animals and potential zoonotic reservoirs of disease [8].

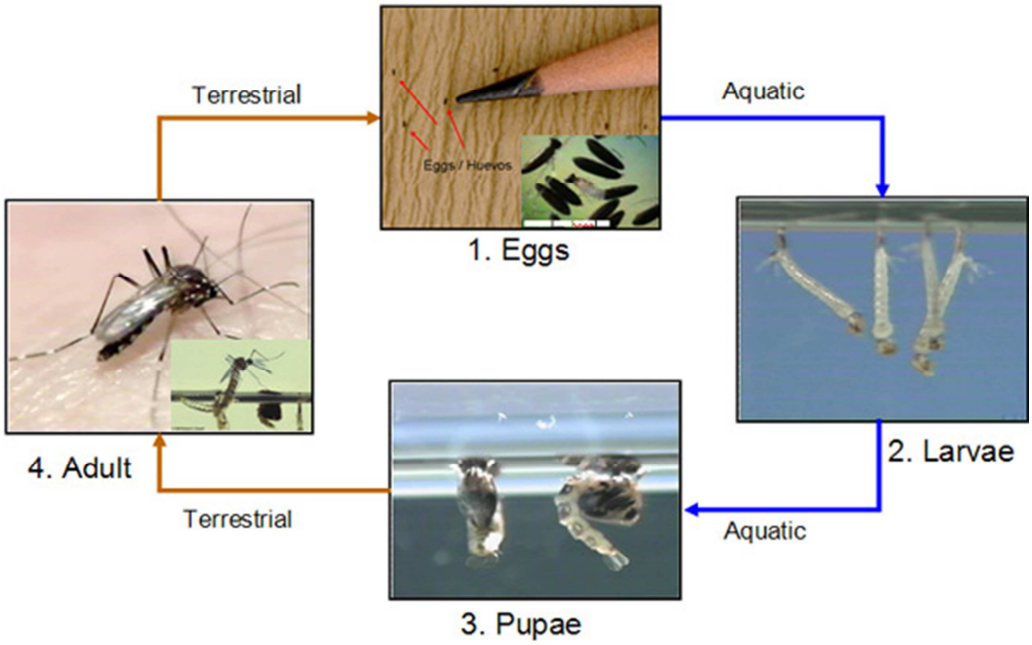

Figure 4: Life Cycle of *Aedes*. Image source, Centers for disease control and prevention. http://www.cdc.gov/dengue/entomologyecology/m_lifecycle.html

1.2. Arboviral diseases spread by *Ae. aegypti* and *Ae. albopictus*

Of the arboviruses transmitted by these two mosquito species, dengue is one of the most important. The WHO reports nearly 50% of the world's population lives in an at risk country, with an estimated 50-100 million cases a year [9]. However a recent project from the Oxford Map group estimates the total dengue burden to be three times higher than current WHO estimates [10]. Dengue virus is a single stranded RNA virus (Genus: Flavivirus; Family: Flaviviridae) and consists of four serotypes (DENV I-IV)[11] with a fifth (V) having potentially been identified recently in Malaysia [12], however this has yet to gain traction in the literature and be confirmed. The common disease manifestations are flu like, with severe joint and muscle pain, leading to the colloquial name "break-bone fever". Reinfection with a second serotype increases the risk of developing the more severe dengue haemorrhagic fever and dengue shock syndrome, which contribute to the 20,000 deaths per year caused by the dengue virus [13]. The Zika virus, another arbovirus spread by *Ae. aegypti* and *Ae. albopictus,*

has recently received attention due to an outbreak in South America and the Caribbean which has been associated with cases of microcephaly in new born infants [14][15]. First identified in Uganda in 1947 [16] the Zika virus is an emerging disease threat, with recent outbreaks likely facilitated by global travel and immunologically naïve populations, coupled with the introduction to high density population centres. Following an outbreak in Micronesia in 2007, the virus spread to Brazil, where in 2015 there were an estimated 440,000 – 1,300,000 cases [17]. Subsequent outbreaks were seen in neighbouring countries and Caribbean islands [18], with its spread northwards and the distribution of *Ae. aegypti* and *Ae. albopictus* it is now considered a threat to the United States of America.

Chikungunya, another emerging arbovirus transmitted by *Ae. aegypti and Ae. albopictus*, saw its first autochthonous transmission in the Americas in November 2013 [19]. This was the start of an epidemic that continues, with the CDC currently reporting local transmission in 25 Caribbean countries [20]. In 2007 there were 197 confirmed cases in Northern Italy, demonstrating the potential for transmission in continental Europe [21]. With symptoms similar to dengue fever, the name chikungunya comes from the Kimakonde language meaning "to become contorted", describing the doubled over appearance of those with severe joint pain [21].

Treatment and preventative prophylaxis against these viruses are sparse and where available, do not offer complete protection. A tetravalent vaccine against dengue has been licenced for use in Mexico under the name Dengvaxia®, however it offers only limited protection of between 43 - 76.9% dependent on serotype [22]. There are no drugs available that specifically target dengue, Zika or chikungunya viruses. Treatment regimens therefore tend to be supportive to alleviate the disease symptoms rather than curative.

1.3. Traditional vector control

With a lack of traditional protective measures, an efficient method of disease control comes through targeting of the mosquito vector. Disease control should be achieved if the mosquito population can be reduced under the level necessary to maintain disease transmission. Understanding the ecology and behaviour of the target species is paramount to the success of a control strategy. For example long lasting insecticide treated bed nets (LLIN) and indoor residual spraying (IRS) have both been shown to reduce the incidence of malaria [23] [24]. The use of LLINs do not offer a suitable strategy against *Aedes* mosquitoes, due to their day time biting behaviour. However *Aedes* are predominantly endophilic (indoor resting) after a blood meal. This indicates that IRS should be effective, and has been demonstrated as a viable method for controlling *Aedes aegypti* populations [25]. However a review of dengue intervention methods found that IRS had no overall impact on dengue transmission [26]. Commonly used entomological indicators of *Ae. aegypti* occurrence, such as container, house and bretau indices, have also been demonstrated to fall caused by the use of insecticide treated curtains and water container lids [27]**.** *Aedes aegypti* and *Ae. albopictus* are predominantly urban vectors, which imposes a logistical constraint on using these techniques for control, due to the large numbers of properties and buildings that would require treatment to have an impact on disease transmission. An alternative technique commonly deployed during epidemic outbreaks is the use of peridomestic space spraying with aerosol insecticides. This has the advantage of being able to cover a wide area with insecticide over a short period of time. A comprehensive review of peridomestic space spraying [28] found that 13/15 studies that met their inclusion criteria reported reductions in immature entomological indices. However, these effects were not sustained, with fast recovery of adult populations to pre-treatment levels or higher. There is to date, very little evidence that space spraying is an effective vector control tool for reducing incidence of dengue [26] [28]. Although this is

acknowledged by the WHO, space spraying remains a recommended control strategy during an epidemic dengue outbreak [29].

An alternative control strategy for *Aedes* includes larval source management. That is reducing the vector population through the removal or treatment of bodies of water suitable for oviposition and larval development. This can be on a large scale with development of water management systems through to community engagement and clean-up projects. Although this too is recommended by the WHO [29], there is little evidence that it on its own, or as part of an integrated vector management program, is effective at reducing incidence of dengue [30].

Where insecticide is used as the basis for control, through IRS, space spraying or larval source treatment, due consideration must be given to resistance. Resistance to the four main classes of insecticide (carbamates, organophosphates, organochlorines and pyrethroids) have been identified for *Ae. aegypti* [31], and the molecular basis for resistance mechanisms are well understood [32]. In *Ae. albopictus* resistance has been documented towards organochlorines and organophosphates, but still appear to be susceptible to carbamates and pyrethroids [33]. Given the resistance mechanisms already in place, and the likely evolution and establishment of further resistance in *Aedes* populations, there is a clear need for novel control techniques that are not based on the use of insecticides.

1.4. Sterile Insect Technique

The sterile insect technique (SIT) involves the release of a target species which is either already sterile, unable to mate successfully or cause sterility in the next generation. If released in suitably large enough numbers, the number of sterile matings will cause the population to reduce. This was proposed as a potential control method for the tsetse fly (*Glossina sp.*) by Potts [34] and Vanderplank [35] in 1944, and trialled successfully by Vanderplank in 1947 [36] against *Glossina swynnertoni* , representing the first field trial of genetic control [37]. The method of sterility used was one of hybridisation, that offspring from a mating of *Glossina*

mortisans and *Glossina swynnertoni* are semi-sterile. By releasing *G. morsitans* into an area populated by *G. swynnertoni*, he observed hybrid matings, and that the more *G. morsitans* released, the higher the number of hybrid matings. He proposed that by release of *G. morsitans* into the target area the *G. swynnertoni* population would be driven out, and the resulting *G. morsitans* population would perish due to unfavourable climatic conditions, which was confirmed by Jackson in 1945 [38].

Development of SIT continued through the 1950s to eradicate the New World screwworm, *Cochliomyia hominivorax*, from North America[39][40][41]. The sterilization process here involved the release of individuals sterilized with ionizing radiation. The released sterile insects mate with their wild counterparts, but produce no offspring. This technique has been met with success in the control of New World screwworm, with its eradication from Libya in 1992 [42] and also from North and Central America, with continued release in Panama to prevent reinvasion from South America [43].

Control of various mosquito species has been attempted through SIT releases, using a variety of sterilization techniques including radiation and chemical sterilization [44]. The mating competitiveness of males is a concern however, being compromised through fitness costs likely incurred from the sterilization treatment. However the use of low dose gamma radiation as the sterilization mechanism for a pilot study in Italy did lead to successful reduction of *Ae. albopictus* [45].

The concept of SIT has recently been repurposed for the control of mosquitoes using advanced genetic manipulation. The mate seeking behaviour of males can be exploited to deliver a genetic mechanism of choice to subsequent generations, in order to bring about a population wide change. This could be one of several methods including the use of endosymbionts to introduce refractoriness to disease; gene drives to distort wild sex ratios and self-limiting genes to reduce populations [46].

1.5. Novel Control Strategies

The ability to replace a population with a targeted genetic change can be made more efficient with selfish genetic elements known as gene drives, which cause biased non-Mendelian inheritance [47]. To be successful the gene drive systems must be able to drive the new genetic element to fixation in the target population and be robust enough to prevent loss of linkage between the gene and the driver mechanism. Where anti-pathogen genes are being driven into a population due consideration must be given to any potential resistance mechanisms to the gene by the parasite [47]. Mechanisms of selfish genes which are neutral or deleterious to an organism have been well characterised [48], and recent research has focussed on exploiting these mechanisms to spread desired traits into populations.

1.5.1. *Wolbachia*

Wolbachia are a group of rickettsia bacteria believed to infect ~17% of all insect species, across all major insect orders [49]. They are found in tissues throughout the host, notably in the reproductive tissues, and are transmitted vertically from mother to offspring through the egg cytoplasm. Their ability to manipulate the host insect's reproductive system allows for a selective advantage to carrying the *Wolbachia* causing a non-Mendelian distribution of inheritance. Known as cytoplasmic incompatibility (CI), males that are infected with *Wolbachia* will not give rise to progeny if they mate with an uninfected female. Infected females however will give rise to progeny regardless of the infectivity status of the male (Figure 5A). This form of unidirectional CI enables *Wolbachia* to spread through a population to fixation.

Bidirectional transfer occurs where the target population is already infected with a different strain of *Wolbachia*. Here different strains of *Wolbachia* lead to incompatibility (Figure 5B), fixation will occur in whichever strain first reaches a majority. In the case of incompatible *Wolbachia* strains, it is possible to generate a superinfected individual, containing both species. In this case a similar model of inheritance is followed, whereby superinfected females

can mate successfully with superinfected males or males carrying only one of the strains, but superinfected males are only compatible with superinfected females (Figure 5C).

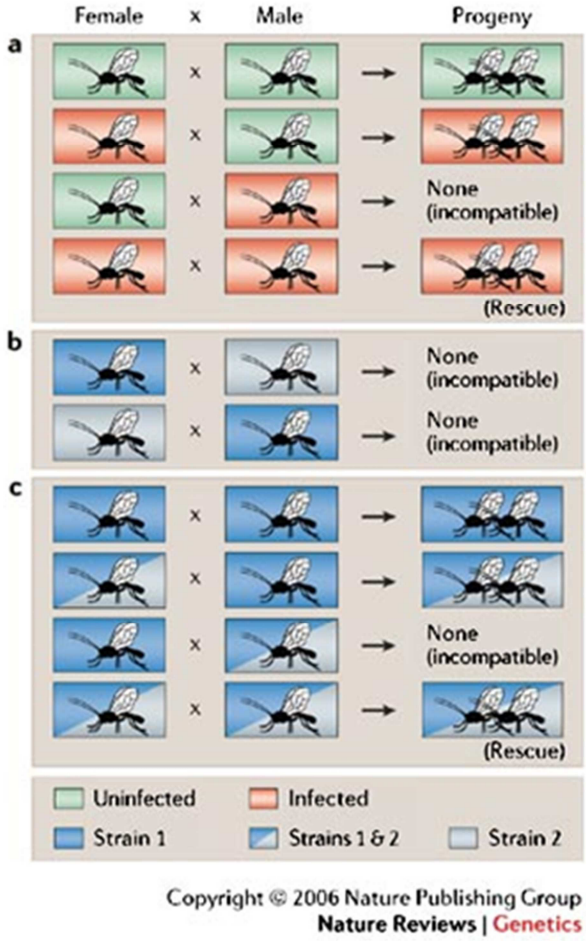

Figure 5: Taken from Sinkins et al. [47] **Panel A**: Unidirectional CI. Infected females (red) can mate successfully with an infected (red) or uninfected (green) male, giving rise to infected progeny. Uninfected females are only able to mate with uninfected males, matings with infected males are not compatible**. Panel B**: Bidirectional CI.: Matings between individuals carrying two different *Wolbachia* strains are incompatible. The larger population will drive the other to extinction. **Panel C**: Bidirectional CI with superinfections. Superinfected females are compatible with males carrying one or both strains of *Wolbachia*. Superinfected males however are only compatible with superinfected females. Superinfections should spread to fixation in a population that contains only of the *Wolbachia* species.

This system of biased inheritance can be exploited to artificially drive desired traits into a population. A specific strain of *Wolbachia* identified in *Drosophila melanogaster*, *w*MelPop, has been shown to reduce the lifespan of the host species [50]. This is of particular relevance for mosquitoes, as any intervention which can impact significantly on adult survival will have a

resultant impact on disease transmission due to the effect adult survival has on vectorial capacity [51]. *Ae. aegypti* artificially infected with *w*MelPop were shown to have a shortened lifespan [52] and increased refraction to pathogens [53]. A population infected with *w*MelPop should therefore have reduced disease transmission as fewer infective females will be present as a result of being resistant to the pathogen and/or not living long enough for the pathogens to develop. *w*MelPop is not without its limitations, with negative effects on general fitness including larval development time, adult size, egg viability, and feeding ability [54][55][56]. A field trial where *Ae. aegypti* infected with *w*MelPop were released in Australia and Vietnam resulted in transient infections of populations at the trial sites, however on cessation of releases the infection in the wild population reduced rather than increasing to fixation [57]. Infection with another *Wolbachia* strain, *w*Mel, has also been shown to induce refractoriness to the dengue virus, but with reduced fitness costs compared to *w*MelPop, with fixation into a naïve population under semi field conditions [58]. Open field trials of the *w*Mel infected *Ae. aegypti* showed good establishment in wild populations [59] which have been sustained for more than 2 years [60]. Whilst this research represents a milestone in the fight against dengue it is important to note that *w*Mel only impacts on DENV-2 serotype [58], and would therefore be limited in its use for dengue control. It has also been reported that wMel impacts on the vector competence of both Zika [61] and Chikungunya [62] viruses, and could therefore be used for targeting multiple diseases.

1.5.2. **Homing endonuclease genes**

A homing endonuclease gene (HEG) encodes an endonuclease enzyme that targets a specific sequence of DNA. The DNA target matches the sequence directly flanking the HEG as if it were not interrupted. It causes a double stranded break in the DNA in the target sequence, resulting in DNA repair using the homologous chromosome containing the HEG as a template, causing it to be replicated into the target DNA sequence (Figure 6).

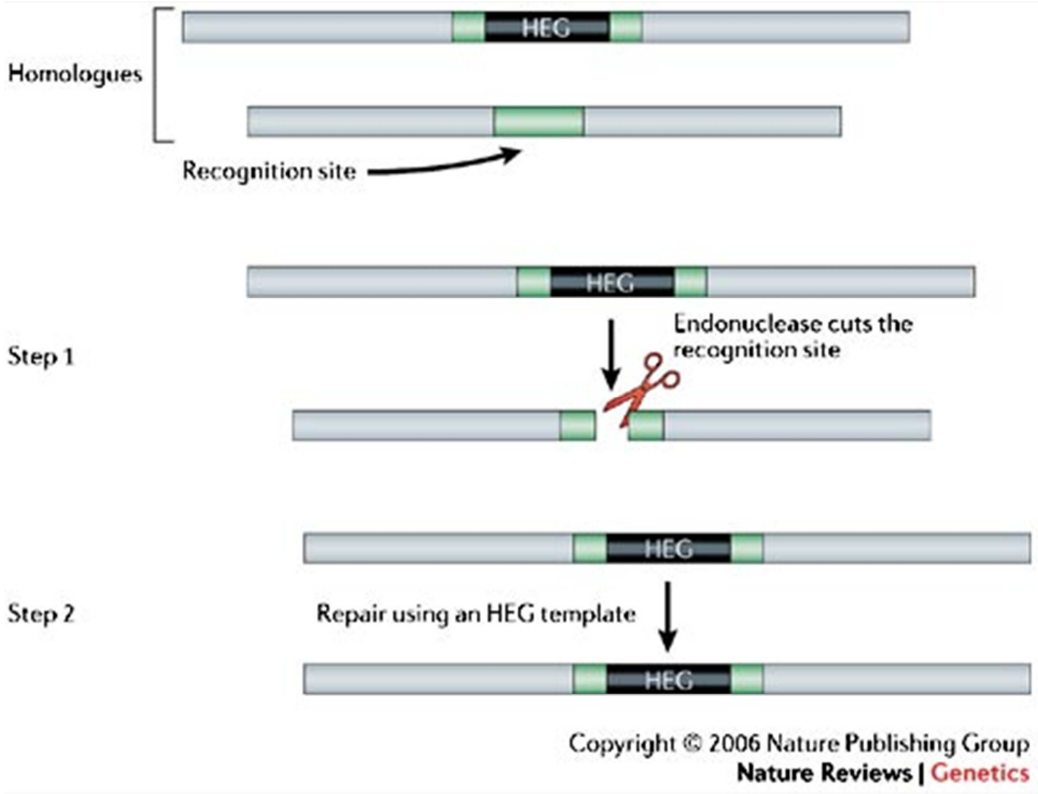

Figure 6: Taken from Sinkins et al. [47]. The Homing endonuclease gene (dark green) encodes an endonuclease that recognises the flanking regions as if they were not interrupted (light green). The endonuclease catalyses a double stranded DNA break in this region (Step 1), which is subsequently repaired with the homologue containing the HEG, causing it to be replicated (Step 2).

This methodology has been exploited in the malaria transmitting mosquito *An. gambiae,* using the HEG *I-PpoI* which targets the ribosomal rDNA repeats on the X-chromosome. It was found that in males expressing the HEG during spermatogenesis X-chromosomes were shredded, resulting in only Y carrying spermatids. However these spermatozoa were infertile, due to the *I-PpoI* being active after fertilization and shredding the maternal X chromosome [63]. A study by Galizi et al. [64] was able to lessen the toxicity of the *I-PpoI* by limiting its activity to spermatogenesis, thus producing fertile males whose progeny are >95% male. They subsequently demonstrated that releases of these males were capable of inducing a population crash in a caged environment. Although a successful technique for *An. gambiae*, the same method could not easily be applied to *Ae. aegypti* due to the lack of allosomes.

However the recent discovery of *nix*, a male determining factor that, when artificially introduced to females, causes the development of male genitalia, provides a potential target for sex ratio distortions [65].

1.6. Self-limiting genes

Whilst the releases of insects carrying either *Wolbachia* or HEGs have potential, neither is as far into development as the release of insects carrying self-limiting genes. The premise is to release male mosquitoes that carry a self-limiting gene which, when inherited by their progeny, will cause mortality before they develop to adulthood [66]. Unlike gene drives, self-limiting genes won't invade a population to fixation, but will limit itself to one (or few) generations. In the laboratory setting it is necessary to repress the self-limiting gene to allow for populations to be maintained and reared for a release program.

1.6.1. Tetracycline mediated gene activation

The release of insects carrying a self-limiting gene uses the tet on / off switch (Figure 7). A minimal promoter is placed upstream of the tetracycline repressible transcriptional transactivator gene tTA (hereafter tTAV). The tTAV protein is able to bind to a tetracycline operator (tetO) placed upstream of the minimal promoter. On binding to tetO, upregulation of the promoter sequence causes a positive feedback loop. The over production of tTAV causes interference with cellular processes, ultimately leading to cell death. This process, termed "transcriptional squelching", occurs as the transactivator sequesters transcriptional components, preventing other essential genes from being transcribed [67]. This system can be regulated by the addition of tetracyclines (or analogues such as chlortetracycline and doxycycline) in the larval rearing water, which binds to tTAV, causing a conformational change that prevents it from binding to tetO [68]. This allows for large numbers of males to be produced in the laboratory setting prior to being released into the field.

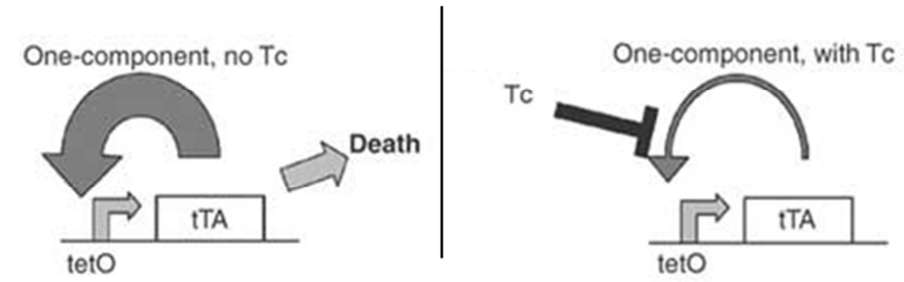

Figure 7: Adapted from Gong et al. [69]. **Left**: In the absence of tetracycline, expression of tTA initiates a positive feedback loop on binding to tetO, producing an overexpression of tTA causing death through transcriptional squelching. **Right**: In the presence of tetracycline, tTA is inactivated through binding of tetracycline, the feedback loop is prevented, tTA expression is limited to a non-lethal level.

The self-limiting system offers substantial control over the expression pattern of the transgene through careful selection of the promoter sequences upstream of the tTAV. This allows for transgenes that are specific, be it to tissues, life stages or sex [70]. An additional component to the self-limiting system is a fluorescent marker. Whilst this does not factor into the functioning of the conditional lethal system, it provides a suitable mechanism for identification, so that transgenic mosquitoes can be easily identified from their wild type counterparts. In the laboratory environment, transgenic individuals can quickly be identified, essential for maintaining and testing transgenic lines. Additionally in the field, the success of a control program can be monitored through the presence of fluorescent individuals caught in ovitraps, which can help direct releases for a more efficient control strategy [71][72][73].

1.6.2. **Self-limiting genes in *Ae. aegypti***

1st Generation Technology

Oxitec Ltd have successfully developed the self-limiting system in *Ae. aegypti.* Termed OX513A, the self-limiting system works as described above (1.6.1), with a fluorescent red identification marker (Figure 8). The bisex nature of OX513A, killing both males and females as juveniles, is an example of Oxitec Ltd's 1st generation technology (1G).

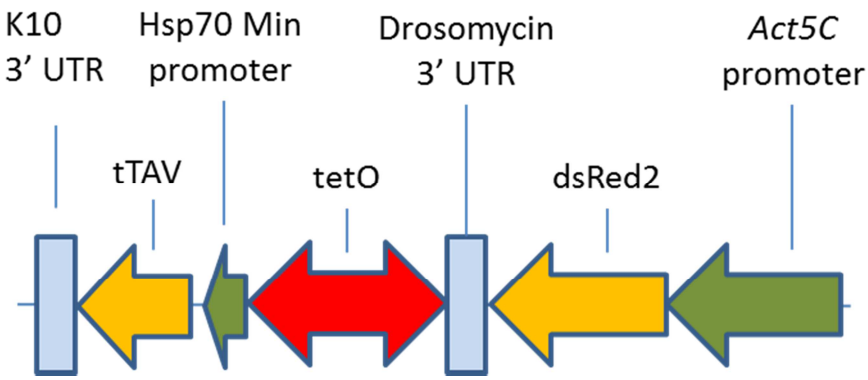

Figure 8: Schematic diagram of construct OX513A. It contains the functional element of tTAV under control of the *Hsp70* minimal promoter, both of which are downstream of tetO. Also present are regulatory 3'UTR regions and a fluorescent marker, DsRed2, under the control of the Actin 5 promoter, *Act5c*.

The OX513A product has been successfully deployed in field trials in Grand Cayman [74], Brazil [75] and Panama [76] with reductions in wild population of 85%, 94% and 93% respectively. The success of OX513A in the field represents a milestone not just in mosquito control, but also in the acceptance and regulation of genetic modification for this purpose.

2nd Generation Technology

Current sex separation of OX513A utilizes a size dimorphism between the sexes. Smaller males are sieved from the larger females prior to a release; however this does carry a contamination risk for the release generation. A female specific, self-limiting strain eliminates this contamination risk, and the costs associated with sorting males from females prior to a release. This concept forms the basis for development of Oxitec Ltd's 2nd generation technology (2G). One of the major advantages of the self-limiting system is the control over expression that can be achieved through selection of the component parts. Genetic sexing systems have previously been developed in mosquitoes that rely on a fluorescent marker under control of the male sex specific promoter β*2-tubulin* [77]. An automated sorting machine is able to then sort males from females for a potential release based on fluorescence. However this system has yet to be tested on a large scale, as would be required for a control

program, which may limit its effectiveness [78]. Sorting through fluorescent markers would also require a secondary stage, such as irradiation, to confer some form of sterility on the males prior to a release. A genetic sexing system that utilises a female lethal element can be used not only to sort females and males, but also as the population control mechanism, limiting the number of females in each subsequent generation after a release [79]. There are currently genetic sexing strains (GSS) under development at Oxitec Ltd in *Ae. aegypti* and *Ae. albopictus.*

A fully functioning GSS, OX5034, has been developed in *Ae. aegypti*. Here the transgene uses sex specific splicing to limit expression to the females. The gene *doublesex* (*dsx*) is well characterised as a sex determining factor, with different mRNA transcripts in males and females produced through an alternative splicing mechanism [80]. This can be exploited in a self-limiting system by combining the sex-specific splicing with the tet on/off system, such that tTAV is only produced in females but not in males, leading to tetracycline mediated lethality in females only.

1.6.3. **Self-limiting genes in *Ae. albopictus***

2nd Generation Technology

Development of self-limiting systems in *Ae. albopictus* is somewhat behind that of *Ae. aegypti*; although a GSS (OX3688) has been developed that utilises the female specific *Actin-4* gene (*AeAct4*) [81]. *AeAct4* is active in the indirect flight muscles of females, with expression starting in the L4 larval stage [82]. The female specificity of *AeAct4*, like *dsx*, is mediated through sex specific splicing. This is exploited in a similar manner by engineering a minigene which restricts production of functional tTAV transcript to females only. Unlike OX5034 the OX3688 uses a two component tet on/off switch (Figure 9). Rather than a positive feedback loop causing transcriptional squelching, the tetO binding domain is placed upstream of a suitable effector, in this case VP16. The tTAV produced, therefore leads to upregulated

production of VP16 which like tTAV causes transcriptional squelching and cell death. The phenotype of OX3688 is a female specific flightless; fully formed adult females which are unable to fly. Because of this the transgene can be considered “lethal” as the affected females cannot fly, feed or mate and for all intents and purposes can be considered dead. It is important to note that this two-part system can be used to drive effectors that do not lead to a lethal phenotype; a core principle for the work discussed in this thesis.

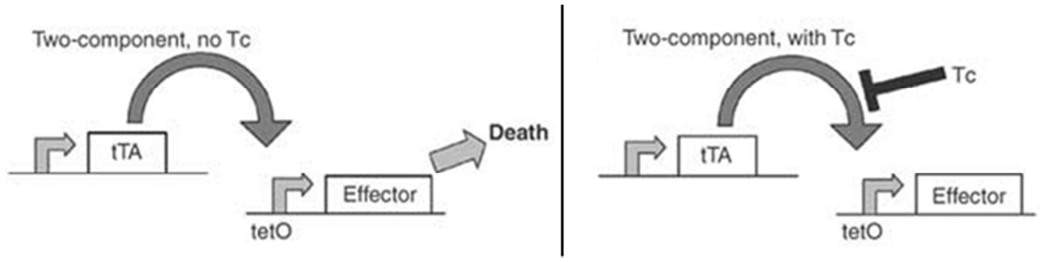

Figure 9: Adapted from Gong et al. [69] **Left**: In the absence of tetracycline tTA is able to bind to the tetO DNA binding domain, situated upstream of an effector. This causes upregulation of the effector, leading to cell death. **Right**: In the presence of tetracycline, tTA is inactivated through binding of tetracycline, tTAV can no longer bind to tetO and the effector is not transcribed.

Prior to approval for field releases, insects carrying a self-limiting system must receive regulatory approval, currently issued on a country by country basis. With the GSS in *Ae. aegypti* and *Ae. albopictus* a potential barrier to regulatory approval could be the potential persistence of the transgene through the male lineage. A solution to this is a sterilization treatment of the males prior to a release, however as discussed previously there are detrimental fitness costs associated with such a process. The work detailed in this thesis aims to provide a genetic sterilization mechanism, to provide a fully self-limiting system that is capable of genetic sexing.

A further barrier to regulatory approval concerns the background strain in which the self-limiting system is developed. There are multiple *Ae. albopictus* wild type strains in colony at

Oxitec Ltd; with selection based on anecdotal evidence of the easiest to maintain in the laboratory. The different wild type strains have been colonised from wild populations from different geographic regions. Given the varying evolutionary pressures applied to these populations there is likely a degree of variation in their genetic backgrounds. Whilst this may have little impact on the research and development of new self-limiting genes it may become an issue when large scale rearing and regulatory scrutiny are applied. For example, it is unlikely that a background carrying insecticide resistance alleles will be cleared for an open release by a regulatory body. The wild type background of *Ae. aegypti* used for production of OX513A is suitably well characterised, however assessment of the wild type *Ae. albopictus* strains is necessary before further transgenic development.

1.7. Thesis aims

The overall aim of the work presented in this thesis is to advance development of Oxitec Ltd's technology in both *Ae. aegypti* and *Ae. albopictus.* This is achieved in two parts, the first is focussed on the development of Oxitec Ltd's 3rd generation technology (3G) in *Ae. aegypti*. 3G aims to combine the fully self-limiting aspect of 1G with the genetic sexing capability of 2G (Table **1**). The second body of work is concerned with characterisation of the wild type strains of *Ae. albopictus* to allow for an informed choice of background in which to develop transgenic strains.

Table 1: Characteristics of Oxitec Ltd self-limiting technologies

Self-limiting technology	Line	Self-Limiting	Genetic Sexing
1st Generation	OX513A	+	-
2nd Generation	OX5034	-	+
	OX3688	-	+
3rd Generation		+	+

Chapters 3 and 4 detail work to identify a suitable self-limiting mechanism and combine it with the 2G, OX5034, in *Ae. aegypti*. Chapter 3 is concerned with the concept of paternal effect (PE), a conditional male sterility phenotype controlled through a two-part tet on/off system. The self-limiting system is used here to control a sterility phenotype rather than a lethal phenotype. Males reared in the presence of tetracycline will be fertile, and those without will be sterile. Suitable promoters and effectors are tested to achieve this phenotype before being combined into an 'all in one' paternal effect construct. In Chapter 4 the paternal effect construct is combined with the genetic sexing construct, OX5034, to make a 3G proof of concept line. In the presence of tetracycline females and males both survive and are fertile to maintain a colony. When tetracycline is withheld, the GSS component will ensure females fail to develop, leaving just the males, which are rendered infertile by the PE component.

Chapter 5 details experiments into three life history characteristics of the *Ae. albopictus* wild type strains, mass production, insecticide resistance and diapause. It is essential that the wild type background chosen is able to be productive when intensively reared. Parameters such as pupal size and adult productivity are examined, along with pupal size dimorphism, currently used as the method for sex separation. The wild type strains were exposed to different insecticides to examine for signs of resistance, and reared under conditions designed to induce diapause (a state of suspended animation), as these traits will likely be a hurdle to gaining regulatory approval for a release.

Chapter 2: General Materials and Methods

2.1. Mosquito Husbandry

Ae. aegypti and *Ae. albopictus* colonies were maintained in separate insectaries, both under a 12 : 12 hour light to dark photoperiod at 26°C (± 1°C) and 80% (± 10%) relative humidity.

2.1.1. *Ae. aegypti* and *Ae. albopictus* background strains

Two background strains of *Ae. aegypti* mosquitoes and four background strains of *Ae. albopictus* were used:

- *Ae. aegypti* WT:
    - Latin Wild Type [LWT], colonised from the Chiapas region of Mexico in 2006, held at Oxitec Ltd since 2006
    - Asian Wild Type [AWT], colonised from Malaysia in 1975 and held at Oxitec Ltd since 2003
- *Ae. albopictus* WT:
    - Malaysian Wild Type [MAL], originated from the Institute of Medical Research in Kuala Lumpur, Malaysia. Received at Oxitec Ltd from Georgetown University, Washington, USA in 2006.
    - Malaysian Wild Type [KLP], originated from the Institute of Medical Research in Kuala Lumpur, Malaysia. Received at Oxitec Ltd from Georgetown University, Washington, USA in 2010.
    - Reunion Wild Type [REU], 2003, colonised from La Reunion, and reared through 2-3 generations before being transferred to Oxitec in 2012.
    - Hawaii Wild Type [HAW], received from Georgetown University, Washington, USA and have been kept in colony at Oxitec Ltd since 2010.

2.1.2. Egg hatching

Mosquito egg papers were submerged in approximately 200-400 ml deionised water in a 500 ml plastic hatch pot. Netting was placed over the top to prevent contamination with other eggs. To stimulate hatching, hatch pots were placed into a vacuum desiccator and a vacuum of ~0.8 bar was applied for between 3-15 hours. In the case of poor hatch rates Liquifry (Interpet Ltd, UK) was added to the water to further stimulate egg hatching.

2.1.3. Larval rearing

Hatched L1 larvae were transferred from the deli pot to a suitable container depending on the number to be reared, either a hatch pot for up to 200 larvae, a plastic container (15 cm x 10 cm x 8 cm) for up to 500 larvae, or a standard rearing tray (30 cm x 15 cm x 5 cm) for up to 1000 larvae. Larvae were typically reared at 1 larva (lar) / ml, or 0.5 lar / ml. At this stage chlortetracycline (hereafter tetracycline or tet) or doxycycline (dox) was added to the rearing water if required. Hereafter rearing in the presence of tet or dox is indicated by “on tet” and “on dox” respectively. Rearing in deionised water is indicated by “off tet” or “off dox” where comparisons are being drawn to on tet or dox rearing. Tet was added at 30 µg / ml or 60 µg / ml and dox was added at 1 µg / ml or 4 µg / ml. The concentration used was dependent on the mosquito line being reared as certain lines require a higher dose to be effective.

Larvae were fed on ground TetraMin Ornamental Fish Flakes (Tetra GmbH, Germany) either as required for line maintenance or to a standard feeding regimen for structured experiments (Table 2).

Table 2: Feeding regimen for *Ae. aegypti* and *Ae. albopictus*

Day	Quantity Tetramin g / 100 larvae
1	0.006
2	0
3	0.008
4	0.016
5	0.032
6	0.032
7	0.032

Larvae typically begin pupation from day 7 onwards, with male pupation peaking on day 8, and females on day 9 or 10 depending on the rearing density. Pupae were removed from rearing trays with a 3 ml pastette and placed in a plastic 100 ml weighboat in ~50 ml of water. Pupae were allowed to eclose in small (15 cm x 15 cm x 15 cm) plastic Bugdorm cages (Megaview, Taiwan), with development from pupa to adult usually taking 48 hours.

If required, pupae were screened for fluorescent markers or sexed prior to eclosion. Pupae were temporarily immobilised by removing the water from the 100 ml weighboat and placing the weighboat onto ice. With the aid of a dissection microscope, pupae could then be separated based on a sexual dimorphism of the end terminal segment (Figure 10).

Figure 10: **Left**: *Ae. aegypti* female pupa, end terminal segment (enlarged) shows a rounded tip. **Right**: *Ae. aegypti* male pupa, end terminal segment (enlarged) shows a pronounced tip. Scale in mm / 0.1 mm breaks.

If required, the immobilised mosquitoes were screened for fluorescence in a dark room using a Leica MZFLIII or Olympus SZX12 fluorescence microscope. The fluorescence marker allows for identification of mosquitoes carrying the transgene of interest. Two fluorescent markers were used, DsRed and AmCyan [83], which were under control of one of two promoters, an all body promoter Hr5iE1 [84], or the eye specific promoter 3xP3 [85] (Figure 11).

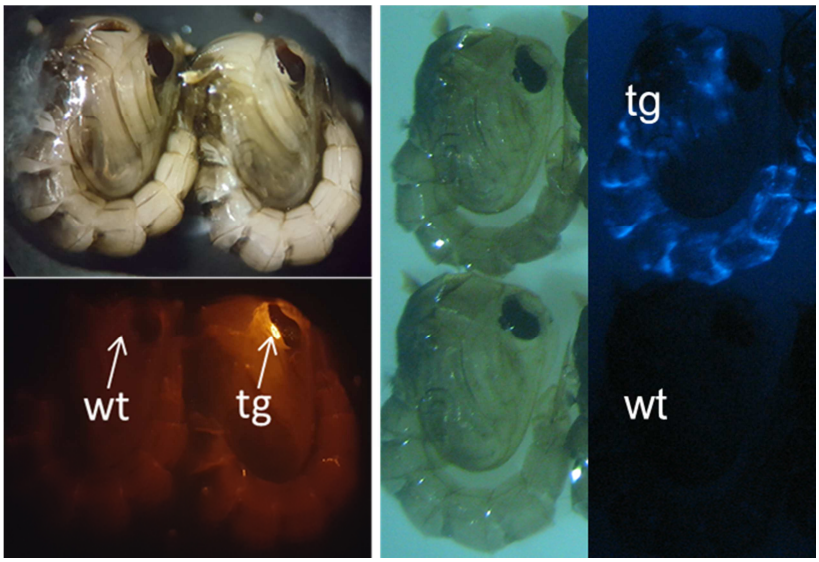

Figure 11: **Left**: *Ae. aegypti* pupae expressing the fluorescent DsRed protein under control of the eye specific 3xp3 promoter (Tg), in contrast to the wild type larvae (wt) visible under fluorescent light (bottom) and under white light (top). **Right**: *Ae. aegypti* pupa expressing the fluorescent AmCyan protein under control of the all over body promoter Hr5iE1 (Tg), in contrast to the wild type pupa (wt) under white light (left) and fluorescent light (right).

2.1.4. Adult cages and blood-feeding

Adult mosquitoes were housed in either small (15 cm x 15 cm x 15 cm) or large (30 cm x 30 cm x 30 cm) Bugdorm cages (Megaview, Taiwan) at a 1 : 1 male to female ratio for colony maintenance. In some experiments male to female ratios varied depending on experimental design, e.g. 1 : 2 or 1 : 5 males to females. Mosquitoes were supplied with a 10% sucrose solution, with either 14 U / ml penicillin and 14 µg / ml streptomycin or 0.2% Nipagin to prevent bacterial growth. The sugar solution was supplied through a cotton wick in a 30 ml reservoir tube and was changed weekly. Mosquitoes were given at least three days post eclosion to mature and mate before being fed on defibrinated horse blood (TCS Bioscience, UK). For *Ae. aegypti* blood was administered through a simple plate feeding system. Parafilm M® (Bemis, USA) is stretched over a metal plate of approximately 10 cm x 10 cm, and ~6 ml of blood is added to the pocket, and manipulated to cover the whole plate. This is placed blood-side down on top of a cage and a beanbag heated to approximately human body temperature

placed on top to stimulate females to take a blood meal. *Ae. aegypti* colony maintenance cages were blood-fed three times per week, experimental cages were blood fed as required. *Ae. albopictus* were fed using a Hemotek (Hemotek Ltd, UK) system, where Hemotek wells are covered with swine intestine, ~3 ml of blood is added and the wells sealed. The blood filled wells are secured into a heating pod, covered with parafilm and placed on top of the cage. Blood feeding was encouraged by gentle blowing into the cage periodically throughout the day.

2.1.5. Egg collection and storage

Egg collection for *Ae. aegypti* and *Ae. albopictus* was performed twice a week. *Ae. aegypti* females were presented with seed germination paper partially submerged in ~50 ml deionised water in a 100 ml weighboat. *Ae. albopictus* were presented with a damp 90 mm Whatmann no. 3 paper on a piece of saturated cotton wool in a 100 ml weighboat. Egg papers were left in cages for three days before being removed, drained and allowed to air dry under insectary conditions. Eggs were stored in labelled sandwich bags under standard insectary conditions. Colony stock strains were hatched every three months. Study strains and transgenic lines were given a minimum of three days to develop before hatching.

2.2. Germline transformation

2.2.1. Injection mix

Germline transformation of *Ae. aegypti* and *Ae. albopictus* was mediated through *piggyBac* transposase. For the transformation procedure an injection mix is prepared, consisting of the DNA plasmid for integration along with the *piggyBac* mRNA helper, H_20 and a 10x injection buffer (1.0 mM of NaH_2PO_4 and 0.05 M of KCL, maintaining a pH of 6.8). Construct OX3081 was linearized using the XbaI restriction enzyme. Capped *piggyBac* mRNA was then transcribed using the mMESSAGE T7 kit (Ambion, USA) and subsequently purified using the MEGAclear kit (Ambion, USA) to a concentration of ~1.6 µg / ml, and stored at -80°C. Plasmid DNA maxipreps

were prepared using an endo-free Plasmid Kit (Qiagen, Germany) according to the manufacturer's instructions and stored at -20°C. Both plasmid DNA and *piggyBac* mRNA were produced by the molecular team at Oxitec Ltd. Plasmid DNA and helper mRNA were mixed to a ratio of 100 ng / µl : 500 ng / µl respectively, although this was modified depending on the expected toxicity of the transgene. In addition, 1 µl of tet (30 µg / ml) or dox (1 µg / ml) was used where it was thought transient expression of the DNA plasmid could be causing lethality. Confirmation of plasmid DNA and helper mRNA in the injection mix was obtained through gel electrophoresis, by running 1 µl of the injection mix in 9 µl of a 10x loading dye on a 1% agarose gel (2.3.1). Two distinct bands were expected, representing the larger plasmid DNA and the smaller helper mRNA. In the absence of either band the injection mix was prepared again; otherwise it was aliquoted into 10 µl samples and stored at -80°C until required.

2.2.2. Preparation for microinjection

Microinjection was performed using fine glass needles, prepared at Oxitec Ltd by heating and pulling a 10 cm aluminosilicate glass filament with a P-2000 Intracel LTD needle-puller (Sutter Instrument Co., UK). A pre-programmed set of variables was used (Heat= 420; Fil=120; Vel=50; DEL=200; PUL=140) to produce a standard needle size, suitable for microinjection of *Aedes* eggs. A 10 µl sample of injection mix was defrosted and spun in a small centrifuge to 4200 rpm. 2 µl was added to an injection needle using a 20 µl microloader pipette (Eppendorf, Germany). The injection needle was loaded onto an Intracel LTD bevelling machine (Sutter Instrument Co., UK), and lowered onto the diamond abrasive bevelling plate for ~10 seconds until the end of the needle was opened for the injection mix to begin running through. The bevelled injection needle was then loaded onto a MN-151 micromanipulator (Narishige, Japan) and connected to a FemtoJet microinjector air-pump (Eppendorf, Germany). The micromanipulator allows for fine control of the needle for microinjection and the FemtoJet allows for control over pressure to the needle to mediate the flow of injection mix out of the

needle. The needle was moved into the field of view on a BA400 Motic light microscope and gently lowered into a drop of semi-permeable halocarbon oil consisting of halocarbon oils 27 and 700 (Sigma-Aldrich, USA) in a 1:9 ratio.

2.2.3. Egg collection for microinjection

The *piggyBac* vector provides a relatively low transformation efficiency of 5-10% and consequently a large number of embryos are required to produce a suitable array of transformation events. Three weeks prior to the planned injection dates approximately 3000 eggs of the relevant wild type background strain are hatched and reared to the standard protocols at approximately 1 lar / ml. Approximately 500 adults are allowed to eclose into a small bugdorm cages and are blood-fed to the appropriate protocol dependant on species. Blood feeding is performed at least three days prior to the injection date, to allow females the necessary time to digest the blood and produce eggs. One hour before injections the cage is presented with a damp 90 mm Whatmann no. 3 filter paper on saturated cotton wool in a petri dish. The cage is placed in a dark cupboard to stimulate egg laying. Egg papers are periodically removed from the cage and examined under a dissection microscope for the presence of eggs. 1-2 hours after oviposition, the egg chorion develops and hardens causing the egg to darken from white to black. Up to 140 light grey eggs were removed from the egg papers and lined up with the posterior ends in the same orientation. Eggs were transferred to double-sided sticky tape on a glass cover slip. The eggs were allowed to desiccate slightly before being covered with the semi-permeable halocarbon oil (2.2.2), to prevent becoming overly desiccated.

2.2.4. Microinjection

Microinjection of the pre-blastoderm eggs was performed as described by Morris [86].The FemtoJet backpressure was adjusted to an equal pressure balance so that when the needle was inserted into an embryo the negative pressure caused by desiccation, pulls out a small

quantity of the injection mixture into the embryo (Figure 12). After all the eggs have been injected the cover slip carrying the eggs is placed vertically into a holder and the eggs submerged in deionised water overnight. This allows the oil to run off the slide and allow the eggs to continue their development. Eggs are then transferred into a high humidity chamber for 3 days before being hatched to standard protocols (2.1.2)

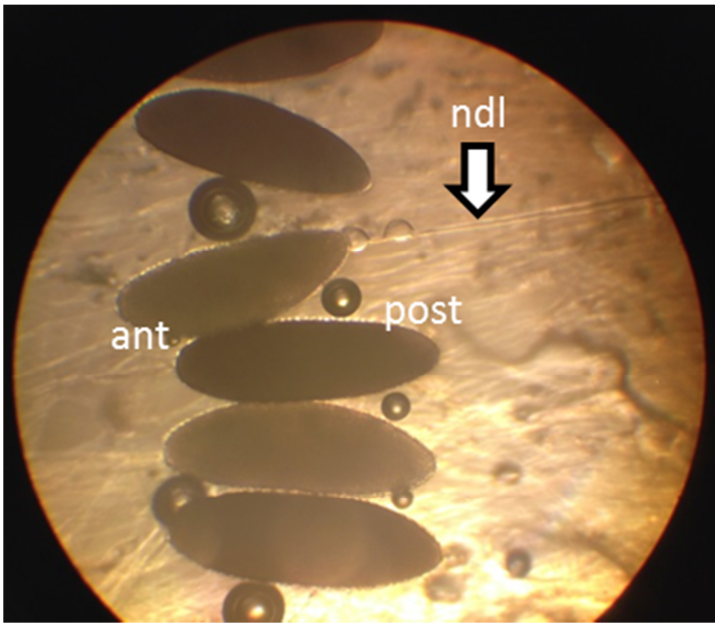

Figure 12: Microinjection of *Ae. aegypti* eggs aligned with the anterior end (ant) on the left and the posterior end (post) on the right. The needle (ndl) can be seen dispensing injection mix next to the posterior end of the central egg.

2.2.5. Transgenic line establishment

Hatched G_0 larvae were transferred to a pot containing deionised water with dox or tet at the relevant concentrations if required. Larvae were reared to standard conditions, being fed as required. Pupae were sexed and placed in pools of either 2 males or up to 10 females in small bugdorm cages. Wild type mates were introduced to the cages at a ratio of 1 G_0 male : 5 WT females or 10 G_0 females : 5 WT males. Blood feeding and egg collection was performed to standard conditions for three gonotrophic cycles. Eggs (G_1) from each gonotrophic cycle were hatched and reared to standard protocols and were screened at the L2/L3 stage for fluorescence as evidence of successful transformation. Line establishment from each G_0 pool

(cage) was presumed to come from a distinct transformation event, and as such treated as a separate transgenic line of the same construct. Each line is given a unique code to determine the construct and the originating G_0 pool e.g. OX513_A represents the injected construct OX513 and the line originating from pool A.

2.3. Molecular Techniques

2.3.1. Gel Electrophoresis

Agarose gels were made by dissolving 1 g of agarose in 100 ml of 1X TAE buffer (for a 0.8% gel 0.8 g of agarose was used). 2 µl of ethidium bromide was added, and the gel allowed to cool and set around suitably sized lane combs for 20 minutes. 1 µl of the nucleic acids to be run on the gel were mixed with 10x loading dye and loaded on to the gel. Nucleic acids were separated by size through electrophoresis at 120 V for 25-30 minutes. The size of the nucleic acid was compared to that of fragments of known size in a DNA Smartladder (Eurogentec, UK) which was run concurrently on the gel.

2.3.2. Reverse Transcription PCR (RT-PCR)

Two Step RT-PCR

Total RNA extractions were performed with a Norgen RNA extraction kit according to the manufacturers instruction. The extracted RNA was quantified on a P300 NanoPhotometer (Spectra, USA), measuring 1 µl of RNA with Lid10. The volume of each RNA sample required to achieve 500 ng/µl was calculated. The calculated RNA was combined with a volume of H_20 to make it up to 11.5 µl. 1 µl of Oligo(dT) (ThermoFisher) was added to each sample, and run in a PCR thermocycler at 65°C for 5 minutes. Then to each sample 4 µl of 5x reaction buffer (ThermoFisher), 0.5 µl of Ribolock (ThermoFisher), 2 µl of 10mM dNTP (ThermoFisher) mix was added. 1 µl of RevertAid (ThermoFisher) was added to all samples except the no-RT controls. The PCR reaction then continued at 42°C for 60 minutes, 70°C for 5 minutes and finally paused at 4°C. cDNA samples were frozen at -20°C.

One Step RT-PCR

Two master mixes were prepared (Table **3**), one with Superscript III and Taq-polymerase platinum (Invitrogen, USA) to perform the RT-PCR, the other with just the Taq-polymerase platinum as a gDNA control. Primers were identified for each endogenous target splice-form (detailed in 4.2.1.1). PCR was performed (Table **4**) with 18.9 µl of the master mix, 0.3 µl of the relevant primer set and 0.5 µl of the RNA samples.

Table 3: One step reverse transcription PCR reagents

Master Mix	Reagent	Quantity per well (µl)
RT-PCR	2 x Reaction Mix	10
	Superscript III / Taq-pol platinum	0.8
	H_2O	8.1
gDNA - PCR	2 x Reaction Mix	10
	Taq-pol platinum	0.2
	H_2O	8.7

Table 4: One step RT-PCR conditions

Step	Temperature (°C)	Time
1	55	Pause
2	55	30 m
3	94	2 m
4	94	15 s
5	55	30 s
6	68	2 m (Back to step 4 * 1 cycle)
7	94	15 s
8	60	20 s
9	68	30 s (Back to step 7 * 37 cycles)
10	68	3 m
11	4	Pause

2.3.3. Quantitative real time polymerase chain reaction

To determine the relative quantity of transcript a quantitative real time polymerase chain reaction (qRT-PCR) was performed. To each reaction in a 96 well plate was added: 12.5 µl of Applied Biosystems Taqman Gene Expression Mastermix (Thermo-Fisher, USA), 1.38 µl H_2O, 2.5 µl of each of the forward and reverse primers and 0.0625 µl of the relevant probes (Table 5). A 1 µl sample of each cDNA sample was added, and the 96 well plate spun in a centrifuge at 4000 rpm for 30 seconds. PCR amplification and detection of fluorescent probes was performed using an Mx3005P real-time PCR machine (Stratagene, USA). The first stage was a heat of 95°C, maintained for 10 minutes 30 seconds. Stage two was a heat of 60°C for 1 minute followed by 95°C for 30 seconds. Stage 2 was repeated for 42 cycles with fluorescence measured at the end of each cycle. The relative quantity of gene transcripts were calculated using the ΔΔ Ct method [87] using the HEX filter for the gene of interest transcript and the 18s ribosomal RNA as an endogenous control with the FAM filter. Negative controls were also included by way of no RT controls as well as H_2O controls.

Table 5: Primers and probes for the qRT-PCR for tTAV expression using the 18S ribosomal RNA as an endogenous control

Transcript	Primers	Probe
tTAV	**857)TaqVp16F2** CCACGCCGATGCCCTGGA **858)TaqVp16R2** GGTGAACATCTGCTCGAACTCGAAATC	**1485)VP16probe2BHQ** HEX-CGGGATTCACCCCGCACGATAGCGC-BHQ1
18s	**1259)18StaqF2** GTATTACGGCGCGAGAGGTG **714)18StaqR** GAAAACATCTTTGGCAAATGCTT	**1260)18Sprobe2** FAM- TTCGTAGACCGTCGTAAGACTAACTAAAGCG- BHQ1

Chapter 3: *Ae. aegypti* - Development of Paternal Effect

3.1 Introduction

This chapter introduces the concept and development of paternal effect (PE), a male specific sterility phenotype. The work detailed here describes the process of identifying suitable genetic components for use in a PE strain, combining them into an 'all in one' genetic construct for transformation of *Ae. aegypti*, through to characterisation and functionality testing.

3.1.1 Paternal Effect

As described previously, SIT is used for control of target insect species. Traditionally it has relied on a number of sterilization techniques: including radiation and chemical application to induce sterilisation. Sterilisation through these techniques likely impose a great fitness cost to the individual, which therefore requires an over flooding of the wild type population to ensure successful control. A number of trials have previously been met with limited success in attempting to reduce target populations of a number of different mosquito species [88][89][90]. Sterilised male competitiveness in the wild is of paramount importance to a successful control program, and was highlighted by Benedict & Robinson [44] as one of the major technical issues contributing to the failure of past mosquito control efforts. A PE transgenic strain would deliver a similar sterility phenotype, whereby the sperm are disabled through genetic means. The goal of a genetic sterilisation is primarily to eliminate the fitness cost associated with radiation, thereby reducing the number of mosquitoes required to achieve success in the field.

As described in Chapter 1, one of the overall aims presented in this thesis is the development of Oxitec Ltd's 3G technology. This technology requires two distinct parts which must work together in order to achieve the goal of a fully functional transgenic line capable of genetic sexing and male sterility. The work detailed in this chapter focuses on achieving one of the 3G

components, transgenic lines that contain the self-limiting aspect, through development of a PE line. The PE concept works in a manner that is more closely aligned to the traditional sterile insect technique than 1G technology such as OX513A. The PE genetic construct will render a male's sperm non-functional; therefore a successful mating with a wild type female should result in no viable progeny. Similar to OX513A, laboratory colonies may be maintained by suppressing the PE construct with the addition of tetracycline in the larval diet.

3.1.2 Requirements for Paternal Effect

OX513A has previously been described as a sterile insect [74] as its progeny fail to mature to adulthood; however this is not a "true" sterility, as eggs are still fertilised and hatch. The PE concept discussed here aims to introduce a true sterility phenotype, whereby embryos are not successfully fertilised. To develop an effective PE strain a number of factors must be considered when designing a genetic construct. The construct expression must be limited to males only, and specifically to the germline. Off target expression is likely to lead to overall adverse effects on fitness, negatively impacting male competitiveness, longevity and female egg production. The PE construct should be fully repressible, giving comparable egg hatch rates to a wild type strain when reared on tetracycline, but total egg hatch suppression when reared in its absence. The desired phenotypic outcome of the PE construct is to have normal sperm production in so far as structure is concerned, but that are ultimately inviable. As *Aedes* mosquitoes are known to be polyandrous [91][92], it is essential that wild type females believe they have received a viable sperm packet from the PE male, this will help prevent re-mating with a wild type, which would lower the efficiency of the control program.

To successfully achieve these requirements, suitable promoter and effector units must be identified that are active with both spatial and temporal facets which can facilitate the desired paternal effect qualities.

3.1.3 Sperm specific promoters

Spermatogenesis is the process by which mature spermatozoa are formed. At the proximal end of the male testes a primary spermatogonium cell divides through mitosis to two primary spermatocytes. Bands of spermatocysts containing spermatocytes at various stages of development can be observed in the testes as they undergo meiotic division to form fully motile spermatozoa at the distal ends of the testes [93]. A large number of gene products are involved in a complex interaction to produce spermatozoa, many of which exhibit expression that is limited to the testes only. For the purpose of producing a PE construct the desired promoter sequences will need to be based on genes that are active during spermatogenesis, and which are not expressed elsewhere. This should allow for accumulation of a suitable effector product to render the spermatid sterile whilst limiting off target effects. A review by White-Cooper [94] identified a number of genes involved throughout spermatogenesis in Drosophila, many of which will have homologues in *Ae. aegypti.* Two drosophila genes of interest include: *matotopetli* (hereafter: *topi*), an *aly*-class gene involved in DNA binding and transcriptional regulation [95]; and β*2-tubulin* which is involved in meiotic spindle and tail axoneme formation in immature spermatocytes [96]. As part of the testis meiotic arrest complex, *topi* is active in the primary spermatocytes, prior to meitoic division [97] and is germline specific, with no expression in females (Figure 13). β*2-tubulin* has been previously demonstrated as testis specific [96] and was used successfully as a sex separation and sperm marker in *Ae. aegypti* [98]. Both of these promoters therefore demonstrate features that make them ideal candidates for use in a PE strain.

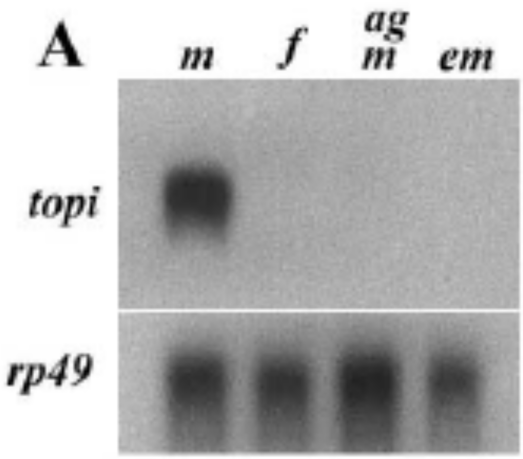

Figure 13: Taken from Perezgasga L. et al. [95]. Northern blot of poly A+ mRNA from *Drosophila melanogaster* samples, using *topi* (top) and *rp49* (bottom) probes. A 2.7 kb topi transcript was detected in RNA from whole males (m), but not from females (f), agametic males (ag m) or embryos (em). rp49 was detected at similar levels in all the samples.

3.1.4 Pre-existing transgenic lines

The PE concept works via a two component tet on/off system (Figure 14) described by Gong et al. [69]. The transcriptional transactivator (tTAV) gene is placed under the control of a sperm specific promoter. A suitable effector component, placed downstream of the DNA binding site tetO, is upregulated by tTAV binding to tetO. Rather than lethality, the effector will lead to sperm that are rendered inviable through the action of the effector. The addition of tetracycline in the larval diet prevents upregulation by binding to tTAV, thereby repressing the system and allowing functional sperm to be produced.

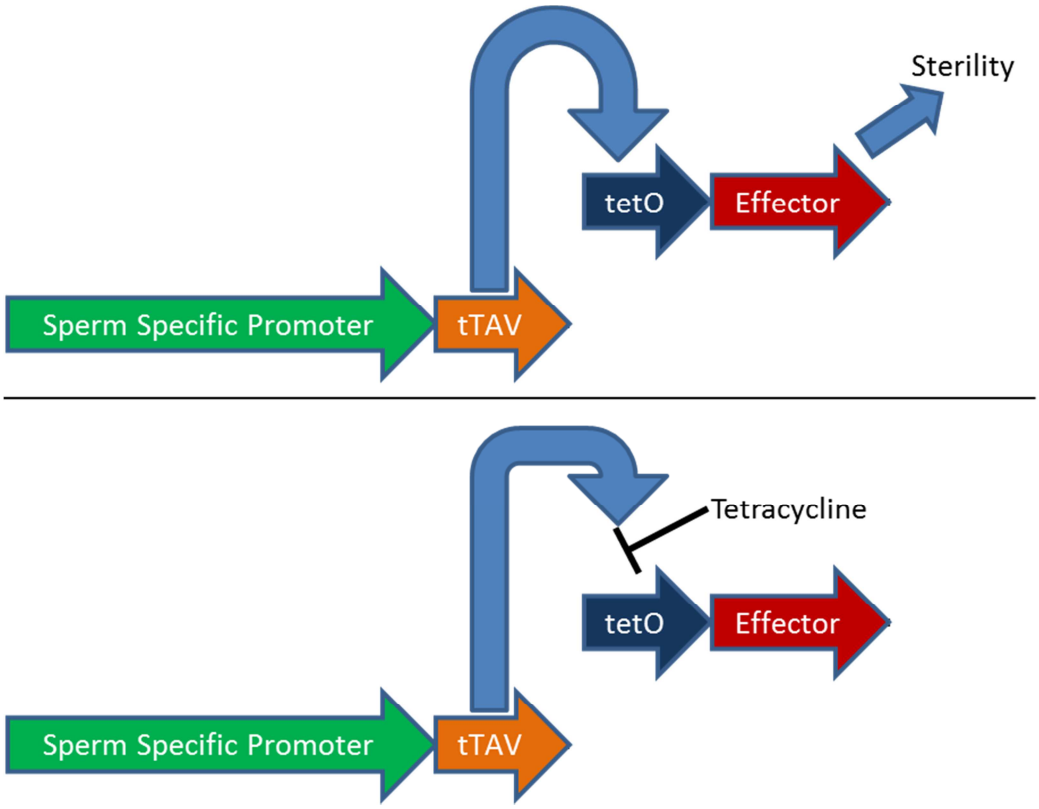

Figure 14: (adapted from Gong et al. [69] Two-component tet on/off system. **Top**: In the absence of tetracycline, tTAV, under the control of a sperm specific promoter, is able to bind to tetO, driving expression of an effector molecule leading to sterility. **Bottom**: In the presence of tetracycline, tTAV binds to tetracycline and can no longer bind to tetO thereby inactivating the system.

The two sperm specific promoter regions discussed above were previously incorporated into genetic constructs [99] as potential candidates for the first part of the PE system (Figure 15 A, B). The second component of the PE system is an effector gene downstream of the tetO binding site. Previous work by M. Bliski [99] revealed that a potential effector could be a DNA binding nuclear protein, protamine, fused to an endonuclease, which was subsequently built into a genetic construct (Figure 15 C). Protamine replaces DNA bound histones late in spermatogenesis [100] and should target the FokI cleavage domain (CD) to the DNA. As a restriction endonuclease, FokI is able to recognise a specific DNA sequence, 5'-GGATG-3', and cleaves the DNA a short distance away [101]. Protamine was selected for use in the genetic construct due to its late activation during spermatogenesis. It is predicted that this will allow

for targeting of the DNA (by the FokI-CD) prior to being condensed and packaged, whilst allowing for complete maturation of the spermatozoon structure.

Males that inherit either the *topi*-tTAV and tetO-*fokI-protamine* constructs or the β*2-tubulin*-tTAV and tetO- *fokI-protamine constructs* will express the FokI-Protamine in the male germline. It is predicted that this will result in inviable spermatozoa when the restriction endonuclease cleaves the DNA, rendering the males sterile, if reared in the absence of tetracycline. A sterility phenotype was demonstrated previously [99] (Appendix A: Figure 66, Figure 67) using the constructs detailed above and forms the basis of the work presented here. The pre-existing PE lines are tested for potential toxicity effects and the sterility phenotype reconfirmed through egg hatch assays. In this chapter, an 'all in one' PE construct was successfully transformed into *Ae. aegypti*, assessed for suitability and tested for functionality. The results of this chapter lay the foundation for future work on the 3G technology, combing the PE with a GSS.

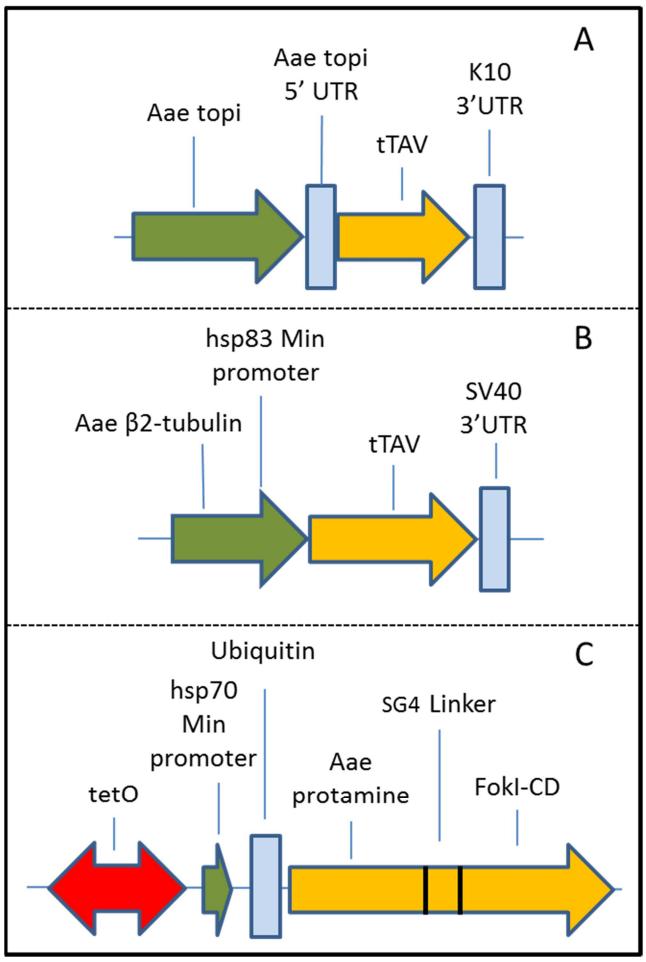

Figure 15: Schematic diagrams of previously built genetic constructs: **A) OX4286**: The sperm specific promoter *Aae topi* drives production of tTAV. Identified by the eye specific promoter 3xP3 driving dsRed expression. **B) OX4635**: The sperm specific promoter *Aae β2-tubulin* drives production of tTAV. Identified by the all body promoter Hr5ie1 driving dsRed expression. **C) OX4627**: The *Aae-protamine* fused to the *fokI* cleavage domain is under control of the DNA binding site tetO. Marked by the all body promoter Hr5Ie1 driving dsRed expression.

3.2 Materials & Methods

3.2.1 Toxicity of paternal effect components – rearing and crossing

Enriched stocks (a mixture of heterozygous and homozygous individuals) carrying the promoter or effector cassettes were reared at approximately 1 larva (lar) / ml. Transgenic pupae of each strain were selected through fluorescent screening and outcrossed in cages of 100 fluorescent males with 200 AWT females or 200 fluorescent females to 100 AWT males. Cages were blood-fed and maintained to standard rearing protocols (2.1.4). The heterozygous progeny were then hatched and reared as before at 1 lar / ml. Heterozygous pupae (determined through fluorescent screening) were placed into small Bugdorm cages (Megaview, Taiwan), with 100 males carrying the promoter cassette crossed to 200 females carrying the effector cassette. A standard protocol for egg collecting was followed (2.1.5). Eggs were hatched under vacuum and the resultant L1 larvae were aliquoted into 6 pools of 400 and reared at 1 lar / ml. Three pools were reared with the addition of tet at 30 µg / ml. Larvae were fed to a standard rearing regimen (2.1.3) and screened for fluorescence at the pupal stage.

3.2.2 Male backcrosses and egg hatch assay

Male mosquitoes that inherited both OX4627 and OX4286 PE transgenes were placed in pools of up to two in deli pots with AWT females a ratio of 1 male : 2 females. Individuals from the on/off tetracycline treatments were pooled with mosquitoes from the same replicates. AWT male and female crosses were performed as a control group under the same conditions, reared off tetracycline. AWT females in the deli pots were blood-fed in a similar manner to feeding those in cages. A small filter paper (Whatmann 3, Diameter 55 mm) was placed in the bottom of the deli pots in a weigh boat with a small piece of damp filter paper as an oviposition substrate. After 4 days the egg papers were collected, counted and hatched

overnight in deionised water under vacuum. Hatch rates were calculated by counting the number of L1 larvae that hatched, represented as a proportion of the initial number of eggs. Due to poor egg collection for the first gonotrophic cycle, damp cotton wool balls were used as an alternative oviposition substrate for the second gonotrophic cycle. Collected eggs were allowed to mature for three days before being transferred to a weigh boat to dry for one week prior to hatching. All mosquitoes were supplied with a 10% sucrose solution throughout the experiments.

Females that inherited both the *topi*-tTAV and tetO-*fokI-protamine* constructs were allowed to eclose in cages to which males were added at a ratio of 1 male: 2 females. All females were blood-fed and where possible 10 were selected from each cage to be placed in to individual drosophila tubes. Small filter papers (Whatmann 3, diameter 55mm) were placed in the tube on damp cotton. The tubes were sealed and females allowed three days to oviposit before being removed. Eggs were allowed three days to mature before being removed and left to dry for one week before hatching.

3.2.3 OX5056 Transformation

Transformation was achieved through microinjections of pre-blastoderm LWT embryos as described in Chapter 2 (2.2.4). An injection mix of 100 µg / µl DNA and 500 µg / µl *piggyBac* helper mRNA was made. A further mix was made containing 1 µl of tetracycline (at 30 µg / µl). G_0 mosquitoes were pooled and outcrossed, before being blood-fed and allowed to oviposit for up to three gonotrophic cycles. Each batch of eggs was allowed at least three days to develop before being hatched and screened for fluorescence as evidence of a successful transformation event.

3.2.4 OX5056 Line Assessment

To establish a sufficient number of transgenic lines for functionality testing up to three single male outcrosses to LWT were performed from each of the positive pools. The resultant

offspring were examined for normal Mendelian inheritance of the transgene through screening for the appropriate fluorescent marker and sex.

3.2.5 OX5056 Functionality Testing

Heterozygous eggs (G_3) of suitable lines were hatched under vacuum and aliquoted into lunchboxes of 300, either on or off tetracycline reared at 0.5 lar / ml. Larvae were fed to the standard feeding regimen and were sexed and screened for fluorescence at the pupal stage. Twenty males from each line were separated at this point and allowed to eclose individually in small deli pots. They were allowed three days to mate with three LWT females. Females were removed and placed in individual Drosophila tubes, from which they were blood-fed. Three days post blood-feeding, females were transferred to a second drosophila tube with a suitable oviposition substrate. Females were given three days to oviposit, before the egg papers were removed, counted and then dried for 1 week prior to hatching in order to assess any sterility effects.

3.2.6 OX5056 Sperm transfer evaluation

Twenty heterozygous males were reared either on or off tetracycline to standard rearing conditions, and were allowed to mate up to two wild type females over three days in a deli pot. Female spermathecae were manually dissected and crushed under a cover slip before being examined for the presence of sperm under a light microscope. Females were scored as either positive or negative depending on the presence or absence of sperm. (Credit to Ilona Flis, Oxford University, for performing dissections).

3.2.7 OX5056 Homozygous viability

Heterozygous males and females were mated together at a ratio of 1 : 2 in small bugdorm cages. Progeny from these crosses were aliquoted into three replicates of 400 and reared in the presence of doxycycline (1 µg / ml). Larvae were reared to the standard feeding regimen and were screened for fluorescence and sexed as pupae.

3.2.8 Molecular characterisation

Heterozygous eggs of OX5056 were hatched and reared to the standard protocol at 1 lar / ml both on (1 µg / ml) and off dox. Pupae were removed from the tray on day 8 and discarded. Pupae were picked the following day, screened for fluorescence and sexed, and then left for 24 hours to ensure they were between 24-48 hours old. This was repeated throughout the week to collect suitable numbers of samples, 9 Males & 9 Females (for both on and off dox cohorts). Male end terminal segments were removed under a dissection microscope with a scalpel (Figure 16). Samples (male end segments, the remaining male pupa or whole female pupa) were pooled in samples of 3 and frozen at -80°C. A two-step RT-PCR was performed as described in Chapter 2 (2.3.2). A 1 µl sample of each cDNA was used for in a qRT-PCR, as described in Chapter 2 (2.3.3). Quantities of transcripts were calculated relative to one of the on dox female samples used as a calibrator.

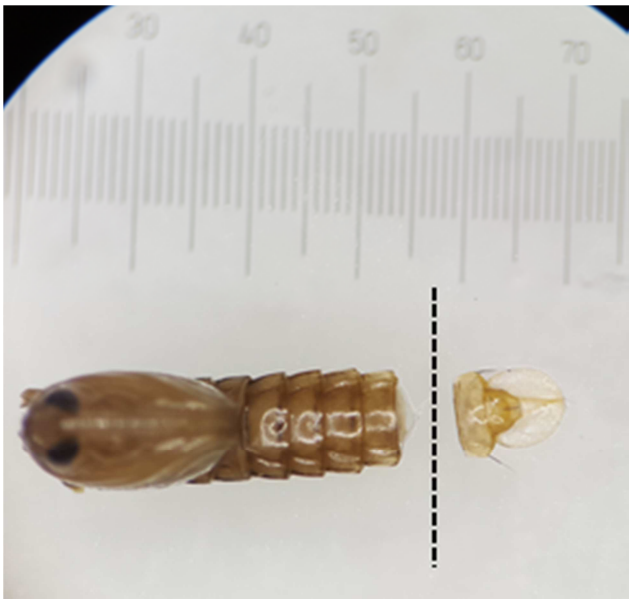

Figure 16: Male pupa with the end terminal segment dissected (right of dashed line). Scale in mm / 0.1 mm breaks (40 - 50 is equivalent to 1 mm).

3.2.9 Statistics

All statistics were carried out in Microsoft Excel (2010) or R studio (Version 0.98.1091).

A two-way ANOVA was performed examining the effect of phenotype, sex and their potential interaction on the total number of individuals from the crosses examining toxicity of the PE promoter and effector constructs. Where significant differences occurred, a TukeyHSD post-hoc analysis was performed to determine the root cause and magnitude of the differences.

One sample t-tests were performed to investigate egg hatch assays, comparing off tet cohorts to the required baseline egg hatch rate of 1% and the on tet cohorts to wild type controls in the preliminary hatch rates (3.3.2) or a required baseline hatch rate of 80% in the OX5056 hatch rate experiment (3.3.5). Full statistical report can be found in Appendix A: Table 29 and Table 30.

A fisher's exact test was performed on the sperm transfer data (3.3.6) due to the low counts of failures (<5).

A repeated G-test for homogeneity was used to test for differences between the numbers of fluorescent pupae counted to the expected numbers from the progeny of the heterozygous crosses of OX5056 (3.3.7). If the total G- score was significant a post-hoc analysis was performed to ascertain if the observed finding was due to differences between the replicates (G-test for independence) or between the counted and expected numbers (pooled G-test). Full statistical analysis can be found in Appendix A: Table 31 to Table 36.

3.3 Results & Discussion

3.3.1 Toxicity of combining paternal effect components

Whilst a paternal effect has been previously reported through genetic crosses of the constructs mentioned previously (3.1.4), it is important to replicate these finding and to ascertain whether there were any adverse effects as a result of carrying one or both of the transgenic cassettes; as this may have implications further into product development. Three genetic constructs were tested: two contained a promoter region (*topi* or β*2-tubulin*) driving tTAV production; the third cassette contained an effector region (*fokI-protamine*) under the control of tetO. Lines carrying promoter or effector transgenes were crossed together to assess any toxic effects to an individual inheriting both constructs (Figure 17). These progeny were then used to determine whether a sterility phenotype was produced where both constructs were inherited.

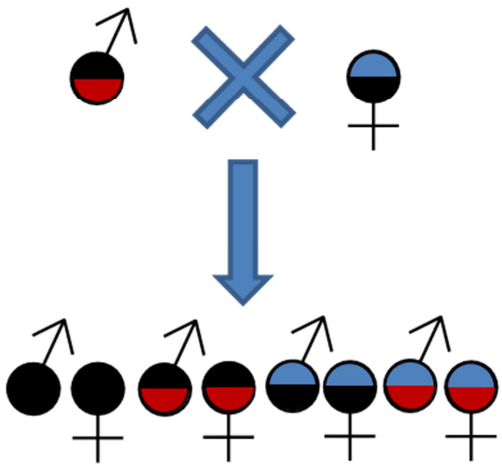

Figure 17: Heterozygous cross of males carrying promoter transgene to females carrying effector transgene. Black: WT allele, Red: Promoter Transgene; Blue: Effector transgene. Expected distribution of progeny would be 25% WT, 25% Promoter, 25% Effector, 25% both promoter and effector.

Progeny from crosses of males carrying the *topi* promoter driving tTAV (OX4286), to females carrying tetO-f*okI-protamine* (OX4627), were screened as pupae for fluorescence markers for evidence of each transgene. The expected 25% distribution between the phenotypes was not split evenly between males and females (Figure 18A). The skewed distributions of each phenotype between sexes is shown through a significant interaction of transgene and sex on the proportion of pupae for both on (df=3, f= 189.430 p=1.04e-12) and off (df=3, f=342.68, p= 1.01e-14) tet cohorts. Low numbers of males inheriting both the *topi*-tTAV promoter and the tetO-*fokI-protamine* effector cassettes were observed, however the total numbers of males and females do not indicate that one cohort is dying, but that there is a biased distribution of the transgene. *Ae. aegypti* lack allosomes, and instead have a Y-chromosome like region that contains a male dominant sex determination factor known as the M-locus [102]. The biased distribution indicates that the *topi*-tTAV cassette (OX4286) has inserted close to the female locus (opposite the male determining locus), resulting in few male individuals inheriting the promoter construct or both the promoter and effector constructs. To confirm this, the reciprocal cross of females carrying the *topi* promoter cassette (OX4286) crossed to males carrying the *fokI-protamine* effector cassette (OX4627) was performed (Figure 18B). The progeny of the reciprocal cross exhibited even proportions of all individuals, at ~12.5% with no evidence of linkage as shown by the lack of interaction between sex and transgene for both on (df=3, f=1.419 p=0.274) and off (df = 3, f = 0.058, p= 0.981) tet cohorts.

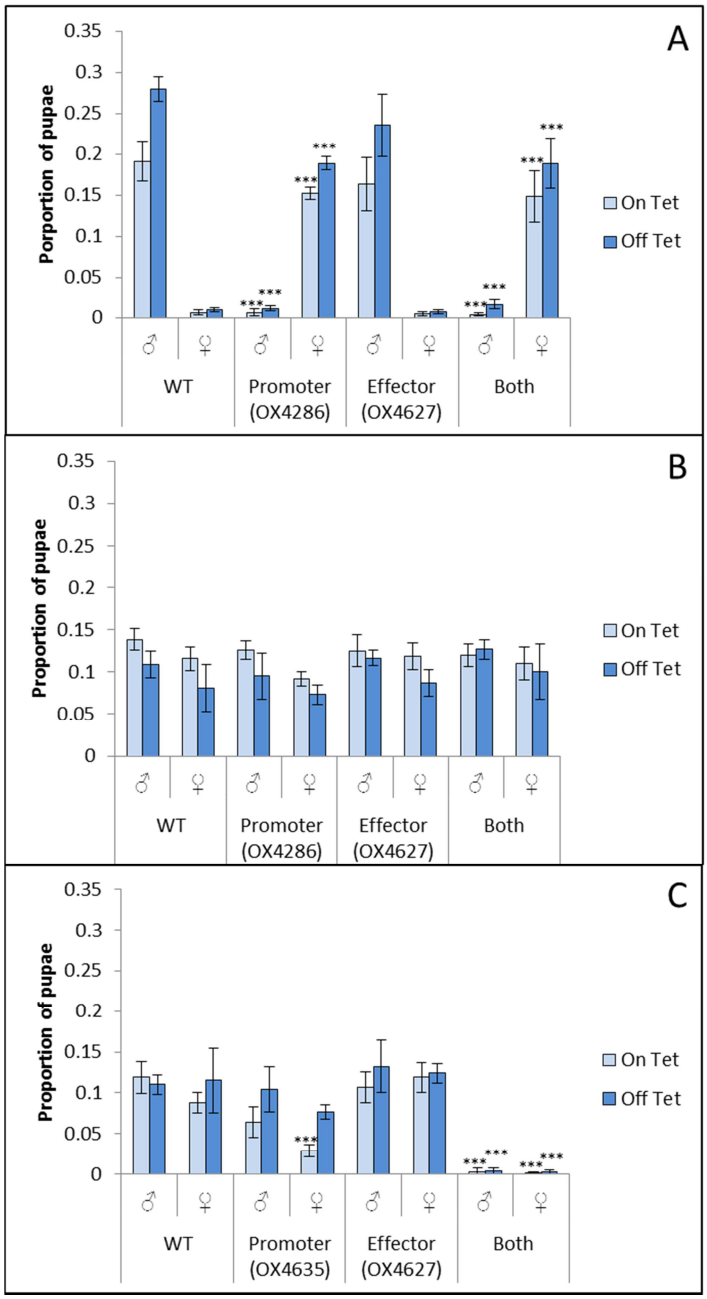

Figure 18: Mean proportions (with 95% CI) of progeny from the following genetic crosses reared on or off tet. **A)** Males heterozygous for the *topi*-tTAV promoter cassette (construct OX4286) crossed to females heterozygous for the tetO-*fokI-protamine* effector cassette (construct OX4627). **B)** Reciprocal cross of A, males heterozygous for the *fokI-protamine* cassette (OX4627) crossed to females heterozygous for the *topi*-tTAV promoter cassette (OX4286). **C)** Males heterozygous for the β*2-tubulin*-tTAV promoter cassette (construct OX4635) crossed to females heterozygous for the tetO-*fokI-protamine* effector cassette (construct OX4627). ANOVA results indicate significant differences indicated by: $p < 0.05$ *; $p < 0.01$ **, $p < 0.001$ ***, are to the corresponding wild type.

Although significant differences were seen between the number of males and females both on ($f = 12.081$, $df = 1$, $p = 0.00312$) and off tet ($f = 12.407$, $df = 1$, $p = 0.00283$), this is generally to be expected. The larval rearing water tends to foul over time due to bacterial growth, which can have a detrimental impact to the survivorship of larvae. As females have a longer developmental time than males, they spend a greater period of time in the larval rearing water, affecting their survivorship. No differences were seen between the number of pupae in each phenotypic cohort, either on ($f = 2.282$, $df = 3$, $p = 0.11826$) or off tet ($f = 2.719$, $df = 3$, $p = 0.07908$). This suggests that no mortality occurred as a result of inheriting either of the transgenes or a combination of both. It was therefore concluded that *topi*-tTAV and tetO-*fokI-protamine* would be good candidates to test for a sterility phenotype in individuals inheriting both constructs.

Males carrying the β*2-tubulin*-tTAV promoter construct (OX4635) were crossed to females carrying the tetO-*fokI-protamine* construct (OX467). Their progeny were sexed and screened for fluorescent markers as evidence of the transgenes (Figure 18C). There was a marked reduction observed in individuals inheriting construct OX4635 from the on tetracycline cohort, where it would appear the β*2-tubulin* construct was impacting survival in both males (5.5% reduction, $p=0.0014$) and females (5.9% reduction, $p<0.001$) compared to the AWT control. This fitness cost was exacerbated in males and females inheriting both the OX4635 promoter construct and OX4627 effector construct, where survivorship was significantly reduced in both the on and off tet cohorts (Table 6).

Table 6: Statistical differences between the percentages of pupae inheriting both the β*2-tubulin*-tTAV promoter construct (OX4635) and the tetO-*fokI-protamine* effector construct (OX467) compared to the corresponding WT cohort, reared either on or off tetracycline.

Treatment	Sex	% Difference to WT **Mean** [95% CI]	p-value
On Tet	Female	**8.6** [5.1 – 12.2]	P<0.001
	Male	**11.5** [8.0 – 15.2]	P<0.001
Off Tet	Female	**11.25** [5.9-16.7]	P<0.001
	Male	**10.58** [5.2-16.0]	P<0.001

This demonstrates that the β*2-tubulin* promoter does not meet the requirements laid out for an effective PE strain. It is expressed in females, as seen by lower than expected numbers inheriting just the β*2-tubulin*-tTAV promoter construct (OX4635) alone. When combined with an effector construct, off target expression leads to death in both females and males, indicating that expression is also not limited to the spermatocytes in males. This is an unusual result as the sperm specific manner in which β*2-tubulin* is expressed is well documented. Smith et al. [98] describes the use of β*2-tubulin* in sperm marking and creation of a sex separation marker. They detected weak expression of β*2-tubulin* in late female pupae and adults, but it was unclear as to whether this was contamination or weak expression. If weak expression of β*2-tubulin* is to be assumed, it would indicate the sensitivity of individuals to off target expression of the FokI-Protamine, with minimal expression required to induce lethality. As so few individuals inherited both constructs it was not possible to test for the sterility phenotype. It is however, unlikely that β*2-tubulin* would make a good candidate for a PE line due to its toxicity.

3.3.2 Male backcrosses and egg hatch assay

In order to assess the sterility effect, males from the toxicity experiment (3.3.1) which inherited both the *topi*-tTAV promoter transgene (OX4286) and the tetO-*fokI-protamine* transgene (OX4627) were mated to AWT females. One group of males, from the off tet cohort,

were used to assess the sterility phenotype, the other group of males, from the on tet cohort, to assess the transgenes' repressibility. Mated females were blood-fed and allowed to oviposit. Eggs were then counted and hatched in an egg hatch assay to assess any sterility effect. Sterility in this case was considered as a hatch rate of 1% or less. This is thought to be an acceptable hatch rate for heterozygous individuals at the research and development stage. As progress continues the acceptable sterility level may change dependent on regulatory input. Likewise, an 80% hatch rate when reared on tet was considered to be fully repressed, as this is representative of a normal WT hatch rate in research and development, but this may be altered in the future depending on the mass rearing approach to appropriate hatch rates. A preliminary egg hatch assay was conducted with male progeny from the cross of males carrying OX4286 to females carrying OX4627 cross. Due to the biased distribution of the progeny, the number of males inheriting both constructs was small. A total of 4 males reared on tet and 10 off tet were tested. Males with both constructs reared off tet exhibited a significantly reduced hatch rate that was not significantly different to 1% ($t = 2.4855$, $df = 5$, p-value = 0.056). Those reared in the presence of tetracycline also exhibited a reduced hatch rate of 40.2%, which was significantly lower than the control group ($t = -6.4239$, $df = 4.977$, p-value = 0.001). A further hatch rate analysis was conducted on individuals from the reciprocal cross of OX4286 and OX4627. A total of 20 males reared off tetracycline and 20 on tetracycline were mated to 5 AWT females in pools of 2. A total of 16 resulted in successful oviposition by the females, 8 in each of the on and off tetracycline cohorts. The off tet cohort had a hatch rate which was not significantly different to 1%, and the on tet cohort exhibited a reduced hatch rate of 45.28%, significantly different to the wild type (Figure 19). Both egg hatch rate experiments showed a significant reduction in hatch rates from males carrying both the *topi*-tTAV promoter and tetO-*fokI-protamine* effector constructs. The sterility effect was partially repressible with the addition of tetracycline in the larval diet. This partial repressibility is most likely due to the specific insertion locations of the promoter and effector transgenes, which

can have significant influence over their expression and tetracycline mediated repressibility [103]. Whilst not an ideal trait for a PE line to have, at this stage of development the focus is primarily on functionality as opposed to product optimisation, which would inevitably come further into product development.

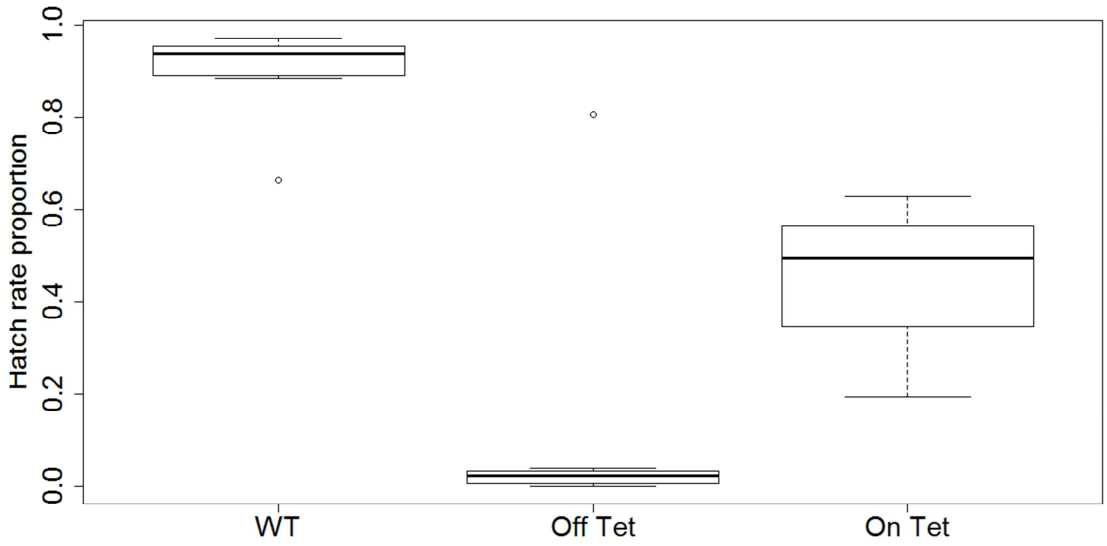

Figure 19: Box and whisker plot demonstrating the egg hatch rates of male mosquitoes inheriting both OX4286 and OX4627 transgenes. Males were reared either on tet (30 µg / ml) or off tet and mated to AWT females which were blood-fed and allowed to oviposit. Hatch rates were calculated as the proportion of hatched L1 larvae from the total number of eggs. The off tet cohort was not significantly different to a 1% hatch rate (t = 1.0694, df = 7, p-value = 0.3204), the on tet cohort was significantly different from the AWT control group (t = -7.174, df = 11.411, p-value = 1.484e-05), calculated through one sample t-tests.

The desired outcome of the performed genetic crosses was to demonstrate that the previously identified PE components could work to provide the desired sterility effect whilst adhering to the requirements listed previously (3.1.2). The desired sterility effect was demonstrated in males which had inherited both the *topi*-tTAV (OX4286) and the tetO-*fokI-protamine* (OX4627) transgenes when reared in the absence of tetracycline. This effect was partially repressible through the addition of tetracycline in the larval diet. The presence of the two constructs did not exhibit adverse off target expression in either males or females and did not affect female

fecundity (Appendix A: Figure 68). Having confirmed functionality of the *topi*-tTAV and tetO-*fokI-protamine* combination, development of PE progressed to an 'all in one' genetic construct, OX5056.

3.3.3 Transformation of the 'all in one' PE construct OX5056

OX5056 is an 'all in one' PE construct containing the transcriptional transactivator tTAV downstream of the sperm specific promoter, *topi,* and the endonuclease effector *fokI* fused to *protamine* downstream of the DNA binding site tetO (Figure 20). A total of 2542 pre-blastoderm embryos were injected as described previously (2.2.4 and 3.2.3). A total of 49 surviving larvae developed to adulthood giving an overall survival rate of 1.9%. Surviving G_0s were pooled together as up to 2 males or 10 females and were backcrossed to wild type at a ratio of 1 male : 2 females. A total of 13 male pools and 3 female pools were blood-fed and allowed to oviposit for up to three gonotrophic cycles. G_1 eggs were hatched under vacuum and reared to L2/3 before being screened for fluorescence as evidence of successful transformation. A total of 7 pools (OX5056_A, OX5056_D, OX5056_F, OX5056_J, OX5056_L, OX5056_M & OX5056_P) were positive for transformants, however line L was discontinued owing to a weak fluorescent phenotype.

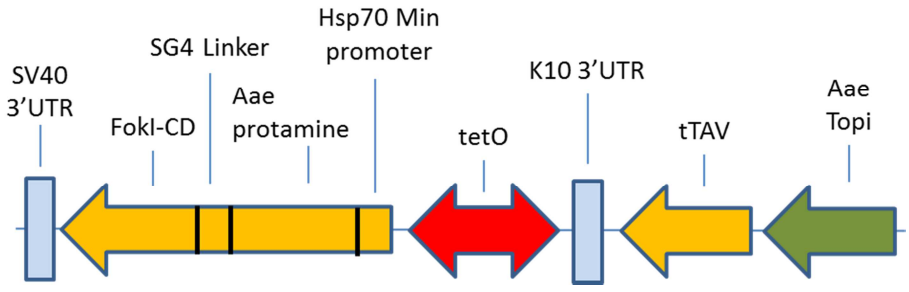

Figure 20: Schematic diagram of construct OX5056. It contains the functional elements of tTAV under control of the *Aae topi* promoter, and *fokI-protamine* downstream of tetO. Also present are regulatory 3'UTR regions and a fluorescent marker, AmCyan, under the control of the all body promoter Hr5iE1. Construct built by Tarig Dafa'alla at Oxitec Ltd.

3.3.4 OX5056 Line assessment

Transgenic lines must be suitably assessed before it is possible to test for functionality. Multiple insertion events of the transgene are likely to impact on functionality, and whilst insertion events near the male determining locus are unlikely to affect functionality, it is easier to work with a transgenic line which exhibits normal Mendelian inheritance.

Three single male outcrosses were performed from pools OX5056_ A, OX5056_M, OX5056_ D & OX5056_ J. Due to lower numbers of surviving G_1 larvae to adulthood only two single male outcrosses were performed from pool OX5056_P, and one single male and three single female outcrosses were performed from pool OX5056_ F. The progeny from these outcrosses were hatched under vacuum, reared under standard conditions and screened for fluorescence as pupae (Figure 21). Lines OX5056_A3, OX5056_M2, OX5056_M3, OX5056_D1, OX5056_D2, OX5056_J2, OX5056_J3, OX5056_P1 & OX5056_P2 were deemed suitable to work with as the proportions of pupae were approximately 25% for each phenotype in stark contrast to lines OX5056_A1, OX5056_A2, OX5056_D3, OX5056_F1 & OX5056_J1 where the sex-biased distribution of progeny suggests an insertion event close to the M-locus.

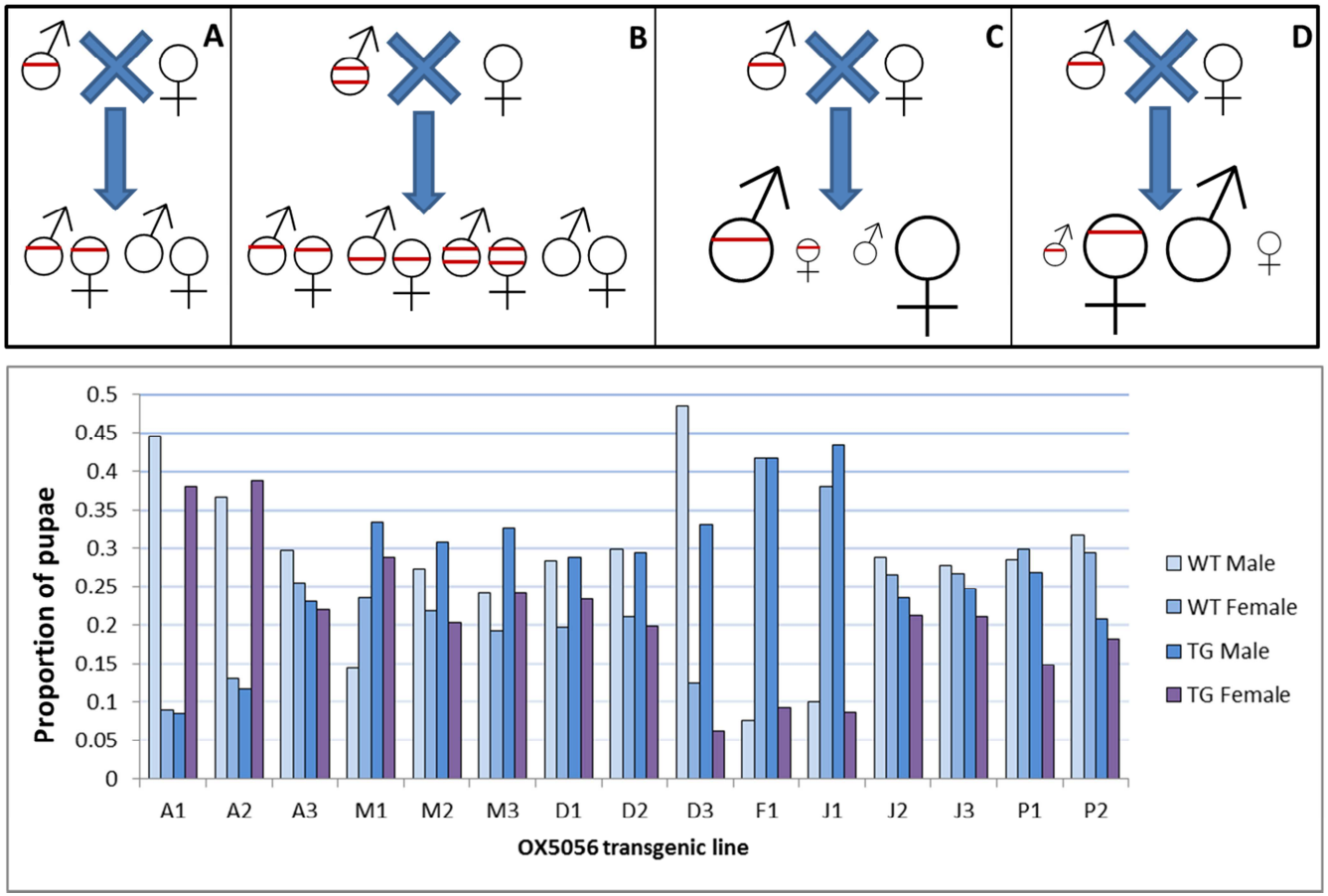

Figure 21: **Top:** Potential distributions of fluorescent and wild type pupae resulting from a single male cross. Red line indicates one insertion of a transgene. **A)** Assuming a single insertion which is not sex linked, normal Mendelian inheritance should be seen (1:1:1:1). **B)** Assuming a multiple insertion which is not sex linked, normal Mendelian inheritance should be seen (3:3:1:1). **C)** Assuming a single insertion which is sex linked to the M-locus, non-Mendelian inheritance will be seen, biased towards towards fluorescent males and wild type females. **D)** Assuming a single insertion which is sex linked opposite the M-locus, non-Mendelian inheritance will be seen, biased towards fluorescent females and wild type males. **Bottom:** Proportion of fluorescent and wild type male and female pupae from single male crosses of OX5056 positive G_0 pools. Letters denote original G_0 pool, numbers represent the single male parent.

3.3.5 OX5056 Functionality

One line from each of the original pools deemed suitable (OX5056_J2, OX5056_A3, OX5056_M2, OX5056_P2 & OX5056_D1) were tested for functionality. Up to 20 single male crosses were performed for each line. The resulting eggs were collected and hatched to assess the sterility effect of each OX5056 line and its repressibility with tetracycline. Figure 22 demonstrates that lines OX5056_J2, OX5056_A3 and OX5056_P2 reared off tetracycline were found to have a hatch rate not significantly different to 1%; and lines OX5056_ J2 and OX5056_A3 reared on tetracycline were found to have hatch rates not significantly different from 80% (Full statistical analysis Appendix A: Table 29 and Table 30). Of the 5 OX5056 transgenic lines tested three confirmed the expected sterility phenotype, which was fully repressed in line OX5056_A3, confirming that OX5056 is a viable PE construct. This proof of concept is an important milestone in transgenic approaches to insect control. It demonstrates the first complete transgenic construct which acts in male specific manner to induce sterility in which the tet on-off switch could be used for control. Further to confirming the sterility phenotype the next stages of development are to ensure that the sterility mechanism is a result of the proposed mechanism, through successful transfer of sterile sperm to females. It is also pertinent to assess the construct for homozygous viability, that there is no ill effect when two copies of the transgene are inherited.

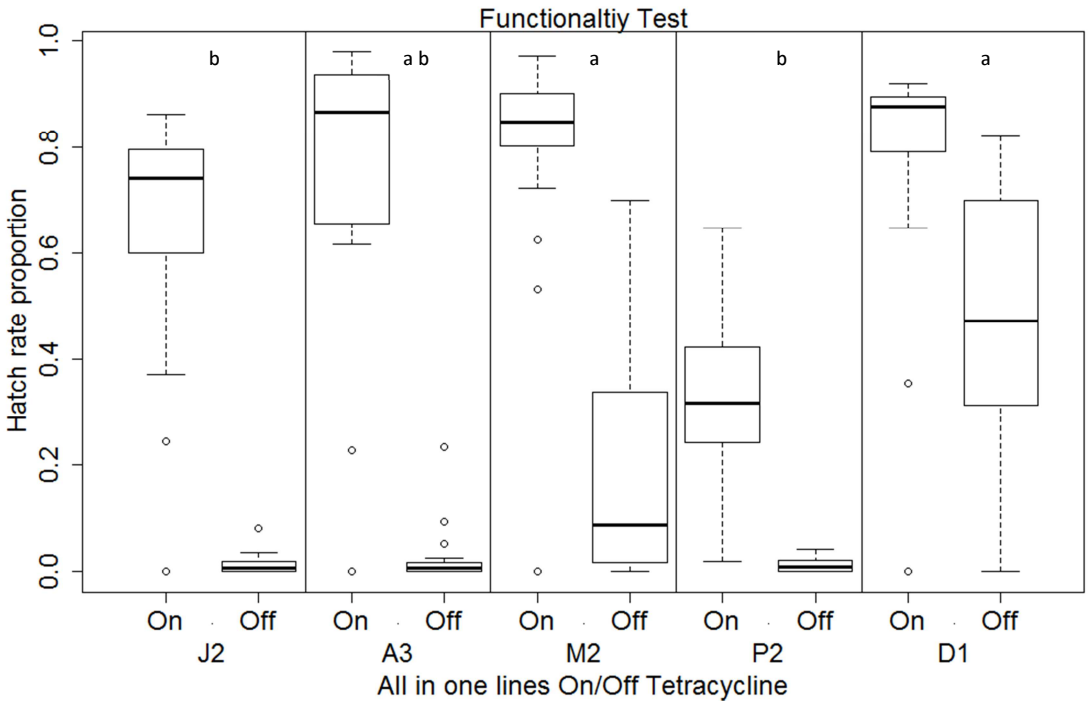

Figure 22: Box and whisker graphs demonstrating the hatch rates of OX5056 transgenic lines reared on/off tetracycline. a) Hatch rate of eggs, fertilised by OX5056 males reared on tetracycline, that are not significantly different from 80% ($p>0.05$) b) Hatch rate of eggs, fertilised by OX5056 males reared off tetracycline, that are not significantly different from 0.1% ($p<0.05$).

3.3.6 OX5056 Sperm transfer evaluation

Although the PE cassette is expected to cause the DNA packaged into male sperm to be fragmented by the nuclease, spermatogenesis could be disrupted through an unknown and less targeted mechanism, resulting in no functional sperm being passed to the female. The passing of bad sperm packets from male to female can inspire females to seek a further mate [104], which is undesirable for SIT-like approaches. As such sperm transfer to females during copulation was assessed. If the female is in receipt of a viable sperm packet, it can be assumed the sterility phenotype is due to the sperm not being viable. It has been documented in the medfly *Ceratis capita* that a sterility phenotype was due to immotile sperm causing a blockage, as opposed to being infertile (personal communication Ryan Turkel 2014). Whilst this sterility phenotype still leads to the desired effect, it does not appear to be brought about through the proposed genetic mechanism, which may have an impact with a regulatory submission.

Female *Ae. aegypti* store sperm in a spermathecae after mating, fertilising the eggs as they are oviposited. On dissecting females and examining the spermathecae it is possible to assess their status by the presence or absence of sperm. If sperm transfer from the OX5056 males is successful it is likely that the sterility phenotype is due to those sperm being infertile, rather than an alternative mechanism.

Twenty individual transgenic OX5056 males (of the A3 and J2 lines) were allowed to mate with up to two female wild types. If either female was successfully inseminated the male counted as a success, if both were negative for the presence of sperm the male was counted as a failure. There was a >90% success in matings of OX5056 males reared off dox, suggesting that infertile sperm is the mechanism of sterility. No significant difference was seen between the three lines tested ($p = 0.7662$).

Table 7: Number of successful matings by OX5056 males reared off dox, resulting in sperm transfer to the female spermathecae. Latin wild type males used as a control.

Line	Positive mating	Total
OX5056_A3	18	20
OX5056_J2	19	20
LWT	19	19

3.3.7 OX5056 Homozygous viability

In order for PE to move from a proof of concept to a working product it must be homozygous viable; that is, an individual inheriting two copies of the transgene does not exhibit adverse fitness effects. In the factory setting, homozygous lines are essential for cost effective colony maintenance, as it eliminates the requirement to screen individuals for fluorescence prior to a field release or refreshing the colony. In order to test for homozygous viability a heterozygous cross of males and females is performed. If normal Mendelian inheritance is followed and there is no impact of the transgene on survival then 75% of the progeny of a heterozygous cross should exhibit fluorescence (25% homozygous and 50% heterozygous), split evenly

between the sexes (37.5 %). There was a significant reduction in all cohorts to the expected 37.5% value (Appendix A: Table 31 - Table 36), except for OX5056_A3 males (Figure 23). It was noted that a slight difference in fluorescence was observed in individuals, most likely due to the number of copies of the transgene (Figure 24). This difference in fluorescence can be an aid to development; by crossing brighter individuals together the chance of establishing a homozygous line are increased. This fluorescence difference indicates that in OX5056_A3 some homozygous individuals were present; however overall survival of the lines is clearly compromised.

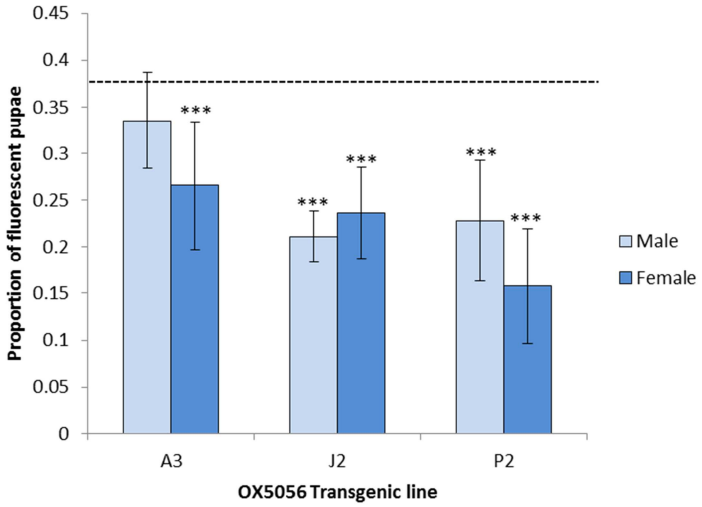

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Figure 23: Mean proportion (with SD) of fluorescent pupae from a heterozygous OX5056 cross, adjusted to WT survival. Dashed line represents the expected 37.5% from a heterozygous cross.

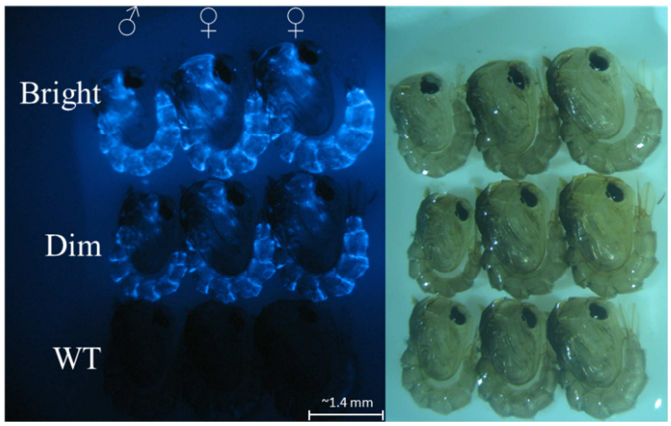

Figure 24: OX5056_A3 under blue filter (Left) and white light (Right). Fluorescent intensity is assumed to be linked to genotype, brighter individuals being homozygous and the dimmer individuals being heterozygous.

Lethality from the heterozygous crosses was seen at both larval and pupal stage, evidenced by dead individuals in the rearing water. Pupal stage mortality exhibited some extreme morphological abnormalities (Figure 25). These abnormalities have been observed in all three of the transgenic lines containing OX5056 indicating that it is unlikely caused by a positional effect but more a direct consequence of the transcriptional products of the construct. Two potential causes are that the *topi* promoter may have a role outside of the male germline (particularly in females where higher mortality rates are seen), or that low level basal expression of the FokI-Protamine is enough to cause mortality. The fact that these abnormalities are seen in both the off and on dox cohorts would suggest that it is the FokI-Protamine being effective at low levels of basal expression.

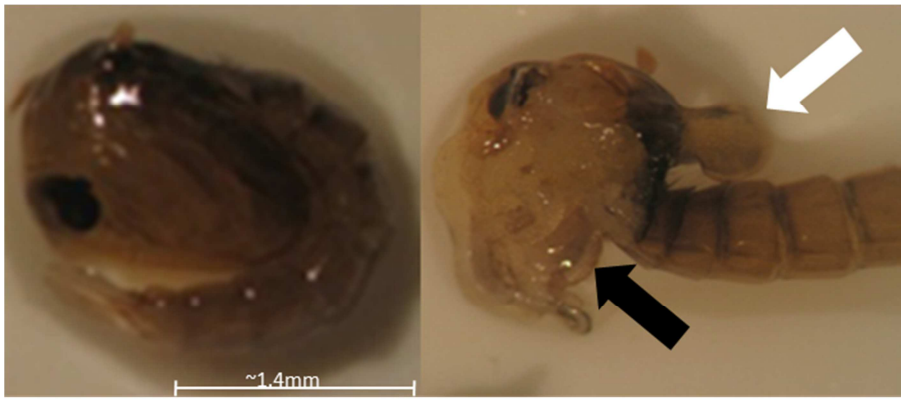

Figure 25: **Left**: Normal *Ae. aegypti* WT pupa. **Right**: Morphological abnormalities occasionally witnessed in *Ae aegypti* pupae carrying the OX5056 construct. The pupal casing around the head has come away (white arrow), exposing the interior (black arrow).

Although female lethality was observed in the OX5056_A3 homozygous viability cross, the results were marginally higher than the 25% fluorescence one would expect if all homozygous individuals were compromised. Coupled with the presence of bright female pupae, it can be assumed that a proportion of homozygous individuals survived, and that it therefore has the potential to be made homozygous.

To generate a homozygous line, OX5056_A3 was enriched for 5 generations by removing WT pupae from each generation. Detection of fluorescence brightness was performed at the L4 stage, where 20 individuals were selected to be mated individually. Two of the 10 crosses produced enough surviving progeny to generate and maintain transgenic lines. These have been kept separate for 4 generations with no presence of the WT allele detected. However molecular confirmation would be required to confirm a homozygous line. The absence of the WT allele for these generations allows a reasonable conclusion that the lines are homozygous; however, evidence of fitness costs persists, with high levels of pupal mortality seen during rearing.

3.3.8 Molecular confirmation of OX5056

A qRT-PCR was performed to examine the relative transcripts of tTAV in male and female samples. The levels of tTAV being expressed in males and females may help to explain the fitness costs associated with carrying two copies of the OX5056 transgene.

The qRT-PCR demonstrated high levels of tTAV transcript in male samples in both the on and off dox cohorts (Figure 26). These values are relative to the quantity of tTAV transcript in one of the off dox female samples. Whilst a large amount of variation is present across the results, they nevertheless demonstrate that tTAV transcript is present in males and not in females as would be expected. There is minimal expression of tTAV detected in one of the female off dox samples. This could be the root cause of the fitness issues seen, compounded in homozygous individuals. Even low levels of tTAV expression could be enough to promote production of lethal quantities of FokI-Protamine. The presence of tTAV in male whole pupa samples and the numbers from the homozygous viability study suggests that either this is not the case, or that males are more resilient to off target FokI-Protamine expression.

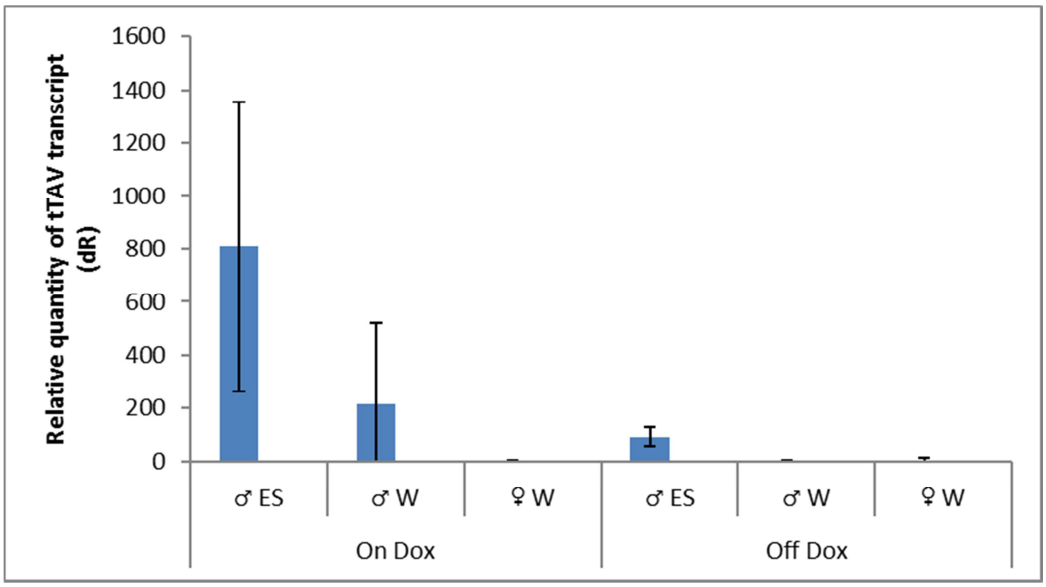

Figure 26: Relative quantities of tTAV2 transcript from male and female pupae reared either on or off doxycycline (with standard deviations). Samples tested included whole female pupae (♀W) and male pupae dissected to remove the end terminal segments (♂ ES) and the remaining body of the pupae (♂ W). As OX5056 functionality should be restricted to the testes, tTAV2 expression should only be seen in samples of male end terminal segments in the off dox cohorts. All values are relative to the female samples reared on dox, set to 1.

3.4 Conclusions

The work presented in this chapter has demonstrated a fully functional PE all-in-one construct. Continuing on from previous work at Oxitec Ltd, promoter and effector constructs were retested to ensure their suitability for use in a PE line. β*2-tubulin* was discarded as a potential promoter due to its lethal effects when combined with the *fokI-protamine* effector using the tetO on/off switch. A highly penetrant and repressible male sterility phenotype was demonstrated in males inheriting both the *topi*-tTAV promoter construct and the tetO-*fokI-protamine* effector construct. A complete 'all in one' construct, OX5056, was built utilising these components, and was successfully transformed into the *Ae. aegypti* LWT background. After characterisation for evidence of sex linkage and single insertion events, the construct was assessed for functionality. A highly penetrant and repressible male sterility phenotype was demonstrated, with confirmation of functionality shown by successful transfer of sperm to females by males reared off dox. Evidence of off target effects were seen, demonstrated

through a heterozygous cross of males and females, where lower than expected proportions of fluorescent individuals were recovered. This leads to the conclusion that individuals are compromised when inheriting two copies of the transgene. Molecular characterisation confirmed the presence of tTAV in heterozygous male pupae, both in the end terminal segments containing the testes and the rest of the pupa. Limited expression of tTAV was noted in females reared off dox. It is currently unclear as to whether the off target fitness costs are due to basal expression of the *fokI-protamine* or upregulated expression via tTAV production. However due to the lack of tTAV present in on-tet female samples, it is more likely the former. Further molecular analysis is required to confirm relative quantities of *fokI-protamine* transcript. The work described in this chapter forms the basis for building a 3rd generation product. The next stages of development, detailed in Chapter 4, are to combine the PE construct with the GSS in an 'all in one' 3G construct; and to develop the PE construct, in an attempt to mitigate the off target effects seen in females.

Chapter 4: *Ae. aegypti* – Development of 3rd Generation Technology

4.1. Introduction

The work detailed in Chapter 3 was concerned with producing a working PE transgenic line. This was achieved in the form of construct OX5056_A3, which when reared in the absence of tetracycline produced sterile males that could be rescued through rearing in the presence of tetracycline. This chapter focuses on further development of the PE, testing it in *Ae. albopictus*, combining it with the genetic sexing technology and attempting to mitigate the adverse fitness effects described at the end of Chapter 3.

4.1.1. 3rd Generation Technology

As discussed previously, 3G technology is the combination of genetic sexing with PE. When reared in the absence of tetracycline, tTAV is produced in both females and males. In females a positive feedback loop is initiated, causing death through transcriptional squelching; in the males, tTAV production is restricted to the germline rendering them infertile, but allows the males to otherwise develop fully (Figure 27).

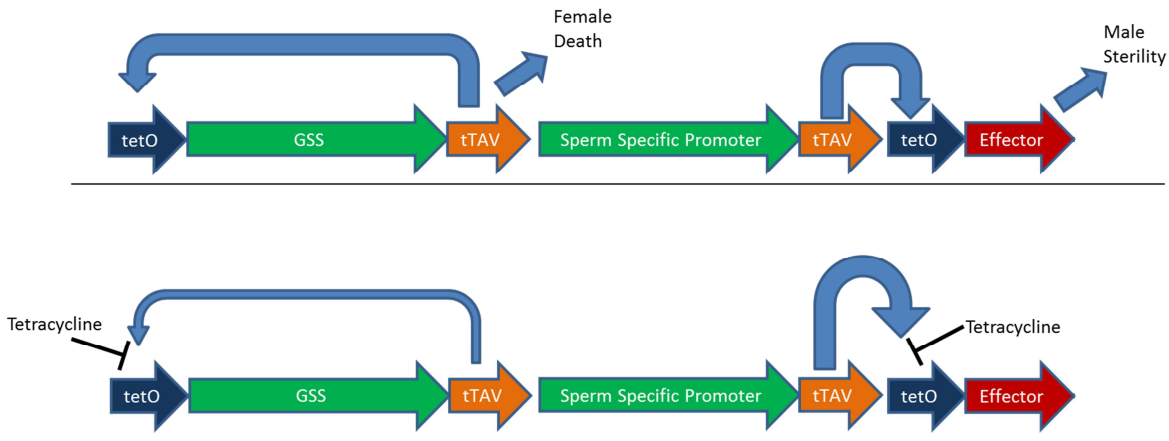

Figure 27: **Top**: In the absence of tetracycline the GSS section produces tTAV in females. This initiates a positive feedback loop, leading to death through transcriptional squelching. The PE section is active in the testes, where production of tTAV causes upregulation of the effector resulting in inviable sperm. **Bottom:** In the presence of tetracycline tTAV is prevented from binding to tetO. This results in females surviving as no positive feedback loop is initiated and fertile males as there is no production of the effector.

4.1.2. Sex-Specific Genes

Sex determination in insects varies across the insect orders, but commonly involves a gene cascade involving many sex-specific genes [105], which are often conserved or contain orthologues across species. The *doublesex* (*dsx*) and *transformer* (*tra*) genes are two such genes [106] [107] which have been exploited recently in genetic sexing strains. Both these genes use a sex-specific splicing mechanism that produces different transcripts in males and females from the same gene. This can be exploited by building constructs using parts of these genes to make expression of tTAV sex-specific. In *Ceratis capitata, tra* was successfully used to produce a GSS [108]; and *dsx* was exploited to produce GSS in two species of moth, the diamondback moth *Plutella xylostella* and pink bollworm *Pectinophora gossypiella* [109]. The *Ae. aegypti dsx* gene was successfully characterised in 2011 [110] and is described in Figure 28. The *dsx* gene, is too large to use in its entirety so the sections of the gene responsible for the sex-specific splicing have been identified and isolated. Exons that are not required are excluded and large introns are truncated to reduce the size of the construct. Reading frames are designed to be open in female splice-forms and closed in the male splice-form through the introduction of point mutations and stop codons. This is referred to as the *dsx* minigene. The *dsx* minigene is then placed upstream of tTAV so that it is transcribed correctly in females, but not in males. This ensures that the production of tTAV, the positive feedback loop and lethality is limited to females.

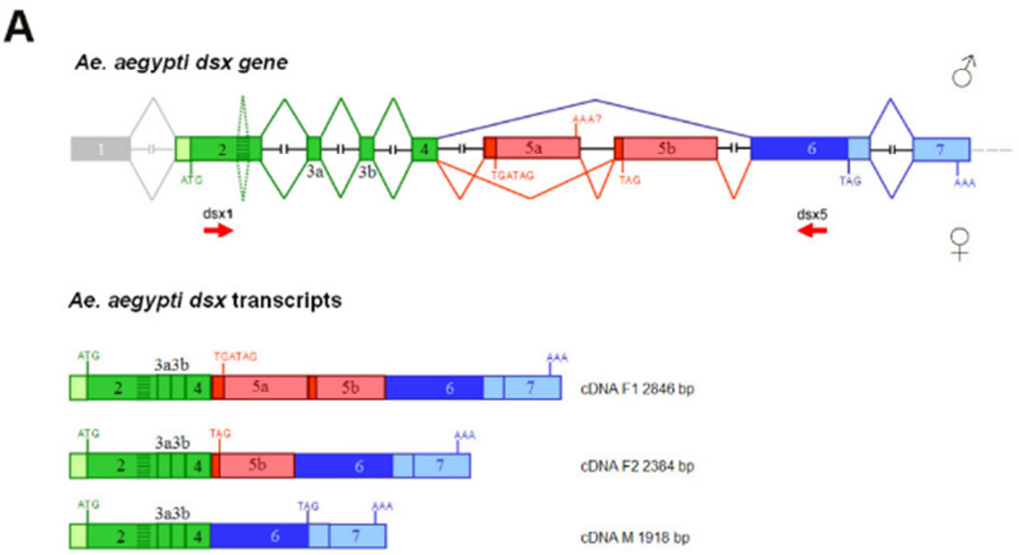

Figure 28: Taken from Salvemini et al. [110]. *Ae. aegypti doublesex* architecture and different transcripts produced through sex-specific splicing. Green boxes represent exons 2, 3a, 3b and 4, which are common to both male and female transcripts. Sex-specific exons are represented by red (female) and blue (male) boxes for the female-specific exons 5a and 5b, and the male-specific exon 6.

Previous work at Oxitec Ltd by Collado [111] aimed to produce a working GSS in *Ae. aegypti,* using the *Ae. aegypti dsx* gene linked with the tet on/off switch. Construct OX4489 contained a *dsx* minigene (Figure 29) in which a frame shift is introduced in females through an artificially introduced ATG start codon to the female-specific exon 5b. This frame shift is in line with the open reading frame for the downstream tTAV component. In females functional tTAV transcript is produced, but in males the 5b exon is spliced out, resulting in the tTAV being read out of frame so no tTAV is produced. The tet on/off switch is therefore restricted to the females, with males unaffected.

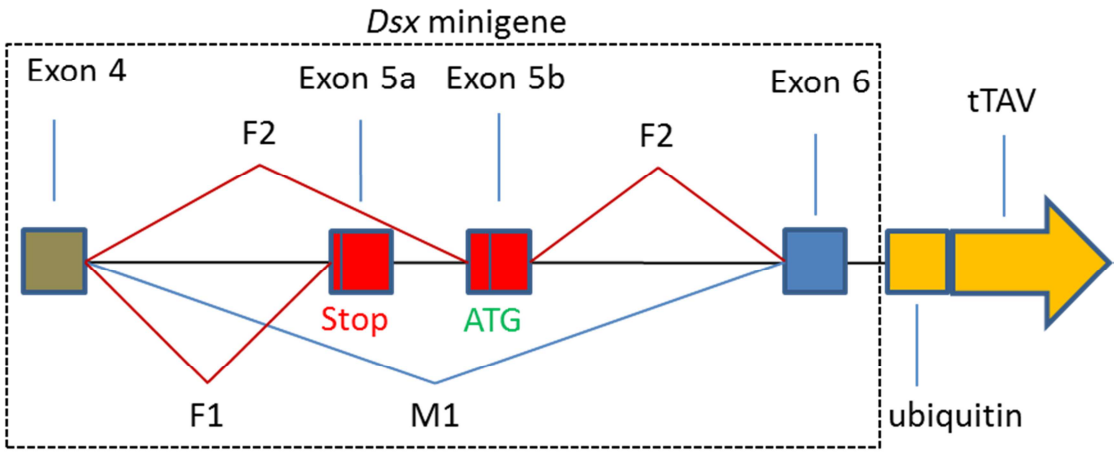

Figure 29: Adapted from Collado [111]. Schematic diagram of construct OX4489. It contains the functional element of tTAV donwstream of the *Aae dsx* minigene, downstream of the DNA binding site tetO (not shown). The *dsx* minigene is a sex-specific splicing mechanism which can produce one of three transcripts. The female F2 transcript consists of the common exon 4 (green), the female-specific exon 5b (red) and the male-specific exon 6 (blue) with shortened introns between them. An artificially introduced ATG start codon in exon 5b causes the downstream tTAV element to be read in frame, resulting in functional transcript in females. The second female splice-form F1 results in a truncated transcript due to the native stop codon at the start of exon 5a. In males the female exons 5a and 5b are spliced out, resulting in tTAV being read out of frame, and so producing a defunct transcript. Also present are regulatory 3'UTR regions (not shown) and a fluorescent marker (not shown), DsRed, downstream of the control of the all body promoter Hr5iE1.

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Female-specific lethality was not observed with construct OX4489 alone, however it was possible to induce it by crossing OX4489 to lines carrying a lethal effector, *reaper* (*rpr*), downstream of tetO. Here females inheriting both the tetO-*dsx*-tTAV and the tetO-*rpr* died at the late pupal stage, demonstrating the female-specific nature of the construct held promise. Further work at Oxitec Ltd aimed at improving OX4489 to function through a positive feedback loop rather than with a separate effector gene. A new construct, OX5034, used a modified *dsx* minigene (Figure 30) with the ATG start codon positioned immediately upstream of exon 4. OX5034 has been successfully shown to deliver a female-specific conditional lethality (data not shown), and is currently undergoing review for field trial releases (personal communication – Sian Spinner 2016).

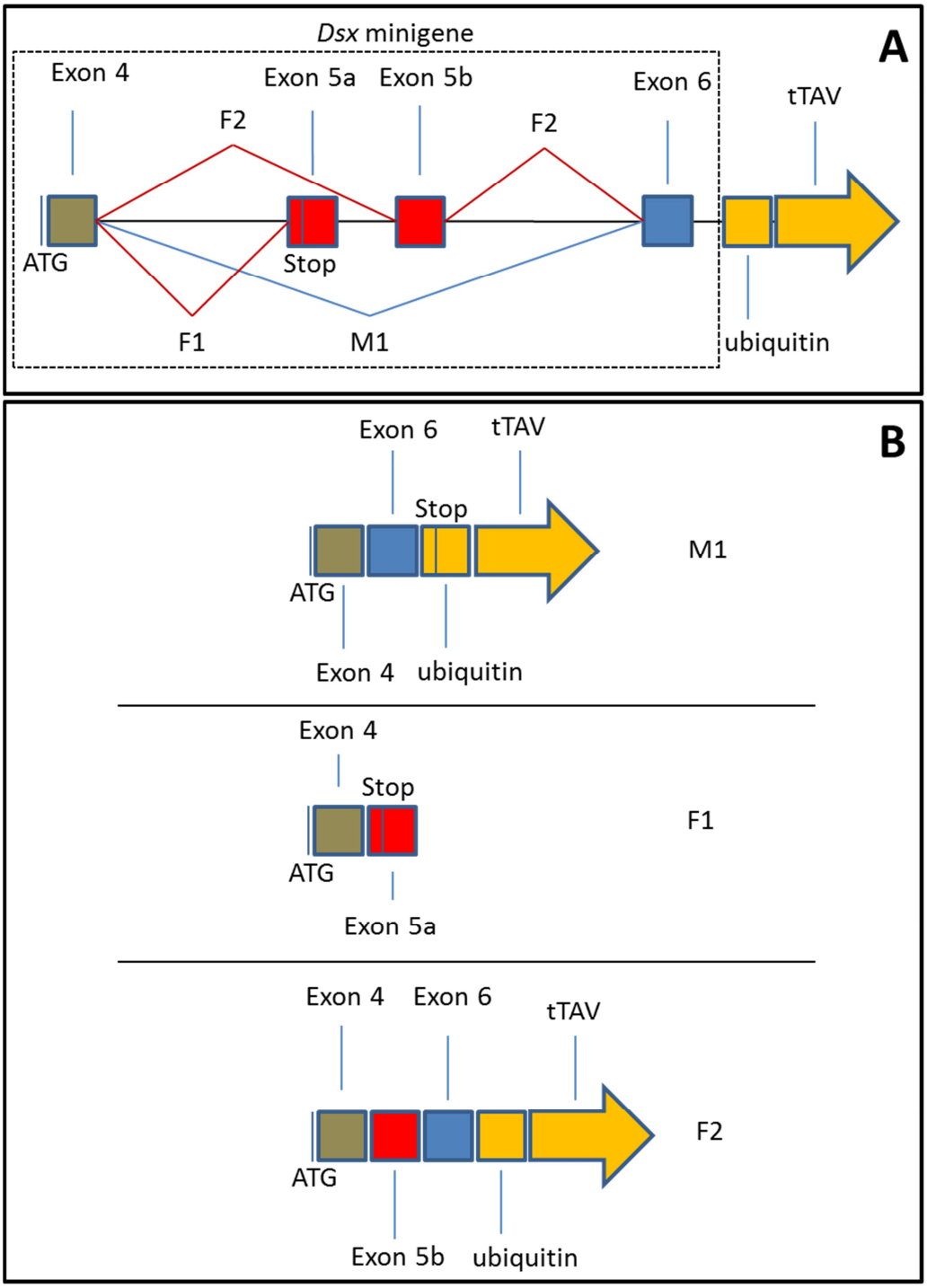


Figure 30: **A)** Schematic diagram of construct OX5034. It contains the functional element of tTAV under control of the *Aae dsx* minigene; A sex-specific splicing mechanism which can produce one male (M1) or one of two female (F1, F2) transcripts. The ATG start codon is placed directly before exon 4 in order to increase the strength of the positive feedback loop. **B)** The three transcripts produced through sex-specific splicing. M1 and F1 both have truncated transcripts and do not result in tTAV transcript. In F1 this is due to the native stop codon in exon 5a and in M1 the combination of exon 4 and 6 causes a stop codon in ubiquitin. Artificially engineered frame shifts in exon 5b produce an open reading frame through to the ubiquitin and tTAV. Also present are regulatory 3'UTR regions (not shown) and a fluorescent marker (not shown), DsRed, under the control of the all body promoter Hr5iE1.

4.1.3. Improving Paternal Effect

The PE construct tested in Chapter 3 demonstrated a suitably penetrant and repressible sterility phenotype. However, there were issues with male and female fitness associated with inheriting two copies of the transgene. This would suggest that the transgene expression is not limited to the male germline or to just the males. This is evidenced by reduced numbers of transgenic individuals as a result of a heterozygous cross (3.3.7). In order to address these fitness costs a similar system to that of the GSS described above is proposed. Sutton [112] identified a number of genes and splice-forms specific to the male germline. These splice-forms work in a similar way to the sex-specific splicing of *dsx* and *tra*, where males and females produce different transcripts from the same gene. It is proposed to include a sex- and tissue-specific splicing cassette to disrupt the open reading frame of the *fokI-protamine* effector (Figure 31). Thus in males where the intron is spliced out, *fokI-protamine* will be encoded in frame, whereas in females, where the intron is not spliced out, *fokI-protamine* will be encoded out of frame resulting in a defunct transcript. The germline specific splicing of the gene should help to localise expression of the transgene to the testes, limiting off target expression and fitness costs in other tissues in both males and females.

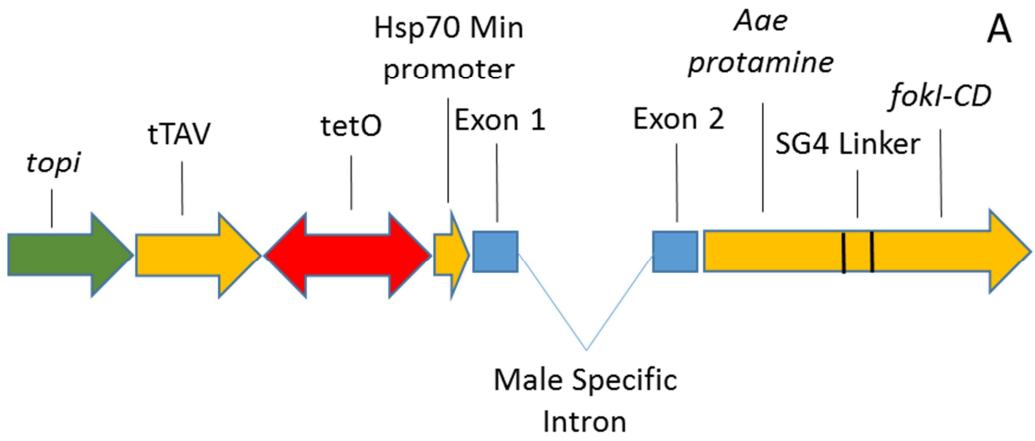


Figure 31: A PE construct in which a sex-specific splice-form is introduced upstream of the *fokI-protamine* effector. The intron is spliced out in the male germline, allowing for correct transcription of the *fokI-protamine.* In females and male somatic cells, the intron is not spliced out, this causes a frame shift resulting in the downstream *fokI-protamine* being transcribed out of frame and defunct.

4.1.4. Chapter Aims

The primary aim of the work presented in this chapter is to combine the GSS OX5034 with the PE OX5056 identified in Chapter 3 to produce a fully functional 3G technology product. This is achieved through transformation of a new construct OX5197 (Figure 32). Transformants are assessed for suitability, and subsequently tested for functionality.

Additional aims are to address the fitness effects seen in the PE line OX5056. This involves identifying a suitable male splice-form, confirming previous results from Sutton[112]. A new construct, OX5244 is constructed by incorporating a suitable male-specific splice-form to the PE construct OX5056. This construct is then transformed into *Ae. aegypti* and assessed for suitability before being tested for functionality and homozygous viability.

As certain genes are often conserved across insect species, it was thought that the *Ae. aegypti topi* promoter may retain its functionality in the OX5056 PE construct when transformed into *Ae. albopictus*. Transformation of OX5056 and functionality testing was performed to assess the PE in this species.

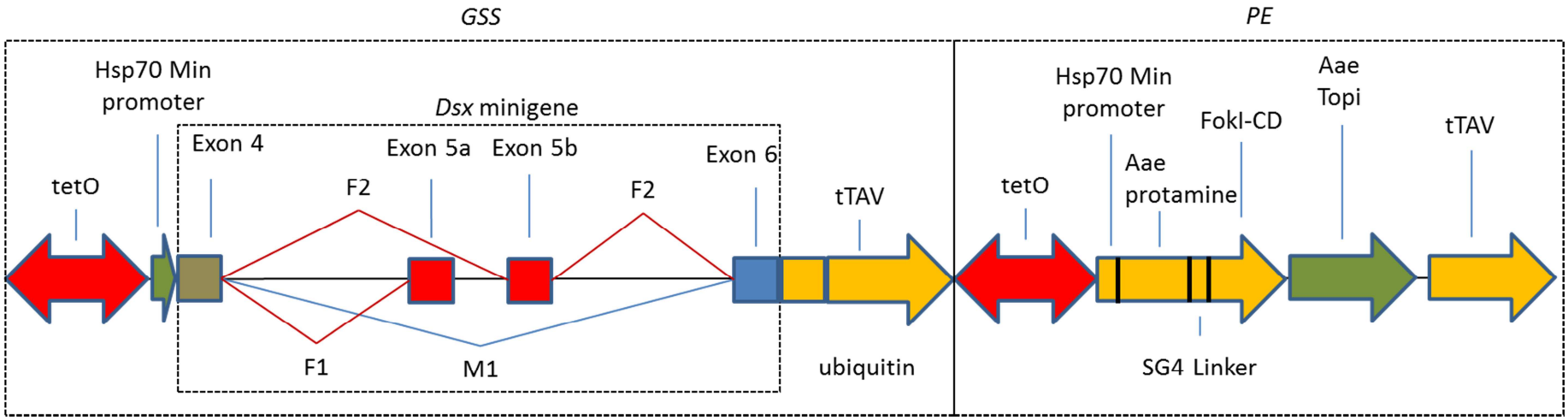


Figure 32: Schematic diagram of construct OX5197. It contains two distinct sections based on OX5034 (GSS) and OX5056 (PE). The two functional elements of tTAV are under control of the *dsx* minigene and the *Aae topi* promoter. The *dsx* minigene is placed downstream of tetO to produce a positive feedback loop in females when reared in the absence of tetracycline. A further tetO binding domain is upstream of the *fokI-protamine* to produce the male sterility phenotype from the tTAV under control of the sperm specific *Aae topi* promoter. Also present are regulatory 3'UTR regions (not shown) and a fluorescent marker DsRed, under the control of the all body promoter Hr5iE1 (not shown). Construct built by Tarig Dafa'alla at Oxitec Ltd.

4.2. Materials and Methods

4.2.1. New Splice-Form Identification

4.2.1.1. Reverse Transcription PCR

Samples of RNA extracted from male testis and female pupae were supplied by Tarig Dafa'alla (Oxitec Ltd). One step reverse transcription PCR (RT-PCR) was performed with the following primers for each gene of interest (Table 8).

Table 8: Forward and reverse primer IDs and sequences for male splice-forms of interest

Gene ID	Gene	Forward Primer	Reverse Primer
1	**AAEL012262**	**Aa_021240_35_1** CAGTGCCAGCAAAGCAGAAG	**Aa_021240_S2** TGAGCTGCTTCCATCTGTGT
2	**AAEL018211**	**4164)Aa-18562-F1** CCGATTTTCATCAAGTCTGCCTTG	**Aa_018562_IT_q1** ATCAAAGCCGCATAGCTTGG
3	**AAEL001898**	**Aa_016105_IO_q2** ACCCAACAGCCATCGATACG	**Aa_016105_IT_q1** TTCAGTGTGCTTTGGAGGTGT
4	**AAEL011153**	**4162)Aa-18622-F1** CTGTCGTTCCACACATATTTGTCC	**Aa_018622_20_EJ1** TCTGCCTTGCCAGTGATTGA
5	**AAEL008428**	**Aa_012248_3_1** GGGACTTTGACTCGCACAGA	**Aa_012248_3_2** AGCAGCAATTCGGTTTTGCAT

4.2.1.2. DNA ligation

3 µl of the cDNA PCR product was run on a 0.8% agarose gel and the resultant bands cut from the gel, ensuring their weight was ~100 mg. DNA was excised from the gel and purified using GeneJet PCR purification kit according to the manufacturer's instructions and eluted in 20 µl elution buffer. 3.5 µl of DNA product was mixed with 2 x reaction buffer (5 µl) and DNA blunting enzyme (cloneJET PCR cloning kit, ThermoFisher) (0.5 µl), incubated at 70°C for 5 minutes before being ligated with pJET 1.2/blunt cloning vector (0.5 µl) and T4 DNA ligase (0.5 µl) for 30 minutes at room temperature.

4.2.1.3. DNA Transformation

XL10 – Gold ultracompetent cells (150 µl) for transformation were mixed with XL10 – Gold – β - mercaptoethanol (6 µl). The DNA ligation mix (2 µl) was mixed with the ultracompetent cell /

β – mercaptoethanol (12 µl) and incubated on ice for 30 minutes. The mix was subsequently heat shocked at 42 °C for 30 seconds before returning to ice for 2 minutes. Transformation medium (200 µl) was added to each mix and incubated at 37 °C with vigorous shaking for 1 hour. IPTG (5 µl) was added to an ampicillin agar plate followed by 100 µl of transformed cells which were then incubated at 37 °C overnight.

A PCR master mix was created (Table 9) and 10 µl added to each well of a 96 well plate. A corresponding flat bottomed 96 well plate had 100 µl of Lysogeny broth (LB) to aid bacterial growth. Bacterial colonies were picked from the agar plates using a pipette, dipped into the PCR plate and the corresponding position on the LB plate. PCR was then performed on the PCR plate (Table 10) to check the correct band size had been ligated. The corresponding bacterial colonies required were added to LB broth and incubated overnight in a shaking incubator @ 37°C, 250 rpm.

Table 9: Reagents for a PCR master mix to confirm expected DNA ligation

Reagent	**Quantity / µl**
PCR Bio Buffer	200
pJet Forward Primer ACTACTCGATGAGTTTTCGG	2
pJet Reverse Primer TGAGGTGGTTAGCATAGTTC	2
PCR BioTaq	10
H_2O	786

Table 10: Conditions for the PCR cycle to confirm expected DNA ligation

Step	Temperature	Time
1	95	1 m
2	95	15 s
3	55	40 s
4	72	1 m (back to step 1 * 2 cycles)
5	95	15 s
6	55	30 s
7	72	25 s (back to step 5 * 29 cycles)
8	72	5 m
9	4	pause

Minipreps were created using the GeneJET Plasmid Miniprep Kit (Fermentas) to the manufacturer's instructions. 1 µl of each miniprep was digested using 0.2 µl of BglII restriction enzyme with 7.8 µl of H_2O and 1 µl of FastDigest buffer for 1 hour at 37°C before being run on a 0.8% agarose gel. 20 µl of the minipreps were sent off site for sequencing by GATC Biotech (UK).

4.2.2. OX5056, OX5197 and OX5244 Transformation

Transformation was achieved through microinjections of pre-blastoderm embryos as described in Chapter 2 (2.2.4). OX5197 and OX5244 were transformed into the *Ae. aegypti* LWT background; the OX5056 was transformed into the *Ae. albopictus* KLP wild type background. For each construct an injection mix of 100 µg / µl DNA, 500 µg / µl *piggyBac* helper RNA and 1 µl of doxycycline (at 1 µg / µl) was made. Surviving G_0 mosquitoes were pooled together as 2 males or up to 10 females and outcrossed with their WT counterpart at a ratio of 1 male : 5 females or 2 females : 1 male. Adults were blood-fed and allowed to oviposit for up to three gonotrophic cycles. Each batch of eggs was allowed at least three days to develop before being hatched and screened for fluorescence as evidence of a successful transformation event.

4.2.3. OX5056, OX5197 and OX5244 Line Assessment

To establish a sufficient number of transgenic lines for functionality testing up to three single male outcrosses to LWT were performed from each of the positive pools. The resultant offspring were hatched under vacuum and aliquoted into pots and reared to standard conditions on dox (1 µg / ml). Pupae were screened for the appropriate fluorescent marker and sex to check for normal Mendelian inheritance. Single male outcrosses were performed for OX5056, however due to time constraints, the lines were not assessed for normal Mendelian inheritance prior to functionality testing.

4.2.4. OX5197 Functionality Testing

Heterozygous eggs (G_3) of suitable lines were hatched under vacuum and aliquoted into 6 pots of 100 reared at 0.5 larvae / ml. Three pots were reared on dox (1 µg / ml) three off dox. Larvae were fed to the standard feeding regimen and were sexed and screened for fluorescence at the pupal stage. Forty transgenic males from each line (20 on dox, 20 off dox) were separated at this point for sterility phenotype testing, to the same protocol as outlined in Chapter 3 (3.2.5).

4.2.5. OX5056 and OX5244 Functionality Testing

Heterozygous eggs (G_3 for OX5244, G_6 for OX5056) of suitable lines were hatched under vacuum. Lines of OX5244 were aliquoted into two pots of 100 and reared at 0.5 larvae / ml either on dox (1 µg / ml) or off dox. Due to low numbers of eggs lines of OX5056 were aliquoted with numbers ranging from 11 – 100 larvae into two pots and reared in 200 ml water either on dox (1 µg / ml) or off dox. Larvae were fed to the standard feeding regimen, and were sexed and screened for fluorescence as pupae. Up to forty males were selected for a sterility phenotype assay, which was performed as previously described (3.2.5).

4.2.6. OX5244 Homozygous viability

Heterozygous males and females were mated together at a ratio of 1:2 in small bugdorm cages. Progeny from these crosses were aliquoted into three replicates of 200 (140 for line D2) and reared in the presence of doxycycline (1 µg / ml). Larvae were reared to standard feeding regimen and were screened for fluorescence and sexed as pupae.

4.2.7. Statistics

A Kruskal–Wallis H test was applied to test for a significant difference between the proportions of WT individuals and those inheriting the OX5197 transgene (from a heterozygous OX5197 x WT cross) when reared on and off dox. Where significant the Dunn's Test of Multiple Comparisons Using Rank Sums was applied to examine the significance between the relevant cohorts, i.e. comparing the transgenic males reared on dox compared to the WT males reared on dox.

One sample *t*-tests were performed to investigate egg hatch assays, comparing off tet cohorts to the required baseline egg hatch rate of 1% and the on tet cohorts to a required baseline hatch rate of 80% for both OX5197 (4.3.3.2) and OX5244 (4.3.8).

A repeated G-test for homogeneity was used to test for differences between the numbers of fluorescent pupae counted to the expected numbers from the progeny of the heterozygous crosses of OX5244 (3.3.7). If the total G- score was significant a post-hoc analysis was performed to ascertain if the observed finding was due to differences between the replicates (G-test for independence) or between the counted and expected numbers (pooled G-test). Full statistical analysis can be found in Appendix B: Table 37 - Table 46.

4.3. Results Discussion

4.3.1. OX5197 Transformation

OX5197 is a combination of the PE construct OX5056 and the GSS construct OX5034. Due to the alternative splicing of the *dsx* minigene, it is predicted that functional tTAV transcript will be produced in females, causing a positive feedback loop as it binds to tetO, ultimately resulting in death (Figure 33 - Top). Male production of tTAV should be restricted to the germline, due to the specific nature of the *topi* promoter. On binding to tetO, the tTAV should cause upregulation of the *fok*I-*protamine* to induce sterility. Both the GSS and PE components should be repressible through the addition of tetracycline to prevent it binding to tetO (Figure 33 - Bottom).

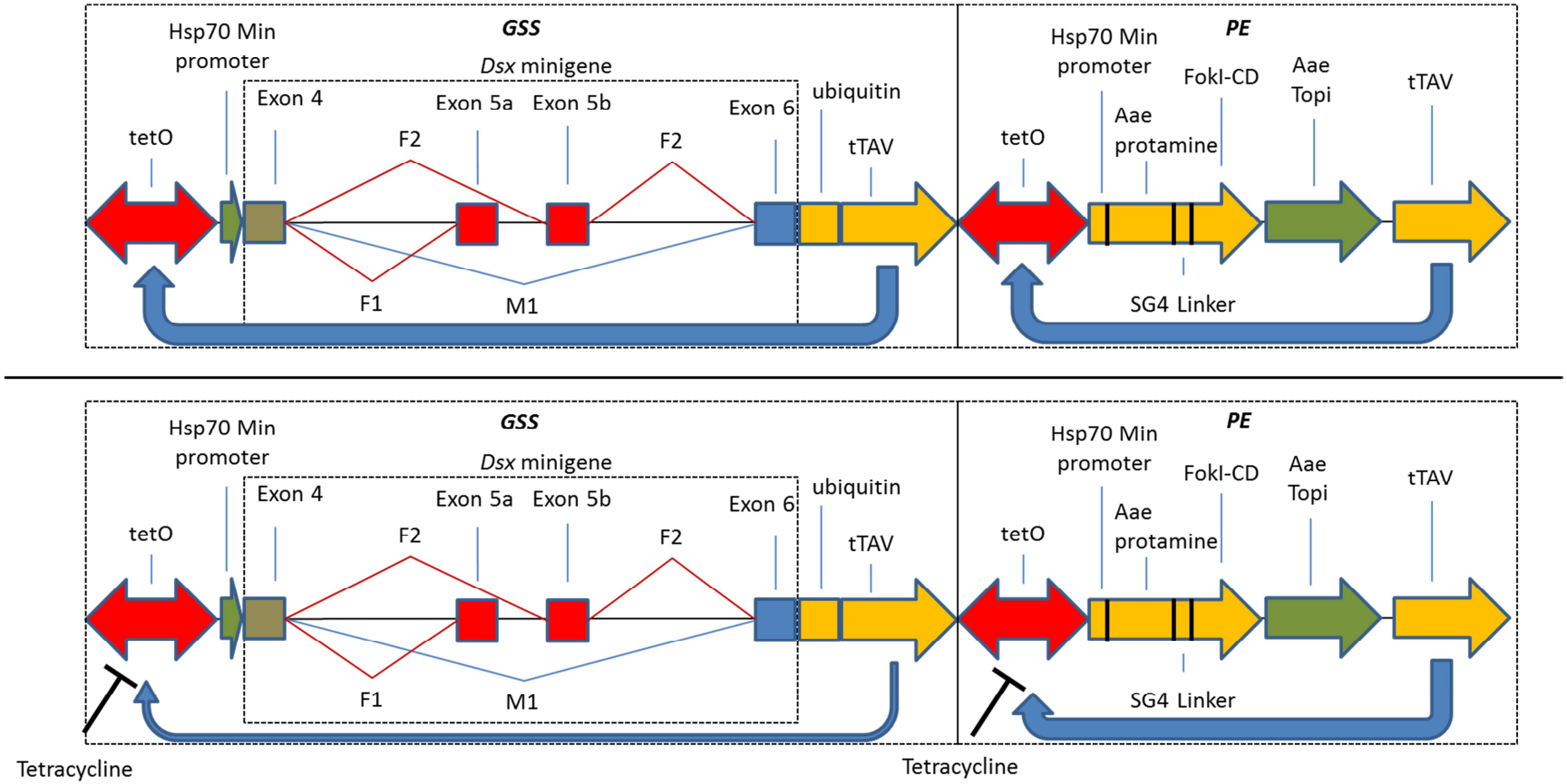


Figure 33: **Top:** When reared in the absence of tetracycline the GSS section based on OX5034 produces tTAV in females, initiating a positive feedback loop, leading to death through transcriptional squelching. The PE section is active in the testes, where production of tTAV causes upregulation of the *fokI – protamine* resulting in inviable sperm. **Bottom:** In the presence of tetracycline tTAV is prevented from binding to tetO. This results in females surviving as no positive feedback loop is initiated, and fertile males as there is no production of *fokI-protamine.*

A total of 5771 pre blastoderm eggs were injected with construct OX5197 as described previously (3.2.3). A total of 39 G_0s survived to adulthood and were pooled together and outcrossed. Screening of G_1 progeny resulted in 3 positive pools (OX5197_F, OX5197_N and OX5197_L) where transformation was successful. Two different fluorescent phenotypes were seen in pool N, which were subsequently separated into N1 and N2.

4.3.2. OX5197 Line assessment

Single male crosses (termed a, b and c) were performed from each positive pool. Multiple insertions were seen in the N1c line (Figure 34), evidenced by higher than expected proportions of transgenic males and females and very few WT males. Linkage of the transgene to the female allele opposite the male determining locus was seen in all three L lines. Five lines were deemed suitable for functionality testing: OX5197_Fa, OX5197_Fc, OX5197_N1a, OX5197_ N1b, OX5197_N2a. Lines OX5197_Fa, OX5197_Fc and OX5197_N2b show reasonably good Mendelian inheritance of the transgene with limited fitness effects in females. Lines OX5197_N1a and OX5197_N1b seem to have detrimental fitness costs associated with the transgene, evidenced by proportions of transgenic males and females lower than the WT counterparts, however they are suited to preliminary functionality testing. Lines OX5197_N2b and OX5197_N2c did not produce sufficient numbers of eggs with which to test functionality.

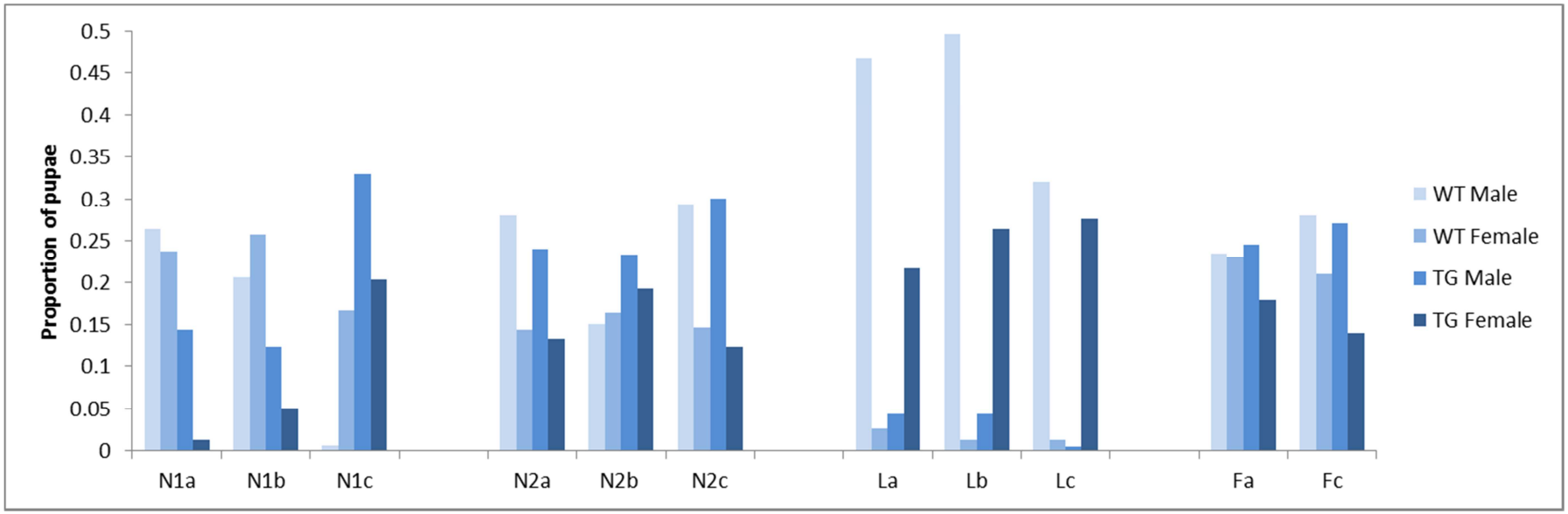


Figure 34: Proportion of fluorescent and wild type male and female pupae from single male crosses of OX5197 positive G_0 pools. Capital Letters (N, L, F) denote original G_0 pool, lower case letters (a, b, c) represent the single male parent.

4.3.3. OX5197 Functionality

4.3.3.1. Genetic Sexing

As the GSS is female specific, it is expected that there would be no reduction in the number of males, when reared either on or off dox. When reared off dox it is expected that the GSS would cause a reduction in the number of females. When reared on dox, the GSS should be repressed resulting in no reduction of females. The five lines deemed suitable for testing were reared both on and off doxycycline to assess female lethality (Figure 35). No differences were seen between proportions of transgenic males and the wild type counterparts when reared on or off dox, with the exception on line OX5197_N1a when reared on dox ($z= -2.727$, $p = 0.0064$). This demonstrates that for four of the lines tested, male survival was unaffected, indicating that the GSS was acting as expected. There were significant differences seen between proportions of transgenic females and their wild type counterparts when reared off dox for lines OX5197_Fa ($z= -2.62$, $p = 0.009$), OX5197_N1a ($z = -2.05$ $p = 0.04$) and OX5197_N1b ($z = -2.80$, $p = 0.005$). This suggests that for lines OX5197_Fa, OX5197_N1a and OX5197_N1b the GSS cassette is working as intended resulting in female lethality when reared off dox. When reared on dox, there is no significant difference in the number of transgenic females compared to wild type females, except for line OX5197_N1a ($z = -2.05$, $p = 0.04$). This suggests that the GSS cassette is repressible with doxycycline, except for line OX5197_N1a, where it is only partially repressible. Although not significant, there appears to be a reduction in the number of females when reared off dox for line OX5197_Fc. This may be due in part to lower than expected numbers of WT females surviving. Lines OX5197_Fa, OX5197_Fc OX5197_N1a and OX5197_N1b were considered for the male sterility functionality test.

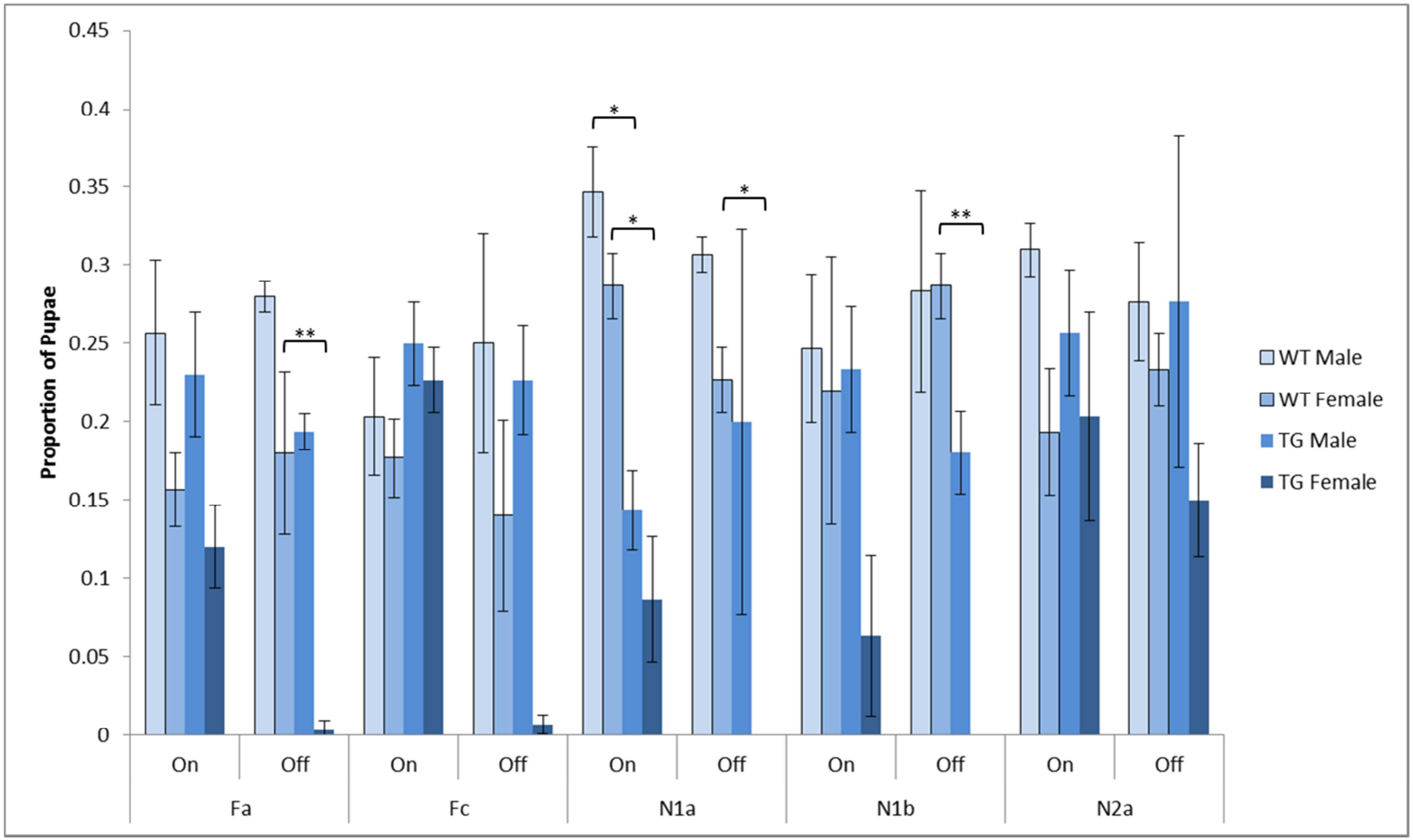


Figure 35: Mean proportions (with SD) of transgenic and wild type pupae from a heterozygous cross of each line of OX5197 to WT reared either off or on dox (1 µg / ml). Dunn's Test of Multiple Comparisons Using Rank Sums shows significant differences ($p < 0.05$ *; < 0.01 **; < 0.001 ***) between the mean proportion of TG pupae and the corresponding WT (i.e. Off Dox TG Males – Off Dox WT Males).

4.3.3.2. Paternal Effect

Egg batches from up to 20 single parent crosses were collected and hatched to assess the sterility effect of each OX5197 line and its repressibility with tetracycline. Figure 36 demonstrates that when reared off tetracycline, hatch rates were not significantly different to 1% for lines OX5197_Fa, OX5197_N1a (Full statistics Table 11; nb OX5197_N1b had 0% hatch rate for all males). Fa and Fc had hatch rates not significantly different from 80% when reared in the presence of doxycycline (Table 12). This demonstrates a highly penetrant and fully repressible PE phenotype in line OX5197_ Fa and a fully penetrant and partially repressible PE phenotype in line OX5197_N1a.

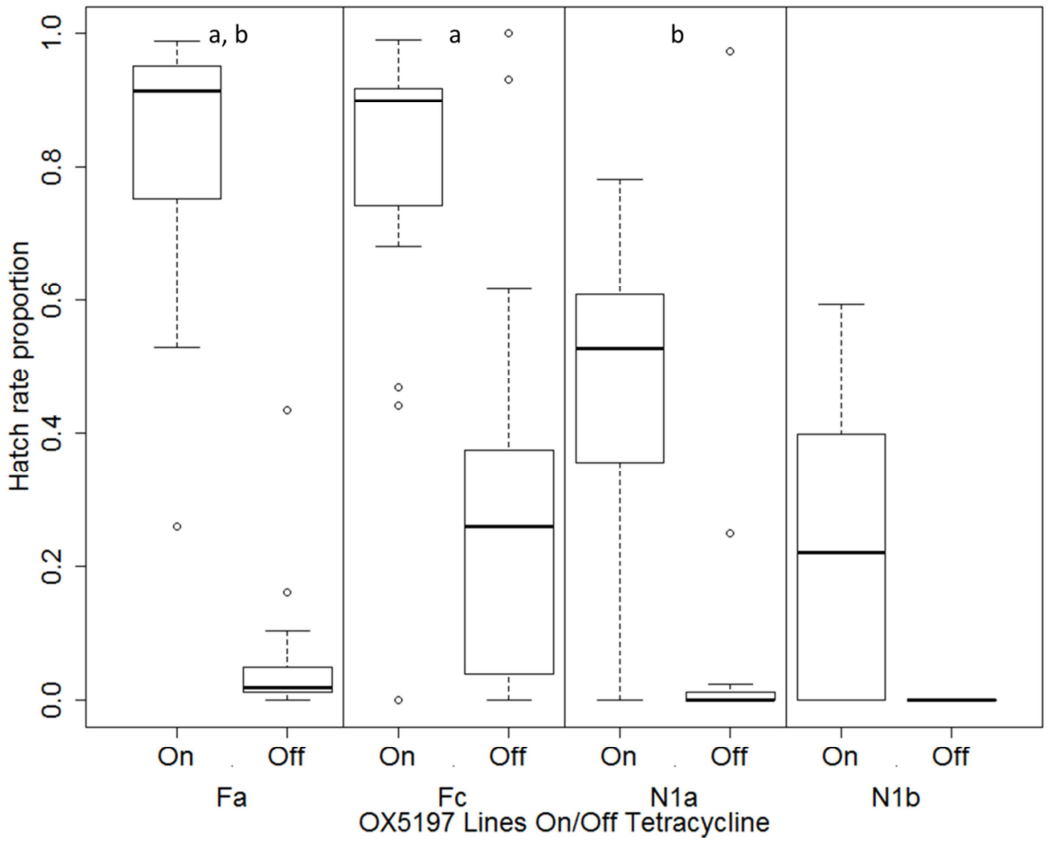


Figure 36: Hatch rates of OX5197 transgenic lines reared on/off tetracycline. **a)** Hatch rate of eggs, fertilised by OX5197 males reared on tetracycline, that are not significantly different from 80% (One sample t-test, $p > 0.05$). **b)** Hatch rate of eggs, fertilised by OX5197 males reared off tetracycline, that are not significantly different from 1% (One sample t-test, $p > 0.05$) (nb OX5197 N1b had 0% hatch rate).

Table 11: Statistical analysis of OX5197 transgenic lines reared off tetracycline compared to baseline hatch rate of 1% using a one sample t-test.

Line	**Mean Proportion** [95% CI]	T value	df	p-value
Fa	**6** [0.6, 11]	1.9805	17	0.0641
Fc	**27** [13, 42]	3.8159	18	0.0013
N1a	**11** [-9, 31]	1.1624	10	0.2721
N1b	**0** [0, 0]	Inf	13	<0.001

Table 12: Statistical analysis of OX5197 transgenic lines reared on tetracycline compared to baseline hatch rate of 80% using a one sample t-test.

Line	**Mean Proportion** [95% CI]	T value	df	p-value
Fa	**83** [74, 92]	0.6741	19	0.5084
Fc	**77** [62, 93]	-0.3632	14	0.7219
N1a	**47** [33, 62]	-4.8895	13	<0.001
N1b	**23** [12, 34]	-10.697	15	<0.001

Taking the GS and PE data together, these results demonstrate that OX5197_Fa has the features of a proof of concept 3G line. It exhibits good female-specific lethality and male sterility when reared without doxycycline, and good repression of the transgene when reared in the presence of doxycycline, evidenced by female survivorship and good hatch rates. N1b demonstrates certain characteristics desired by a 3G line, however it does not exhibit full repression of the transgene, seen by lower than expected female survival and a hatch rate of 47% when reared on dox.

As with OX5056, physical abnormalities were associated with some individuals carrying the OX5197 construct (Figure 37). Minor abnormal growths were detected, associated with strong fluorescence detected in the feeding apparatus in both males and females. Individuals with the abnormal growths were allowed to eclose in a cage to determine if they were still viable.

All individuals were able to eclose however they were unable to feed, presumably due to the malformed feeding apparatus, and subsequently died. It is assumed that the fitness costs associated with the OX5056 construct will be retained with the OX5197 construct. The progeny of a heterozygous cross showed high levels of pupal mortality for both the Fa and N1b lines (data not shown). Work is ongoing to produce homozygous lines of Fa and N1b.

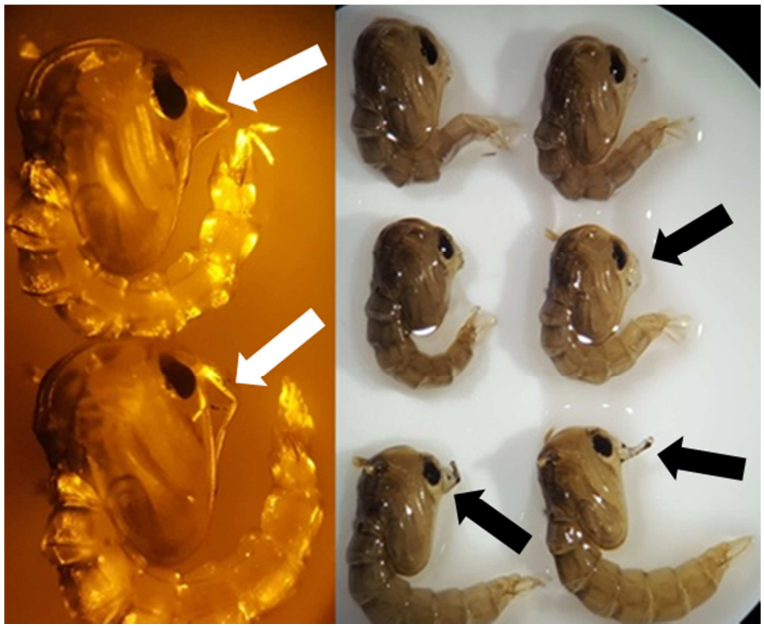


Figure 37: Morphological abnormalities associated with OX5197_N2b line at the pupal stage. Left: Strong fluorescence expression seen in the mouthparts, which can be seen developing incorrectly as they are not straight (white arrows). Right: Irregular growths can be seen (black arrows), likely associated with the abnormal development of the mouthparts. The abnormalities are likely due to off target expression of tTAV causing either a disruption to gene expression or promoting expression of *fokI-protamine.*

4.3.4. Paternal Effect in *Ae. Albopictus*

The OX5056 paternal effect construct showed a good sterility phenotype in *Ae. aegypti* (3.3.5). The same OX506 was transformed into *Ae. albopictus* to see if the sterility phenotype could be replicated in this species.

4.3.4.1. OX5056 Transformation

A total of 1247 pre-blastoderm embryos were injected as described previously (2.2.4). A total of 26 surviving larvae developed to adulthood giving an overall survival rate of 0.02 %. The

initial 6 surviving G_0s were mated individually at a ratio of 1 male : 5 females or 1 female : 1 male, termed (A, B, C, D, E and F). The second group of 20 surviving G_0s were pooled together as up to 2 males or 10 females and were backcrossed to wildtype at a ratio of 1 male : 5 females or 2 females : 1 male (termed G, H, I, J, K, an L). G_0 cages were blood-fed and allowed to oviposit for up to three gonotrophic cycles to standard rearing conditions (2.1.4 / 2.1.5). Three pools gave rise to transformants, B, L and H.

4.3.4.2. OX5056 Functionality

Single male outcrosses were performed for each positive transformed pool (B, L & H), however time constraints did not allow for suitable assessment of these lines for single insertions and sex linkage. Eggs from each line reared on and off dox were hatched in a simple egg hatch experiment. Although the hatch rate conducted was simple in its design, the hatch rates from males reared off dox give an indication that the OX5056 construct is not working in *Ae. albopictus* (Table 13). This is likely due to the *topi* promoter not being well conserved between the species. An *Ae. albopictus* homologue may be required to achieve the same sterility effect as seen in *Ae. aegypti.*

Table 13: Hatch rates of OX5056 lines in *Ae. albopictus* reared on and off dox (1 µg / ml)

OX5056 Line	Dox	Egg Hatch %
B3	on	0
	off	0
B1	on	16
	off	10
LA1	on	2
	off	37
LB1	on	1
	off	28
LB2	on	41
	off	44
LB3	on	2
	off	61

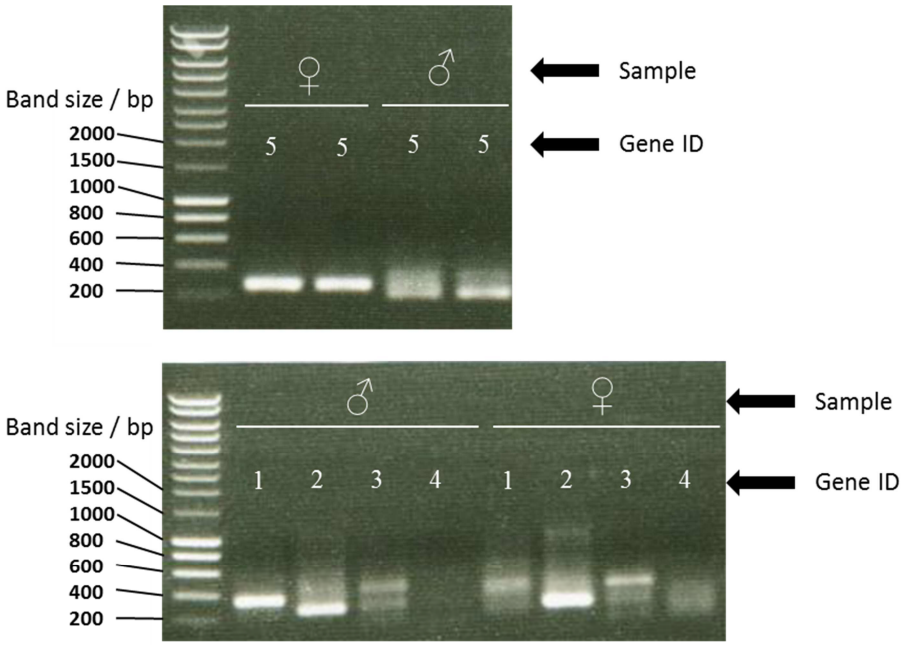
4.3.5. Verifying Testis-Specific Splice-Forms in *Ae. aegypti*

In order to mitigate the negative fitness effects of the PE construct, a male-specific splice-form was identified. Sutton [112] identified several genes that are specifically spliced in the *Ae. aegypti* male germline. Five candidates were selected (Table 14) based primarily on their expression in the male germline with minimal off target expression in tissues of the male and female carcass or ovaries. Selection also considered keeping the intron size to <10,000 bp.

Table 14: Potential gene candidates for male germline specific splicing previously identified by Sutton [112]

Gene ID	Gene	Expected Unspliced size / bp	Expected Spliced size / bp
1	AAEL012262	8502	347
2	AAEL018211	399	420
3	AAEL001898	407	274
4	AAEL011153	956	298
5	AAEL008428	218	165

Testis specific splicing was confirmed by performing a one-step RT-PCR on samples of male testis, and female pupae (Figure 38). The corresponding band sizes associated with correct splicing was seen in male and female cDNA for gene 5. Gene 3 also showed signs of correct splicing, however two bands were present in the males, and possibly also in the females, although less clearly. Samples for gDNA were also run as positive controls.



Sample	Expected band size / bp Gene ID				
	1	2	3	4	5
Male	347	420	274	298	165
Female	8502	399	407	956	218

Figure 38: Amplification of cDNA from RNA extracted from male testis or female pupae run with primers specific to each selected gene, 1-5. Different band sizes are expected for male and female samples if the splicing mechanism is working correctly.

PCR product from the single bands of gene 5 and both bands of gene 3 were extracted and ligated into XL10-Gold ultracompetent cells. Minipreps were generated from bacterial colonies deemed to have the correct cDNA insertion size (Appendix B: Figure 69). Minipreps were generated and sequenced for each band from the male and female samples for genes 3 and 5. Sequence results were compared with the expected outcome and confirmed that for gene 5 the males were correctly splicing out the intron, whilst the females retained it (Figure 39). Results were less clear for gene 3, with unknown sequences reported for both male and female samples. As such gene 5, AAEL008428, was selected for use in an improved PE construct, OX5244.

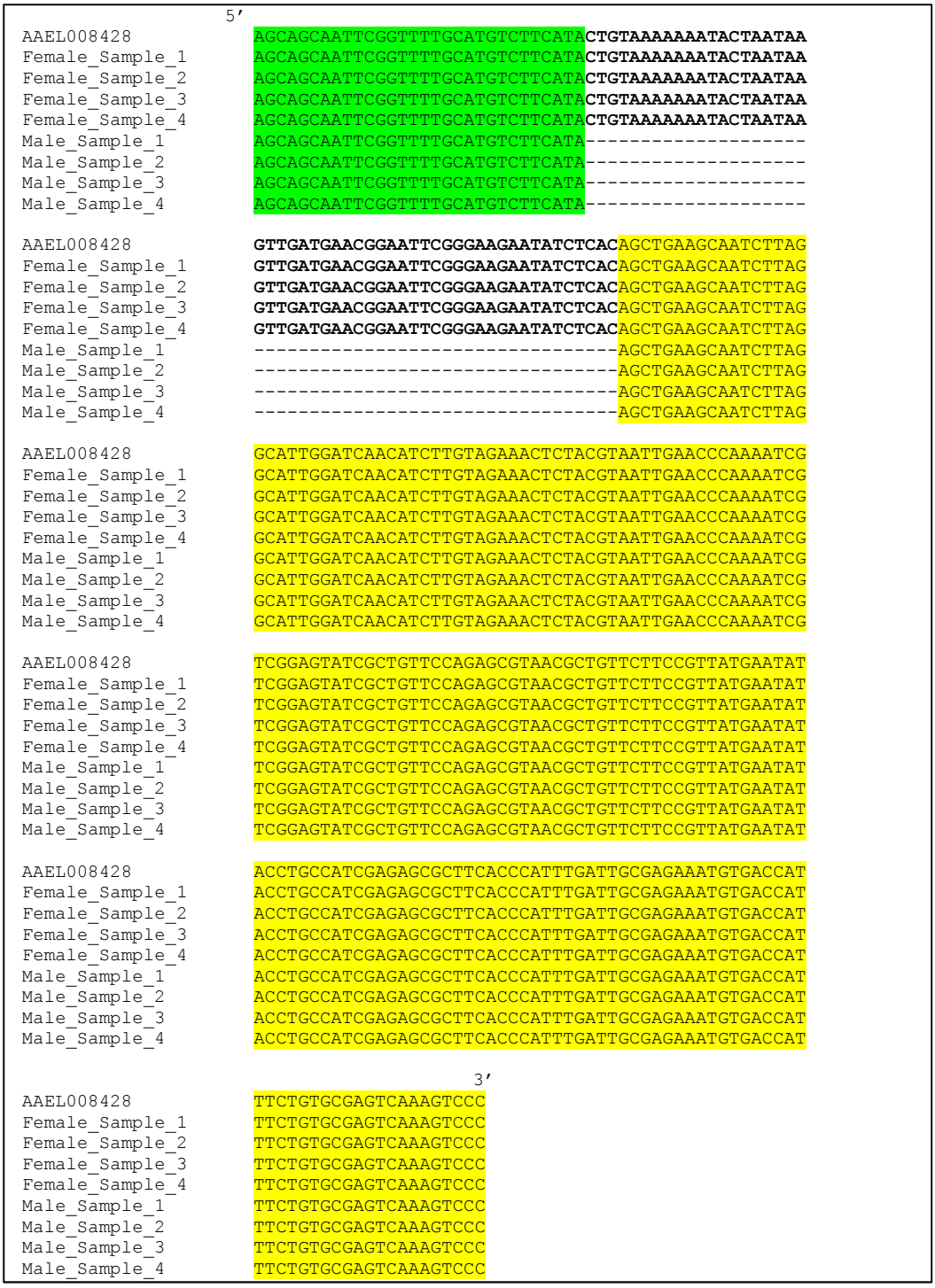


Figure 39: Alignment of the sequencing results for gene 5 (AAEL008428) from male and female samples. Exon 1 (yellow) and exon 2 (green) were both sequenced in male and female samples. The 53bp intron (bold) was sequenced in females, but was absent from the male sequences as expected.

4.3.6. OX5244 Transformation

OX5244 is an improved PE construct (Figure 40) similar to OX5056 described previously. It has an additional sex-specific splicing component upstream of the tTAV, designed to further restrict production of tTAV to testes. The 53 bp intron causes the tTAV transcript to be read out of frame, but when spliced out is read in frame.

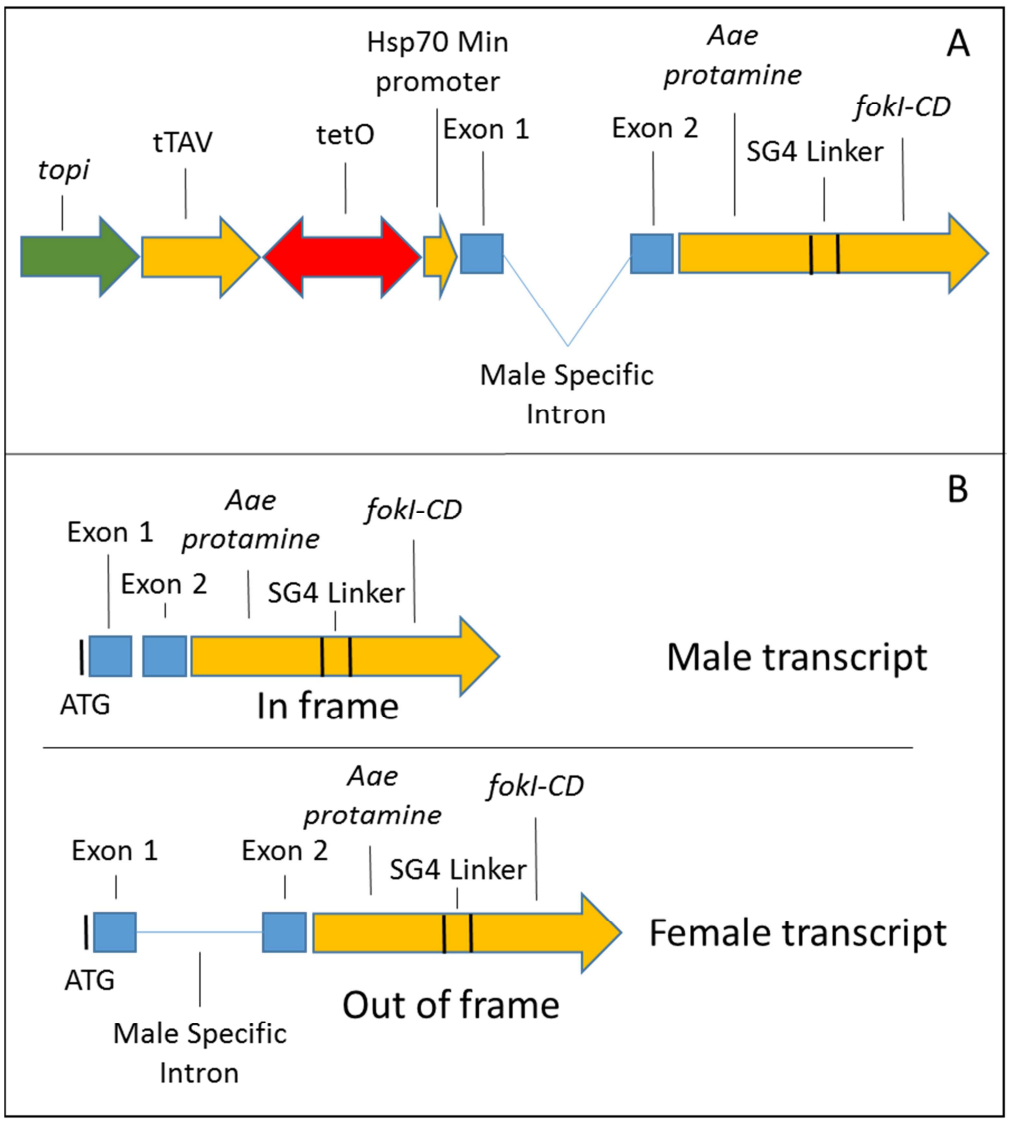


Figure 40: **A)** Schematic diagram of construct OX5244. It contains the functional element of tTAV under control of the *Aae topi* promoter. A sex-specific splicing mechanism based on the endogenous gene AAEL008428 is placed between the tetO and *fokI-protamine*. It contains a 53 bp intron and shortened flanking exons. Also present are regulatory 3'UTR regions and a fluorescent marker, AmCyan, under the control of the all body promoter Hr5iE1. Construct built by Tarig Dafa'alla at Oxitec Ltd. **B)** Predicted male and female transcripts produced from construct OX5244. The male transcript splices out the 53bp intron, producing an in frame *fokI-protamine* transcript. In females the 53bp intron is not spliced out, causing a frame shift, and an out of frame *fokI-protamine* transcript.

A total of 2546 pre-blastoderm embryos were injected as described previously (2.2.4). A total of 30 surviving larvae developed to adulthood giving an overall survival rate of 1.2%. Surviving G_0s were pooled and outcrossed to wildtype. A total of 7 male pools and 2 female pools were blood-fed and allowed to oviposit for up to three gonotrophic cycles. G_1 eggs were hatched under vacuum and reared to L2/3 before being screened for fluorescence as evidence of successful transformation. A total of 8 pools (OX5244_A, OX5244_B, OX5244_D, OX5244_E, OX5244_F, OX5244_G, OX5244_H and OX5244_I) were positive for transformants. Line OX5244_H was split into two separate lines (OX5244_Ha; OX5244_Hb) owing to significantly different fluorescent phenotypes and line OX5244_I was discontinued owing to being sterile.

4.3.7. OX5244 Line assessment

Three single male outcrosses (termed 1, 2 and 3) were performed from each of the 8 positive pools. Due to lower numbers of surviving G_1 larvae to adulthood only one single male outcrosses was performed from pool OX5244_A. The progeny from all outcrosses were reared under standard conditions on tet and screened for fluorescence as pupae. The proportion of each phenotype of the number of pupae screened is displayed in Figure 41. Lines OX5244_B3, OX5244_D1, OX5244_E2, OX5244_F3, OX5244_HB2, were deemed suitable to work with as the proportions of pupae were approximately 25% for each phenotype in stark contrast to line OX5244_Ha1 (where the biased distribution of progeny suggests an insertion event close to the M-locus) or OX5244_E3 where proportions of transgenic pupae appear significantly reduced.

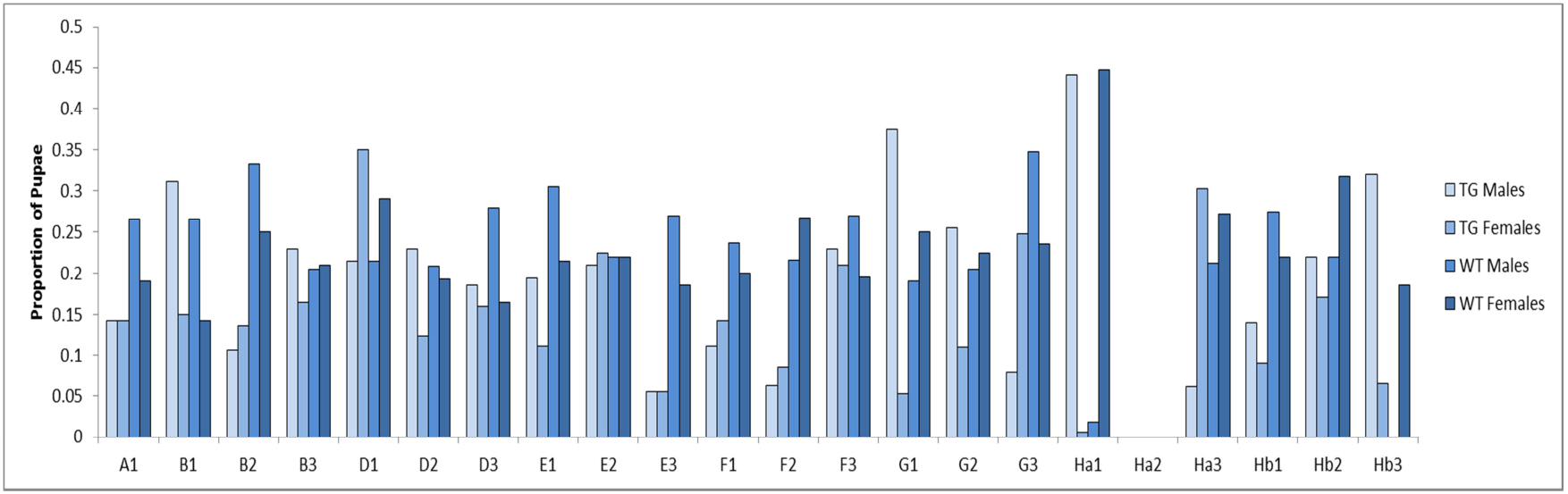


Figure 41: Proportion of fluorescent and wild type male and female pupae from single male crosses of OX5244 positive G_0 pools. Capital Letters denote original G_0 pool, numbers (1, 2, 3) represent the single male parent.

4.3.8. OX5244 Functionality

Lines OX5244_B3, OX5244_D1, OX5244_E2, OX5244_F3 and OX5244_HB2 were deemed suitable for testing based on the proportions of WT and transgenic inheritance. Males of these 5 lines were reared on or off dox and outcrossed to WT females to assess their fertility. No data was collected for line OX5244_HB2 as the mated females did not blood-fed or lay eggs. Figure 42 demonstrates that when reared off tetracycline, hatch rates were not significantly different to 1% for lines OX5244_B3 ($t = -0.9385$, $df = 11$, $p = 0.37$), OX5244_F3 ($t = 2.029$, $df = 19$, $p = 0.057$) and OX5244_E2 ($t = -0.3692$, $df = 13$, p-value = 0.78). When reared in the presence of tetracycline the transgene was repressed to some degree in all four lines, evidenced by a positive hatch rate. Hatch rates were not significantly different to 80% for lines OX5244_D1 ($t = 0.4783$, $df = 18$, p-value = 0.64) and OX5244_E2 ($t = -1.5412$, $df = 12$, p-value = 0.1492). Of the 4 OX5244 transgenic lines tested three confirmed the expected sterility phenotype, which was fully repressed in line OX5244_E2, confirming that the sterility phenotype of OX5056 was unaffected by the introduction of the male-specific splice-form. The next stage is to assess whether the splice-form has tightened up expression of the transgene to the male germline, limiting the off target effects of OX5056.

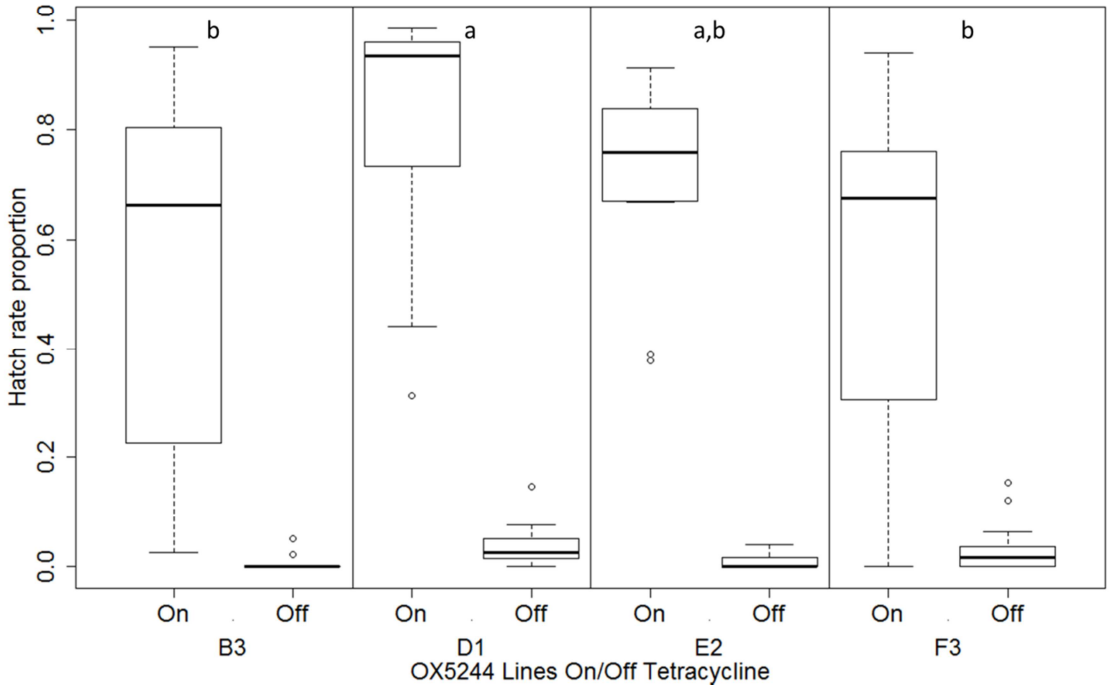


Figure 42 Hatch rates of OX5244 transgenic lines reared on/off tetracycline. a) Hatch rate of eggs, fertilised by OX5197 males reared on tetracycline, that are not significantly different from 80% (One sample t-test, p>0.05) b) Hatch rate of eggs, fertilised by OX5244 males reared off tetracycline, that are not significantly different from 1% (One sample t-test, p>0.05).

4.3.9. OX5244 Homozygous viability

The off target effects of OX5056 became apparent when individuals inherited two copies of the transgene during the homozygous viability study (3.3.7). Therefore a heterozygous cross of OX5244 should indicate if the fitness cost issue has been successfully addressed through the addition of the male splice-form. If normal Mendelian inheritance is followed and there is no impact of the transgene on survival then 75% of the progeny of a heterozygous cross should exhibit fluorescence (25% homozygous and 50% heterozygous), split evenly between the sexes (37.5 %).

There was a significant reduction in males and females to the expected 37.5% value for all lines (Appendix B: Table 37 - Table 46), except for OX5244_F3 males (P = 0.915) and females (P =

0.091), and OX5197_B3 males (P=0.054) which were not significantly different from the 37.5% value (Figure 43).

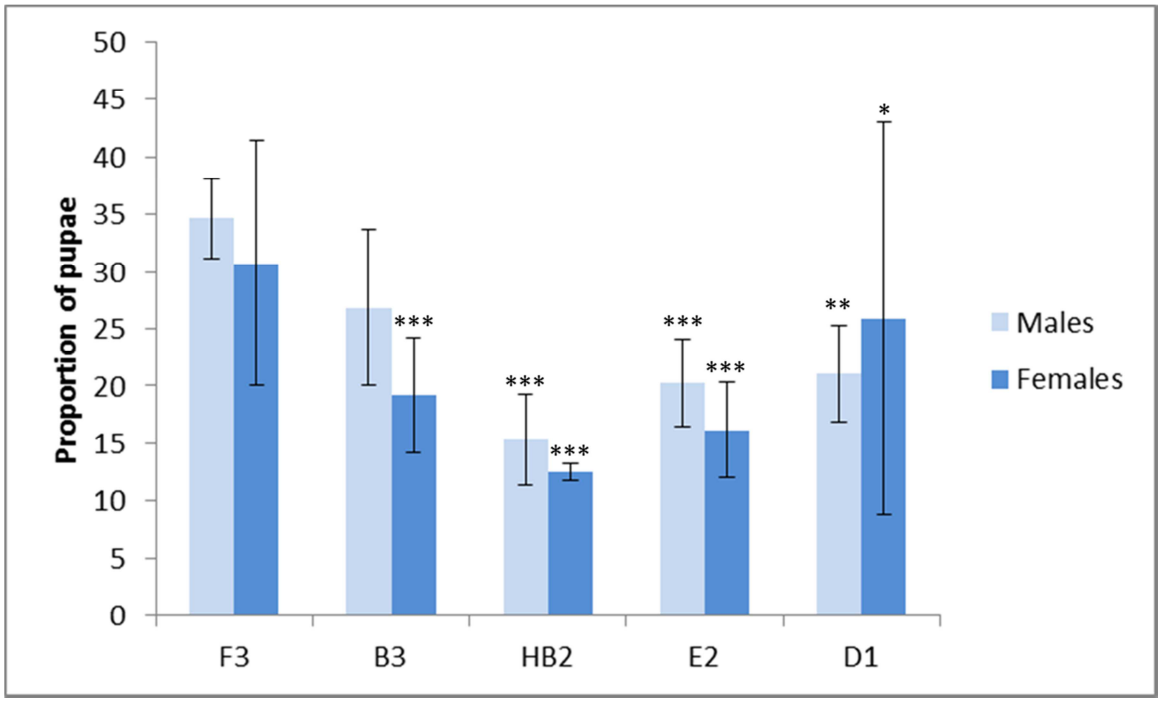


Figure 43: Mean proportion (with SD) of fluorescent pupae from a heterozygous OX5244 cross, adjusted to WT survival. Repeated G-test for homogeneity used to determine significant differences to the expected 37.5%, represented by: p < 0.05 *; < 0.01 **; < 0.001 ***

Four of the five OX5244 heterozygous crosses resulted in fewer transgenic pupae than expected, demonstrating that the male splice-form has not been successful in addressing the associated fitness costs of the PE construct. As with constructs OX5056 and OX5197 morphological deformities were associated with the OX5244 transgene. One male pupa was discovered with multiple abnormal growths (Figure 44), which died before eclosion. It is assumed that this is a direct result of the transgene's expression not being restricted to the male germline, and is further evidence that the new splice-form is not functioning as required.

Figure 44: Morphological abnormalities associated with carrying the OX5244 construct. The abnormalities exhibited were more extreme than those associated with previous constructs, however only one individual has so far been found with the abnormal growths.

4.4. Conclusion

The work presented in this chapter represents a proof of concept 3G technology in *Ae. aegypti* in the form of OX5197_Fa. The previously demonstrated male sterility with OX5056 was combined with the female-specific GSS OX5034. Line OX5197_Fa showed high female lethality and male sterility when reared off dox, and good repression of the transgene when reared on dox. It is believed that tTAV production is limited to females through the sex-specific splicing of the *dsx* minigene, and to the male germline through the use of the testis specific *topi* promoter. This proof of concept represents a milestone in the development of Oxitec Ltd's technology, as it returns the self-limiting aspect of 1G technology to the 2G genetic sexing technology. Whilst a proof of concept has been demonstrated, it should be noted that there are significant limitations to the 3G development presented here. As OX5197 was constructed using the same architecture as OX5056 it would be expected to show similar off target effects as seen in OX5056. Indeed a small enrichment cross of OX5197_Fa showed high levels of pupal death when reared on dox (data not shown), demonstrating the adverse fitness effects have persisted.

In order to mitigate the off target effects of the PE, work was conducted to limit expression further to the male germline, through the use of a sex-specific splice-form. Previous work by Sutton [112] identified several splice-form targets specific to the male germline. These were

confirmed and assessed through RT-PCR and sequencing. Gene fragments from the top candidate, AAEL008428, were positioned in a modified OX5056 PE construct, OX5244, upstream of the sterility effector, *fokI-protamine*. It was predicted that in the male germline the intron would be spliced out, allowing transcription of the *fokI-protamine* to occur, however in females and the rest of the male, the intron would remain causing a frame shift to occur during transcription, as the intron is 53 bp long. This would result in non-functional FokI-Protamine being produced, thereby limiting functionality to the male germline. Whilst functionality of the construct remained, demonstrated by an egg hatch assay, it did not succeed in restricting the effects of the transgene to the male germline; evidenced by lower than expected proportions of transgenic individual from heterozygous crosses of OX5244 lines. Whilst one line (OX5244_F3) demonstrated proportions that were not significantly different from the expected proportions, the remaining four did not. It may be possible to enhance the effect of the male splice-form by manipulating the size of the flanking exons. The flanking exons in OX5244 were shortened, however two other constructs were produced, one with the full exon sequences, and one with just the intron sequence. These constructs are to be injected in the coming months, and it is hoped that they will increase the specificity of the transgene. Additionally, it is planned to examine the gene splicing at the molecular level to assess whether correct splicing of the male-specific splice-form is occurring.

The final part of this chapter investigated the use of OX5056 in *Ae. albopictus*. It has been demonstrated previously that constructs which use well-conserved genes are transferable across species. For example, the construct (OX4358) used the female-specific *Actin-4* promoter from *Ae. albopictus,* and was able to produce a flightless phenotype in *Ae. aegypti* [81]. Although transformation of OX5056 into *Ae. albopictus* was successful, a sterility phenotype was not seen when reared off dox. This demonstrates that should PE be required in *Ae. albopictus* the technology will need to be constructed anew rather than being transferred from another species.

Chapter 5: *Ae. albopictus* - Characterisation of Wild Type

5.1 Introduction

As previously discussed in Chapter 1, *Ae. albopictus* poses a public health risk due to being a vector for a number of arboviruses, primarily dengue and chikungunya [113]. *Ae. albopictus* is not considered as efficient a vector for disease as *Ae. aegypti.* However its incrimination in dengue and chikungunya transmission coupled with its distribution into more temperate climates and contact with potential animal reservoirs of viral disease, all highlight the need for vector control strategies [114]. In the past, WT strain selection for transformation at Oxitec Ltd has been based on anecdotal evidence as to the ease of rearing in the laboratory setting. The work set out in the following chapters aims to provide some empirical evidence on which to base WT strain selection.

5.2. Mass Rearing Characterisation

Oxitec Ltd currently maintains four wild type strains of *Ae. albopictus* as background strains in which transgenic development can be carried out. For transgenic work in the past, choice of background strain was based on those that would feed off the plate feeding system rather than Hemotek feeders (2.1.4); those that would feed well from the top of the cage and those that gave a good number of eggs. Whilst this would make sense from an ease of use perspective during research and development, the choice of background strain should be based on quantitative evidence and consider more than just lab adaptability.

The ability of the background strain to rear well under laboratory conditions is paramount to the final success of a transgenic product. Trial releases of transgenic or irradiated *Aedes* currently require >1000 males / ha / week [45][71], so efficiency in rearing is essential to keep costs low. It is highly likely that mosquitoes of the same species from different geographic backgrounds will behave differently under laboratory adaptation [115]. To make an informed choice with regard to line selection it is necessary to assess how each line will perform under mass rearing conditions. This will have direct implications for many factors associated with the costs of mass production

for a release programme. For example, egg productivity, adult fitness and sex separation will all impact on costs.

5.3. Wild type assessment for regulatory approval

Beyond the ability of a background strain to perform well under laboratory conditions, consideration must be given to the regulatory approval required prior to release trials [73]. A part of the regulatory submission will concern the life history of the background strain in which transgenic development occurred. Background strains which contain 'negative' traits would be unlikely to gain regulatory approval for concern over the negative traits spreading into the wild population. Discussed below are three areas for concern which would require addressing prior to a regulatory submission.

5.3.1. Insecticide Resistance

As discussed in Chapter 1, insecticide use against *Aedes* mosquitoes through insecticide fogging is well documented and widespread [116][117][118]; although there is no clear evidence that it alone is successful in reducing dengue transmission [119]. There are currently four main classes of insecticide that are used for control of mosquitos: Carbamates, Organochlorines, Organophosphates and Pyrethroids. Although other methods such as bacterial insecticides (*Bacillus thuringiensis israelensis* (*Bti*)) and insect growth regulators are available, the four chemical insecticides are more widely used and have a greater range of activity against both larval and adult stages. Organochlorines, such as DDT, are cheap [120] and easy to manufacture due to their simple chemical structure[121]. They work through inhibition of the nervous system; DDT acts on sodium channels, whilst cyclodines act on the receptors for the GABA neurotransmitter. The pyrethroids are arguably the most important insecticide group, being used as the principal component in long lasting insecticide treated bed nets, and proven to have an epidemiological impact on malarial burden [122][123][124]. They work in the same fashion as the organochlorines, and are still effective at low concentrations [125]. Organophosphates, such as

malathion and fenitrothion, work through irreversible binding to acetylcholinesterase, preventing acetyl-choline from being removed at a nerve junction, causing continuous activation of the nerve [121]. Carbamates have a similar mode of action to the organophosphates, however they do not bind irreversibly and do not require activation through monooxygenase in the insect as the organophosphates often do [121].

Vontas et al. [33] discuss the recent worldwide status of insecticide resistance in *Ae. albopictus* from 2008-2012. They report recent instances of resistance to the organophosphate temephos, as a larvicide in China, Cameroon, Greece, Italy and Malaysia and to the organochlorine DDT as an adulticide in Cameroon and Sri Lanka. They report that the pyrethroids deltamethrin and permethrin are both still effective against *Ae. albopictus,* although historically resistance has been seen in the past on the Indian subcontinent. The organophosphate malathion was also seen to still be effective, however resistance has also been noted previously in Sri Lanka [31]. The carbamate propoxur was still seen to be effective across all papers included in the review.

Given insecticide resistance exists in *Ae. albopictus,* it is entirely possible that the colonised strains at Oxitec Ltd originated from resistant populations. It would be inappropriate, and likely a regulatory barrier to release, to develop a transgenic line in a background strain which carries an insecticide resistance genotype. Therefore, background strains should be suitably assessed prior to transgenic transformation.

5.3.2. **Diapause**

A major difference between *Ae. albopictus* and *Ae. aegypti* is the ability of *Ae. albopictus* to withstand harsh environmental conditions such as those experienced during winter in temperate climates. It is able to do this through a mechanism of suspended development known as diapause, in order to delay development until more favourable conditions arise. This has allowed establishment in more temperate areas such as the Czech Republic [126], Belgium [127] and France [128].

The majority of animals in temperate areas use photoperiod as a means of anticipating changing conditions. A critical photoperiod (CPP), the length of daylight required to stimulate diapause, is used to induce a physiological change and enter diapause. Diapause has been noted at varying stages of development including: adult reproductive development (*Culex pipiens*), pupal (*Sarcophaga bullata*), larval (*Wyeomyia smithii*) and embryonic (*Ae. albopictus*) stages [129]. For *Ae. albopictus,* exposure to a shortened photoperiod and cooler temperatures in the late larval and pupal life stages results in female adults producing diapausing eggs [130]. The photoperiodic diapausing nature of *Ae. albopictus* is different to the quiescence of egg hatching seen in both *Ae. aegypti* and *Ae. albopictus;* where eggs will not hatch until submerged in water [131]. Breaking diapause is usually in response to extended photoperiods and cold temperatures [132]; after which eggs enter into a post-diapause quiescence where they will hatch when submerged in water [131].

Diapause in *Ae. albopictus* is associated with rapid evolutionary change in its CPP, seen during its invasion and range expansion into North America [131]. This would suggest that different geographic populations of *Ae. albopictus* have different CPPs and propensity to lay diapausing eggs. It would therefore be pertinent to use a background strain that has a minimal diapause genotype / phenotype, to avoid the introduction of these genes into a population where it may enhance range expansion.

5.3.3. **Vector Competence**

As discussed in Chapter 1, *Ae. albopictus* has been implicated as a competent vector for many arboviruses. The ability of a vector to transmit disease is known as its vectorial capacity, and is a function of a multitude of factors such as vector and host population density, probability of vector / host interaction and daily survival. Alongside this are two other factors, vector competence and the extrinsic incubation period. Vector competence is defined as the proportion of individuals which ingest an infected blood meal and subsequently become infective. The extrinsic incubation

period is the time taken for the pathogen to replicate within the host to a point where it is able to be transmitted. The phases involved in vector competence and the extrinsic incubation period are influenced by the hosts genetic characteristics, which determine physical and chemical factors for the pathogen to overcome to complete its lifecycle [133]. It is therefore highly likely that different populations of the same vector species will have different infection and infectivity rates. Baseline characteristics of the wild type strains will be needed to ensure development does not proceed in a line with a high susceptibility to arboviruses such as dengue or chikungunya which may spread genes which facilitate disease transmission.

5.4. Chapter Aims

The work in this chapter is divided into three parts (5a, 5b & 5c) each of which examine a different aspect of the wild type strains; and a fourth (5d) which considers the available evidence to determine which WT strain is most suitable for use. Chapter 5a examines how the wild type strains perform under mass rearing conditions, examining pupal size and adult productivity as metrics for assessment. Chapter 5b examines the insecticide resistance status of the four wild type lines, using WHO standard tests for each of the four major classes of insecticide. Chapter 5c details work to characterise the diapausing nature of the wild type lines. Whilst not successful in achieving full characterisation, progress was made in protocol development and difficulties in egg handling highlighted areas for future development.

Chapter 5a: Mass Rearing

5a.1 Introduction

This chapter is concerned with characterising the mass rearing potential of the four WT strains currently housed at Oxitec Ltd and an Italian strain [ITA] as a comparison. Adult productivity and larval rearing are considered as metrics for comparison, with pupal size being used to predict the percentage of male recovery for a hypothetical release programme.

5a.1.1 Centro Agricoltura Ambiente "G. Nicoli"

The Centro Agricoltura Ambiente "G. Nicoli" facility (CAA), located in Crevalcore, Italy, has the capability to rear up to 100,000 *Ae. albopictus* adult males per week (personal comm – R. Belini, 2013). They have been involved in a number of mass rearing projects, closely linked with the International Atomic Energy Agency (IAEA), developing a liquid diet [134] and mass rearing cages [135]. Alongside this, they have used irradiated *Ae. albopictus* in a large scale pilot study in Italy for control of *Ae. albopictus* [45]. Funding was awarded from INFRAVEC, a multi-centre European Infrastructure of 31 academic and industrial laboratories operating in the field of mosquito biology. The approved project was to examine the four wild type strains of *Ae. albopictus* currently maintained at Oxitec Ltd under mass rearing conditions at the CAA to assess whether any of the lines had a distinct advantage over the others.

5a.1.2 Pupal size and egg production

It has previously been demonstrated that mosquito size is heavily dependent on several factors including diet, larval rearing density & water quality [136][137][138]. As well as a metric for fitness, pupal size serves a second purpose for a control program. Current use of OX513A depends on the size dimorphism between males and females for sex separation [73]. Male pupae, required for a release, are separated from the female pupae through a fine mesh sieve, calibrated to allow the smaller males to pass through whilst retaining the larger female pupae (Figure 45). It

is therefore necessary to have a definitive size dimorphism to allow this sorting procedure.

Therefore a second metric for consideration is the difference in size between males and females.

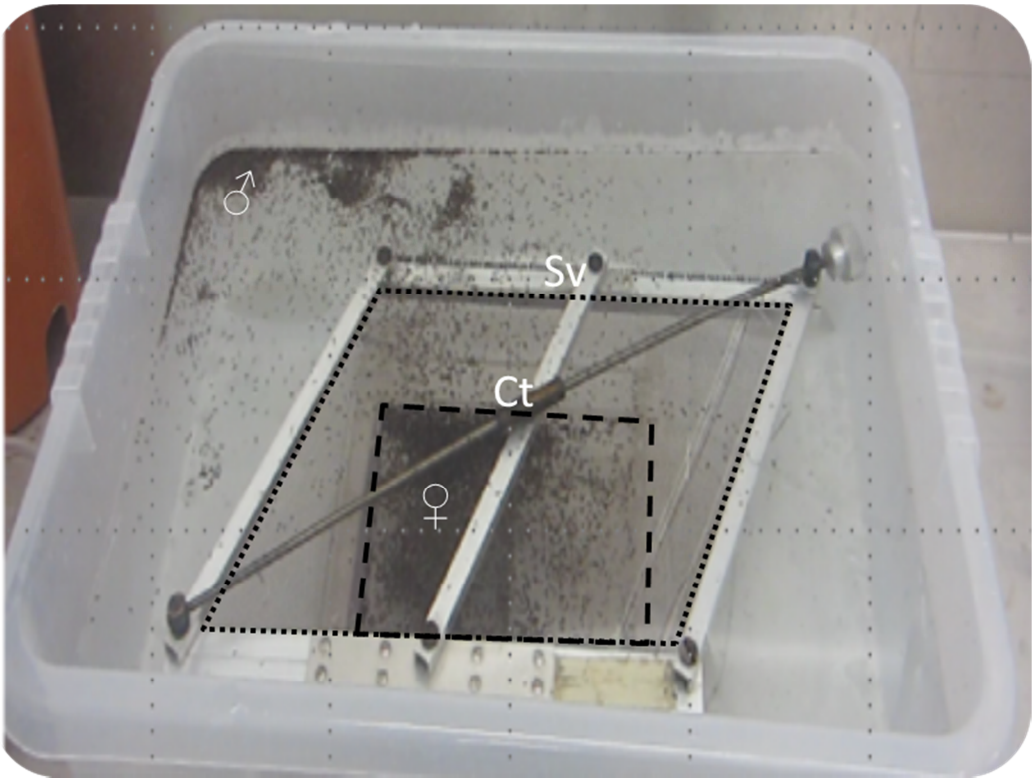


Figure 45: Oxitec Ltd's sex sorter. Mixed pupae are placed in a container (ct) sealed underneath a fine sieve (sv). The entire apparatus is then submerged in water. The smaller male pupae are able to escape through the sieve (♂) whilst the larger females cannot and are retained in the original container (♀).

A further parameter for consideration is larval rearing density. The facilities available at the CAA allow for rearing *Ae. albopictus* at high density at a large scale (something not possible at Oxitec Ltd), and so permit this experiment to be repeated with larvae reared at various densities.

Another life history trait that could impact on line selection is female productivity. From a commercial perspective, productivity is a key component to allow for a cost effective control strategy. Adults housed in high density cages may result in higher egg production, however competition for resources may impact on the output and longevity of a cage and decrease overall

productivity. Understanding how the different wild type strains respond to the pressures of up scaled rearing will provide some evidence on which is an appropriate background strain.

5a.2 Materials and Methods

5a.2.1 Mosquito Husbandry

5a.2.1.1 Mosquito Lines

Four lines of *Ae. albopictus* which are currently maintained at Oxitec Ltd were used, detailed in Chapter 2. Alongside them a further strain [ITA] currently maintained at the CAA facility was used. It was colonised from Rimini, Italy, and reared through more than 40 generations.

5a.2.1.2 CAA Facility

Adult mosquitoes were housed in custom made Perspex cages (40 cm x 40 cm x 40 cm) and supplied with 10% sucrose solution. Blood was administered through a Hemotek Well (Hemotek Ltd, UK), covered with swine intestine and heated in a water bath to ~40°C. This was placed in the bottom of the cage and was reheated periodically throughout the day. Egg collection was performed on coarse white seed germination paper in a 500 ml beaker with 200 ml water. Egg papers were dried overnight and then transferred to a sealed container with a saturated K_2SO_4 solution to maintain humidity at ~100%, and were kept in the insectaries at ~27°C. Eggs were hatched by submergence in 700 ml of hatching solution in a sealed 1 L jar overnight. Hatching solution comprised 0.25 g nutrient broth (OXOID) and 0.05 g brewer's yeast. Larvae for colony maintenance were reared at 2 lar / ml in 2 L deionised water, and were fed with a liquid diet 5% (w/v) comprising: 50% tuna meal, 36% bovine liver powder, 14% Brewer's Yeast + nutrient mix additive of 0.2 g per 100 ml. Larvae were fed for four consecutive days with 16 ml on day 1, 32 ml on day 2, 48 ml on day 3 and 64 ml on day 4. This equates to 0.2, 0.4, 0.6 and 0.8 mg of diet per larva on each day, respectively.

5a.2.1.3 **Egg Paper Analysis**

This methodology is adapted from that proposed by Mains et al. [139]. Egg papers were scanned at 700 dpi on greyscale and saved as a .jpg. Images were processed using ImageJ software (Figure 46). Images were cleaned by cropping out undesirable elements of visible debris such as mosquito body parts, along with any text, areas of shadow and artefacts from the scanner. The contrast of the image was then set to maximum and the image switched to binary. This allows each pixel to register as either black or white. If processed correctly only the image of the eggs should register as black, with the rest of the image being white. The total area of black pixels was then calculated using the Analyze Particles function in ImageJ. Pixel size was set at >5 to exclude small debris missed by image cleaning. The estimated number of eggs was calculated by dividing the total black area by 15, an average number of pixels per egg previously quantified by the CAA.

Figure 46: Egg paper image cleaning. **Left**: Original image. **Centre**: Text, mosquito body parts, dark shadow and scanning artefacts are removed from the image. **Right**: Contrast set to maximum to leave just the image of eggs.

5a.2.1.4 **Aliquoting: Oxitec Ltd method**

L1 larvae are placed in a beaker with 1 L of water. A magnetic stir bar is used to ensure uniform mixing of larvae. Three 2 ml samples are taken and the number of L1s counted manually. The average number of larvae, from the 3 samples, is used to determine the volume needed for the required number of L1 larvae.

5a.2.1.5 **Aliquoting: CAA method**

Egg papers are assessed using the egg paper analysis method (5a.2.1.2) to calculate the number of eggs present on the paper. The number of L1 larvae is then estimated by multiplying the number of eggs calculated by the corresponding hatch rate (depending on the mosquito line). Egg papers are then cut and re-analysed until the required number of L1 larvae has been calculated, which are then hatched out overnight.

5a.2.1.6 **Baseline hatch rates**

Eggs were collected through standard CAA rearing procedures (5a.2.1.2) and left to develop for 3 days. Three egg papers for each line were counted by eye under a dissection microscope before being hatched to the standard CAA protocol. Hatched L1 larvae were counted to determine a baseline hatch rate for each line. As hatch rates were poor, a second hatch rate was conducted, as above, on egg papers that were left to develop for 5 days. The CAA aliquoting protocol was assessed by counting the number of eggs through the scanning protocol. Eggs were then hatched overnight and the number of L1s counted. The actual number of L1 larvae was then compared to the estimated number of L1 larvae the CAA protocol produces.

5a.2.1.7 **Feeding regimen**

The CAA rear larvae on a liquid diet (5% w/v), as described previously (5a.2.1.2). This diet has, however, been specifically tailored to rearing larvae at 2 lar / ml. An adjusted regimen (Table 15) with less food per larva over a longer feeding period was trialled for the higher densities. Diet was

distributed over a longer time period which allowed for acceptable development times of larvae with no spoilage.

Table 15: Feeding regimens for *Ae. albopictus* with a liquid 5% (w/v) diet, adjusted for each density. The 2 lar / ml diet is specifically tailored to that rearing density but was adjusted at the higher rearing densities to avoid spoilage. Feeding at 4 lar / ml was stopped on day 5 as the accumulation of food was causing bacterial growth which would have progressed to the tray spoiling if more diet had been added.

Day	**Volume of 5% (w/v) liquid diet per tray / ml**		
	2 lar / ml	3 lar / ml	4 lar / ml
1	16	6	8
2	32	12	16
3	48	36	48
4	64	72	96
5	N/A	48	64
6	N/A	24	N/A
7	N/A	12	N/A
8	N/A	12	N/A
Total	**160**	**222**	**232**

5a.2.2 **Larval Rearing Experiment**

Mosquito larvae were reared at one of three densities, 2, 3 or 4 lar / ml, in 2 L of deionised water, equating to 4000, 6000 or 8000 larvae per replicate. Three replicates were performed at each density and were run concurrently; however each density was tested separately (Figure 47).

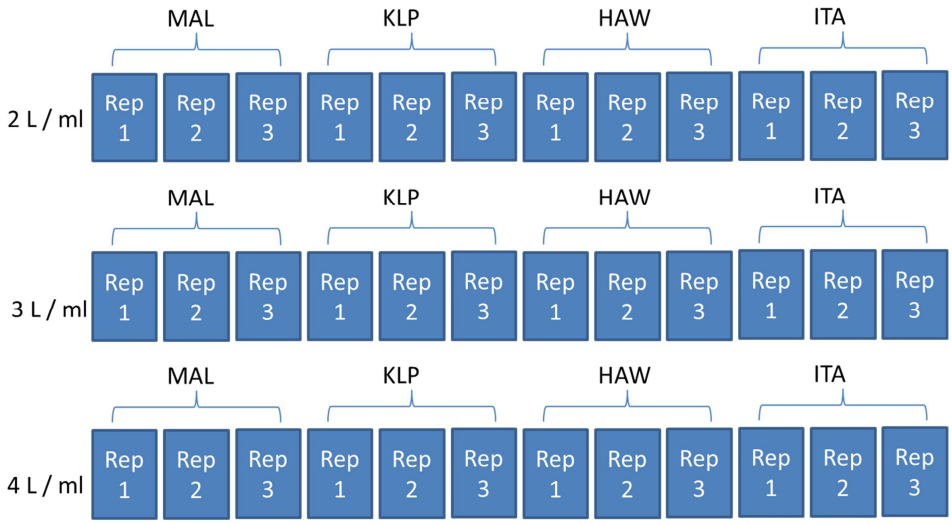


Figure 47: Diagram indicating larval rearing methodology. Each replicate represents an individual tray with the appropriate number of larvae for each density (2 lar / ml – 4000 larvae; 3 lar / ml – 6000 larvae; 4 lar / ml – 8000 larvae). Nb The absence of REU line is due to low numbers of eggs, and being discontinued.

The appropriate number of larvae for each replicate was estimated using the CAA aliquoting methodology (5a.2.1.5). Eggs were hatched according to standard protocols (5a.2.1.2) and subsequently transferred into a plastic tray (41cm x 31cm x 11cm) with 1.25 L of deionised water. Larvae were fed to the appropriate feeding regimen described previously (5a.2.1.7). There were three replicates of each *Ae. albopictus* line tested at each density, with the exception of line REU which was discontinued owing to poor hatch rates.

Pupae collection began 24 hours after the onset of pupation, usually on day 7 after hatching (Day 6 for 2 lar / ml). Pupae were collected daily for four days via a cold sorting method, whereby all pupae and larvae were sieved from the rearing water and placed in a tray containing ice cold water. This temporarily immobilises the insects, causing the larvae to sink and the pupae to float. Pupae can then be manually picked out of the tray with a plastic pipette. Larvae were then sieved and returned to their rearing tray to continue development.

Three samples of pupae from each replicate were taken using a 0.5 ml pupal spoon over four consecutive days. The pupal spoon comprised a 0.5 ml section of a graduated plastic pipette with

a fine mesh secured over one end, allowing water to drain out whilst retaining the pupae. These pupae were subsequently sexed and counted under a dissection microscope before being photographed with a 1 cm graticule to be measured at a later date.

An estimate of the total number of pupae removed each day was calculated by measuring the volume of all pupae in a 10 ml pupae spoon (as above but with a 10 ml section of pipette. The total volume was multiplied by twice the average number of pupae from the 0.5 ml samples. At the end of the four days of sampling the remaining L4 numbers were either counted by hand or if too numerous were estimated in the same manner as estimating pupae numbers.

5a.2.2.1 **Measuring Pupal Size**

Pupal sizes were calculated by measuring cephalothorax width using ImageJ software (Figure 48). A 1 cm graticule was measured to set the scale, and up to 50 males and 50 females were measured per replicate, pooled from the three 0.5 ml samples.

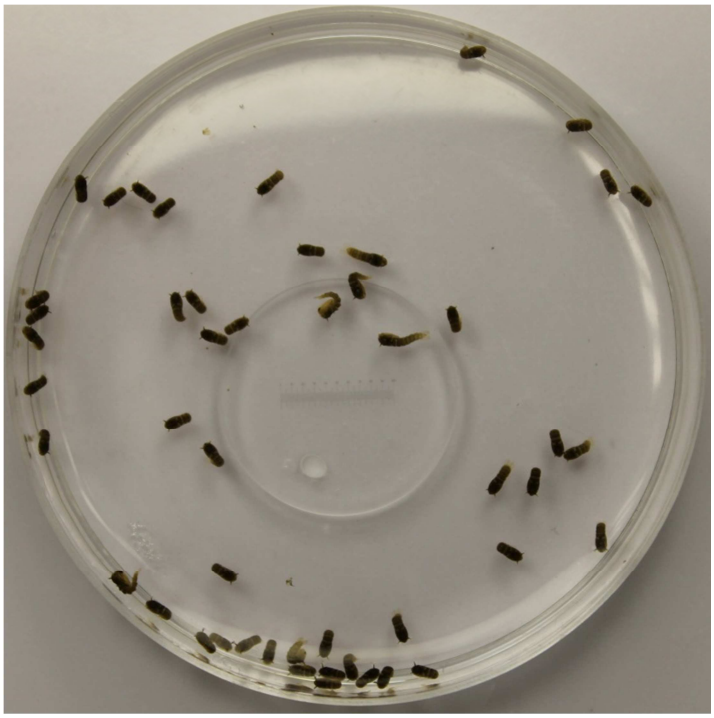


Figure 48: Male HAW pupae photographed with a 1 cm graticule, to be measured in ImageJ

5a.2.3 **Adult density experiment**

Larvae were reared at a density of 3 lar / ml and fed with the adjusted diet regimen (5a.2.1.7). There were three replicates for each line, housed at either high (6000 adults) or low (3000 adults) density. Pupae were picked for ~15 days from the onset of pupation until there were < 50 L4 larvae left.

Due to logistical time constraints, pupae from the larval density experiment were used for the high density adult cages (those reared at 3 lar / ml). As there were 6000 larvae in each tray, all pupae were added to the cages for the high density replicates (ensuring a sex ratio of 1:1). To produce the low density cages, two trays of larvae were reared at the same density (3 lar / ml) and protocol as before. The pupae from one tray were split each day evenly into two cages; the same procedure was followed for the second tray except here 50% of the pupae were discarded. This ensures a population of ~3000 adults (at a sex ratio of 1:1) in each cage for the low density replicates. Pupae were left for a minimum of three days after the final pupae were added to the cages, to allow for eclosion and mating to occur.

The total number of adults for each cage was estimated to the same protocol as used for the larval rearing, counting the number of pupae in three 0.5 ml samples and measuring the total ml of pupae collected each day.

5a.2.3.1 **Cages**

Adult mosquitoes were housed in large bugdorm cages (Megaview, Taiwan). Mosquitoes were supplied with a 10% sucrose solution, replaced weekly. Mosquitoes were fed three times during the week, Wednesday – Friday, to standard feeding procedures (5a.2.1.2). Hemotek wells were filled with ~3 ml of blood, covered with swine intestine and heated in a water bath to 40°C. Two wells were presented inside each cage, and were reheated twice during each day.

5a.2.3.2 **Egg Production**

Egg collection was performed until production dropped to below 10% of that of the first gonotrophic cycle (GC), 4 GCs for low density cages and 5 GCs for the high density cages.

Each cage was presented with an oviposition paper in 250 ml water between Friday-Wednesday. Oviposition papers were subsequently dried in the insectary for 4 days before being scanned onto the computer for egg numbers to be estimated in ImageJ as previously described (5). The number of eggs per female for each cage was then calculated based on the estimated number of mosquitoes in each cage.

5a.2.4 **Statistics and modelling**

5a.2.4.1 **Pupal size – mixed effects model**

A mixed effects model was fitted to the pupal size data. Pupal size was set as the response variable, a single fixed interaction term (Int) combining Day, Sex and Line (e.g. H.F.2 – HAW Females on Day 2) was the main explanatory variable and a random effect explanatory variable was included, as Tray, describing the replicate the pupae came from (in effect giving each replicate a unique identifier).

The experimental design meant that rearing at different densities occurred concurrently rather than in parallel. Density was not included in the model due to experimental design, the data were instead separated into three distinct data sets, one for each density, and the model subsequently applied to each data set. As no constant control was used, results from one density cannot statistically be compared to another, as pupal size is extremely sensitive to environmental conditions.

The model was assessed for suitability and the diagnostic plots can be found in Appendix C. The explanatory variable Int (Day*Sex*Line) was tested for significance by comparison to a model which had the variable omitted. It was confirmed as significant through AIC, BIC and Chi sq

results. Post hoc analyses of the Int explanatory variable were conducted by simultaneous tests for general linear hypotheses.

5a.2.4.2 **Estimating male recovery**

A 'for loop' was constructed in R (Appendix D) which examined male pupae on days 1 and 2 only. For each line it calculated the number of male pupae that were smaller than a given value, representing the number of males that would be collected through a sieve of that gauge. The number of pupae retrieved was returned as a percentage of the total number of day 1 & 2 male pupae. This is referred to as the "Male recovery" percentage. Also recorded was the number of female pupae smaller than the sieve size value tested. This represents a female contamination, and was reported as the number of females as a percentage of the number of males recovered.

This was repeated for size values between the smallest and largest pupal sizes, increasing by 0.01 mm for each iteration.

A five parameter log logistic (LL.5) dose response model (Appendix E) was fitted to the calculated percentages of male recovery and female contamination for each line at each dose. The model was found to be superior through AIC model comparisons to 4 (LL.4) and 3 (LL.3) parameter log logistic dose response models. Diagnostic plots of the model were performed when fitted to each density data set (Appendix E).

Three female contamination rates were used (0.2%, 1% and 2.5%) for the 2 lar / ml data, chosen as they lie between the 0.2% to 5% contamination rates used by Oxitec Ltd. The hypothetical sieve size was calculated based on the 1% female contamination rate, and was subsequently used to predict the proportion of male pupae recovered from days 1 and 2, and report that as a percentage of the total number of males from the tray for each line at each density.

5a.2.4.3 **Adult density – mixed effects model**

A mixed effects model was fitted to the adult density data. The number of eggs per female was set as the response variable, a single fixed interaction term combining GC, Line and density (e.g. KLP at high density, 1st GC) was the main explanatory variable and a random effect explanatory variable was included as cage, describing the replicate the data came from. The model was assessed for suitability and the diagnostic plots can be found in Appendix G. Post hoc analyses of the Int explanatory variable were conducted by simultaneous tests for general linear hypotheses.

5a.3 Results and Discussion

5a.3.1 **Preliminary wild type characterisation**

The preliminary experiments were designed to set baseline characterisation of the wild type strains to allow for efficient testing. Before the larval and adult density experiments could commence it was essential to determine hatch rates for each line so that they could be suitably aliquoted to achieve the correct rearing densities. A preliminary larval rear at 4 lar / ml using the standard CAA feeding regimen, adjusted to reflect higher numbers, indicated the feeding regimen to be inappropriate, as evidenced by high levels of spoilage and larval death (Data not shown). It was therefore necessary to use an adapted feeding regimen when rearing at high density.

5a.3.1.1 **Hatch Rates / Aliquoting**

To determine the larval density, there are a number of techniques that can be employed. Commonly used at Oxitec Ltd is an aliquot method (5a.2.1.4). However the equipment at the CAA was deemed inappropriate for use, as high L1 mortality was caused. It is believed this was due to the magnetic stirrer's slowest setting being too fast, resulting in larval death from being mixed too quickly.

It was decided therefore to use the CAA methodology for aliquoting L1 larvae (5a.2.1.5). However before that protocol could be used, a baseline hatch rate for each WT line was required. Initial hatch rates were performed but returned poor results (Table 16). As such, egg papers were left to

develop for a further 2 days (5 days in total) before being hatched. The second hatch rate returned more favourable results (Table 16), however the REU strain was still performing poorly. It was decided at this point to discontinue work with the strain, as it would be difficult to collect sufficient eggs to perform the planned experiments given the tight time restrictions on the project.

Table 16: Baseline hatch rates of the 5 *Ae. albopictus* wild type strains

Line	1st Hatch Rate **Mean** [95% CI]	2nd Hatch Rate **Mean** [95% CI]
ITA	**0.6086** ± 0.037	
REU	**0.2821** ± 0.017	**0.4703** ± 0.035
MAL	**0.6031** ± 0.031	**0.8090** ± 0.046
HAW	**0.6296** ± 0.078	**0.7666** ± 0.047
KLP	**0.6373** ± 0.014	**0.8267** ± 0.086

The aliquoting protocol was validated by using the second calculated hatch rates to predict the number of L1s from a sample egg paper (left for 5 days) for each line. The eggs were counted manually and were subsequently hatched and the L1s counted. The predictions (Table 17) were deemed satisfactory to continue with this methodology for aliquoting larvae.

Table 17: Aliquot validation through estimating the number of L1s from the corresponding hatch rate, compared to the actual number that hatched determined through manual counting.

Line	Estimated egg number	Prediction of L1s **Mean** [95% CI]	Manual count of L1s
ITA	456	**306** [195, 323]	329
REU	537	**252** [110, 271]	149
MAL	1604	**1298** [990, 1372]	1073
HAW	1295	**993** [714, 1053]	761
KLP	1628	**1346** [997, 1486]	1104

5a.3.2 **Larval Rearing Density**

Pupae were sampled and measured over four consecutive days as described previously (5a.2.2 / 5a.2.2.1). The estimated total number of pupae and remaining L4 larvae of each tray indicated that at least 75% of the initial number survived to L4, at all three rearing densities. Spoilage occurred in two rearing trays meaning only two full replicates were conducted for MAL reared at 2 lar / ml and HAW reared at 4 lar / ml.

At 2 lar / ml there are distinct peaks in male and female pupal development on days 2 and 3 respectively for the MAL, KLP and HAW lines; whereas the ITA females peak on days 2 and 3 (Figure 49). At 3 lar / ml (Figure 50) and 4 lar / ml (Figure 51) male pupation peaks at day 1, and decreases over the 4 days, for all strains. Females are seen to outnumber males from day 3 onwards in every scenario. The overall sex ratio for all four lines was approximately 50 : 50 at 2 lar / ml, whilst at 3 and 4 lar / ml it was around 60 : 40 (data not shown). This is to be expected, as at higher densities development rate is slower. Females take longer to emerge and therefore fewer of the overall number would have been sampled. If the high density trays had been left to develop to completion it is likely the overall sex ratio would be close to 50:50.

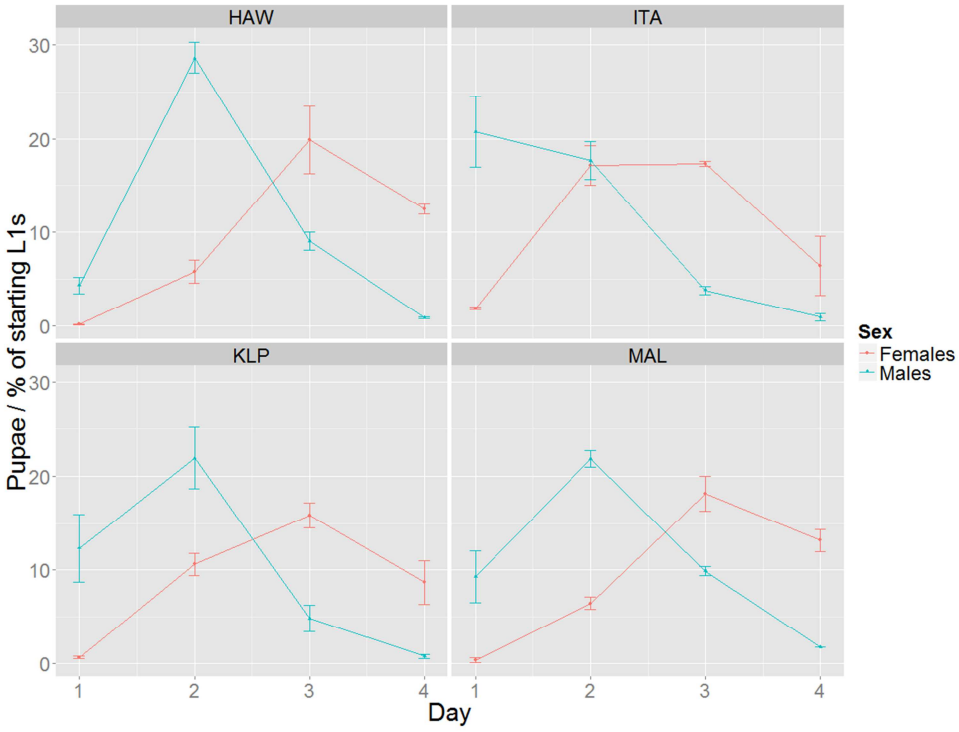


Figure 49: Male and female pupal development when reared at 2 lar / ml; displayed as a percentage of the starting number of L1s (4000). Mean of three replicates with standard deviation shown (Exception MAL, n=2). Top left: HAW, Top right: ITA, Bottom left: KLP, Bottom right: MAL. Day represents days 24 hours after the onset of pupation.

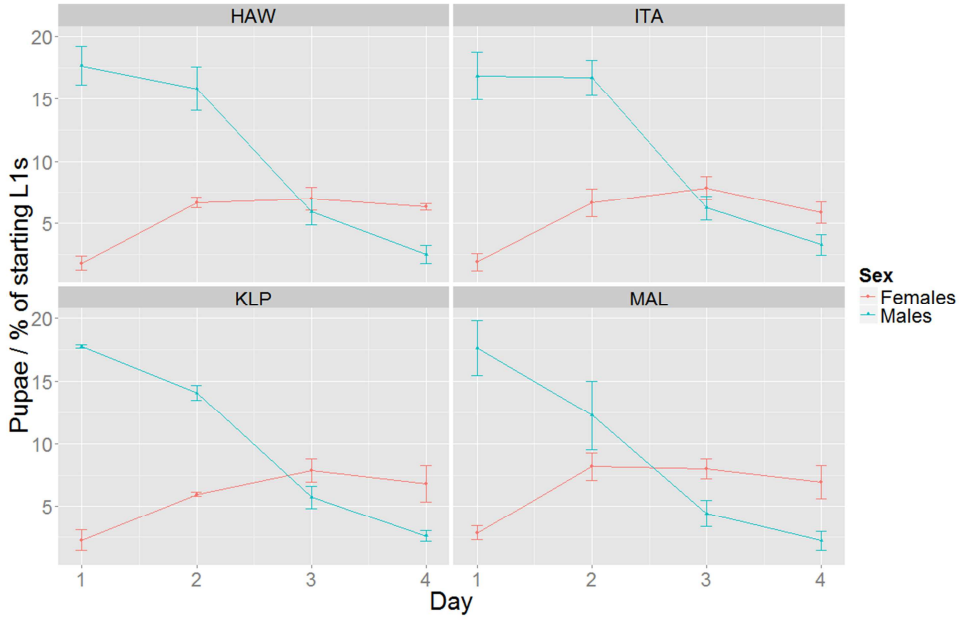


Figure 50 Male and female pupal development when reared at 3 lar / ml; displayed as a percentage of the starting number of L1s (6000). Mean of three replicates with standard deviation shown. Top left: HAW, Top right: ITA, Bottom left: KLP, Bottom right: MAL. Day represents days 24 hours after the onset of pupation.

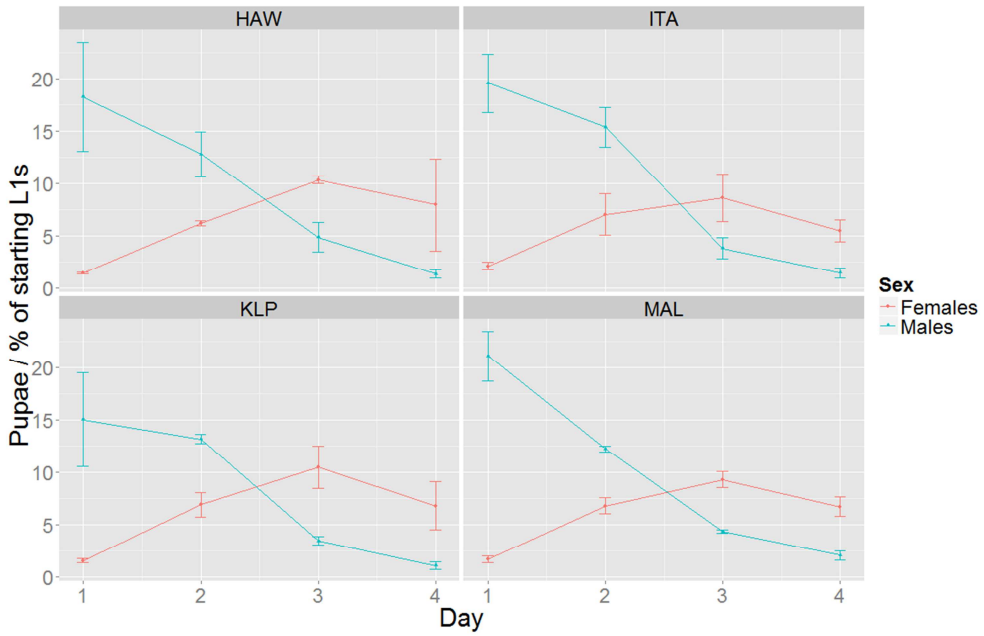


Figure 51 Male and female pupal development when reared at 4 lar / ml; displayed as a percentage of the starting number of L1s (8000). Mean of three replicates with standard deviation shown (Exception HAW, n=2). Top left: HAW, Top right: ITA, Bottom left: KLP, Bottom right: MAL. Day represents days 24 hours after the onset of pupation.

5a.3.3 *Ae. albopictus* pupal size at different rearing densities

Whilst experimental design does not allow for statistical comparisons to be drawn between the different densities, it is nevertheless interesting to note that average pupal size decreased for every line as rearing density increased (Table 18).

Table 18: Average pupal size of males and females for the four WT *Ae. albopictus* lines when reared at one of three rearing densities

Line	Sex	Size / mm		
		2 lar / ml	3 lar / ml	4 lar / ml
HAW	M	0.98	0.91	0.90
	F	1.09	1.06	1.04
MAL	M	0.98	0.90	0.87
	F	1.11	1.07	1.01
KLP	M	0.99	0.90	0.88
	F	1.12	1.05	1.03
ITA	M	0.96	0.90	0.86
	F	1.08	1.04	0.99

Pupae are currently sorted for a release based on the size dimorphism between males and females. It is therefore essential to have a detailed understanding of the changing size of pupae through development. From the graphical output of pupal size it is apparent that, across all lines reared at all three densities, males are inherently smaller than females, and pupal size decreases with time (Figure 52, Figure 53 Figure 54). This was confirmed through the application of mixed effects models.

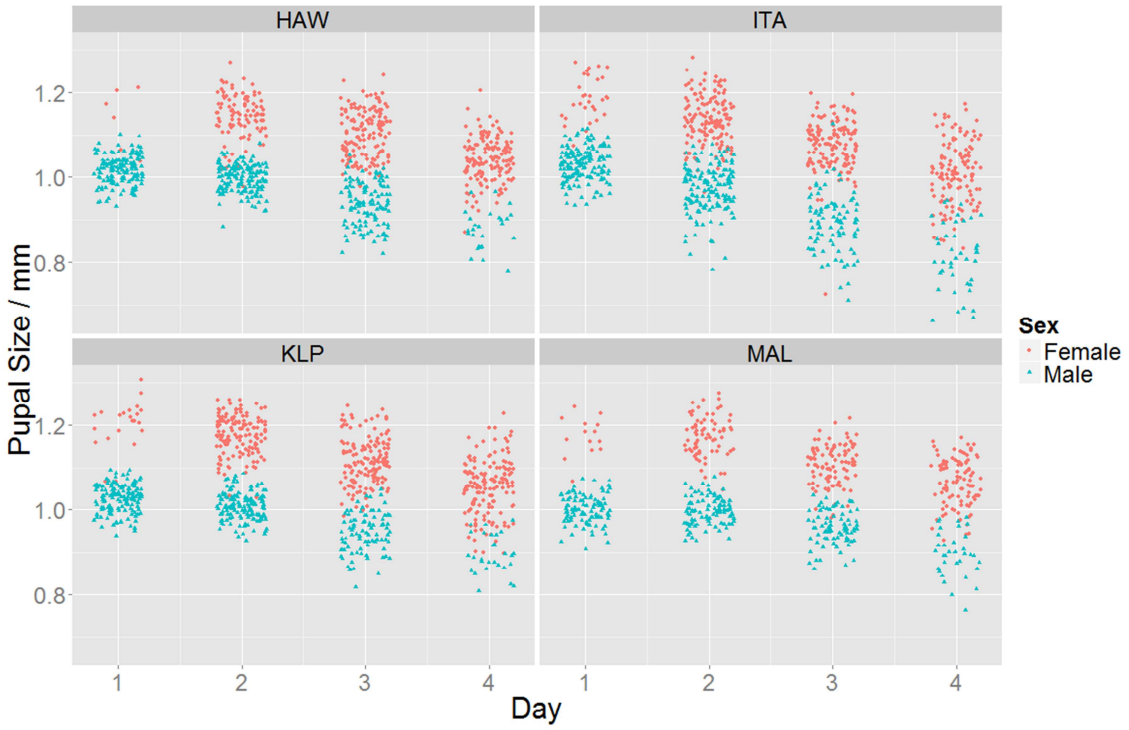


Figure 52: Male and female pupal sizes over four days, from samples of larvae reared at 2 lar / ml. Top left: HAW, Top right: ITA, Bottom left: KLP, Bottom right MAL.

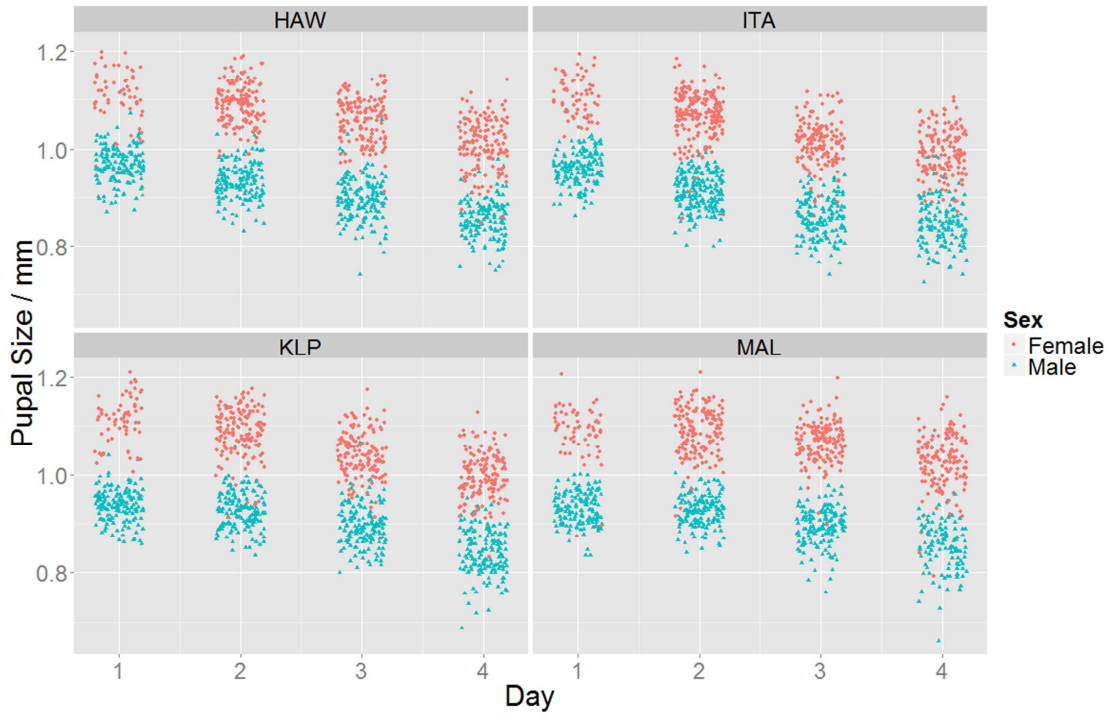


Figure 53: Male and female pupal sizes over four days, from samples of larvae reared at 3 lar / ml. Top left: HAW, Top right: ITA, Bottom left: KLP, Bottom right MAL.

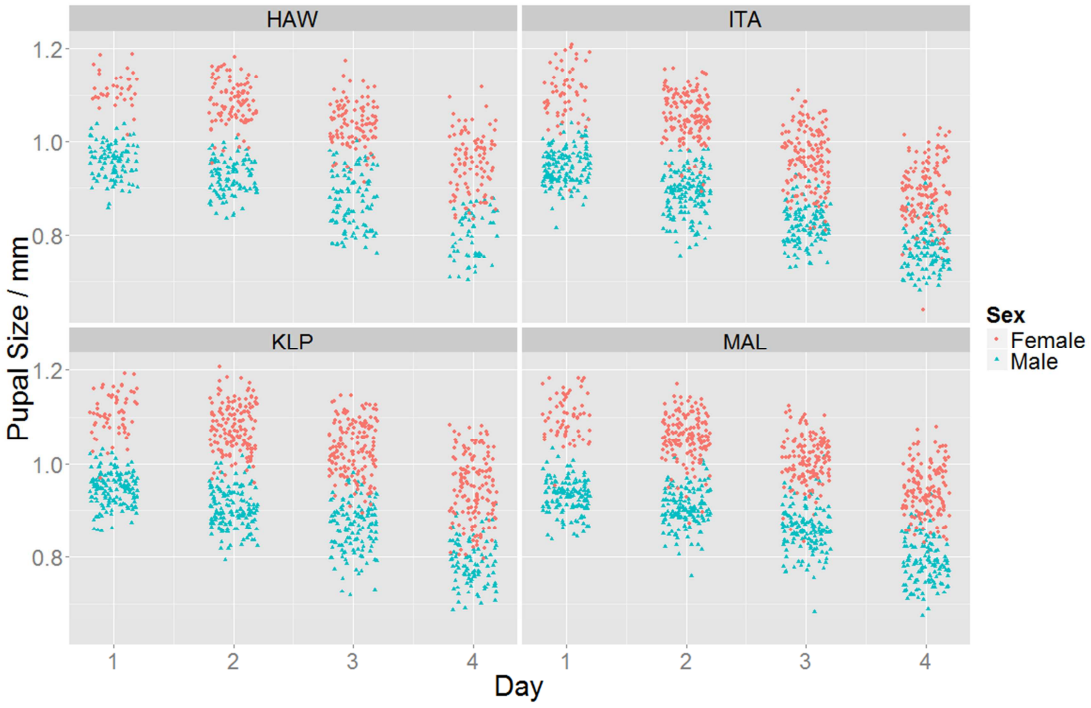


Figure 54: Male and female pupal sizes over four days, from samples of larvae reared at 4 lar / ml. Top left: HAW, Top right: ITA, Bottom left: KLP, Bottom right MAL.

A linear mixed effects model was fitted to the pupal size data set. This was done separately for data from each rearing density. Simultaneous tests for general linear hypotheses were carried out on the fixed interaction term of Day, Sex and Line. The first hypothesis looked at the difference in size between males and females on each day for each line and at each density. It was found that for all lines at all rearing densities, that males were significantly smaller than females on each day (Appendix F: Figure 97, Figure 98 and Figure 99). This was to be expected, as the morphological size difference between the sexes is well documented [140].

The second hypothesis looked at the difference in size between consecutive days for each sex, for each line at each density (i.e. are the HAW female pupae on day 1 different in size to HAW female pupae on Day 2 when reared at 2 lar / ml?) (Appendix F: Figure 100, Figure 101 and Figure 102). Overall, pupal size was seen to decrease over the 4 days, for both males and females of all lines at all densities. However all lines showed no significant differences between day 1 and day 2 for at

least one rearing density. For HAW it was females at 2 and 4 lar / ml; for KLP it was females at 2 lar / ml and males at 3 lar / ml, for MAL it was males and females at 3 lar / ml and for ITA it was males and females at 2 lar / ml. This may be expected for the ITA strain at 2 lar / ml, as this is the standard rearing density at the CAA. Selection pressures on the line from being reared at this density mean it is well adapted to these specific conditions. For HAW it was only the females that did not change significantly in size between days 1 and 2. This may be in part due to the small sample size of female pupae on day 1, and therefore the lack of statistical power to detect a significant difference. The same could be said for the KLP females at 2 lar / ml. The lack of a significant difference between days 1 and 2 for the MAL males and females and KLP males at 3 lar / ml may suggest that this is an optimum rearing density for these lines, however further research would be needed to confirm that this is the case.

Based on these data, ideally male pupae for a release would be selected on days 1 or 2 of pupation, during the male pupation peak. The male pupae are highest in numbers compared to females at this point, lowering the risk of contamination. The more constant that pupal size remains across those 2 days, the more efficient the sorting process will be. The results here suggest that given the right conditions it is possible for male and female pupae size to be consistent over the first two days, which would in theory be an aid to sex separation for a release program.

The third hypothesis tested was to look at overall male and female pupal size between the lines. At 4 lar / ml there were no significant differences seen in average female or male pupal size when comparing across strains (Appendix F: Table 49). At 2 lar / ml KLP males were significantly larger than the ITA males (Difference = 0.0463, $p < 0.0339$ *), but no other differences were seen (Appendix F: Table 47). At 3 lar / ml a number of significant differences were seen, summarised in Table 19 (full statistical results Appendix F: Table 48). The ITA females were consistently smaller than the other lines when reared at this density, and the males significantly smaller than the HAW

males. However it should be noted that the differences seen are relatively small, at ~0.02 mm, which is ~1 - 2 % of an average pupa size.

Table 19: Statistical differences seen between male or female pupae between different lines reared at 3 lar / ml.

Comparison	Estimated difference / mm	Std. Error / mm	Z value	Adjusted p value
HAW females vs ITA females	0.0229	0.0039	5.859	<0.001 ***
HAW males vs ITA males	0.0202	0.0036	5.565	<0.001 ***
HAW males vs KLP males	0.0130	0.0036	3.572	0.0039 **
HAW males vs MAL males	0.0115	0.0037	3.140	0.0177 *
KLP females vs ITA females	0.0153	0.0039	3.939	<0.001***
MAL females vs ITA females	0.0237	0.0039	6.111	<0.001 ***

The exploratory data presented here shows a few minor differences between the four lines tested. The faster development of males and females of the ITA line at 2 lar / ml compared to the other three lines is a curious result. It is proposed that this is due to the standard rearing conditions of the CAA facility, whereby pupae are only picked on days 1-3 of pupation. The selection pressure applied on early development makes it likely that pupae which develop faster are selected for, thereby leading to broader peaks of male and female pupae across days 1-3. This would make it difficult to use in a mass rearing program, as the more females that are present during the sex sorting process the higher the risk of contamination.

From these exploratory data it is difficult to suggest which line may be preferable over another. Size as a metric of fitness is consistent across the lines at each density (except for minor differences at 3 lar / ml) and does not offer any firm conclusions of line choice.

As discussed previously one of the features of interest is not the size of the pupae per se, but the difference in size between males and females, specifically on days 1 and 2, where male pupation peaks and female pupation is still relatively low. To explore this further the pupae size data was

used in a predictive model to assess male recovery and female contamination rates using hypothetical values for sieve gauges.

5a.3.4 **Predictive modelling**

A “for loop”, previously described (5a.2.4.2), was written to represent a hypothetical sorting procedure, in which the number of males smaller than a given value was calculated. This is analogous to sex-sorting, by sieving the males with a sieve of a set gauge, the smaller males will pass through, whilst the others will not. The “for loop” can therefore calculate the male recovery percentage, as the number of males smaller than the given sieve size out of all the males. The loop ran sieve sizes between the size of smallest and largest pupa, increasing by 0.01 mm for each iteration. This was performed on data from days 1 and 2 for each mosquito line at each density (Figure 55, Figure 56 and Figure 57)

Alongside this, the number of females smaller than the sieve gauge was also calculated, returning a “female contamination” percentage, which is the number of females as a percentage of the number of recovered males. This was also repeated for sieve gauges between the size of the smallest and largest pupae, increasing by 0.01 mm for each iteration. The dose response model was subsequently fitted and plotted with the data for each line at each density (Figure 55, Figure 56 and Figure 57).

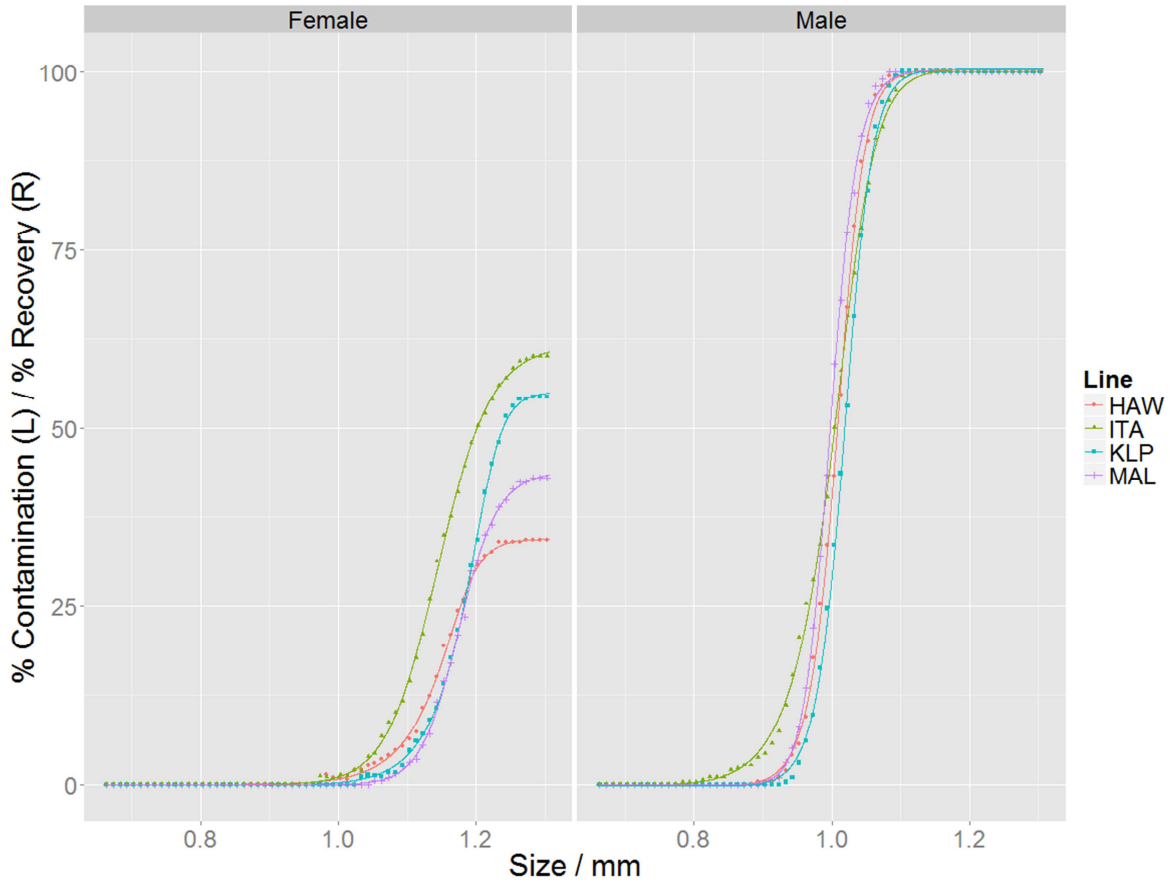


Figure 55: The predicted female contamination rates (Left) and male recovery rates (R) from a dose response model using a range of potential sieve sizes for pupae reared at 2 lar / ml.

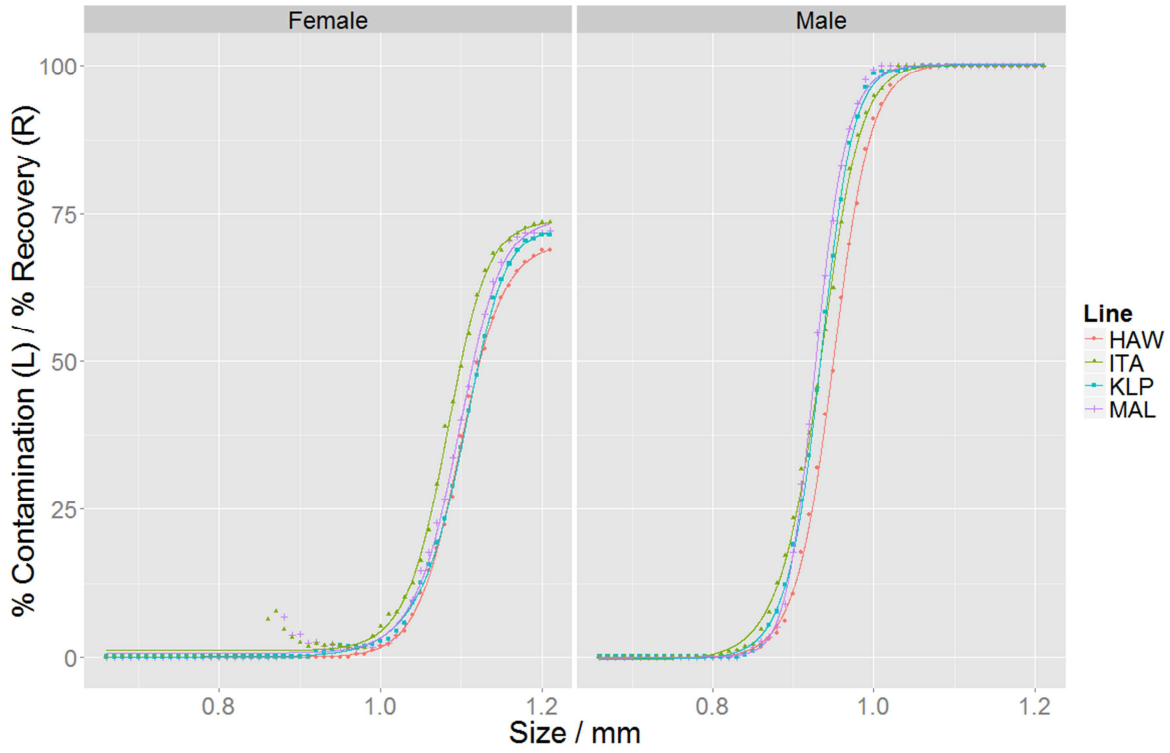


Figure 56: The predicted female contamination rates (Left) and male recovery rates (R) from a dose response model using a range of potential sieve sizes for pupae reared at 3 lar / ml.

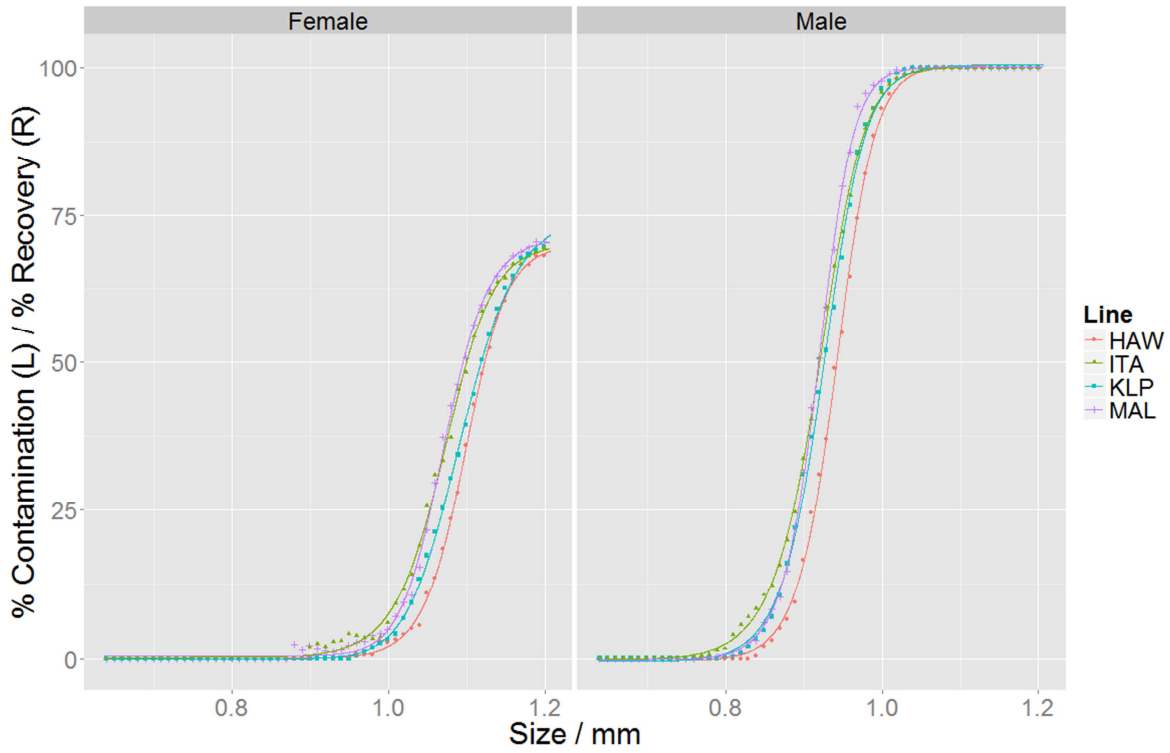
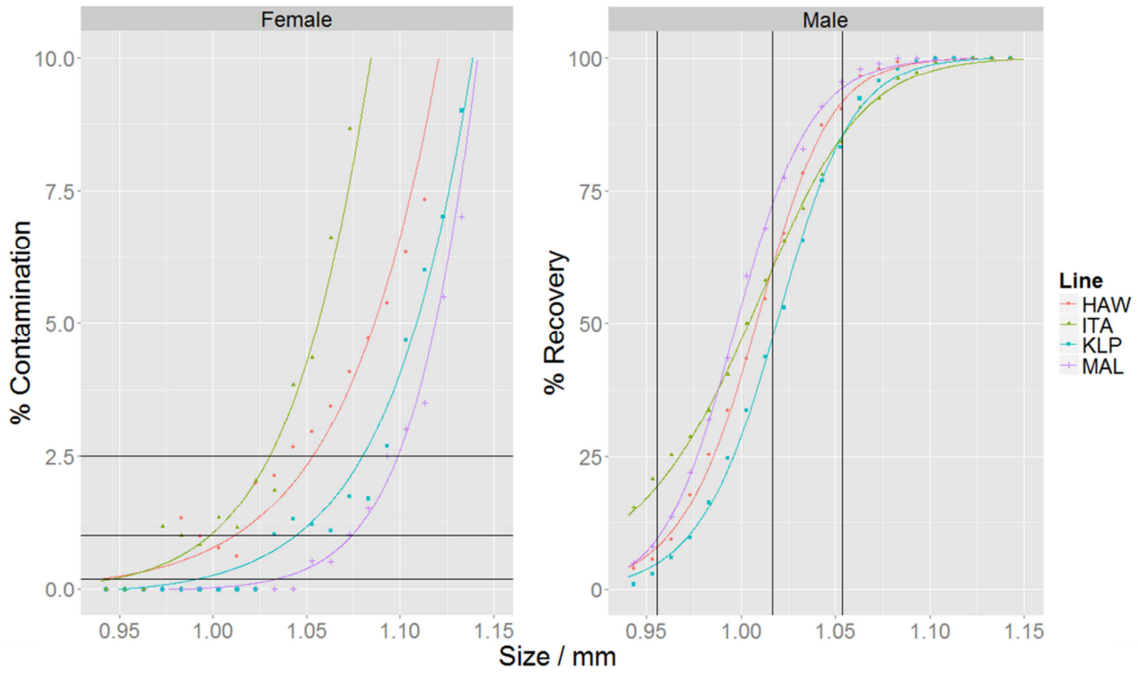


Figure 57: The predicted female contamination rates (Left) and male recovery rates (R) from a dose response model using a range of potential sieve sizes for pupae reared at 4 lar / ml.

Using these models, it is possible to look at the problem from another angle. That is to say, given a set female contamination rate, what would the percentage of male recovery be? This was achieved by using the Estimated Dose (ED) function to assess three different contamination rates (Figure 58) for the 2 lar / ml data. The contamination rates of 0.2%, 1% and 2.5% were set, and the model returned the number of male pupae which would have been recovered on days 1 and 2 (represented as a percentage of all male pupae on days 1 and 2).



Female Contamination %	Mean % Recovery of male pupae on days 1 and 2 [95% CI]			
	MAL	KLP	ITA	HAW
0.2	**67** [42, 85]	**9.4** [4.6, 18]	**2.2** [0.82, 5.7]	**0.33** [0.04, 1.3]
1	**94** [89, 97]	**73** [62, 81]	**30** [19, 43]	**15** [7.0, 30]
2.5	**98** [97, 99]	**94** [92, 96]	**69** [60, 76]	**59** [44, 73]

Figure 58: **Top Left**: Female contamination rates at 2 lar / ml, as calculated by the predictive model. Black lines represent desired contamination rates of 0.2%, 1% and 2.5%. The required sieve sizes to obtain these contamination rates can be calculated for each line. **Top Right**: The sieve sizes calculated for the set female contamination rates can be used to assess the male recovery percentage. As an example, the black lines demonstrate the sieve sizes calculated for KLP to allow for 0.2%, 1% and 2.5% female contamination. **Bottom**: The mean male recovery percentages (with 95% CI) are displayed for each line for the given female contamination rates of 0.2%, 1% and 2.5%

It is interesting to note that the MAL line consistently returns a higher percentage of males than the other lines. However these data alone are insufficient to fully assess the lines, as they do not take pupal development time into account. If very few males pupate on days 1 and 2 then even a high recovery percentage will only return few pupae; whereas if the majority of males pupate on days 1 and 2 a large number could be retrieved even with a low recovery rate. The male recovery percentage for days 1 and 2 was calculated for each line at each density using the 1% female contamination threshold. Given the estimated numbers of male pupae on each day it is possible to calculate the total number of pupae that would have been collected on days 1 & 2. This can then be expressed as a percentage of the total number of male pupae from the starting number of male L1s. This was performed for each line at 2 lar / ml (Table 20), 3 lar / ml (Table 21) and 4 lar / ml (Table 22).

At 2 lar / ml, the overall male recovery for HAW and ITA was significantly lower (11% and 25% respectively) than for MAL and KLP (62% and 55% respectively) at the 95% significance level.

At 3 lar / ml there was a slight reduction in overall male recovery for KLP when compared to HAW (54% and 71% respectively) at the 95% significance level.

At 4 lar / ml, overall male recovery for ITA (36**%**) was significantly less than for MAL, KLP and HAW (65%, 60% and 68 % respectively) at the 95% significance level.

Over the three larval rearing densities two lines, MAL and KLP, perform well and consistently show an overall male recovery rate at over 50%. ITA performed poorly at two of the rearing densities, only recovering more than 50% when reared at 3 lar / ml. The HAW line performed poorly when reared at 2 lar / ml, with only 11% of the overall males being recovered.

Table 20: The estimated male recovery with a 1% female contamination rate from larvae reared at 2 lar / ml. Male recovery is expressed as the percentage of males recovered on days 1 and 2, and as an overall percentage of the number of starting male L1 larvae.

WT Line	Male recovery percentage for days 1 & 2 **Mean** % [95% CI]	Overall recovery percentage of males **Mean** % [95% CI]
MAL	**94**	**62**[a]
	[89, 97]	[59, 64]
KLP	**73**	**55**[a]
	[62, 81]	[47, 62]
ITA	**30**	**25**[b]
	[19, 43]	[16, 36]
HAW	**15**	**11**[b]
	[7.0, 30]	[4.9, 20]

Table 21: The estimated male recovery with a 1% female contamination rate from larvae reared at 3 lar / ml. Male recovery is expressed as the percentage of males recovered on days 1 and 2, and as an overall percentage of the number of starting male L1 larvae.

WT Line	Male recovery percentage for days 1 & 2 **Mean** % [95% CI]	Overall recovery percentage of males **Mean** % [95% CI]
MAL	**85**	**67**[ab]
	[72, 92]	[57, 72]
KLP	**72**	**54**[a]
	[55, 83]	[42, 63]
ITA	**82**	**70**[ab]
	[71, 90]	[60, 76]
HAW	**93**	**71**[b]
	[87, 96]	[66, 73]

Table 22: The estimated male recovery with a 1% female contamination rate from larvae reared at 4 lar / ml. Male recovery is expressed as the percentage of males recovered on days 1 and 2, and as an overall percentage of the number of starting male L1 larvae.

WT Line	Male recovery percentage for days 1 & 2 **Mean** % [95% CI]	Overall recovery percentage of males **Mean** % [95% CI]
MAL	**83**	**65**[a]
	[74, 90]	[57, 70]
KLP	**83**	**60**[a]
	[72, 91]	[52, 66]
ITA	**46**	**36**[b]
	[32, 61]	[25, 48]
HAW	**91**	**68**[a]
	[85, 95]	[64, 71]

The overall male recovery is a useful metric as it combines information from tray development and pupal size into a measurable outcome. The predictive model used to calculate female contamination and male recovery requires refinement, particularly at the lower and higher sizes where the residuals plotted against the predictors are consistently either above or below 0, rather than evenly distributed (Appendix E). Whilst it comes with certain caveats as with any data based on a model, it does highlight some stark contrasts between the lines, and is a useful aid to distinguishing between them.

5a.3.5 **Adult Density**

Adult mosquitoes were housed at two different densities, either 3000 or 6000 per cage at a 1 : 1 sex ratio. Eggs were collected on a weekly basis, and a mixed effects model was fitted to this data to examine differences between the lines at these two densities using simultaneous tests for general linear hypotheses.

The first hypothesis examined differences between the total number of eggs between the low and high density cages for each gonotrophic cycle (GC). No significant difference was seen between high and low densities for the 1st (z value = 0.042, p =1.00.) or 4th (z = 1.014, p = 0.762) GC; however a significant difference was seen between the high and low densities for the 2nd (z = 8.848, p < 0.001) and 3rd (z = 4.869, p < 0.001) GC (Figure 59).

Given the high density cages contained twice as many adults as the low density cages, one would expect significantly more eggs from the high density cages. Whilst this is the case overall, with an average of 7250 more eggs in the high density cages (z = 5.814, p<0.001), they showed the same productivity for the 1st and 2nd GC. This would suggest a limiting factor on productivity, likely to be either blood availability or oviposition substrate.

The second hypothesis examined differences in the number of eggs between the lines, for the first and second GC at each density. At high density the KLP line produced significantly fewer eggs

than the MAL (z = -4.759, p < 0.001) and ITA (-5.117, p < 0.001) lines for the first GC, but no other significant differences were seen. At the low density no significant differences were seen between the lines (Appendix G: Table 50 and Table 51).

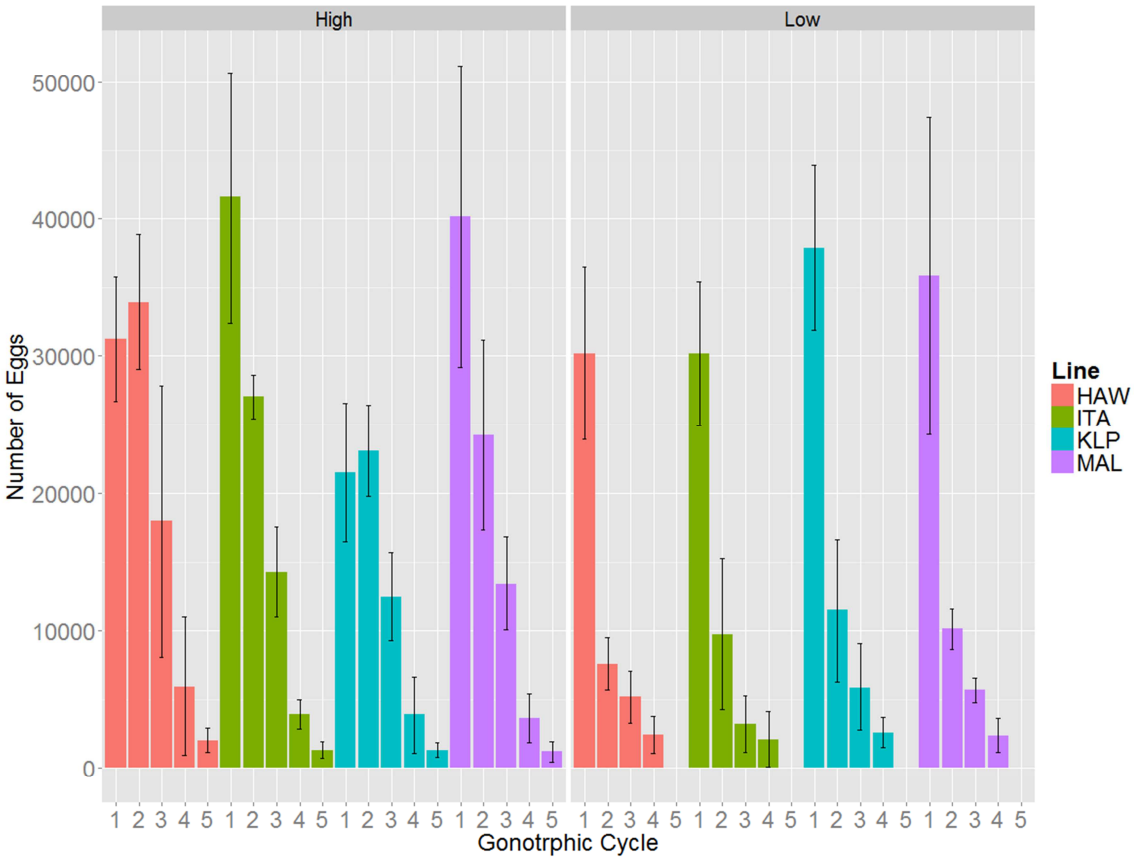


Figure 59 Mean number of eggs (± SD) housed at either High or Low density for 4 or 5 gonotrophic cycles respectively, for each wild type strain.

5a.3.6 **Conclusions**

From the mass rearing data, clear male and female peaks in pupation were seen when reared at 2 lar / ml, these peaks were lacking for the higher densities, particularly with respect to females. It is highly likely that this is due to water quality, as at the higher densities water conditions will be worse due to the increased quantity of diet, larval excrement and the resulting bacterial growth. However a refined feeding regimen and water replacement should be able to reduce the stress caused. All lines demonstrated good size dimorphism between males and females, and

reasonably stable pupal sizes between days 1 and 2 for at least one of the rearing densities tested. Some small differences in pupal size were seen between lines, when reared at 3 lar / ml. However these differences were small at ~0.01 – 0.02 mm and are unlikely to have a large impact on overall fitness.

The pupal size data was used to determine the number of males recovered from the total number of starting males, with an acceptable female contamination rate of 1%. This is a more interesting metric, as it combines information on tray development and pupal size dimorphism. MAL and KLP both performed well at each density tested, consistently recovering over 50% of the overall males. HAW performed poorly when reared at 2 lar / ml, but well at 3 and 4 lar / ml. ITA only performed well at 3 lar / ml.

The results of the adult density experiment highlight two interesting points, the first being that egg production in the high density cages was limited for the first GC, evidenced by similar egg production to the low density. Further experiments would need to supply more resources in terms of surface area for blood feeding and oviposition. Overcrowding did not appear to significantly impact production, as the high density cages produced a higher number of eggs than the low density cages overall, however a more rigorous experimental design would be required to fully assess the impact of density on egg production.

With regard to comparisons between the lines, the only significant differences seen were at the high rearing density where KLP produced fewer eggs than the MAL and ITA lines for the first GC.

Chapter 5b: Insecticide resistance

5b.1 Introduction

As discussed previously (1.6.3), it is necessary to determine if any of the *Ae. albopictus* lines currently held at Oxitec Ltd have insecticide resistance. To achieve this, WHO insecticide bioassays were used to calculate the estimated dose required to kill 50% of the exposed mosquitoes (ED50). This was performed with four insecticides, one from each of the four classes commonly used for mosquito control, against each of the four wild type strains. Due to the nature of the work, the experiments were conducted at the London School of Hygiene and Tropical Medicine (LSHTM), where there are suitable facilities for working with insecticides. The work detailed here describes an initial range finding experiment to determine a rough estimate of the ED50s, and a full experiment testing 5 different concentrations of each insecticide to fully assess the ED50s.

5b.2 Materials and Methods

5b.2.1 Mosquito Lines

The four lines of *Ae. albopictus* which are currently maintained at Oxitec Ltd were used, detailed in Chapter 2 (2.1.1). Mosquitoes were reared at 1 lar / ml to standard rearing procedures outlined in Chapter 2 (2.1.3 / 2.1.4 / 2.1.5).

5b.2.2 Insecticides

Due to the sensitive nature of insecticides, the work presented here was performed at the LSHTM, where suitable facilities were provided to work with toxic chemicals. One of each of the four classes of insecticides: organochlorines, organophosphates, carbamates and pyrethroids were used: 4, 4'- DDT 98%, Malathion (analytical grade), Propoxur (analytical grade) and Permethrin (analytical grade) all ordered from Sigma Aldrich.

5b.2.3 **WHO Bioassay**

The WHO bioassay tubes consist of two cylindrical chambers open on one end and sealed at the other with a fine metal mesh secured in place with a plastic ring. The two tubes are connected on the open ends by way of a plastic slider which can be open or closed (Figure 60).

Figure 60: WHO insecticide resistance bioassay tubes. Top tube (red dot) is the test chamber, lower tube (green dot) is the rest chamber, separated by a plastic slider to either open or close the tubes to each other. The ends are secured by mesh, held in place by a plastic cap.

The tubes are marked with a red or green dot to indicate a test or rest chamber, respectively. Filter papers are secured inside the cylinders with two metal springs, an untreated piece in the rest chamber and an insecticide treated one in the test chamber. Twenty unfed virgin females, 3-5 days old, were aspirated using a plastic mouth aspirator, into the rest chamber and the slider closed. The test chamber was then secured into place and the females gently blown from the rest chamber into the test chamber. Once the adults are in the test chamber the slider is closed and females are exposed for 1 hour. After the 1-hour exposure the females were blown back into the rest chamber, and mortality was recorded after 24 hours. Mosquitoes were supplied with 10% sugar solution and kept at 25°C & 90% relative humidity during the 24 hour recovery period.

Mortality rates were adjusted to control mortality using Abotts formula where control mortality was greater than 5% [141].

5b.2.4 **Insecticide papers**

Insecticide papers were made to WHO standards at the LSHTM [142]. Filter paper was cut from a roll of Whatmann filter paper No.1 to be 50 cm x 50 cm. Insecticides were first dissolved in acetone, and mixed with a suitable oil before application to the paper. The oil carrier ensures an even distribution of insecticide across the paper and prevents crystallisation.

A total of 648 mg of oil was applied to each paper. The quantity of insecticide in mg was calculated to produce the relevant concentration to oil (% w/v). A total of 2.66 ml Oil/Insecticide/Acetone mix was applied evenly across each paper by use of a p1000 Gilson pipette. Papers were dried for 24 hours and stored in aluminium foil at 5°C. Each insecticide paper was used for no more than 5 exposures.

For the preliminary range finding experiment, each insecticide concentration was made individually (Table 23). For the full experiment a stock solution of each insecticide was made (Table 24) and dilutions performed to achieve the desired concentration (Table 25).

Table 23: Composition and concentration of range finding insecticide solutions for making insecticide treated papers. Concentrations displayed are of insecticide % to oil (w/v). Silicone oil (density 0.98) was used as the carrier for DDT and Permethrin and Olive oil (density 0.92) for Malathion and Propoxur. All concentrations were made in 10 ml of Acetone solvent.

Insecticide	Quantity Insecticide, mg	Concentration of insecticide % (w/v)
DDT	9.9	0.3
	33	1
	99	3
Malathion	8.59*	0.3
	28.63*	1
	85.89*	3
Propoxur	1.057	0.03
	3.522	0.1
	10.57	0.3
Permethrin	0.99	0.03
	3.3	0.1
	9.9	0.3

* µl at a density of 1.23

Table 24: Composition of stock insecticide solutions, concentration based on the percentage of insecticide to oil (w/v). Silicone oil (density 0.98) was used as the carrier for DDT and Permethrin and Olive oil (density 0.92) for Malathion and Propoxur.

Insecticide	Quantity Insecticide, mg	Insecticide Concentration, % (w/v)	Volume Acetone, ml	Volume Stock, ml
DDT	165	2	25	33.25
Malathion	260.47 *	4	18.5	25.22
Propoxur	8.8	0.1	25	33.25
Permethrin	59.4	0.8	22.5	29.925

* 211.77 µl at a density of 1.23

Table 25: Dilutions of stock solutions of insecticide, with oil and acetone; to achieve solutions with the desired quantity and concentration.

Insecticide	Stock Concentration %	Dilution Factor	Volume Stock Solution	Quantity Oil	Quantity Acetone	Total Volume	Concentration % (w/v)
Malathion	4	10	1.352	3.168	9	13.52	0.4
Malathion	4	4	3.38	2.64	7.5	13.52	1
Malathion	4	2	6.76	1.76	5	13.52	2
Propoxur	0.1	10	1.352	3.168	9	13.52	0.01
Propoxur	0.1	5	2.704	2.816	8	13.52	0.02
Propoxur	0.1	2.5	5.408	2.112	6	13.52	0.04
Propoxur	0.1	1.25	10.816	0.704	2	13.52	0.08
Permethrin	0.8	8.00	1.6625	2.8875	8.75	13.3	0.1
Permethrin	0.8	4.00	3.325	2.475	7.5	13.3	0.2
Permethrin	0.8	2.67	4.9875	2.0625	6.25	13.3	0.3
Permethrin	0.8	2.00	6.65	1.65	5	13.3	0.4
DDT	2	10.00	1.33	2.97	9	13.3	0.2
DDT	2	5.00	2.66	2.64	8	13.3	0.4
DDT	2	2.50	5.32	1.98	6	13.3	0.8
DDT	2	1.25	10.64	0.66	2	13.3	1.6

5b.2.5 **Statistics and modelling**

5b.2.5.1 **Calculating ED50 of insecticides**

A four parameter log logistic dose response model was fitted to the insecticide data. Dose was the primary explanatory variable, with an interaction term of line and insecticide used to group the data. The model was assessed and deemed to be superior to 3 (LL.3) and 5 (LL.5) parameter log logistic models through AIC model comparison. The model fit was assessed using diagnostic plots (Appendix H). Parameter comparison for the ED50 (estimated dose for 50% lethality) of insecticides was performed using the CompParm function, at the 95% significance level.

5b.3 Results and Discussion

5b.3.1 Insecticide Range Finding

To determine the ED50 for each insecticide, a range finding experiment is necessary to narrow down the range of concentrations to test. To perform this, mosquitoes were exposed to a wide range of concentrations (over at least one order of magnitude) to roughly indicate the insecticides ED50. Mosquitoes were then tested fully under a narrower range of concentrations to better estimate the ED50.

A range finding set of concentrations was produced for each insecticide based on a search of the current literature and WHO standards (Table 26).

Table 26: Concentrations of insecticides from the literature and the WHO diagnostic dose, used to define initial range finding concentrations.

Class	Insecticide	WHO DD LC99 (%)	Vontas et al. (2012)[1]	Initial Range Finding
Organochlorines	DDT	4%	4%	0.3% 1% 3%
Organophosphates	Malathion	0.8% [b]	0.8%-5%	0.3% 1% 3%
Carbamates	Propoxur	0.1% [a]	0.1%-0.3%	0.03% 0.1% 0.3%
Pyrethroids	Permethrin	0.25% [b]	0.25%-0.9%	0.03% 0.1% 0.3%

[a] Reported for *Ae aegypti.* [b] Reported for *Aedes.*

Five insecticide papers were made (5b.2.4) of each range finding concentration for each insecticide (Table 23). Two replicates of each concentration were performed for each mosquito line along with two control groups exposed to treated paper with oil and acetone only. Adjusted mortality rates after 24 hours were plotted and fitted with a dose response curve (Figure 61) to

assess the ED50 (Table **27**). Due to low numbers of adult females for the HAW line, only two insecticides were tested.

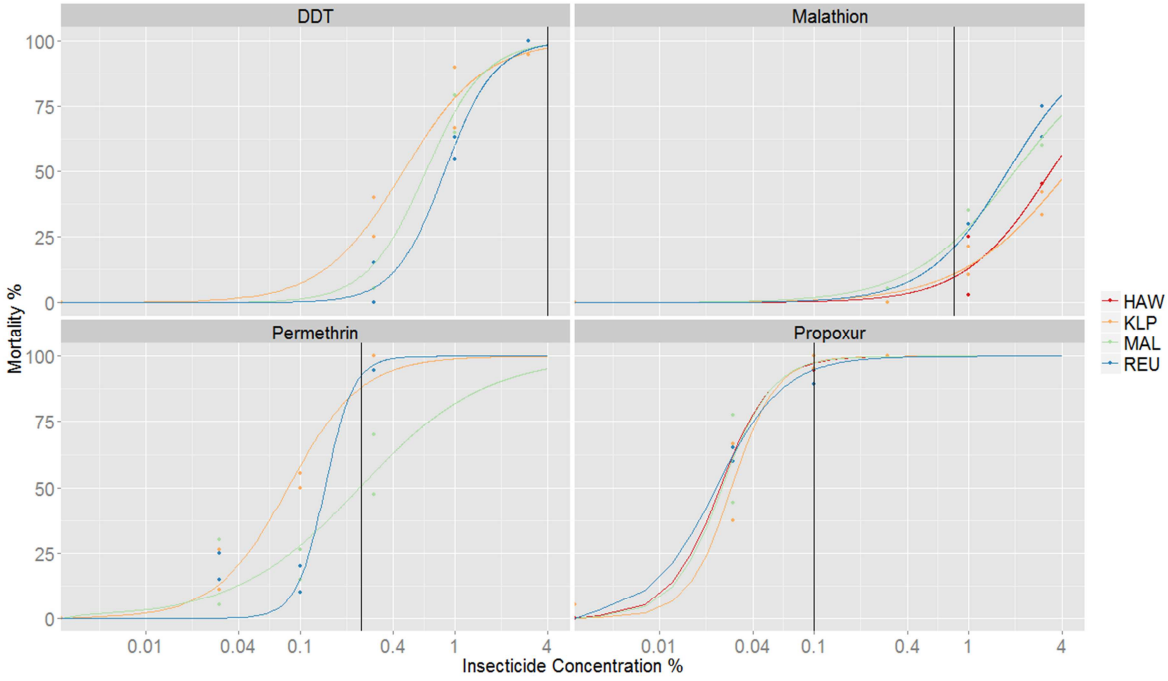


Figure 61: Dose response curves of range finding concentrations of four insecticides for each of the four WT *Ae. albopictus* lines. Nb Due to low numbers of adult females only two insecticides were tested on the HAW line. Vertical black lines represent the WHO DD concentration.

Table 27: ED50 with [95% CI] of each insecticide for each WT *Ae. albopictus* line.

Insecticide	Line **ED50** [95% CI]			
	MAL	HAW	KLP	REU
DDT	**0.65** [0.50, 0.80]		**0.46** [0.35, 0.58]	**0.86** [0.69, 1.03]
Propoxur	**0.025** [0.017, 0.034]	**0.025** [0.016, 0.034]	**0.029** [0.024, 0.034]	**0.023** [0.014,0.032]
Permethrin	**0.24** [0.15, 0.33]		**0.084** [0.063, 0.10]	**0.15** [0.10, 0.19]
Malathion	**1.99** [1.4, 2.57]	**3.39** [2.23, 4.55]	**4.42** [1.97, 6.87]	**1.79** [1.35, 2.23]

Nb Due to low numbers of adult females only two insecticides were tested on the HAW line.

5b.3.2 **Insecticide resistance bioassay**

From the range finding results a set of 5 concentrations for each insecticide was produced (Table 25). Due to the quantity of Malathion available the highest concentration possible was 4%.

Female mosquitoes were tested in a similar fashion to the range finding experiment, 5 replicates of each concentration was performed. As with the range finding data, dose response models were fitted to the data and plotted with the original data points (Figure 62).

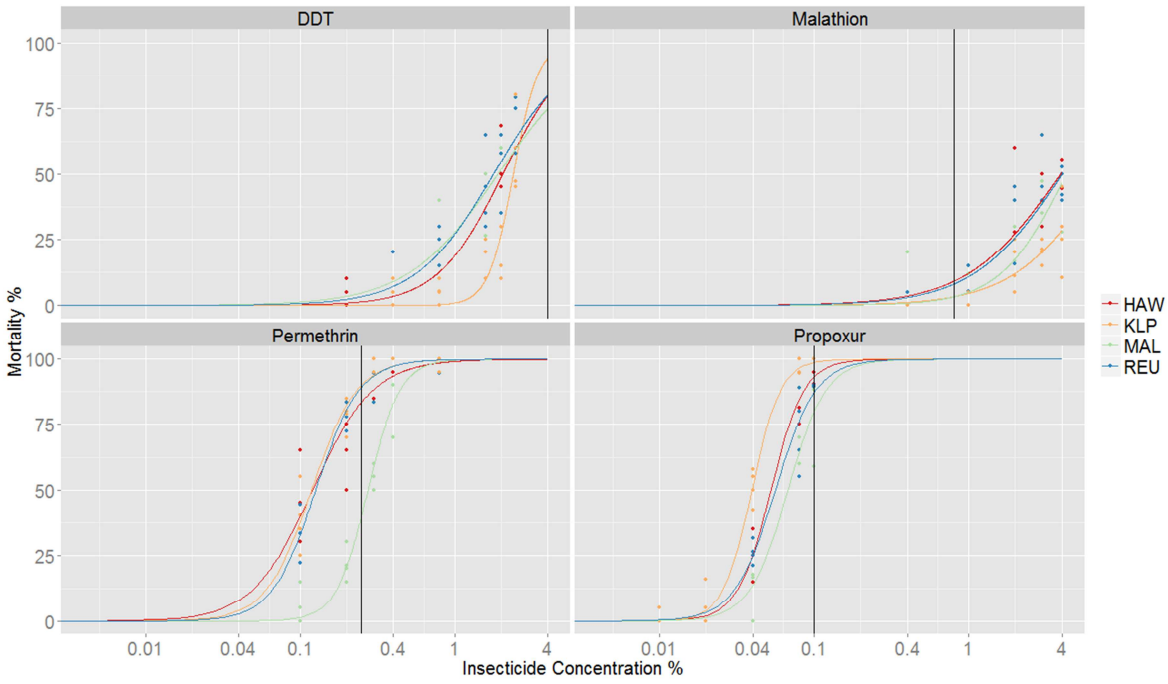


Figure 62: Fitted dose response models, with original data, for each insecticide for each of the four WT *Ae. albopictus* lines. Vertical black lines represent the WHO DD concentration.

The ED50 for each insecticide for each line was calculated (Figure 63). There were slight differences noticed between lines in susceptibility to DDT and propoxur, however all remained below the WHO diagnostic dose of 4% and 0.1%, respectively. No differences were seen between lines with regard to malathion; however the estimate for KLP lies outside of the tested concentration range, and as a result has a large error surrounding the mean. Had testing at higher concentrations been performed it is likely the ED50 would have been significantly higher than the

other lines, however this cannot be determined from these results. Regardless all lines tested were above the WHO DD of 0.8% for malathion. The MAL background showed a level of resistance to Permethrin, with the ED50 above the WHO DD of 0.25%, all other lines had significantly lower mortalities below this level.

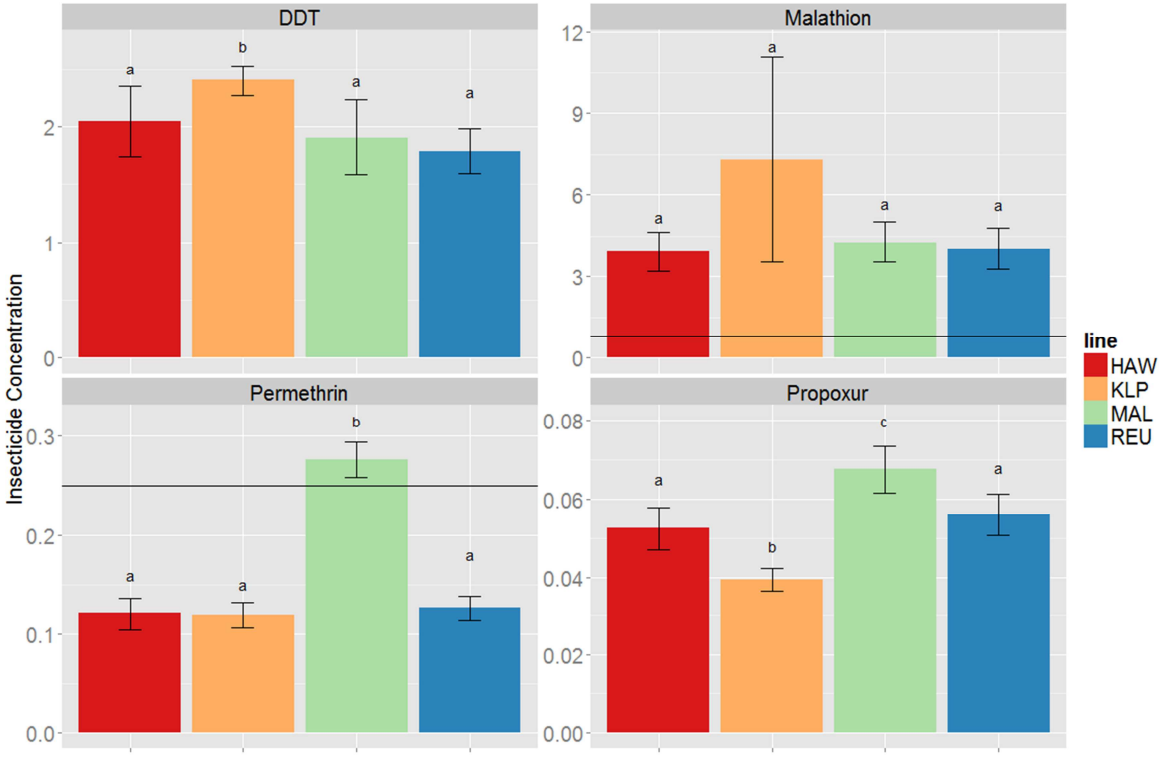


Figure 63: LD50 of each insecticide for each of the four WT Ae. albopictus lines. Horizontal black lines represent the WHO DD concentration (not shown for DDT (4%) or Propoxur (0.1%) as they are off the scale). Different letters indicate a significant difference at the $p < 0.05$ level.

5b.4 Conclusions

There were no differences seen between the four lines for the ED50 of malathion. This may be due in part to the concentrations tested not being high enough to accurately estimate the ED50 for the KLP line, as demonstrated by the large error bars (Figure 63). Regardless, all four lines are above the 0.8%WHO DD, suggesting resistance to malathion. All lines were below the diagnostic dose for DDT and permethrin, suggesting full susceptibility to those insecticides. MAL showed possible resistance to permethrin, with the ED50 above the WHO diagnostic dose.

Chapter 5c: Diapause

5c.1 Introduction

Diapause is a developmental delay in a state of induced suspended animation until more favourable conditions arise. It is hormonally regulated in advance and is not immediately terminated when conditions become favourable, but rather after exposure to specific conditions, e.g. an extended cold interval and extended photoperiod indicating spring has come. The diapause mechanism in *Ae. albopictus* is present at the egg stage, where they are able to withstand the harsh winter conditions. However, the induction of diapause begins with the females prior to laying eggs. *Ae. albopictus* females that are exposed to a shortened photoperiod (autumnal), undergo a response to lay diapausing eggs. The exact mechanism that causes diapausing eggs to be oviposited is not yet fully understood, although transcriptome sequencing has identified several genes associated with diapausing [143]. Alongside shorter photoperiods, temperature also impacts on inducing diapause. Whilst not able to induce diapause by itself, cooler temperatures do increase the diapausing incidence [144]. As shown in Chapter 1, *Ae. albopictus* can be found across the world, in both tropical and temperate climates. Those populations that have spread into temperate climates require a diapausing phenotype in order to survive; those in the tropics however do not, and as might be expected, do not often have a diapausing phenotype [145]. Because populations of *Ae. albopictus* from different geographic regions have different diapausing tendencies it is necessary to examine the wild type strains at Oxitec Ltd for the presence of diapause prior to any field releases. The use of a background strain that has a diapausing phenotype is likely to be denied regulatory approval. If the diapausing phenotype is passed into a non-diapausing population it may facilitate range expansion of that population into more temperate regions. The work detailed in this chapter sets out the experimental design to test for diapause, which although unsuccessful in practice, highlights the protocols to be used, and the difficulties which were encountered.

5c.2 Materials and Methods

5c.2.1 Mosquito Lines

The four lines of *Ae. albopictus* which are currently maintained at Oxitec Ltd were used, detailed in Chapter 2 (2.1.1).

5c.2.2 Mosquito Rearing

Mosquitoes were reared at 0.5 lar / ml under standard rearing conditions outlined in Chapter 2 (2.1) at either an extended photoperiod 16: 8 light: dark at 27°C (± 1°C) and 80% (±10%) relative humidity or at a reduced photoperiod and temperature of 8:16 light: dark at 21°C (± 1°C) and 80% (±10%) relative humidity. Emerging pupae were sexed and allowed to eclose in a cage at a ratio of 1 male : 5 females, with two cages per WT line, one for each rearing condition. Adult mosquitoes were housed at the same conditions they were reared at. Adults were blood-fed daily using the hemotek feeding system, and egg papers were collected weekly. Three egg papers were collected, to be hatched as pseudo-replicates. They were housed for one week under the same conditions that the adults were reared.

5c.2.3 Estimating Diapause

Eggs of the four WT *Ae. albopictus* lines were hatched to standard protocols (2.1.2) and the resulting L1 larvae counted. The remaining eggs are bleached to remove the chorion to identify if an unhatched egg is viable [146]. Sodium hypochlorite 10-15% (Sigma Aldrich, UK) was diluted in deionised water to a concentration of 1-1.5%. This was tested on embryos over three exposure time periods: 1, 4 and 12 hours, to assess the potential to bleach the chorion. Eggs deemed viable but unhatched would be scored as diapausing, with the final percentage of diapausing eggs being those viable eggs which didn't hatch as a percentage of all viable eggs (hatched and unhatched). This "diapausing percentage" can then be compared between mosquitoes reared under standard conditions

5c.3 Results and Discussion

5c.3.1 Estimating Diapause

To examine the diapausing nature of the WT lines, mosquitoes were reared under conditions reported to induce diapause and under extended non-diapause inducing conditions as a control [147]. Diapausing eggs can be defined as the percentage of viable eggs that do not hatch after favourable hatching conditions have been experienced. If a line does come from a diapausing background, there should be a higher proportion of diapausing eggs from the shortened photoperiod conditions than from the standard control conditions.

5c.3.2 Process Development: Bleaching

To assess whether an unhatched embryo is viable and diapausing (rather than unfertilised and non-viable), the chorion can be bleached to allow the embryo to be examined for the presence of an unhatched larva. Eggs were exposed to a sodium hypochlorite solution and left for 12 hours at room temperature. At the end of the exposure all embryos appeared to have either hatched or had the protective chorion and vitellin membrane layers dissolved as evidenced by bleached L1 larvae in the solution. It was believed this may have been a result of the temperature however a further test where embryos were kept at 4°C experienced the same issue.

A much reduced exposure time of 1 hour was trialled at 4°C (Figure 64) which resulted in partial bleaching of the chorion. By increasing this to four hours complete bleaching of the chorion was achieved with very few L1 larvae present in the solution.

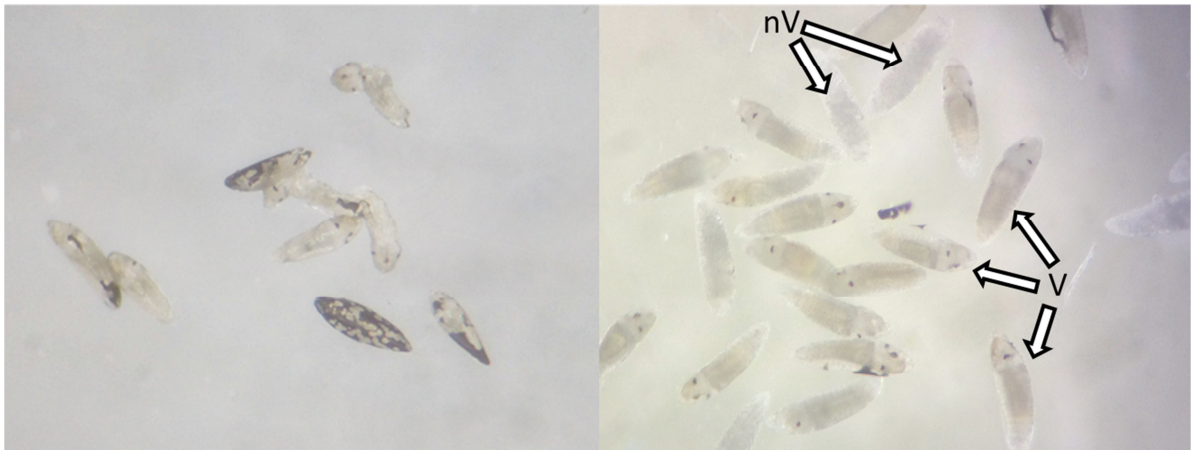


Figure 64: Left: Mosquito embryos after exposure to 1-1.5% sodium hypochlorite for 1 hour at 4°C. Right: Mosquito embryos after exposure to 1-1.5% sodium hypochlorite for 1 hour at 4°C. Viable (V) and non-viable (nV) embryos can be seen.

5c.3.3 **Initial Diapausing Experiment**

Eggs from the diapausing and non-diapausing conditions were counted and subsequently hatched under standard conditions (Figure 65). Whilst it may appear that hatch rates from the short photoperiod were lower than for the long photoperiod, it should be noted that hatch rates were generally poor and inconsistent across all lines. Particularly when compared to the hatch rates reported by Suman et al. [147], where non-diapausing hatch rates were reported to be >95%. It is believed that egg storage conditions are the reason behind such poor hatch rates. Work is ongoing at Oxitec Ltd to improve egg storage in order to deliver consistently high hatch rates. The number of eggs hatched was also extremely low at ~100 eggs per pseudo-replicate (though some were as low as 10 eggs), which also will have contributed to the larger than expected variability in hatch rates. Due to the poor hatch rates experienced, unhatched eggs were not assessed for viability as it was thought any data on egg viability at this point would not be meaningful enough to determine the diapausing status of each line. Due to time constraints a further hatch was not attempted.

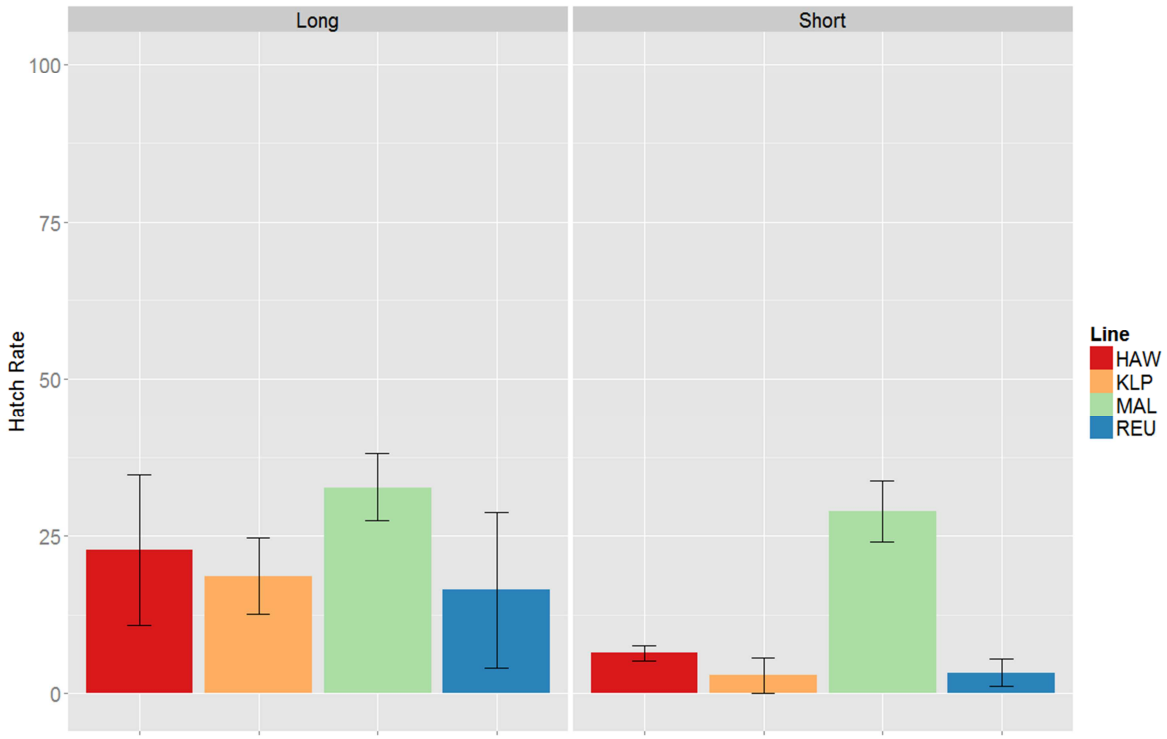


Figure 65: Hatch rates (mean ± standard error) of the four WT *Ae. albopictus* lines reared under a long (left) or short (right) photoperiod at 27°C or 21°C respectively.

5c.4 Conclusions

Unfortunately, no firm conclusions can be drawn with regards to the diapausing nature of the four WT *Ae. albopictus* lines. Poor hatch rates meant that any comparisons drawn would not have the power to stand up to statistical scrutiny. Current work at Oxitec Ltd is examining egg storage under different temperatures and humidity, primarily in order to determine the most efficient conditions for long term storage. Through this experiment it should be possible to calculate the ideal storage conditions for short term use. Once in place this should allow the diapausing experiment to be completed fully, as the bleaching protocol described has been successfully developed.

Chapter 5d: Conclusions

Transgenic work in *Ae. albopictus* at Oxitec Ltd has historically been performed in the MAL genetic background [81]. This choice was based on its lab adaptability, able to feed well off the simple plate feeders (2.1.4) resulting in high egg production. The results from the mass rearing experiment confirm that the MAL line is also capable of rearing well under the pressures of mass production. Whilst the MAL line performed well on all fronts from size dimorphism through to adult productivity, there were issues with the other lines tested. The WT strain colonised from La Reunion had extremely poor hatch rates from the outset, and was unable to provide sufficient larvae from which to collect data. In contrast the KLP and HAW WT strains were both adequate at rearing; however the HAW line performed poorly with regard to predicted male pupae recovery, and KLP had poor female productivity compared to the other lines when reared at a high density. Nevertheless, it is likely that these issues could be addressed by tailoring diet and rearing density specifically to the chosen line. From these results, the MAL WT would remain the preferable choice of background strain, however KLP and HAW would also be suitable.

With regard to the life history parameters pertinent to regulatory approval, the work presented highlight concerns regarding the insecticide resistance status of the WT lines currently being held at Oxitec Ltd. Potential resistance was found in all four WT strains to malathion, with the ED50 for each line well above the 0.8% WHO DD. However it should be noted that there remains some confusion over the correct DD for malathion against *Ae. albopictus.* The 0.8% figure stated is from the WHO as the DD against *Ae. aegypti* [148], although recently this has been extended to all *Aedes* mosquitoes [149]. However in the literature the DD is commonly referred to as 5% for malathion [150], and it is unclear as to why this benchmark is routinely used. One suggested source is the DD for malathion against *Culex quinquefasciatus* is also 5%. Whilst the data here suggest resistance mechanisms are in place against malathion, molecular confirmation of the mode of action will be required to confirm actual resistance. There was also potential resistance

found in the MAL line against permethrin, with the ED50 above the 0.25% WHO DD. This is of concern as permethrin is arguably one of the most important insecticides available. Its toxicity at low concentrations coupled with its slow release from long lasting insecticide treated bed nets has made it a crucial tool for combating malaria. Recent developments have attempted to use permethrin in insecticide treated clothing in an attempt to target day biting *Aedes* mosquitoes [151]. The potential resistance discovered in MAL WT suggest that it should not be considered for use as a background strain for transgenic development.

The current diapause status of the four WT lines remain unknown at the present time, however development of a suitable bleaching protocol and ongoing work into egg storage should allow for this to be determined in the near future. Vector competence, mentioned previously, was not explored due to time constraints. This will certainly require attention prior to submission for regulatory approval. It is proposed that a bioassay be developed based on that of Richards et al. [152], to determine vector competence of the wild type strains for both dengue and chikungunya virus. Ideally all four dengue serotypes would be considered, however resources and logistics may be an issue. Suitable facilities capable of handling the virus would also be required, such as those at the Pirbright Institute (UK).

Research and development at Oxitec Ltd continues into development of transgenic products in *Ae. albopictus.* Although there are still unknowns with regard to certain life history parameters, it is the recommendation of this body of work that future transgenic work be conducted in the KLP WT background, owing to potential insecticide resistance in the MAL WT background. This has already been put into effect, (4.2.2) and other projects are similarly now using the KLP WT background.

Chapter 6: Summary and Final Conclusions

6.1. Introduction

The public health burden caused by arboviral diseases transmitted by *Ae. aegypti and Ae. albopictus* is ever increasing. With potentially more than 50% of the world's population currently living at risk of dengue fever [9][10] and recent epidemic outbreaks of Zika [17] and chikungunya [19] there is an urgent requirement for novel vector control tools to tackle this growing problem. Oxitec Ltd's self-limiting technology is an adaptation of classical SIT, whereby individuals are made to be genetically "sterile" by passing on a lethal gene to the next generation. Oxitec Ltd's 1G technology, the self-limiting strain OX513A, has been shown to reduce populations of *Ae. aegypti* by more than 90% in field trials in three different countries [71][75][76]. Only males are required for a release programme (as they are not vectors of disease), and this is currently achieved through exploiting a size dimorphism between males and females. However the process is costly and results in a small contamination of females in the releases. Oxitec Ltd's 2nd generation technology (2G) has been under development to produce a GSS, OX5034. This will reduce costs and the female contamination risk by killing only the females in the final generation prior to a release. The work presented in this thesis aimed to further the development of Oxitec Ltd's technology in *Ae. aegypti* and *Ae. albopictus.* In *Ae. aegypti* this involved development of the PE concept, a true male sterility phenotype and its combination with a GSS to form Oxitec Ltd's 3G technology. In *Ae. albopictus* development progressed in characterization of the life histories of the wild type strains; a necessary endeavour prior to transgenic development.

6.2. Principal Findings

The work presented in this thesis can be broadly split into two sections, one details the development of PE and 3G technology in *Ae. aegypti;* the other is concerned with characterization of the wild type strains of *Ae. albopictus* currently maintained at Oxitec Ltd.

Section one begins with Chapter 3 and the initial development of PE, confirming the sterility phenotype seen previously [99]. A subsequent 'all in one' PE construct was transformed into *Ae. aegypti* and the repressible sterility phenotype confirmed. In Chapter 4 the PE construct was combined with the GSS construct OX5034 into a single 'all in one' construct. This was transformed into *Ae. aegypti* and successfully tested for functionality; confirming that when reared in the absence of tetracycline only sterile males were produced.

Section 2 is described in Chapter 5, which is split into three separate sub-chapters, each concerned with a different life history characteristic. Chapter 5a is concerned with the mass rearing potential of the WT lines. It was discovered that the REU line performs poorly, but that the other three WT lines all performed to an acceptable standard, with minor differences between them. Chapter 5b characterised the insecticide resistance status of the WT strains. It was discovered that all lines tested showed potential resistance to malathion, and the MAL line showed potential resistance to permethrin. Chapter 5c was unsuccessful in characterizing the diapausing nature of the WT lines, but a working protocol for bleaching eggs was developed which can be employed at a later date.

6.3. Summary and relevance of results

The principal result from Chapter 3 was the development of a functional and repressible PE strain in *Ae. aegypti.* As described previously this was based on the two component tet on/off switch [69], using the sperm specific *topi* promoter to drive tTAV production, and an endonuclease (*fokI*) effector fused to *protamine* effector, downstream of the DNA binding site tetO. Egg hatch assays demonstrated that males that inherited both the promoter and effector constructs were sterile if reared off tet, but fertile if reared on tet. An 'all in one' PE construct (OX5056) containing both these components was transformed into *Ae. aegypti* and exhibited the desired sterility phenotype. It was noted however that there were significant fitness costs associated with the transgene when two copies were inherited. The PE effect construct demonstrated in Chapter 3

represents one of the first 'true' sterility phenotypes using Oxitec Ltd's technology. This is an important milestone as it develops the technology beyond the one component tet on/off system, towards a more tailored and specific approach. OX513A uses a minimal promoter from drosophila (*hsp70*) to drive production of tTAV [66]. As this is downstream of tetO, a positive feedback loop is initiated in the absence of tetracycline, leading to death during development. Other constructs using this architecture were able to restrict expression through careful selection of the promoter used to drive tTAV. The *Actin-4* promoter was able to limit expression to the indirect flight muscles in females in both *Ae. aegypti* [153] and *Ae. albopictus* [81]. Following that success, the PE construct has demonstrated that not only can the choice of promoter be used to specify the expression pattern of the transgene, but that it can also drive phenotypes other than a lethal, in this case sterility.

The primary aim of Chapter 4 was to deliver 3G technology and an improved PE, using sex-specific splicing. Sex-specific splicing involves a gene that contains at least one intron that is spliced depending on the sex of an individual; this results in different transcripts in males and females. This feature can be combined with the tet on/off switch to ensure that tTAV production is limited to only one of the sexes. In the case of 2G technology, OX5034, the sex-specific splicing mechanism is based on the sex determination gene *dsx*. This limits production of tTAV transcript (and the positive feedback loop) to females, resulting in a strain where females die during larval development but males are unaffected. As discussed previously (1.6.2), the major advantage of this technology involves reducing contamination by females prior to a release and cost savings at the operational level. Primarily this is brought about by no longer having to sort male pupae from female pupae prior to a release. A further cost benefit may be seen after a release, whereby the transgene will persist for a number of generations through the male lineage. Whilst this should allow for a more efficient control program through smaller releases, it may be considered an undesirable trait by a regulatory body, and prevent approval for release. The combination of PE

with GSS (3G technology) addresses this issue, as it re-introduces the self-limiting trait associated with 1G technology. A 3G construct (OX5197) was successfully transformed into *Ae. aegypti,* comprising both the PE construct, OX5056, and the GSS construct, OX5034. It was demonstrated that it retained both of the desired traits, a highly penetrant female lethality and male sterility phenotypes, both of which were repressible when reared on tet. Alongside 3G technology, sex-specific splicing was used in an attempt to further limit expression of the PE to the male germline. Off target expression was seen with the original PE construct, OX5056, with lethality seen in both males and females. This is assumed to be a direct result of inheriting two copies of the gene. To address this, a gene was identified which demonstrated sex-specific splicing, specifically to the male germline. This could therefore be used to restrict the production of anything downstream of it to the male germline. After confirming the sex specific nature of gene AAEL008428, it was included in a new PE construct, OX5244. It was predicted that its placement upstream of the *fokI-protamine* component should further restrict expression of the effector to the male germline. Whilst a repressible sterility phenotype was retained, the construct failed for the most part to improve on the fitness effects seen in OX5056. It is unclear at present why this is the case, but molecular analysis should determine whether the transgene is splicing as intended. Two further constructs based on the same architecture have been built. One includes longer flanking exons of the male specific intron, the other contains just the male specific intron, as it is believed the length of the flanking exons may determine how effective the splicing mechanism is.

The demonstrated proof of concept for 3G is an important result in the development of Oxitec Ltd's technology. In the first instance it represents a potentially new product which could be deployed if restrictions are placed on 2G technology. Furthermore it demonstrates that multiple tet on/off switches can be used in a single organism to achieve multiple phenotypic effects. Perhaps most significantly however, is the potential technology transfer to other insect species. In *Ae. aegypti* the PE concept is primarily geared towards addressing a potential regulatory issue. In

agricultural pest species the PE concept is potentially more valuable. Pest species such as *C. capitata* and *Drosophila suzukii* cause huge economic loss through infestation of a wide range of high value fruits [154][155]. The damage of these pests comes primarily during the larval stages as they feed on the crop, though secondary bacterial and fungal infections also cause damage. 1G technology is unsuitable for control of these pests, as size dimorphism cannot be used to separate males from females. Whilst 2G technology has been successful in reducing insect pest populations [71][156], agricultural pests controlled in this manner will still result in damaged fruits. The 2G technology will only affect females at the late larval stage, meaning crops damaged from female and male larvae will still be an issue. A PE construct should exhibit the same level of control, but also reduce the damage to fruits and crops by preventing larvae from hatching. The success of 3G technology in *Ae. aegypti* demonstrates the potential for use in other species, and sets out the potential mechanisms and components that could be used.

The results from Chapter 5 highlight characterization of certain life history traits of the wild type strains of *Ae. albopictus* currently maintained at Oxitec Ltd. As discussed previously, this is a necessary step in product development, to ensure the genetic WT background used for transformation does not contain any negative traits that would be deemed a barrier to release by a regulatory body. The first major project, detailed in Chapter 5a was designed to discern any differences between the wild type strains when reared under mass rearing conditions. Three of the four WT strains performed well under these conditions, any of which could be considered for use as a background strain. The mass rearing project included data collection on pupal development and size during the first four days of pupation. This allowed for construction of a predictive model to assess the predicted female contamination rates and male recovery rates with different gauges of sieve, to represent a sex sort prior to a field release. Whilst this was used to assess differences between lines by comparing the recovery of male pupae, it could also be used to assess changes to the mass rearing protocol. Where sex sorting is necessary (OX513A for

example), any changes in rearing protocols, such as diet or rearing density, are likely to impact on pupal size and development time, both of which will affect the efficacy of the sex sorting system. This model could be used to demonstrate what effects those changes may have and if they should be implemented.

The second project, detailed in Chapter 5b, was designed to examine for signs of insecticide resistance in the four WT *Ae. albopictus* lines. It was demonstrated that all four WT lines appeared to show resistance to the organophosphate malathion. The molecular mechanism of this resistance requires characterisation, but likely comes from elevated carboxylesterase activity or target site resistance in acetylcholinesterase [32] [157]. The MAL WT line was also found to exhibit potential resistance to the pyrethroid permethrin. Molecular confirmation of this resistance is also required, and could come from a number of mechanisms including: upregulation of Cytochrome P450-dependent monooxygenases or Glutathione S-transferases; or target site resistance in the voltage-gated sodium channel [32]. Whilst not a definitive assessment of the insecticide resistance status of the WT lines, these results do demonstrate that insecticide resistance mechanisms can potentially persist in laboratory colonies over many generations. Based on the mass rearing data and the insecticide resistance data it is recommended that any transgenic work be continued in the KLP background rather than the MAL background. However it must be stressed that these results are not a definitive characterization of the WT strains, and further work will be required prior to a regulatory assessment.

The third project detailed in Chapter 5c, attempted to determine the diapausing nature of the WT strains. Poor hatch rates, likely caused by poor egg storage, meant that characterization could not take place. However protocol development for bleaching of the chorion was successful. This should allow a full assessment to take place once suitable egg storage protocols are in place.

6.4. Recommendations for future work

Whilst a number of advancements have been highlighted with regard to Oxitec Ltd's technology, there remain several areas which require further investigation. A number of these are described in Table 28, along with recommendations to address these concerns.

Table 28: Suggestions for further work to address concerns arising from the projects summarised above.

Area of Work	Recommendations
Paternal effect development Off target expression in both females and males remains.	Two further PE constructs have been built which include modified versions of the sex-specific splice-form identified in Chapter 4. One includes the full flanking exon regions and the other includes only the male specific intron. These will require transformation, functionality testing and homozygous viability studies to determine if they have addressed the issue. Additional identification of new sex-specific splice-forms may be required if the two new constructs do not address the off target effects. Further molecular confirmation is needed to address the source of the off target effects. This would involve qRT-PCR of the tTAV and *fokI-protamine* transcripts to

	determine if *topi* is active outside the male germline or if there is low basal expression of the *fokI-protamine*.
3rd Generation technology	
The proof of concept lines will likely exhibit the same off target effects seen in PE lines.	The proof of concept lines require characterization through a homozygous viability study.
***Ae. Albopictus* WT characterisation**	
Poor hatch rates prevented a full diapause characterization from taking place.	Ongoing work at Oxitec Ltd is examining optimum conditions for storing *Aedes* eggs. Once completed a full diapause characterisation of the WT lines can be carried out.
Vector Competence has yet to be characterized.	Vector competence is an important characteristic that requires attention. Project planning is required to identify suitable facilities and collaborators to carry out a full assessment of the vector competence of the WT strains; with an initial focus on Dengue and Chikungunya.
Technology Transfer	
Work to develop GSS in other pest species is ongoing, but development of PE is not as advanced.	The advancement of GSS in other pest species requires the additional development of PE in order to produce 3G technology in other species. Suitable promoter homologues to *topi* should be investigated, particularly with regard to PE development in *Ae. albopictus*, given the success with *Ae. aegypti.*

6.5. Conclusions

This thesis has demonstrated major advancements of Oxitec Ltd's technology. It considers the product development process from the early selection of background wild type strains, through to the proof of concept of new technology. Presented here is the first working example of 3G technology, a fully self-limiting, genetic sexing strain. This technology represents not only a new tool against *Ae. aegypti,* but potentially to other vectors and agricultural pest species.

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Appendix A

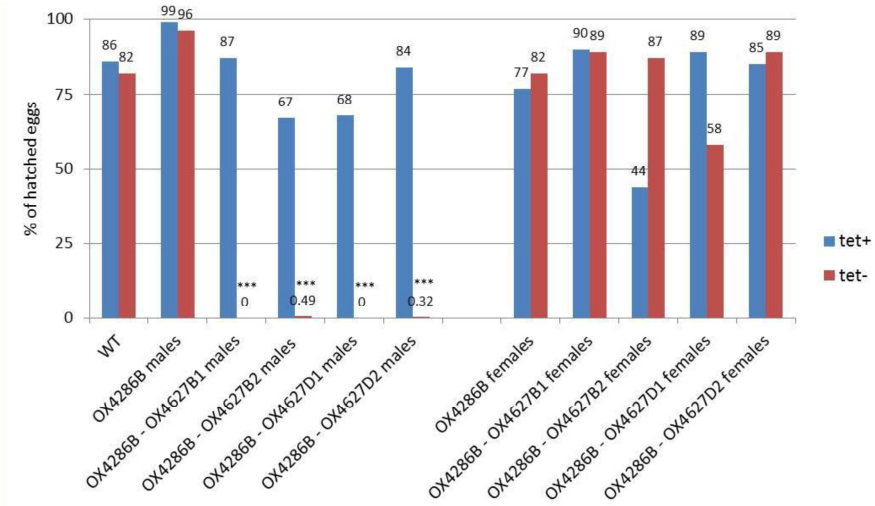


Figure 66: Adapted from Bliski [99]. Hatch-rate assay of *Aedes aegypti* lines carrying both *Topi*-tTAV and tetO-*fokI-protamine* alleles. Progeny of crosses between OX4286B line and OX4627 lines was reared either on a diet with (tet+) or without tetracycline (tet-). Males (or females) carrying both driver and effector alleles were crossed to the wild type and the hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type and OX4286B males' crosses with wild type females were used as controls. Crosses where highly significant male sterility was observed (chi-squared test, P<0.0001) are marked with asterisks.

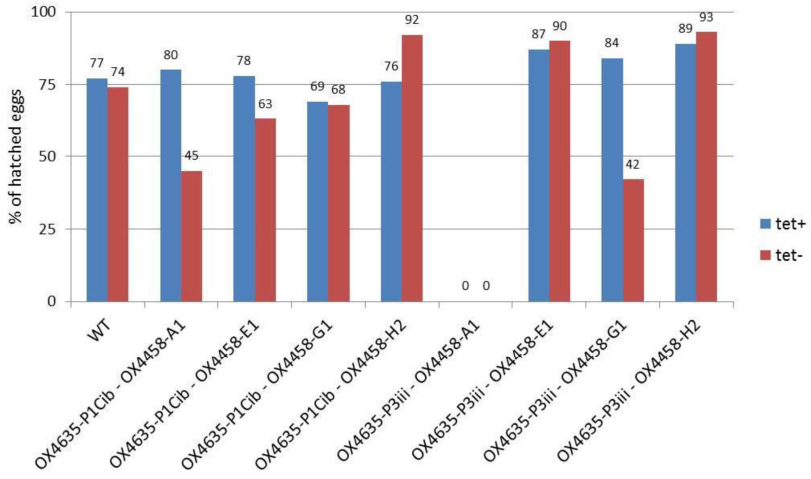


Figure 67: Adapted from Bliski [99]. Hatch-rate assay of *Aedes aegypti* lines carrying both β2-*tubulin*-tTAV and tetO-*Dm-protamine-fokI* alleles. Progeny of crosses between OX4635 and OX4458 lines was reared either on a diet with (tet+) or without tetracycline (tet-). Males carrying both driver and effector alleles were crossed to the wild type females and the hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type males' crosses with wild type females were used as controls.

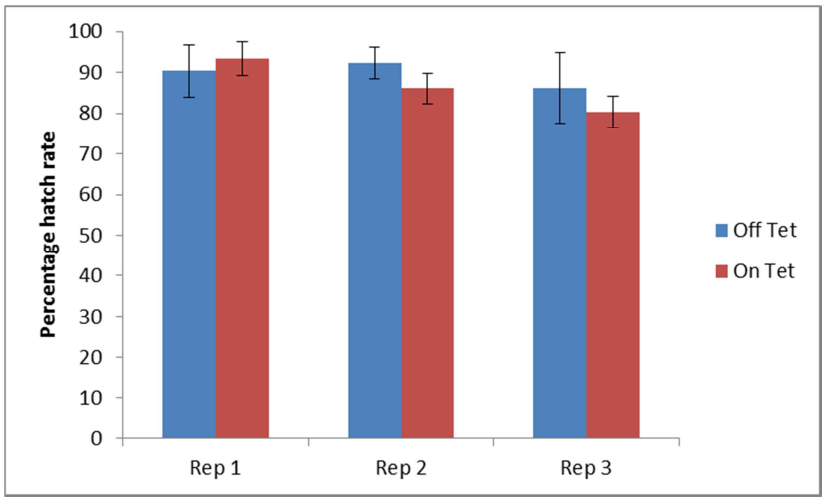


Figure 68: Egg hatch rates of females carrying both the *topi*-tTAV and tetO-*fokI-protamine* paternal effect constructs were found to be above 80% for all replicates both on and off tetracycline, indicating little to no effect on female fecundity.

Table 29: Statistical analysis of OX5056 transgenic lines reared off tetracycline compared to baseline hatch rate of 1%

Line	**Mean hatch percentage** [95% CI]	T value	df	p-value
J2	**1.64** [0.44 - 2.83]	1.1155	18	0.279
A3	**2.46** [-0.22 - 5.13]	1.1418	18	0.269
M2	**18.25** [9.00 - 27.50]	3.9044	19	<0.001
P2	**1.33** [0.63 - 2.03]	0.994	16	0.335
D1	**48.61** [37.02 - 60.20]	8.6646	17	<0.001

Table 30: Statistical analysis of OX5056 transgenic lines reared on tetracycline compared to baseline hatch rate of 80%

Line	**Mean hatch percentage** [95% CI]	T value	df	p-value
J2	**64.49** [53.43 - 75.55]	-2.9461	18	0.009
A3	**71.91** [56.01 - 87.80]	-1.0741	17	0.2978
M2	**78.63** [68.11 - 89.15]	-0.274	18	0.7872
P2	**33.80** [26.48 - 41.13]	-13.2508	18	<0.001
D1	**72.65** [56.91 - 88.38]	-0.9908	16	0.3365

Table 31: Individual and total G tests for homogeneity of fluorescent OX5056_A3 males from a heterozygous cross, adjusted to WT controls.

A3 Males	TG	WT	G-value	d.f.	P value
Trial 1	122	50	1.434	1	0.231
Trial 2	125	50	1.156	1	0.282
Trial 3	155	50	0.041	1	0.840
		total G		3	0.452

Table 32: Individual and total G tests for homogeneity of fluorescent OX5056_A3 females from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

A3 Females	TG	WT		G-value	d.f.	P value
Trial 1	110	50		3.214	1	0.073
Trial 2	81	50		11.160	1	<0.001
Trial 3	129	50		0.836	1	0.361
			Total G		**3**	**0.0016**
Pooled	320	150	**Pooled G**	**11.5454**	**1**	**<0.001**
			Heterogeneity G	3.6643	2	0.1601

Table 33: Individual and total G tests for homogeneity of fluorescent OX5056_J2 males from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

J2 Males	TG	WT		G-value	d.f.	P value
Trial 1	92	50		7.230	1	0.007
Trial 2	73	50		14.290	1	<0.001
Trial 3	88	50		8.716	1	0.003
			Total G		**3**	**0.0017**
Pooled	253	150	**Pooled G**	**29.3493**	**1**	**<0.001**
			Heterogeneity G	0.886	2	0.6421

Table 34: Individual and total G tests for homogeneity of fluorescent OX5056_J2 females from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

J2 Females	TG	WT		G-value	d.f.	P value
Trial 1	102	50		4.815	1	0.028
Trial 2	107	50		3.740	1	0.053
Trial 3	75	50		13.529	1	<0.001
			Total G		**3**	**<0.001**
Pooled	284	150	**Pooled G**	**19.801**	**1**	**<0.001**
			Heterogeneity G	2.2832	2	0.3193

Table 35: Individual and total G tests for homogeneity of fluorescent OX5056_P2 males from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

P2 Males	TG	WT		G-value	d.f.	P value
Trial 1	44	25		4.435	1	0.035
Trial 2	35	25		7.742	1	0.0054
Trial 3	58	25		1.114	1	0.291
			Total G		**3**	**0.004**
Pooled	137	75	**Pooled G**	**11.2938**	**1**	**<0.001**
			Heterogeneity G	1.9963	2	0.3686

Table 36: Individual and total G tests for homogeneity of fluorescent OX5056_J2 females from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

P2 Females	TG	WT		G-value	d.f.	P value
Trial 1	32	25		9.402	1	<0.001
Trial 2	21	25		18.526	1	<0.001
Trial 3	42	25		4.943	1	<0.001
			Total G		**3**	**<0.001**
Pooled	95	75	**Pooled G**	**29.4149**	**1**	**<0.001**
			Heterogeneity G	3.4551	2	0.1777

Appendix B

The statistics summarized here relate to repeated G-tests for homogeneity to test for differences between the numbers of fluorescent pupae counted to the expected numbers from the progeny of the heterozygous crosses of OX5244 (3.3.7).If the total G- score was significant a post-hoc analysis was performed to ascertain if the observed finding was due to differences between the replicates (G-test for independence) or between the counted and expected numbers (pooled G-test).

Table 37: Individual and total G tests for homogeneity of fluorescent OX5244_D1 males from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

D1 Males	TG		WT		G-value	d.f.	P value
Trial 1		28	17.5		4.201	1	0.040
Trial 2		25	17.5		5.382	1	0.020
Trial 3		36	17.5		1.562	1	0.211
				Total G		**3**	**0.0017**
Pooled		89	52.5	**Pooled G**	**10.2947**	**1**	**0.0013**
				Heterogeneity G	3.6643	2	0.1601

Table 38: Individual and total G tests for homogeneity of fluorescent OX5244_D1 females from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

D1 Females	TG		WT		G-value	d.f.	P value
Trial 1		31	17.5		3.051	1	0.081
Trial 2		16	17.5		11.665	1	<0.001
Trial 3		62	17.5		0.429	1	0.512
				Total G		**3**	**0.0017**
Pooled		109	52.5	**Pooled G**	**4.6256**	**1**	**0.0315**
				Heterogeneity G	**10.2947**	**2**	**0.0052**

Table 39: Individual and total G tests for homogeneity of fluorescent OX5244_F3 males from a heterozygous cross, adjusted to WT controls.

F3 Males	TG		WT		G-value	d.f.	P value
Trial 1		74	25		0.003	1	0.956
Trial 2		61	25		0.712	1	0.400
Trial 3		73	25		0.021	1	0.884
				Total G		3	0.865

Table 40: Individual and total G tests for homogeneity of fluorescent OX5244_F3 females from a heterozygous cross, adjusted to WT controls.

F3 Females	TG		WT		G-value	d.f.	P value
Trial 1		55	25		1.641	1	0.200
Trial 2		85	25		0.335	1	0.563
Trial 3		44	25		4.202	1	0.040
				Total G		3	0.103

Table 41: Individual and total G tests for homogeneity of fluorescent OX5244_B3 males from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

B3 Males	TG		WT		G-value	d.f.	P value
Trial 1		68	25		0.164	1	0.686
Trial 2		41	25		5.327	1	0.021
Trial 3		52	25		2.161	1	0.142
				Total G		3	0.054

Table 42: Individual and total G tests for homogeneity of fluorescent OX5244_B3 females from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

B3 Females	TG		WT		G-value	d.f.	P value
Trial 1		47	25		3.273	1	0.070
Trial 2		28	25		12.414	1	<0.001
Trial 3		40	25		5.713	1	0.0170
				Total G		**3**	**<0.001**
Pooled		115	75	**Pooled G**	**19.219**	**1**	**<0.001**
				Heterogeneity G	2.1815	2	0.336

Table 43: Individual and total G tests for homogeneity of fluorescent OX5244_HB2 males from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

HB2 Males	TG		WT		G-value	d.f.	P value
Trial 1		22	25		17.256	1	<0.001
Trial 2		33	25		9.062	1	<0.001
Trial 3		37	25		7.010	1	<0.001
				Total G		**3**	**<0.001**
Pooled		92	75	**Pooled G**	**31.3536**	**1**	**<0.001**
				Heterogeneity G	1.9746	2	0.3726

Table 44: Individual and total G tests for homogeneity of fluorescent OX5244_HB2 females from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

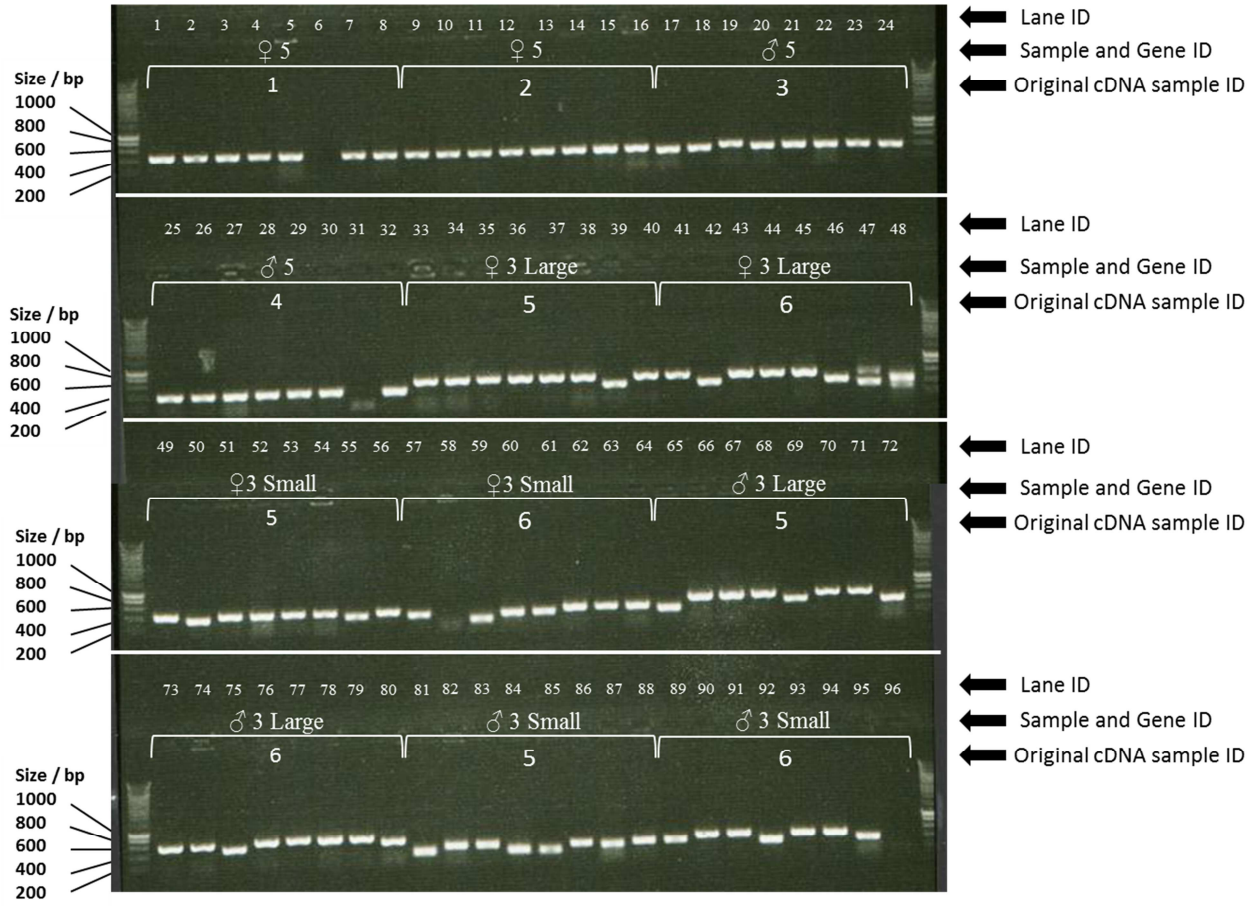
HB2 Females	TG	WT		G-value	d.f.	P value
Trial 1	25	25		14.384	1	<0.001
Trial 2	24	25		15.550	1	<0.001
Trial 3	27	25		13.164	1	<0.001
			Total G		**3**	**<0.001**
Pooled	76	75	**Pooled G**	43.0117,	**1**	<0.001
			Heterogeneity G	0.0868	2	0.9575

Table 45: Individual and total G tests for homogeneity of fluorescent OX5244_E2 males from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

E2 Males	TG	WT		G-value	d.f.	P value
Trial 1	33	25		8.984	1	0.0027
Trial 2	40	25		5.713	1	0.0168
Trial 3	48	25		3.048	1	0.0808
			Total G		**3**	**<0.001**
Pooled	121	75	**Pooled G**	**16.6497**	**1**	**<0.001**
			Heterogeneity G	1.0955	**2**	0.5783

Table 46: Individual and total G tests for homogeneity of fluorescent OX5244_E2 males from a heterozygous cross, adjusted to WT controls. Heterogeneity and pooled G tests were performed to indicate the source of a significant Total G test for homogeneity.

E2 females	TG	WT		G-value	d.f.	P value
Trial 1	36	25		7.600	1	0.0059
Trial 2	29	25		11.636	1	<0.001
Trial 3	46	25		3.644	1	0.0563
			Total G		**3**	**<0.001**
Pooled	111	75	**Pooled G**	**20.7901**	**1**	<0.001
			Heterogeneity G	1.4732	**2**	0.4787



Sample	Expected band size / bp Gene ID		
	3 (Small)	3 (Large)	5
Male	274	407	165
Female	274	407	218

Figure 69: PCR of cDNA inserts from bacterial colonies used to create minipreps for sequencing. The original cDNA sample refers to the lane number of Figure 38 which were ligated into bacterial colonies. For gene ID 3, two bands were seen in Figure 38 and both were ligated into bacteria for sequencing. Small and large refer to the corresponding band ligated. It is assumed that the smaller band has the intron spliced out, and the larger band has not spliced the intron. For gene 5 samples: 1, 2, 9, 10, 17, 18, 25 and 26 were chosen for sequencing. For gene 3 samples: 33, 34, 41, 43, 50, 51, 55, 61, 63, 64, 66, 69, 73, 74, 76, 77, 82, 83, 84, 85, 90, 92, 93 and 95 were chosen for sequencing.

Appendix C

Mixed effect model formula:

$$lmer(Size \sim Int + (1|Tray) - 1)$$

- lmer – Linear mixed effect model
- Size – Pupal Size
- Int – Individual factor combining Sex, day and line into one variable (e.g. HAW Males on Day 3)
- 1|Tray – Random effect of tray (Replicate)

Density was not included in the linear mixed effect model due to experimental design. The fitted model was shown to be meaningful in representing the original data, as seen by the plots of fitted values for the random effect split by the fixed effect predictors for each rearing density (Figure 70, Figure 75 and Figure 80). Tukey Anscombe plots of fitted vs residual values indicate an even variance of the residuals, seen by even distribution around 0, for each density (Figure 71, Figure 76 and Figure 81). QQ plots of the residuals indicate the errors are normally distributed, visualised by a straight line for each density (Figure 72, Figure 77 and Figure 82). Variance of the residuals also appears to be consistent across the fixed effect predictors (Figure 73, Figure 78 and Figure 83) and the random effect (Figure 74, Figure 79 and Figure 84) at each density.

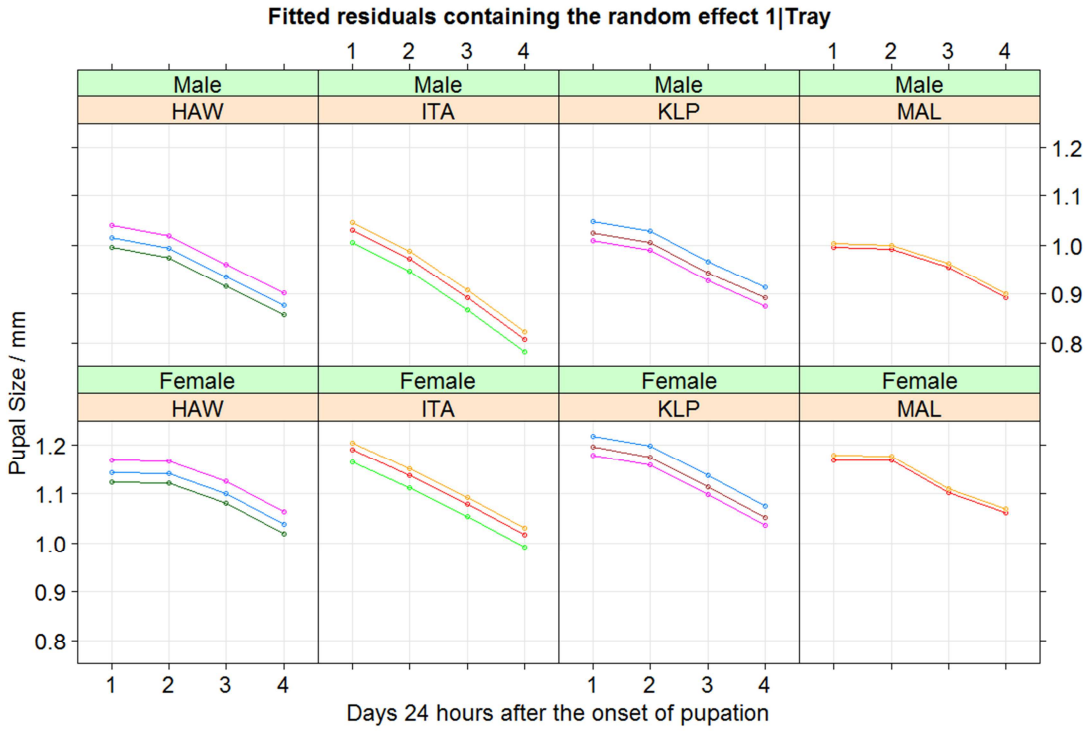


Figure 70: Fitted values of the mixed effects model for the random effect of tray within each set of fixed effect predictors (Day, Line and Sex), from 2 lar / ml data.

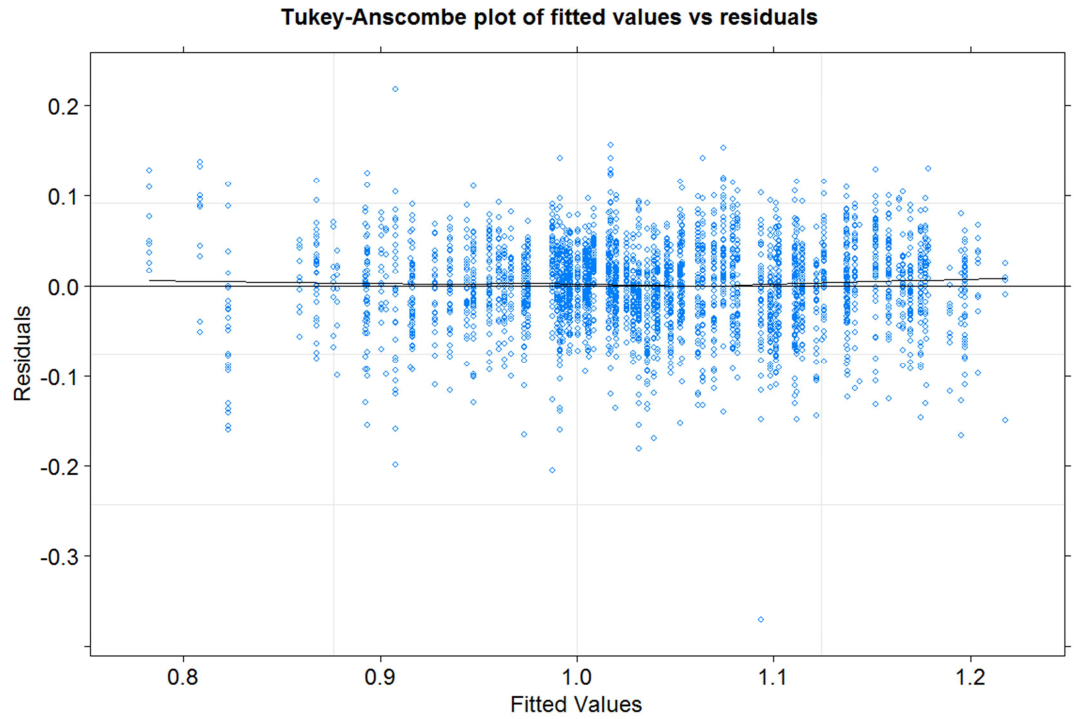


Figure 71: Tukey Anscombe plot of fitted values against residuals for the mixed effects model, from 2 lar / ml data.

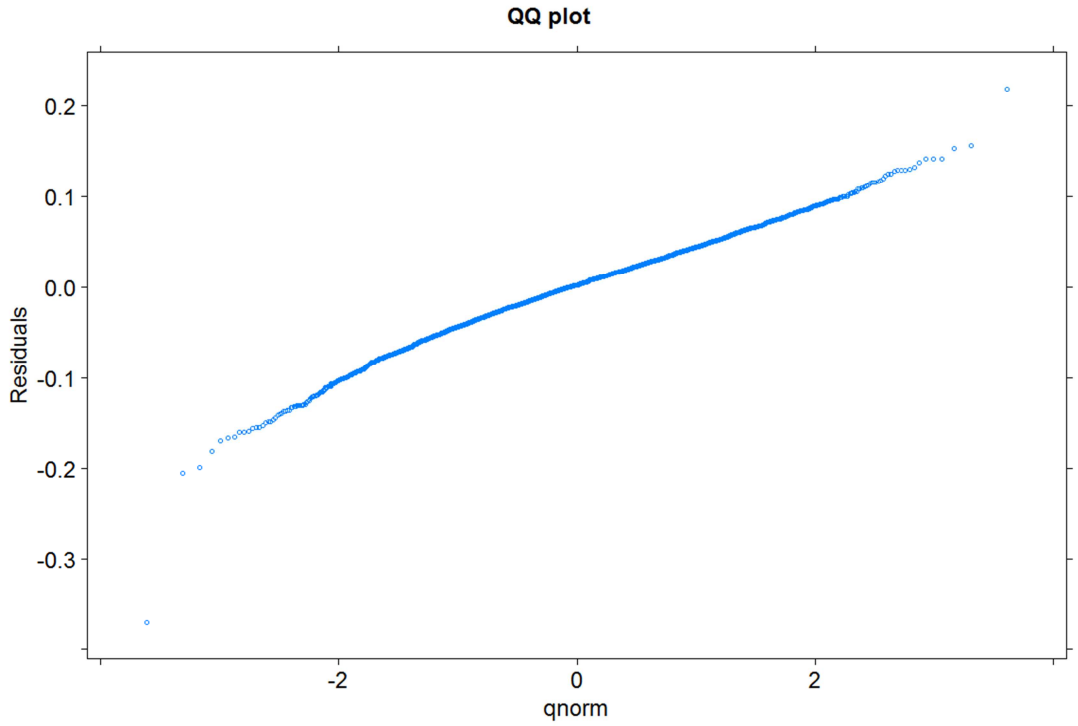


Figure 72: QQ Plot of fitted residuals, from 2 lar / ml data.

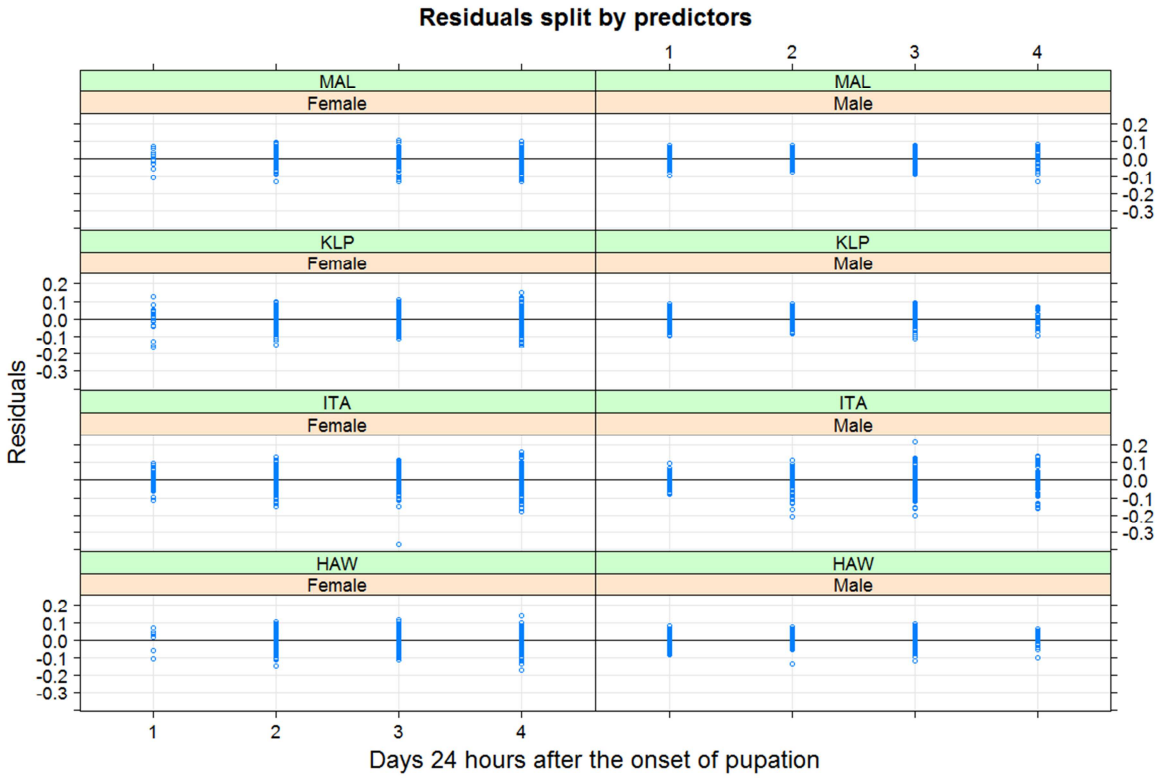


Figure 73: Residuals split by the fixed effect predictors (Day, Line and Sex), from 2 lar / ml data.

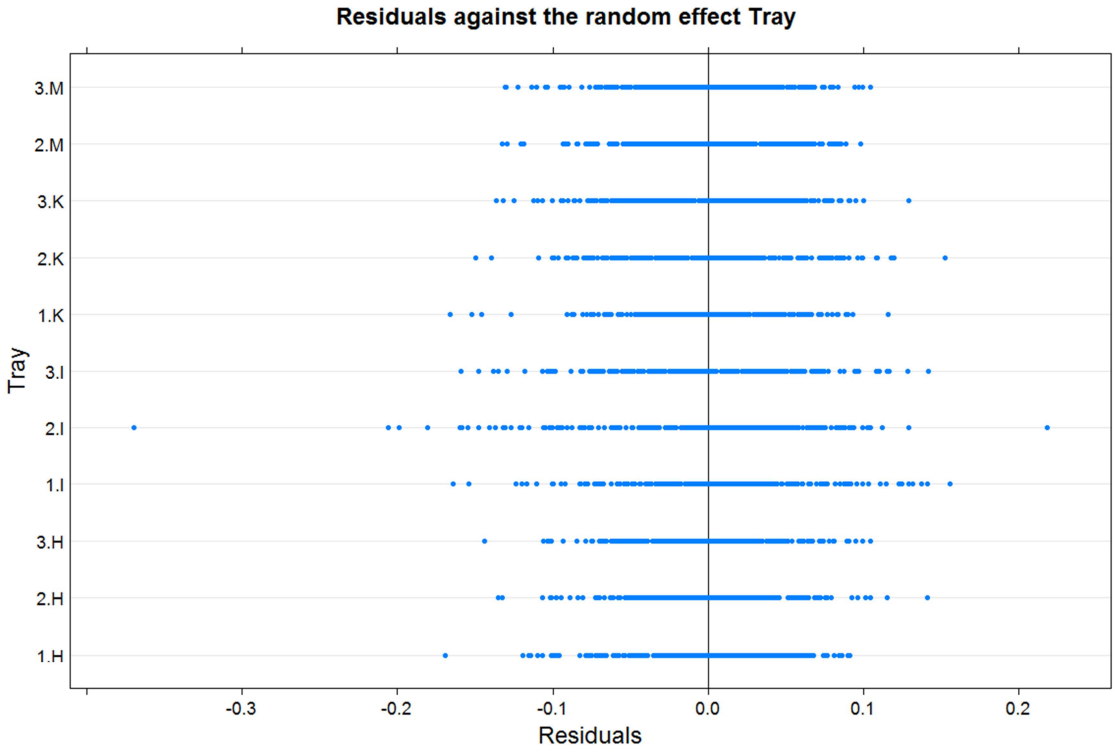


Figure 74: Residuals split by the random effect predictor (Tray), from 2 lar / ml data.

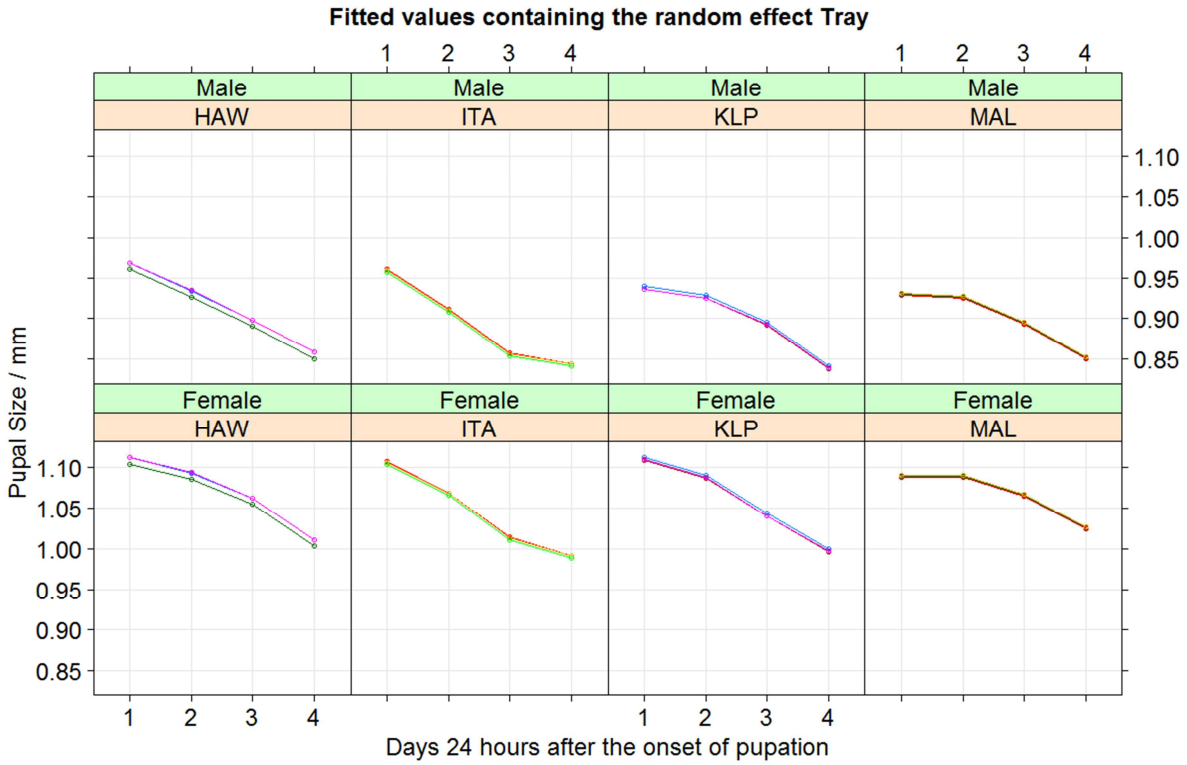


Figure 75: Fitted values of the mixed effects model for the random effect of tray within each set of fixed effect predictors (Day, Line and Sex), from 3 lar / ml data.

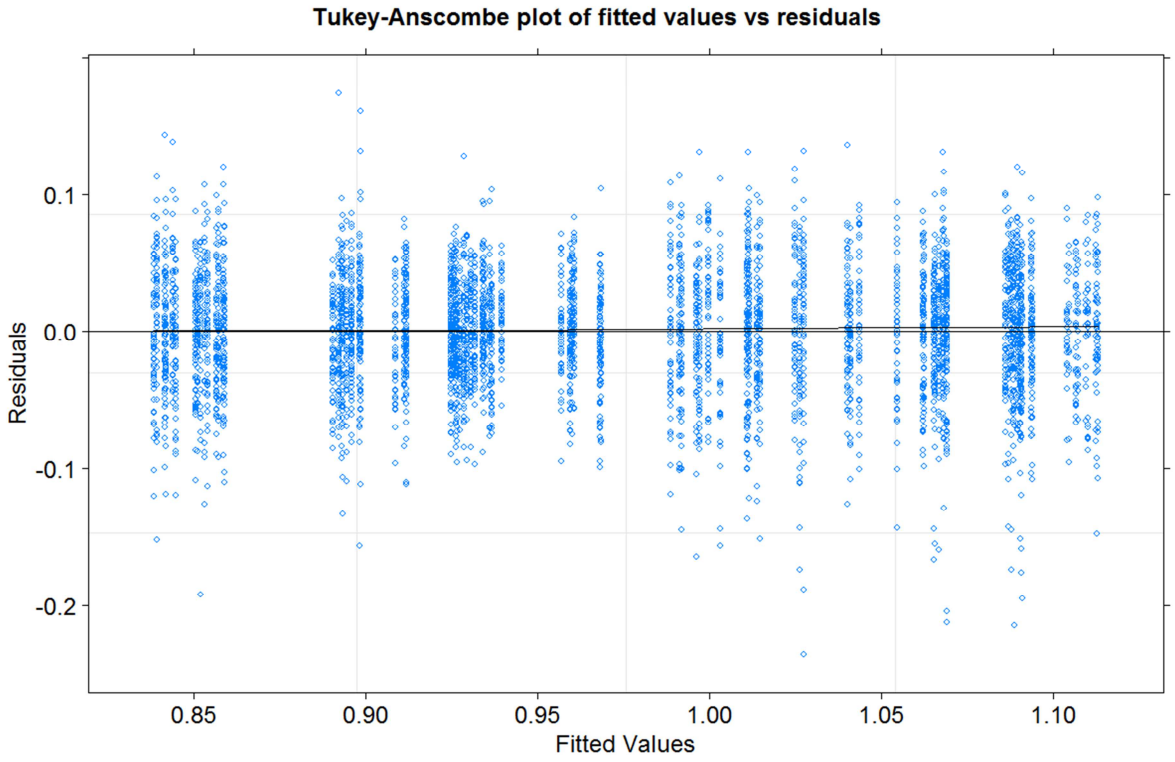


Figure 76: Tukey Anscombe plot of fitted values against residuals for the mixed effects model, from 3 lar / ml data.

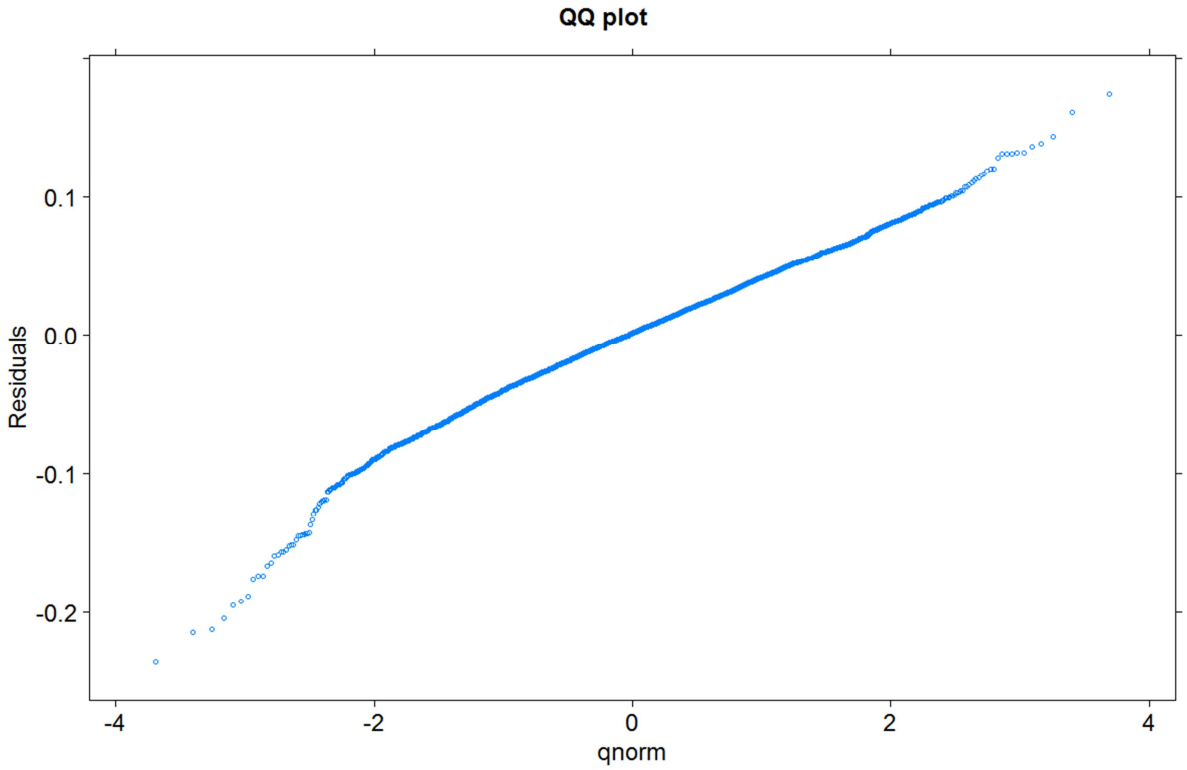


Figure 77: QQ Plot of fitted residuals, from 3 lar / ml data.

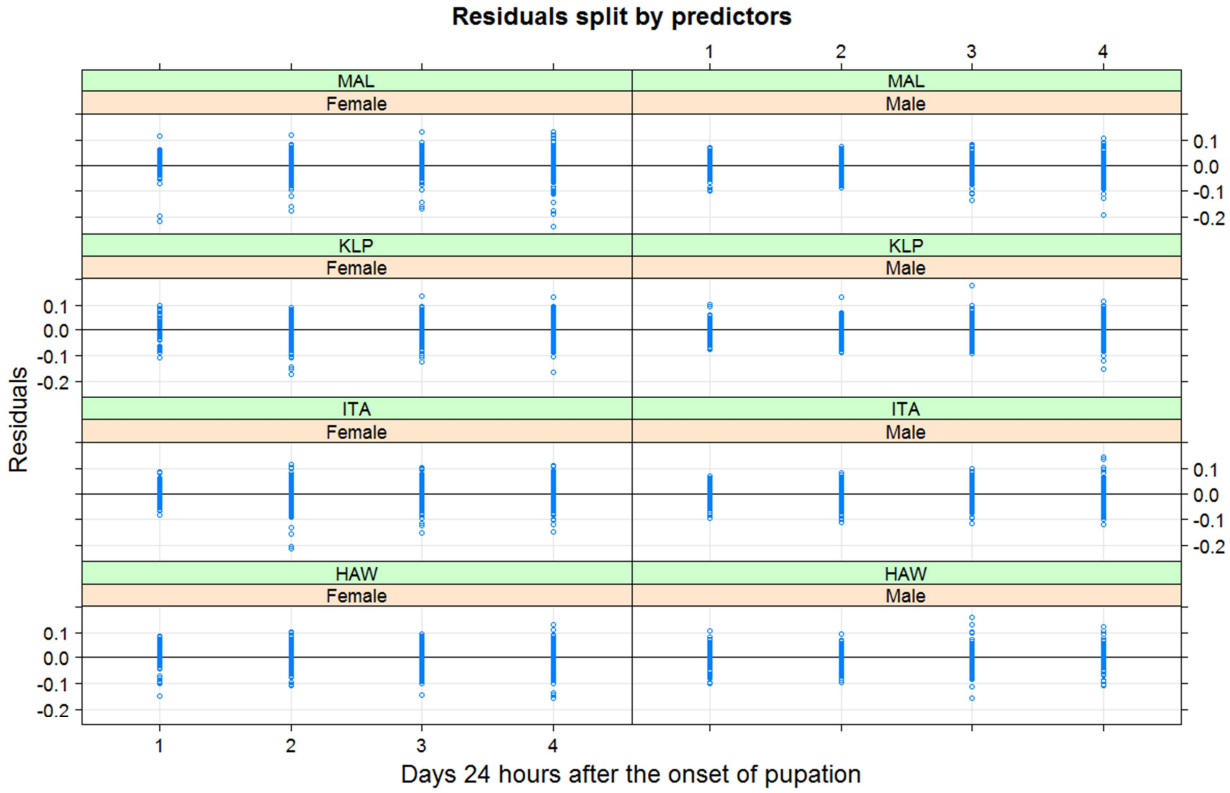


Figure 78: Residuals split by the fixed effect predictors (Day, Line and Sex), from 3 lar / ml data

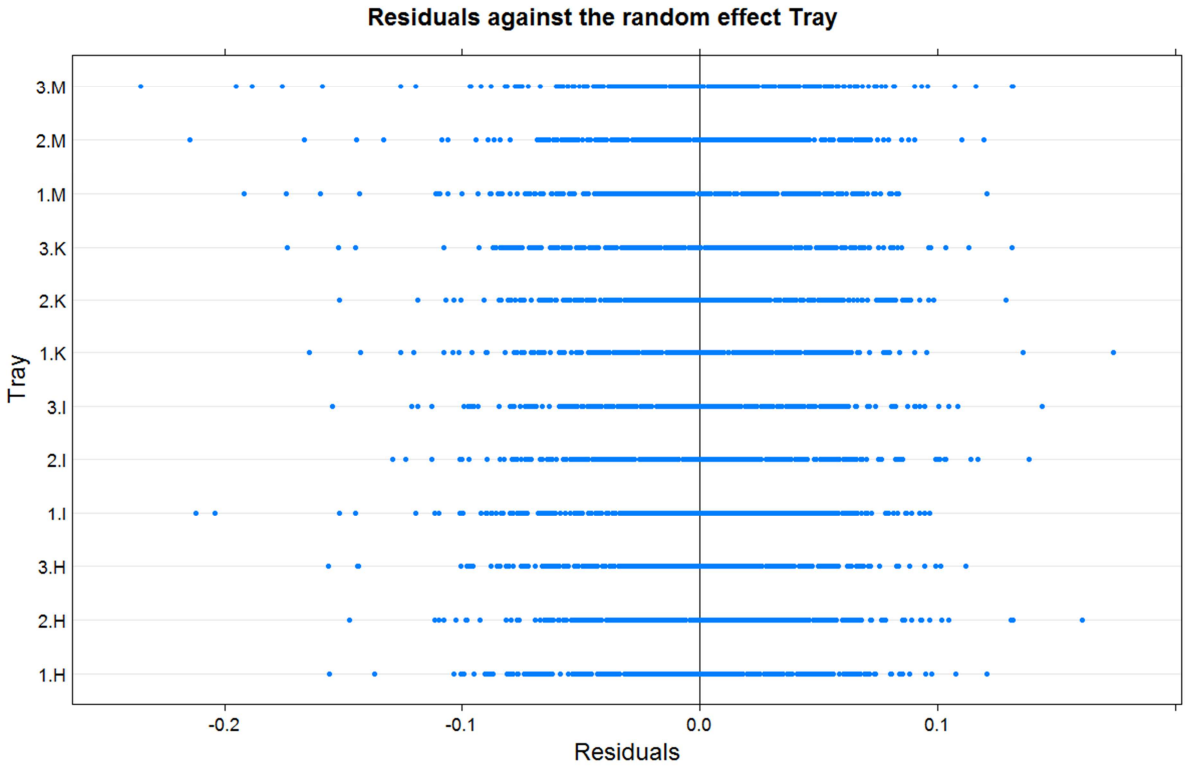


Figure 79: Residuals split by the random effect predictor (Tray), from 3 lar / ml data.

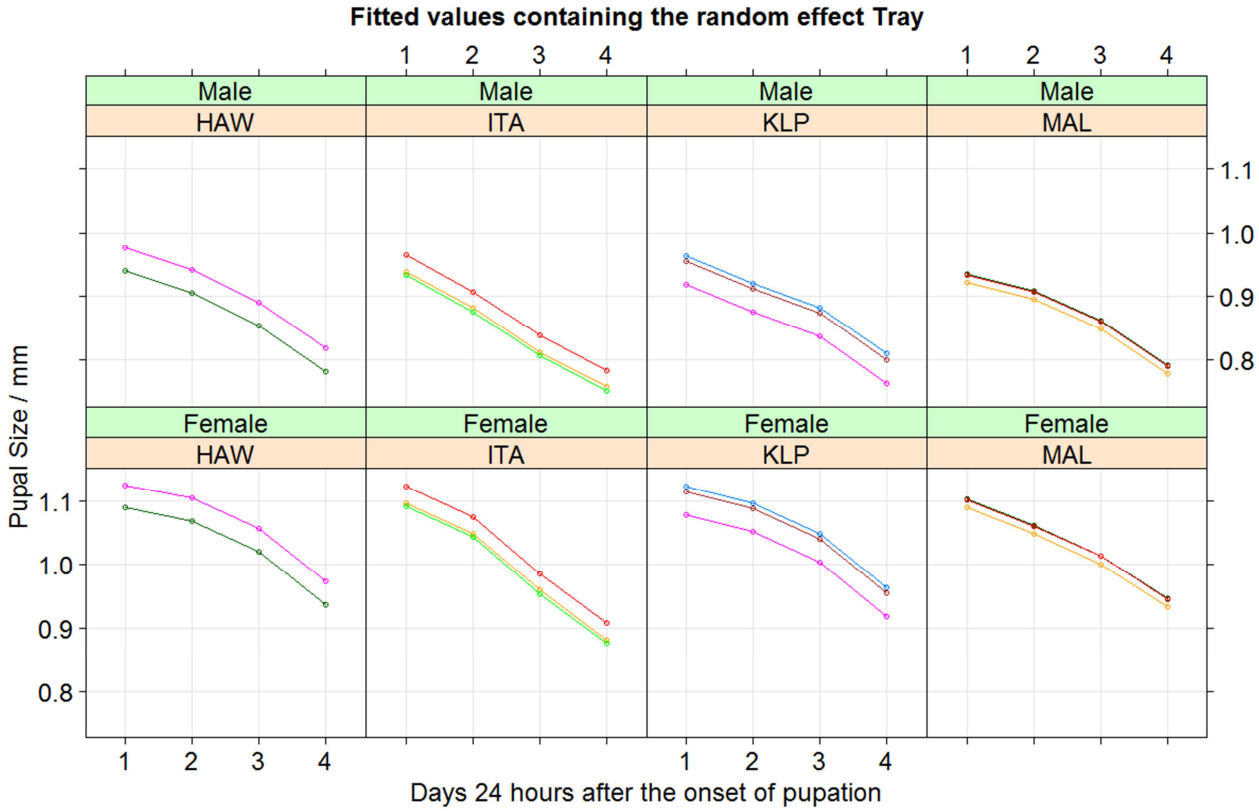


Figure 80 Residuals split by the fixed effect predictors (Day, Line and Sex), from 4 lar / ml data.

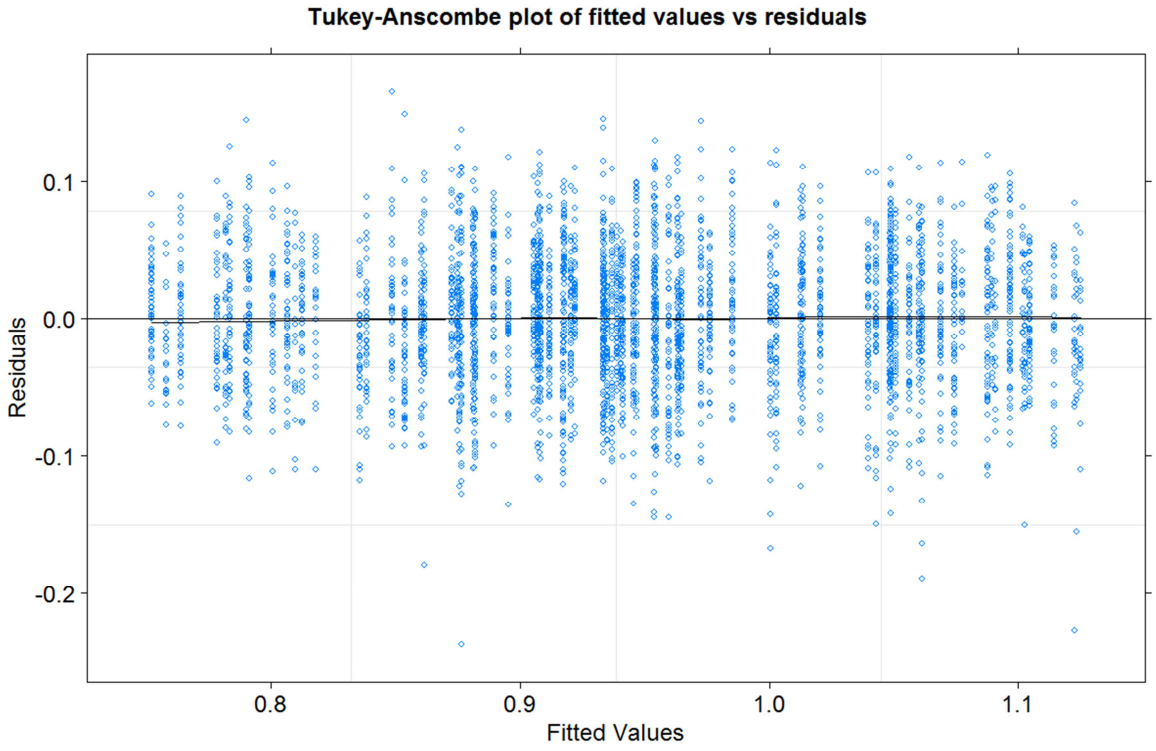


Figure 81 Tukey Anscombe plot of fitted values against residuals for the mixed effects model, from 4 lar / ml data.

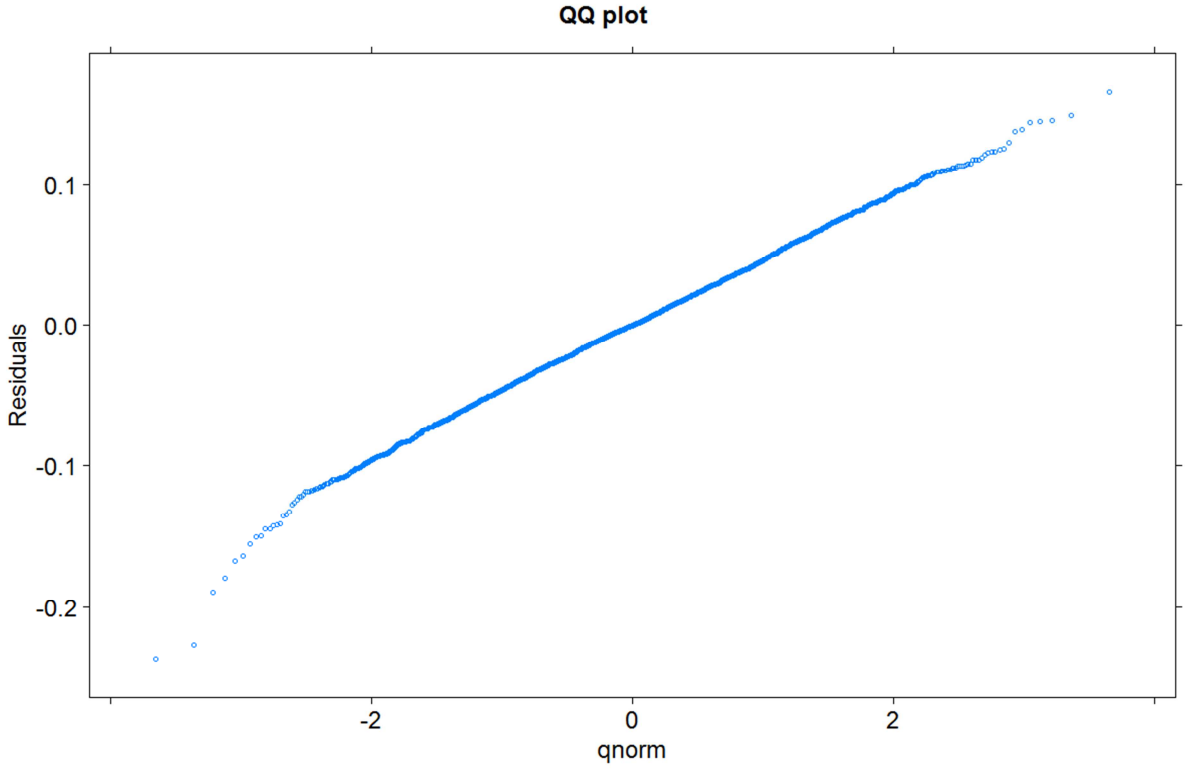


Figure 82: QQ Plot of fitted residuals, from 4 lar / ml data.

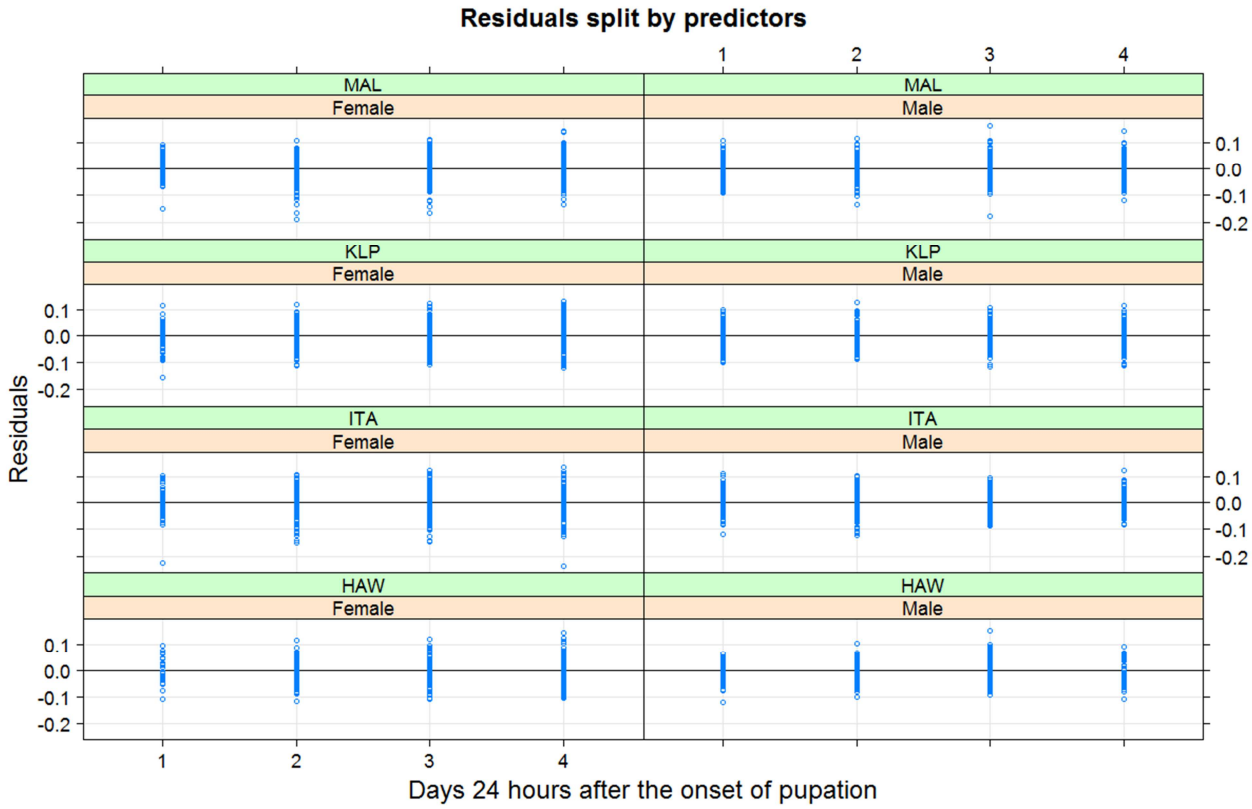


Figure 83 Residuals split by the fixed effect predictors (Day, Line and Sex), from 4 lar / ml data.

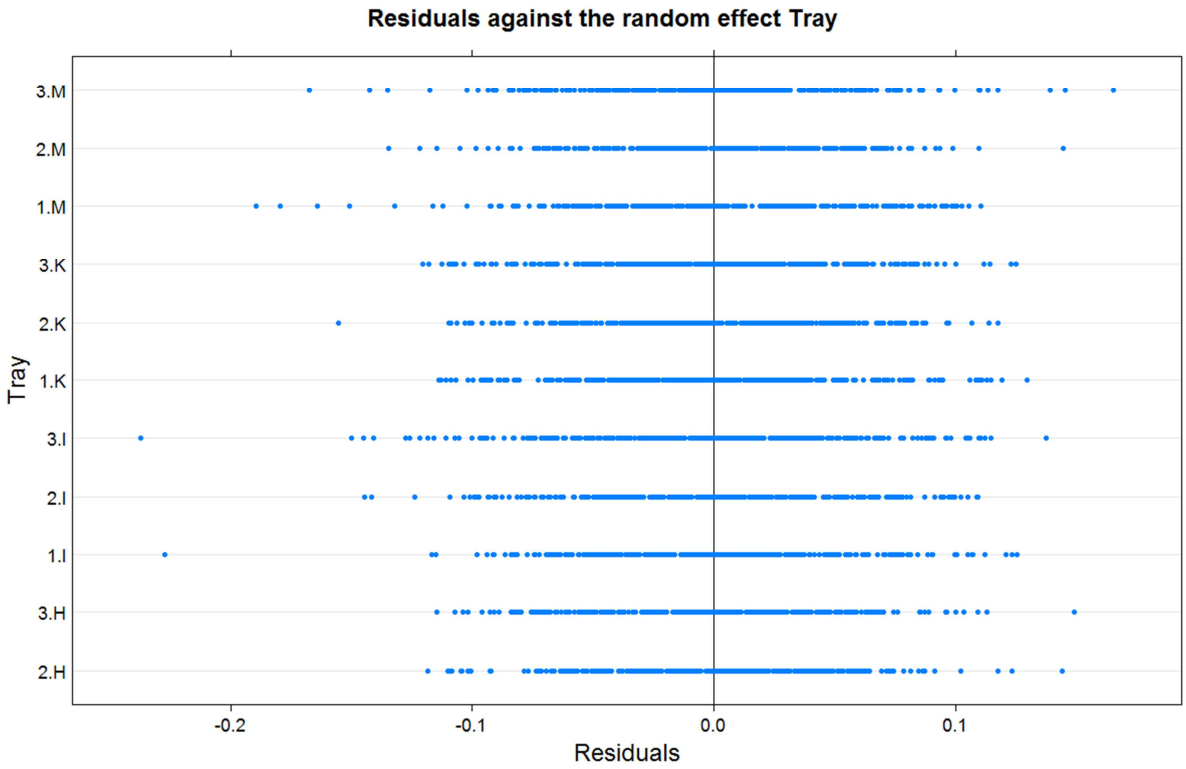


Figure 84: Residuals split by the random effect predictor (Tray), from 4 lar / ml data.

Appendix D

ForLoop.R

Edward Sulston

Thu Jul 14 13:56:25 2016

```
######2 L ml-1
ds<-read.csv("D:/Users/Edward Sulston/Documents/Work/Mosquito Work - Oxi
/Mosquito Work - Oxi/Albo/Infravec/Data/Larval Experiments/2Lml-1/Pupae2
lml.csv")

###### 3 L ml-1
ds<-read.csv("D:/Users/Edward Sulston/Documents/Work/Mosquito Work - Oxi
/Mosquito Work - Oxi/Albo/Infravec/Data/Larval Experiments/3Lml-1/Pupae3
lml.csv")


###### 4 L ml-1
ds<-read.csv("D:/Users/Edward Sulston/Documents/Work/Mosquito Work - Oxi
/Mosquito Work - Oxi/Albo/Infravec/Data/Larval Experiments/4Lml-1/Pupae4
lml.csv")



############ Set the acceptable contamination rate of females in the rec
overed males

Cont<-(0.5)

############ Create Empty Vectors for the For-loop
### Vectors are required for each line + sex
##### M=MAL; K=KLP; I=ITA; H=HAW ------ m = Male; f=Female

Mm<-vector()
Mf<-vector()
Hm<-vector()
Hf<-vector()
Km<-vector()
Kf<-vector()
Im<-vector()
If<-vector()

MaleM<-vector()
FemaleM<-vector()

MaleK<-vector()
FemaleK<-vector()

MaleI<-vector()
```

```
FemaleI<-vector()

MaleH<-vector()
FemaleH<-vector()


########## Set the Day (1-4) of pupae to be tested
Day.Set=c(1,2)
#################
#### Total number of males needs to be known to calculate % recovered th
rought the sieve

Mtot<-subset(ds,ds$Line=="M" & (ds$Day==Day.Set[1] | ds$Day==Day.Set [2]
)  & ds$Sex=="M")
Ktot<-subset(ds,ds$Line=="K" & (ds$Day==Day.Set[1] | ds$Day==Day.Set [2]
) & ds$Sex=="M")
Htot<-subset(ds,ds$Line=="H" & (ds$Day==Day.Set[1] | ds$Day==Day.Set [2]
) & ds$Sex=="M")
Itot<-subset(ds,ds$Line=="I" & (ds$Day==Day.Set[1] | ds$Day==Day.Set [2]
) & ds$Sex=="M")

min<-min(ds[,6])
max<-max(ds[,6])


#########################################################################
#
####################    Begin Loop
#################################
#### For Loop, runs values of smallest to largest pupae to represent dif
ferent guages of sieve.

i = seq(min,max, by=0.01)
for (n in 1:length(i)) {

  #############
  ############ Number of males smaller than the sieve size calculated
  Mm[n] = nrow(subset(ds,ds$Line=="M" & (ds$Day==Day.Set[1] | ds$Day==Da
y.Set [2]) & ds$Sex=="M" & ds$Size<i[n]))

  #############
  ############ % of males recovered calculated
  MaleM[n]<-Mm[n]/nrow(Mtot)*100

  ################
  ################ Number of females smaller than sieve size calculated
  Mf[n] = nrow(subset(ds,ds$Line=="M"& (ds$Day==Day.Set[1] | ds$Day==Day
.Set [2]) & ds$Sex=="F" & ds$Size<i[n]))

  #############
  ############# Female contamination % of recovered males calculated
  FemaleM[n]<-(Mf[n]/(Mm[n]))*100
```

```
  ##############
  ############## Repeat process for each line

  Km[n] = nrow(subset(ds,ds$Line=="K" & (ds$Day==Day.Set[1] | ds$Day==Da
y.Set [2]) & ds$Sex=="M" & ds$Size<i[n]))
  MaleK[n]<-Km[n]/nrow(Ktot)*100

  Kf[n] = nrow(subset(ds,ds$Line=="K"& (ds$Day==Day.Set[1] | ds$Day==Day
.Set [2]) & ds$Sex=="F" & ds$Size<i[n]))
  FemaleK[n]<-(Kf[n]/(Km[n]))*100

  Hm[n] = nrow(subset(ds,ds$Line=="H" & (ds$Day==Day.Set[1] | ds$Day==Da
y.Set [2])& ds$Sex=="M" & ds$Size<i[n]))
  MaleH[n]<-Hm[n]/nrow(Htot)*100

  Hf[n] = nrow(subset(ds,ds$Line=="H"& (ds$Day==Day.Set[1] | ds$Day==Day
.Set [2]) & ds$Sex=="F" & ds$Size<i[n]))
  FemaleH[n]<-(Hf[n]/(Hm[n]))*100

  Im[n] = nrow(subset(ds,ds$Line=="I" & (ds$Day==Day.Set[1] | ds$Day==Da
y.Set [2]) & ds$Sex=="M" & ds$Size<i[n]))
  MaleI[n]<-(Im[n]/nrow(Itot)*100)

  If[n] = nrow(subset(ds,ds$Line=="I"& (ds$Day==Day.Set[1] | ds$Day==Day
.Set [2]) & ds$Sex=="F" & ds$Size<i[n]))
  FemaleI[n]<-(If[n]/(Im[n]))*100
}
############################
############################ End Loop

sessionInfo()

## R version 3.1.2 (2014-10-31)
## Platform: x86_64-w64-mingw32/x64 (64-bit)
##
## locale:
## [1] LC_COLLATE=English_United Kingdom.1252
## [2] LC_CTYPE=English_United Kingdom.1252
## [3] LC_MONETARY=English_United Kingdom.1252
## [4] LC_NUMERIC=C
## [5] LC_TIME=English_United Kingdom.1252
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## loaded via a namespace (and not attached):
##  [1] digest_0.6.8     evaluate_0.9     htmltools_0.3.5  knitr_1.12.3
##  [5] magrittr_1.5     Rcpp_0.12.4      rmarkdown_0.3.11 stringi_1.0-1
##  [9] stringr_1.0.0    tools_3.1.2
```

Appendix E

Dose response model of female contamination and male recovery from different hypothetical sieve sizes.

$$drm\left(Value \sim Size, Int, data = df, fct = LL.5\left(fixed = c(NA, NA, NA, NA, NA)\right)\right)$$

- drm: dose response model
- Value: Female contamination or Male recovery percentage
- Size: Hypothetical sieve size
- Int: Interaction factor of Line and Sex
- df: Data frame containing: Value, Size and Int
- LL.5: Log logistic 5 parameter model, with unspecified parameters.

Diagnostic plots

Across all three densities the fitted model was shown to be meaningful in representing the original data, as seen by the plots of fitted values for the predictors for each rearing density (Figure 85, Figure 89 and Figure 93). Tukey Anscombe plots of fitted vs residuals indicate normal distribution that is heavily weighted at fitted values 0 and 100 for each rearing density (Figure 86, Figure 90 and Figure 94). QQ plots of the residuals indicate the errors are normally distributed, visualised by a roughly straight line for each rearing density (Figure 87, Figure 91 and Figure 95). Visualization of the residuals split by the predictors highlights the issue of not normally distributed residuals for high and low values of size in males and low values of size in females (Figure 88, Figure 92 and Figure 96).

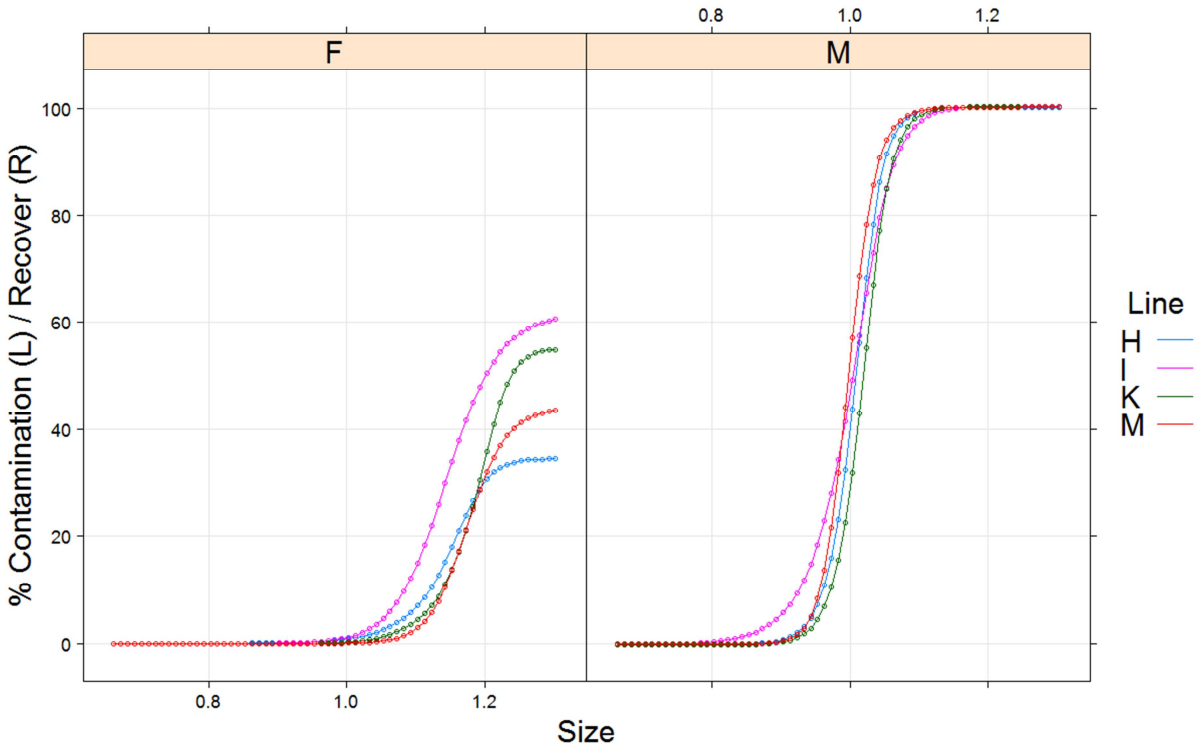


Figure 85: Fitted values of the dose response model for the predictors (Size, Line and Sex), from 2 lar / ml data.

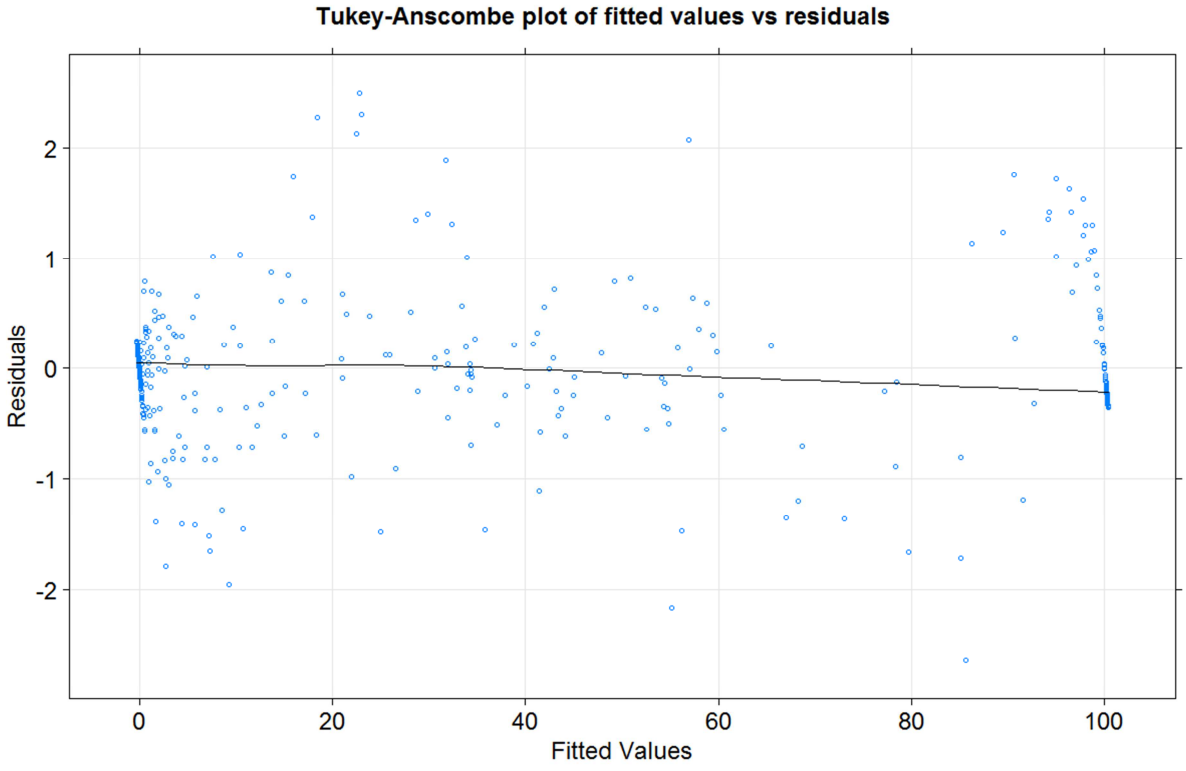


Figure 86: Tukey Anscombe plot of fitted values against residuals for the mixed effects model, from 2 lar / ml data.

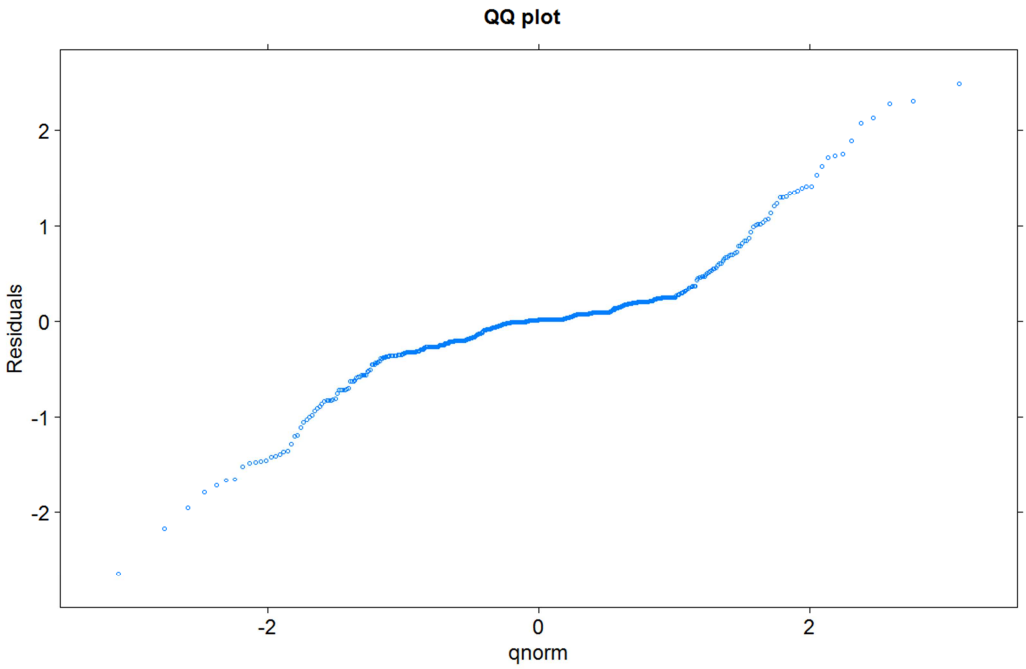


Figure 87: QQ Plot of fitted residuals, from 2 lar / ml data.

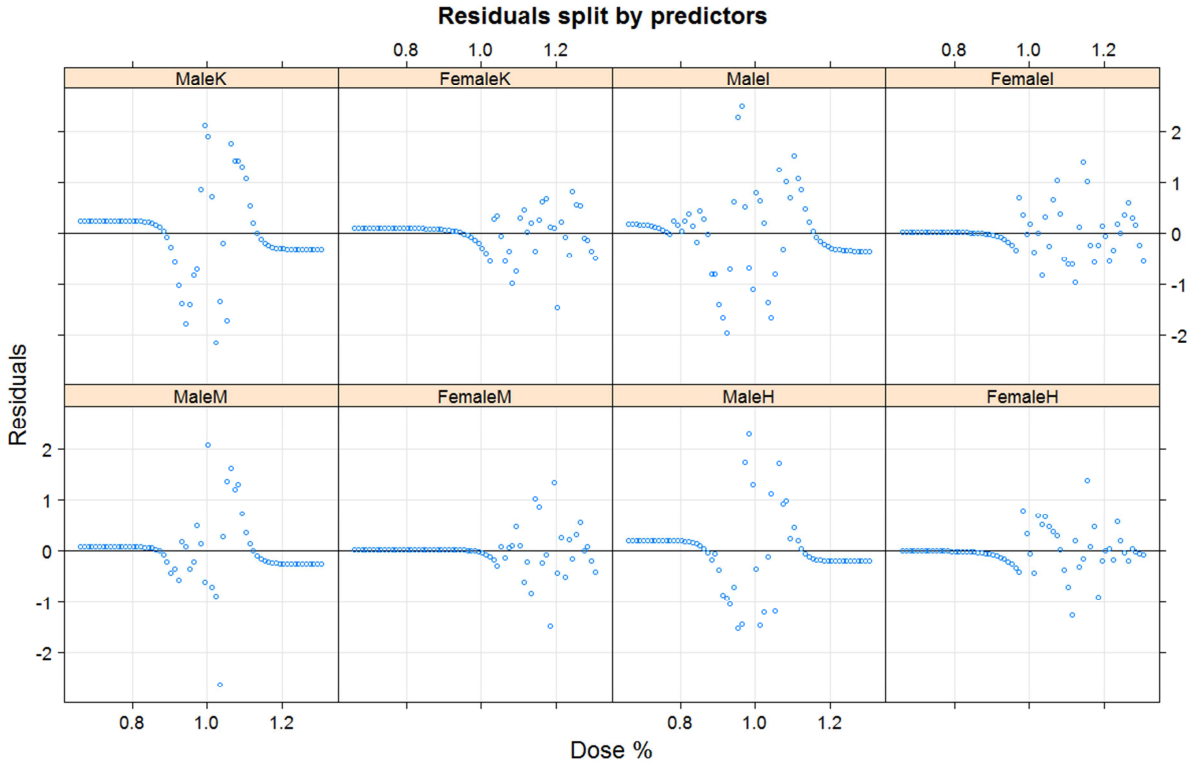


Figure 88: Residuals split by the fixed effect predictors (Size, Line and Sex), from 2 lar / ml data.

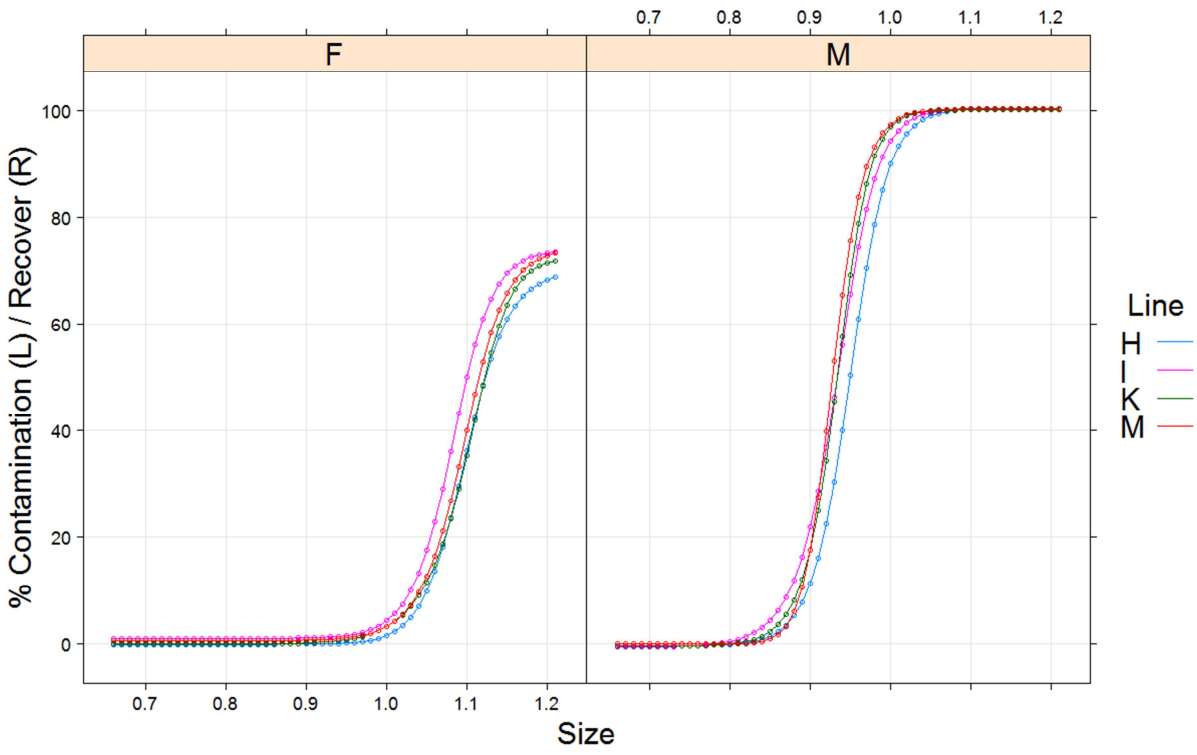


Figure 89: Fitted values of the dose response model for the predictors (Size, Line and Sex), from 3 lar / ml data.

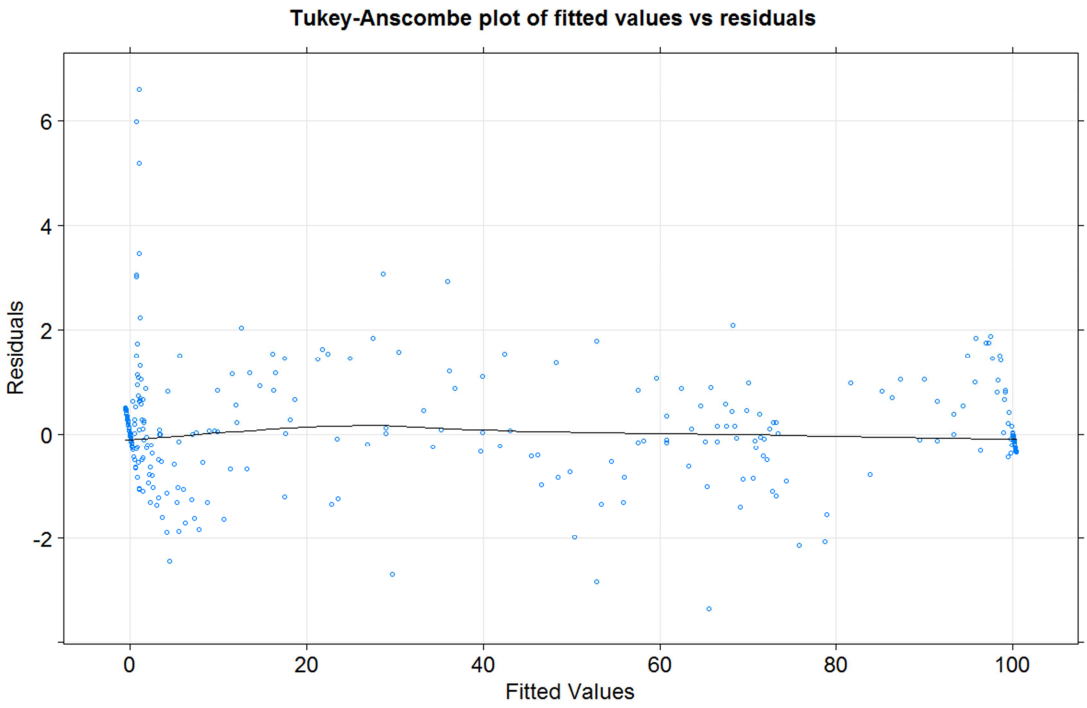


Figure 90: Tukey Anscombe plot of fitted values against residuals for the mixed effects model, from 3 lar / ml data.

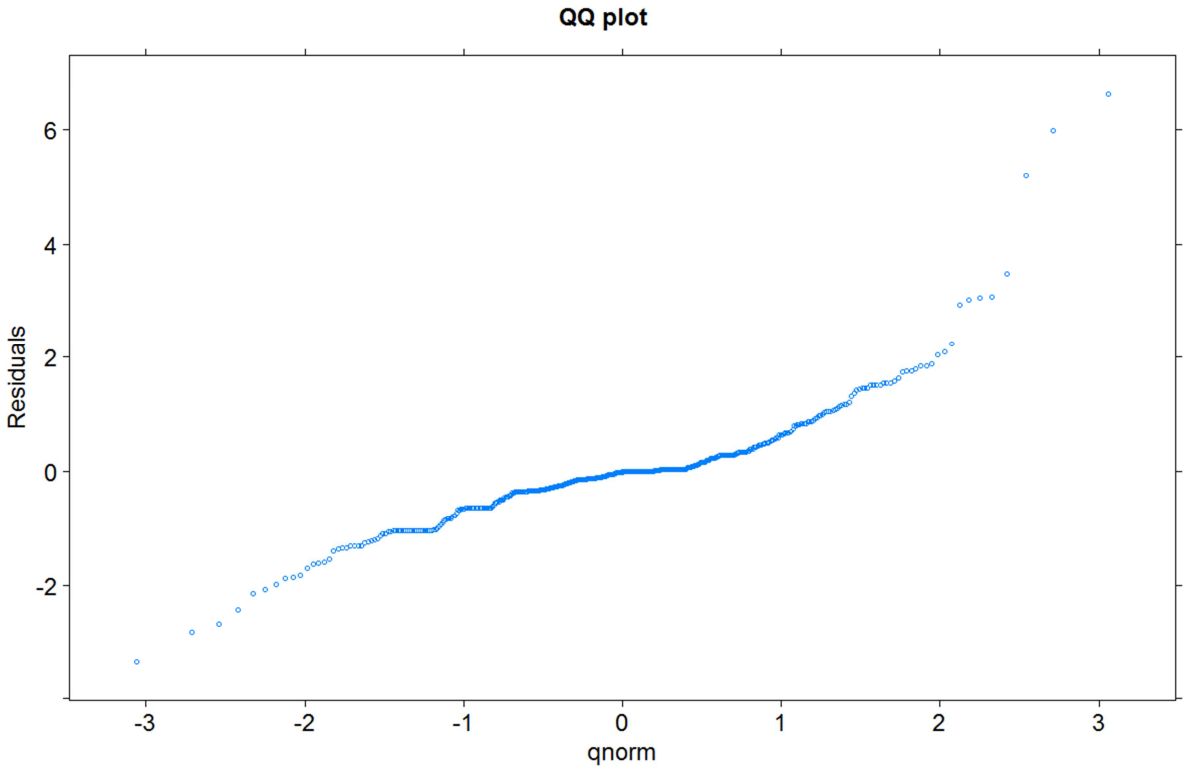


Figure 91: QQ Plot of fitted residuals, from 3 lar / ml data.

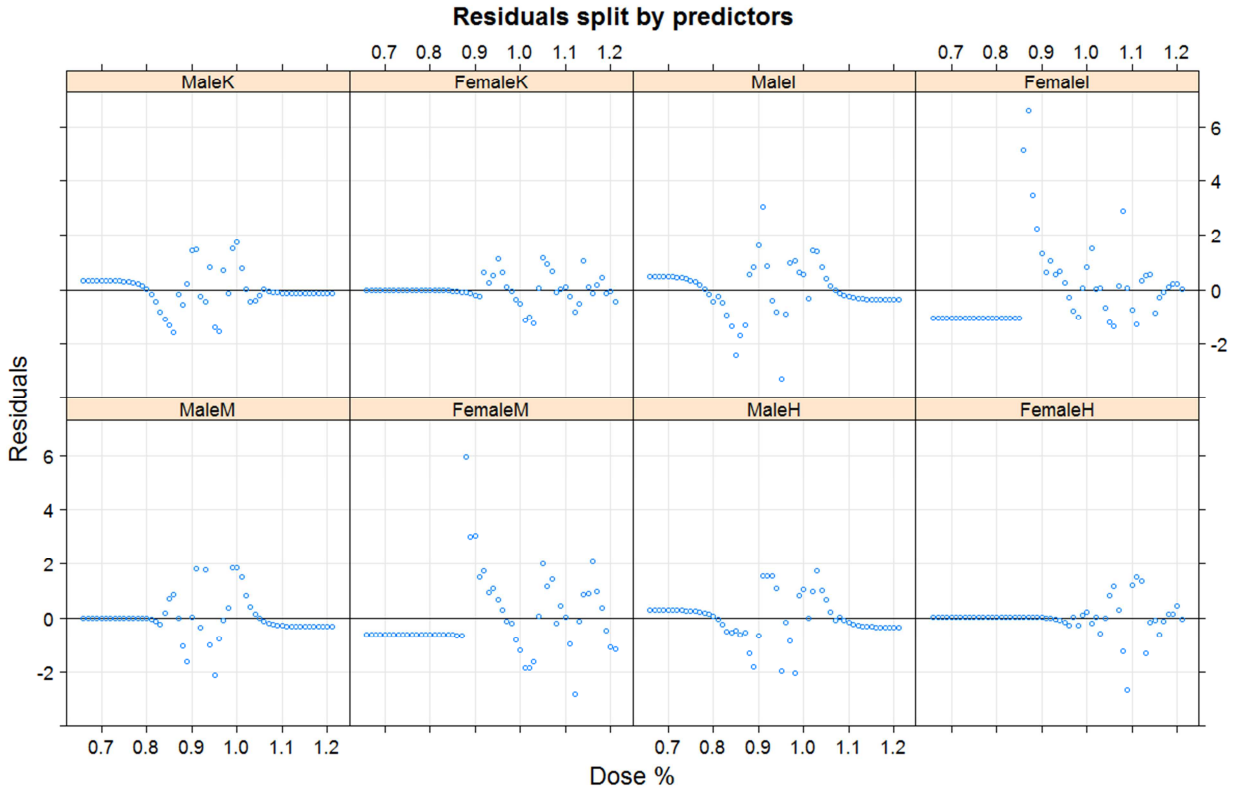


Figure 92: Residuals split by the fixed effect predictors (Size, Line and Sex), from 3 lar / ml data

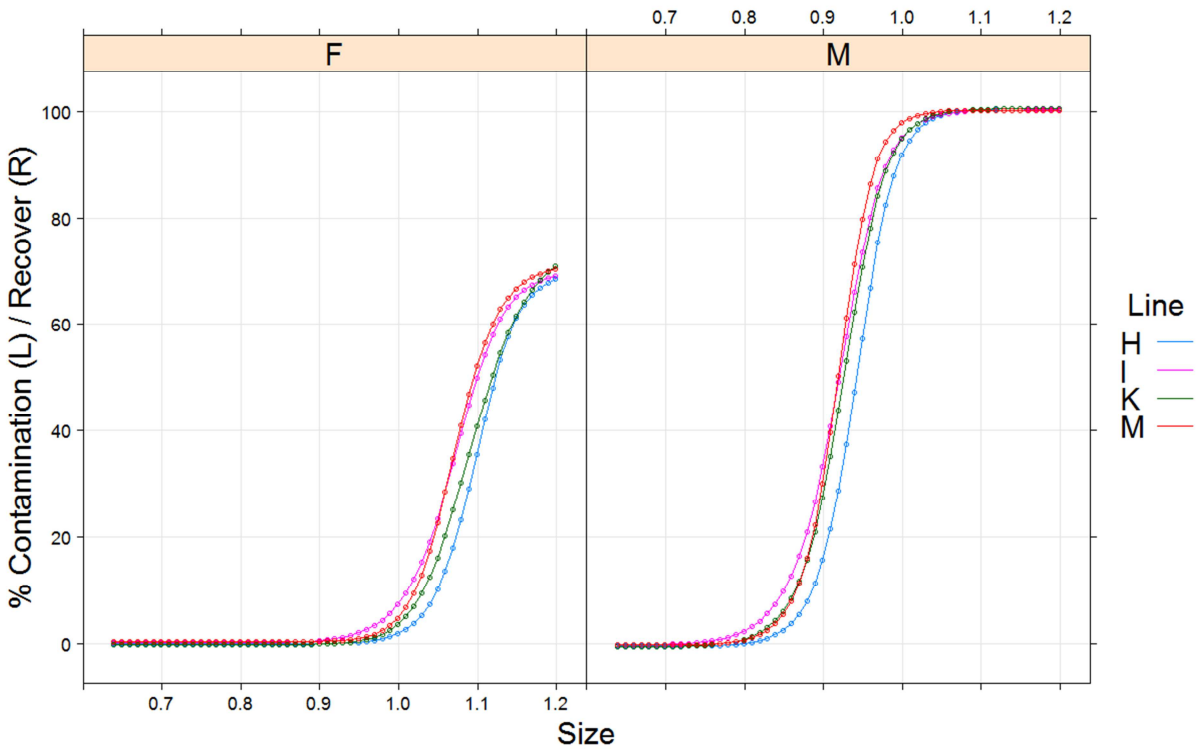


Figure 93: Fitted values of the dose response model for the predictors (Size, Line and Sex), from 3 lar / ml data.

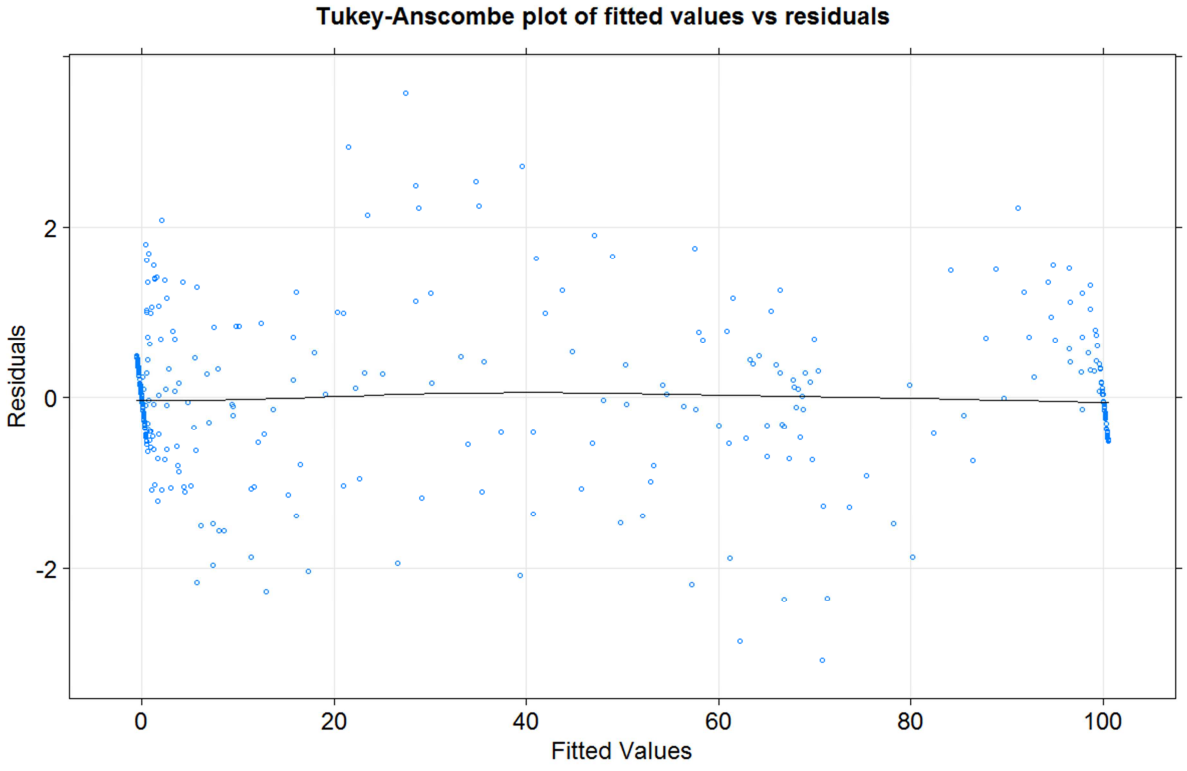


Figure 94: Tukey Anscombe plot of fitted values against residuals for the mixed effects model, from 4 lar / ml data.

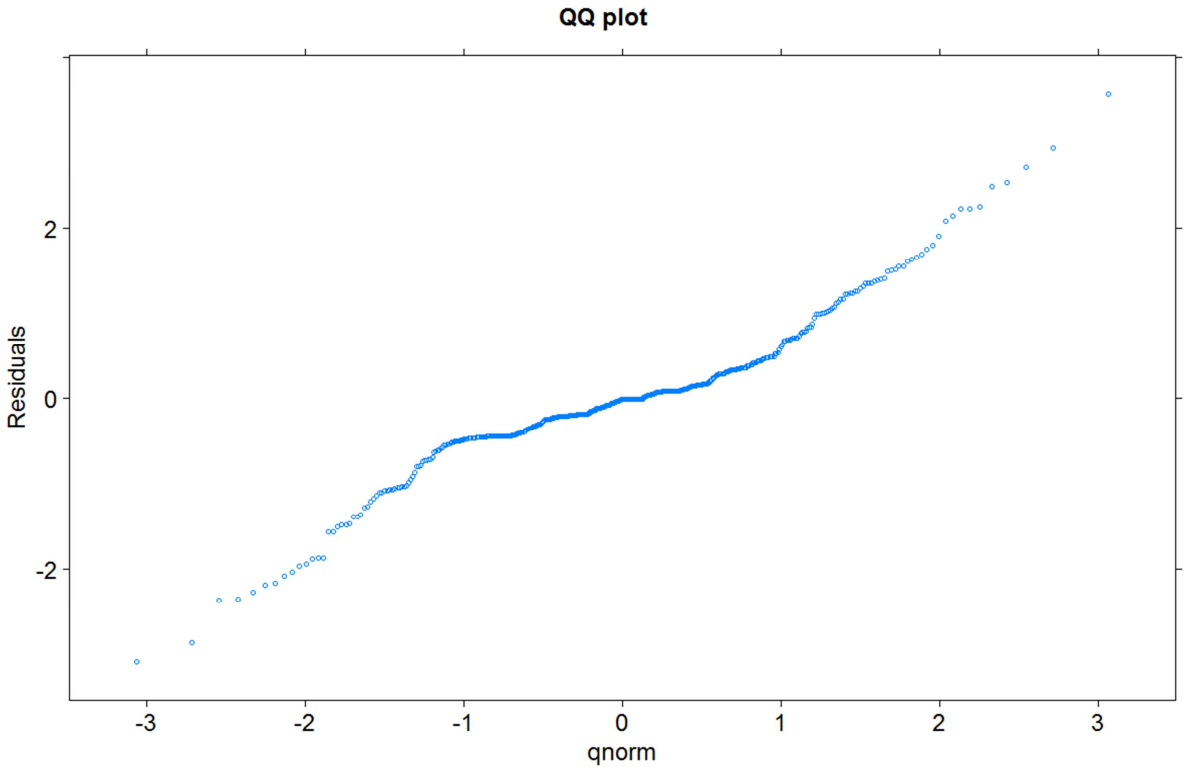


Figure 95: QQ Plot of fitted residuals, from 4 lar / ml data.

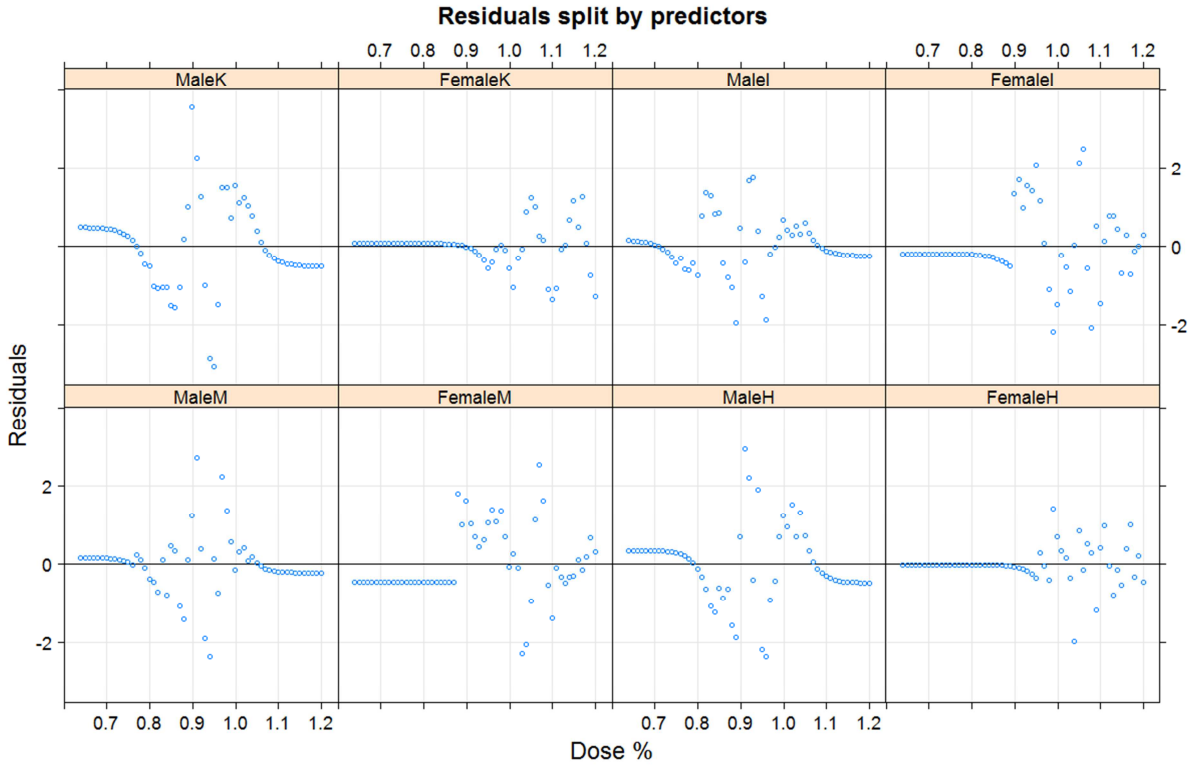


Figure 96: Residuals split by the fixed effect predictors (Size, Line and Sex), from 4 lar / ml.

Appendix F

No differences were seen between male and female pupal size on each day for any of the lines tested at each density (Figure 97, Figure 98 and Figure 99).

Pupal size was seen to decrease across the four days for both males and females, for each line at each density (Figure 100, Figure 101 and Figure 102). The exceptions being between days 1 and 2 for HAW females at 2 and 4 lar / ml (Figure 100 and Figure 102); for KLP females at 2 lar / ml and males at 3 lar / ml (Figure 100 and Figure 101), MAL males and females at 3 lar / ml (Figure 101) and ITA males and females at 2 lar / ml (Figure 100).

Simultaneous tests for general linear hypothesis were performed to examine differences between male and female pupae of each line at each of the rearing densities (Table 47, Table 48 and Table 49).

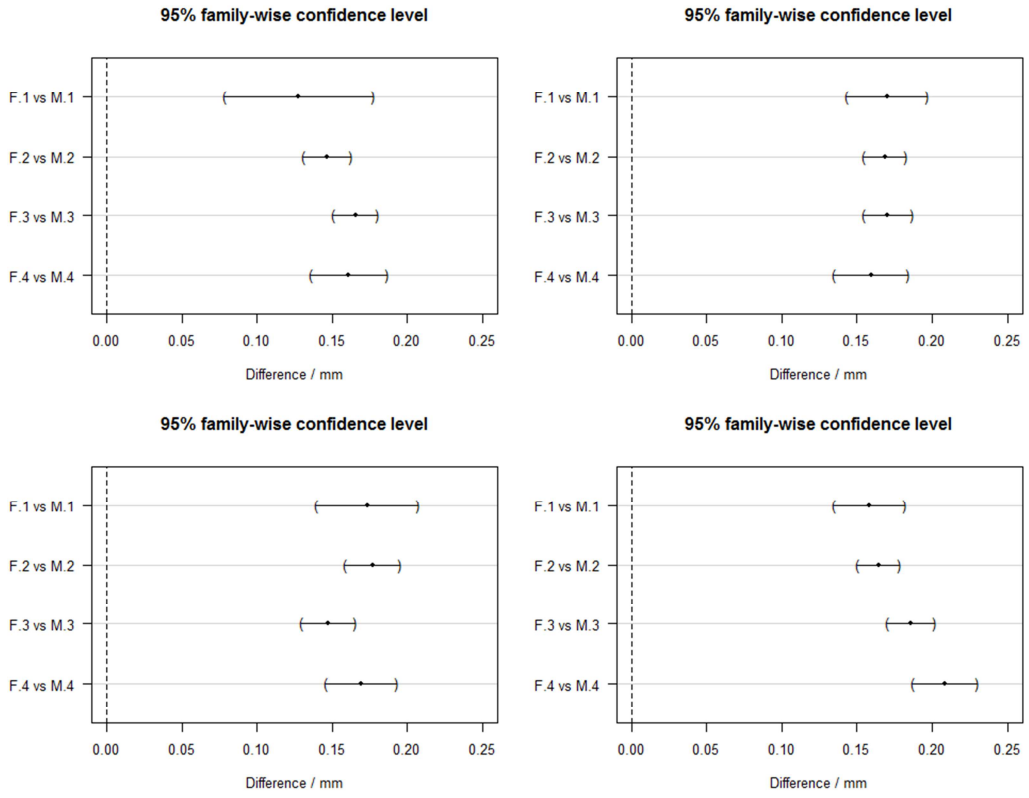


Figure 97: Differences between average female and male pupal sizes on days 1 -4 of testing reared at 2 lar / ml. Top Left: HAW, Top Right: KLP, Bottom Left: MAL, Bottom Right: ITA. (e.g. F1 vs M1: Females on Day 1 vs Males on Day 1)

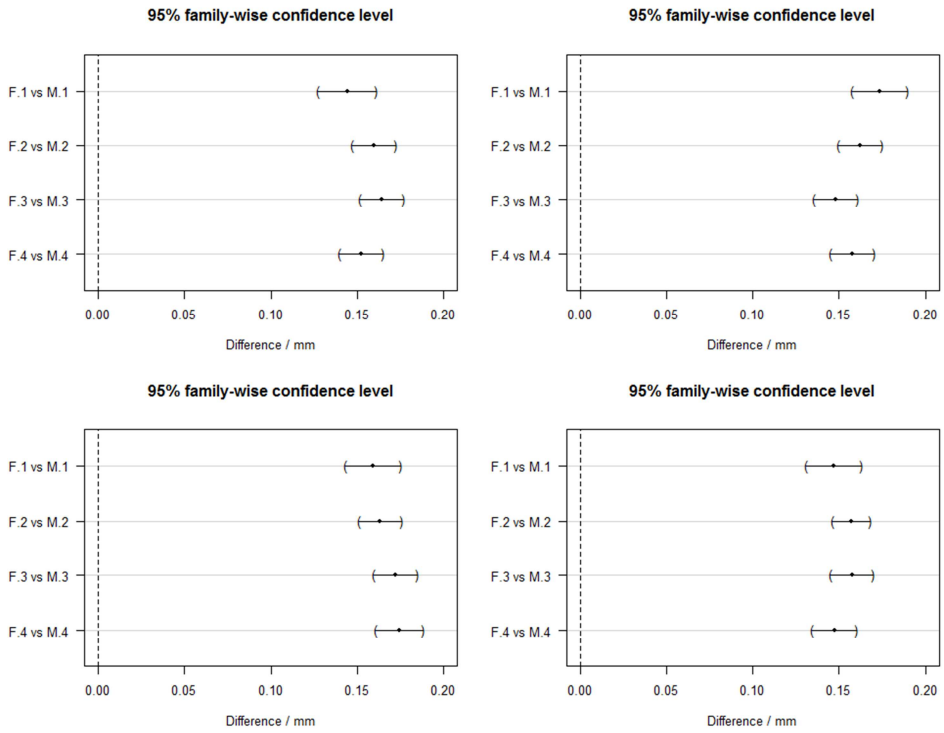


Figure 98: Differences between average female and male pupal sizes on days 1 -4 of testing reared at 3 lar / ml. Top Left: HAW, Top Right: KLP, Bottom Left: MAL, Bottom Right: ITA. (e.g. F1 vs M1: Females on Day 1 vs Males on Day 1)

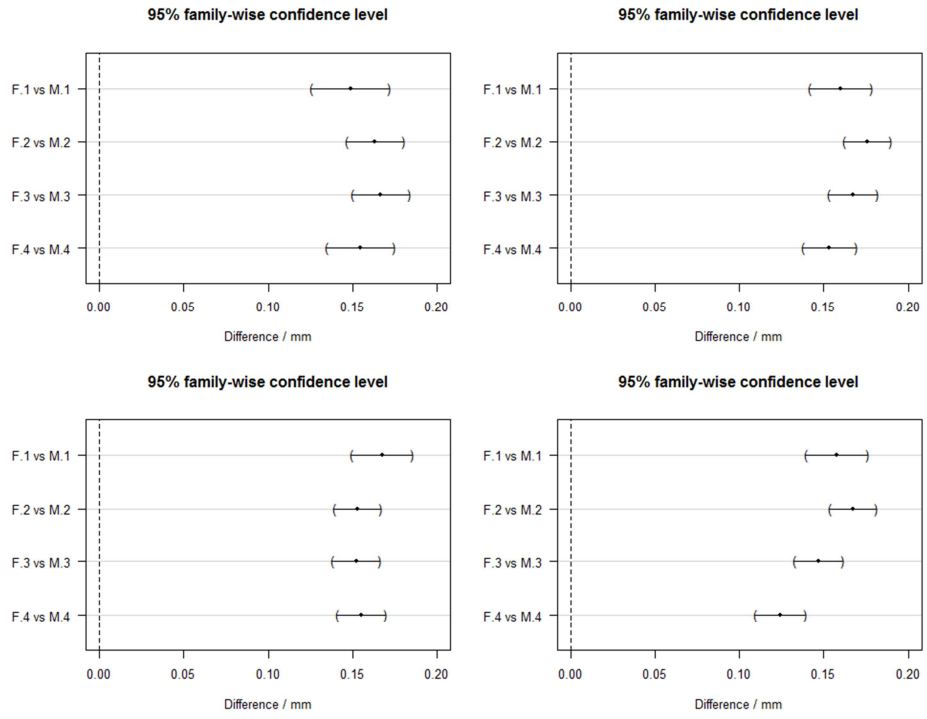


Figure 99: Differences between average female and male pupal sizes on days 1 -4 of testing reared at 4 lar / ml. Top Left: HAW, Top Right: KLP, Bottom Left: MAL, Bottom Right: ITA. (e.g. F1 vs M1: Females on Day 1 vs Males on Day 1)

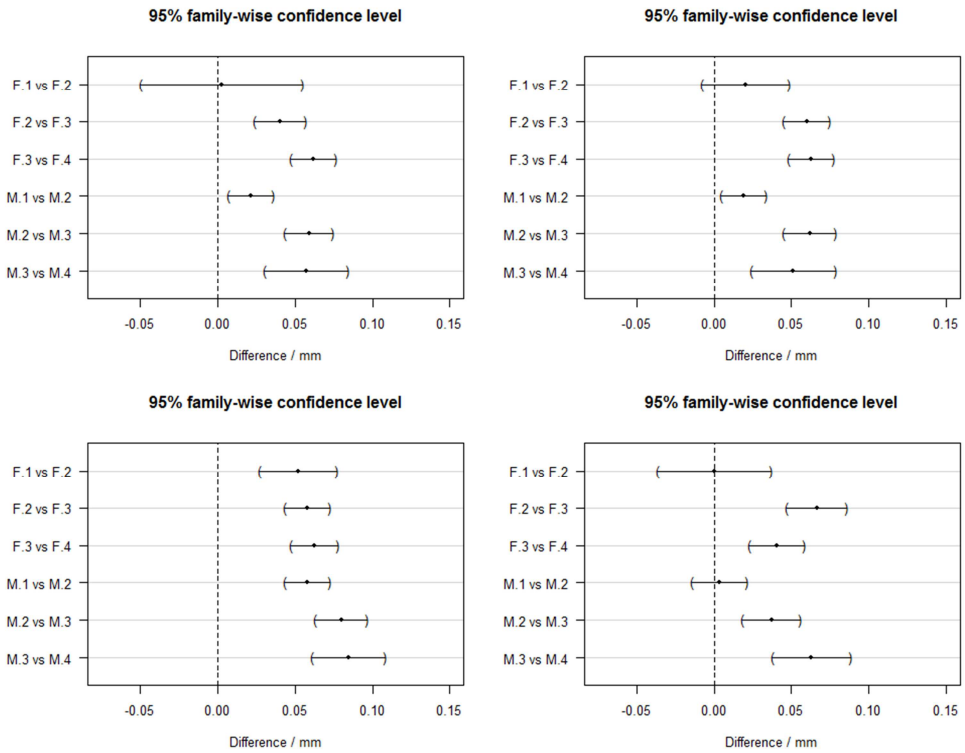


Figure 100 Differences between average pupal sizes between consecutive days for either females or males reared at 2 lar / ml. Top Left: HAW, Top Right: KLP, Bottom Left: MAL, Bottom Right: ITA. (e.g. F.1 vs F.2: Females on Day 1 vs Females on Day 2)

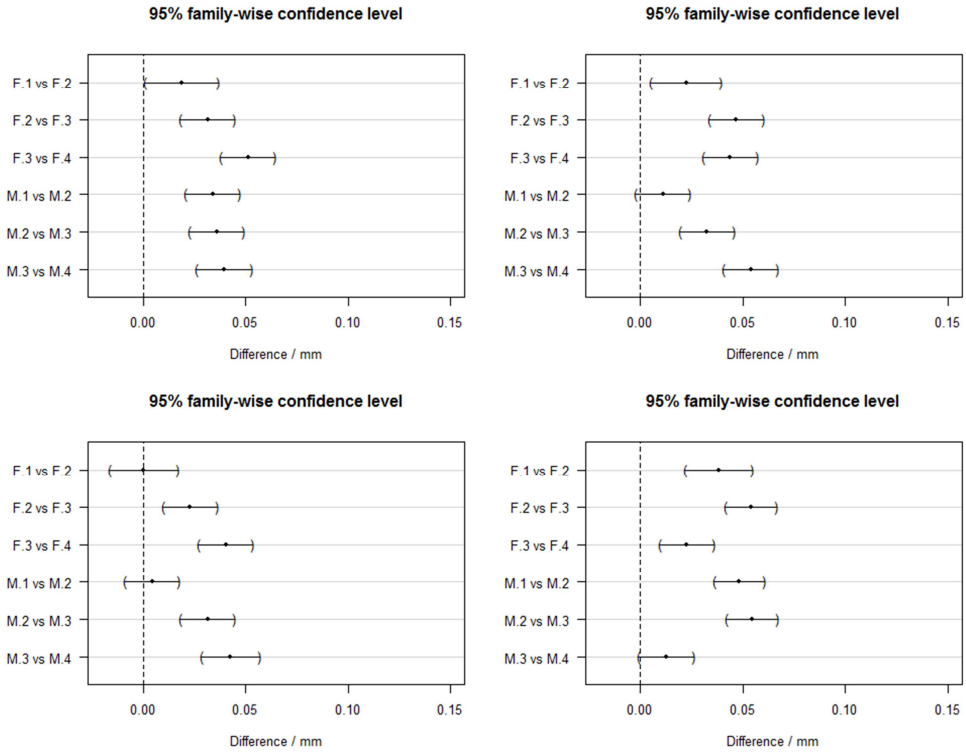


Figure 101: Differences between average pupal sizes between consecutive days for either females or males reared at 3 lar / ml. Top Left: HAW, Top Right: KLP, Bottom Left: MAL, Bottom Right: ITA. (e.g. F.1 vs F.2: Females on Day 1 vs Females on Day 2)

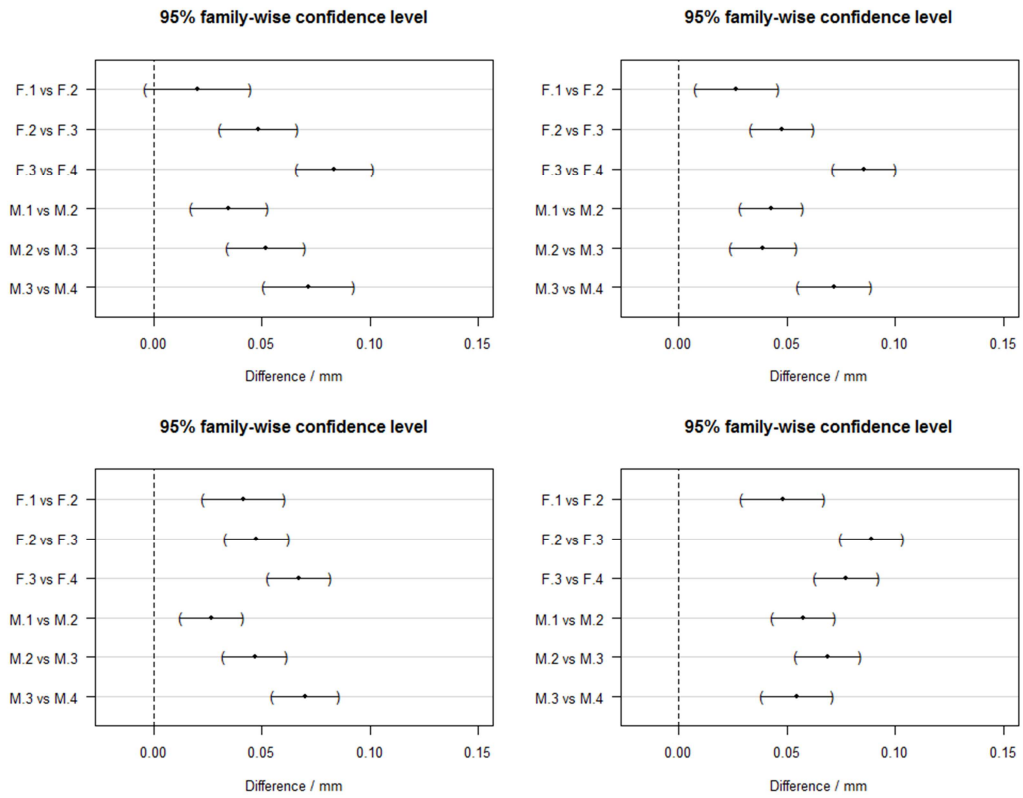


Figure 102: Differences between average pupal sizes between consecutive days for either females or males reared at 4 lar / ml. Top Left: HAW, Top Right: KLP, Bottom Left: MAL, Bottom Right: ITA. (e.g. F.1 vs F.2: Females on Day 1 vs Females on Day 2).

Table 47: Simultaneous tests for general linear hypothesis of male or female pupal size between lines, reared at 2 lar / ml

Comparison	Estimate	Std. Error	Z value	Adjusted p value
HAW females vs ITA females	0.0059	0.0169	0.350	0.9974
HAW females vs KLP females	-0.0282	0.0170	-1.660	0.4242
HAW females vs MAL females	-0.0217	0.0189	-1.148	0.7479
HAW males vs ITA males	0.0351	0.0164	2.146	0.1823
HAW males vs KLP males	-0.0112	0.0164	-0.682	0.9532
HAW males vs MAL males	-0.0052	0.0182	-0.285	0.9991
KLP females vs ITA females	0.0341	0.0164	2.080	0.2079
KLP females vs MAL females	0.0065	0.0184	0.352	0.9974
KLP males vs ITA males	0.0463	0.0164	2.830	0.0339 *
KLP males vs MAL males	0.0060	0.0182	0.328	0.9982
ITA females vs MAL females	-0.0276	0.0184	-1.503	0.5229
ITA males vs MAL males	-0.0403	0.0182	-2.217	0.1570

Table 48: Simultaneous tests for general linear hypothesis of male or female pupal size between lines, reared at 3 lar / ml

Comparison	Estimate	Std. Error	Z value	Adjusted p value
HAW females vs ITA females	0.0229	0.0039	5.859	<0.001 ***
HAW females vs KLP females	0.0077	0.0039	1.946	0.3496
HAW females vs MAL females	-0.0007	0.0039	-0.182	1.0000
HAW males vs ITA males	0.0202	0.0036	5.565	<0.001 ***
HAW males vs KLP males	0.0130	0.0036	3.572	0.0039 **
HAW males vs MAL males	0.0115	0.0037	3.140	0.0177 *
KLP females vs ITA females	0.0153	0.0039	3.939	<0.001***
KLP females vs MAL females	-0.0084	0.0039	-2.151	0.2389
KLP males vs ITA males	0.0072	0.0036	1.982	0.3282
KLP males vs MAL males	-0.0015	0.0037	-0.397	0.9993
ITA females vs MAL females	-0.0237	0.0039	-6.111	<0.001 ***
ITA males vs MAL males	-0.0086	0.0037	-2.361	0.1526

Table 49: Simultaneous tests for general linear hypothesis of male or female pupal size between lines, reared at 4 lar / ml

Comparison	Estimate	Std. Error	Z value	Adjusted p value
HAW females vs ITA females	0.0433	0.0177	2.439	0.0881
HAW females vs KLP females	0.0069	0.0177	0.391	0.9928
HAW females vs MAL females	0.0204	0.0177	1.147	0.7247
HAW males vs ITA males	0.0340	0.0177	1.923	0.2616
HAW males vs KLP males	0.0127	0.0177	0.718	0.9299
HAW males vs MAL males	0.0190	0.0177	1.075	0.7673
KLP females vs ITA females	0.0363	0.0159	2.291	0.1252
KLP females vs MAL females	0.0134	0.0159	0.846	0.8823
KLP males vs ITA males	0.0213	0.0158	1.348	0.5990
KLP males vs MAL males	0.0063	0.0158	0.399	0.9922
ITA females vs MAL females	-0.0229	0.0159	-1.445	0.5363
ITA males vs MAL males	-0.0150	0.0158	-0.950	0.8344

Appendix G

A linear mixed effects model was used to show the relationship between the number of eggs produced and the density of rearing for each WT line over a number of gonotrophic cycles.

$$lmer(Egg\ Number \sim Int + (1|Cage) - 1, data = ds)$$

- lmer – Linear mixed effect model
- Egg Number – Response variable, number of eggs per female
- Int – Individual factor combining Line, gonotrophic cycle and density into one variable. Eg HAW for GC 2 from high density cage.
- 1|Cage– Random effect of cage

The fitted model was shown to be meaningful in representing the original data, as seen by the plots of fitted values for the random effect split by the fixed effect predictors (Figure 103). A Tukey Anscombe plot of fitted vs residual values indicate an even variance of the residuals, seen by even distribution around 0 (Figure 104). A QQ plot of the residuals indicate the errors are normally distributed, visualised by a straight line (Figure 105). Variance of the residuals also appears to be consistent across fixed effect predictors (Figure 106) and the random effect (Figure 107). Full statistical analysis of the difference in the number of eggs between lines at GC 1 and 2 are displayed for the high (Table 50) and low (Table 51) density cages.

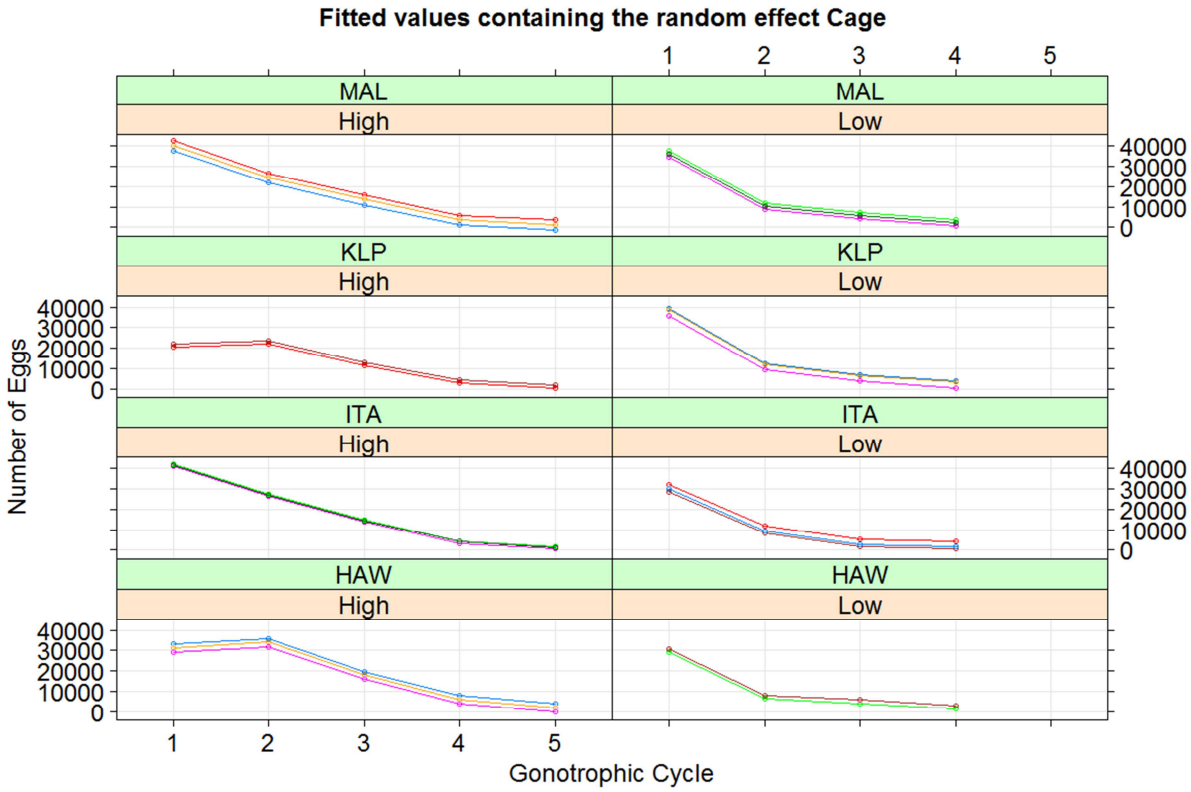


Figure 103 Fitted values of the mixed effects model for the random effect of cage within each set of fixed effect predictors (Line, GC and Density).

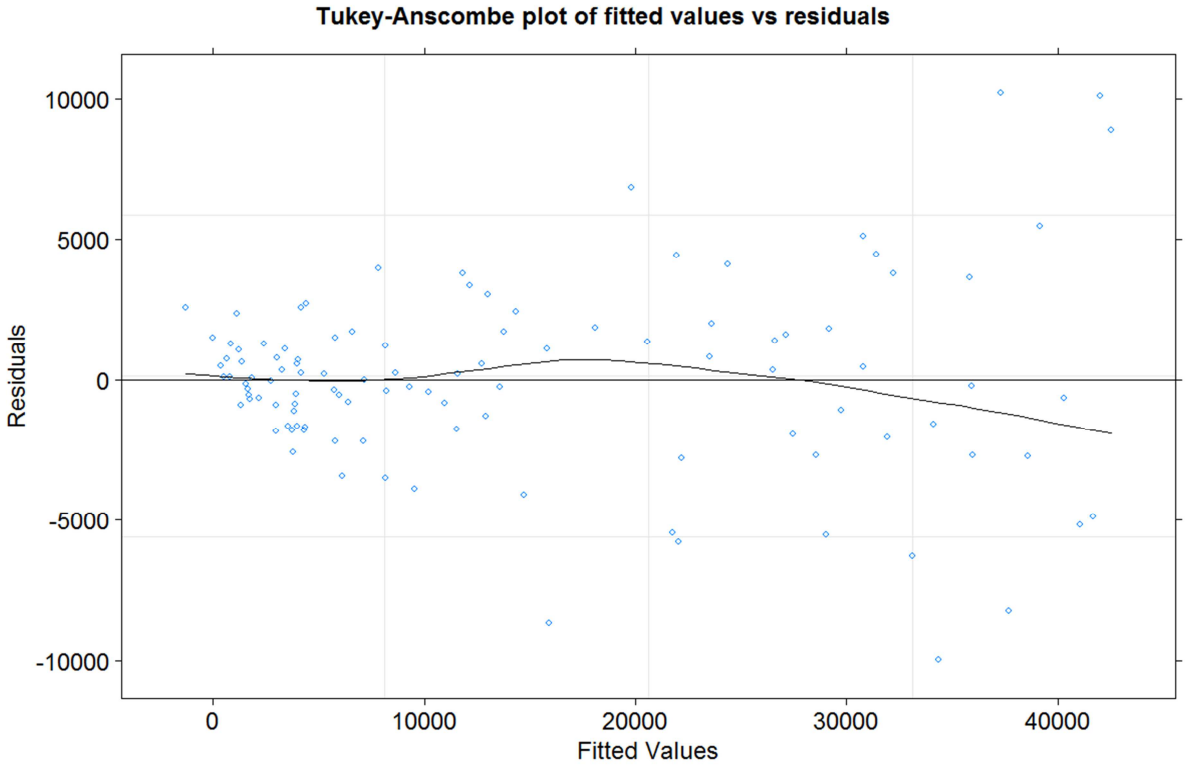


Figure 104: Tukey Anscombe plot of fitted values against residuals for the mixed effects model.

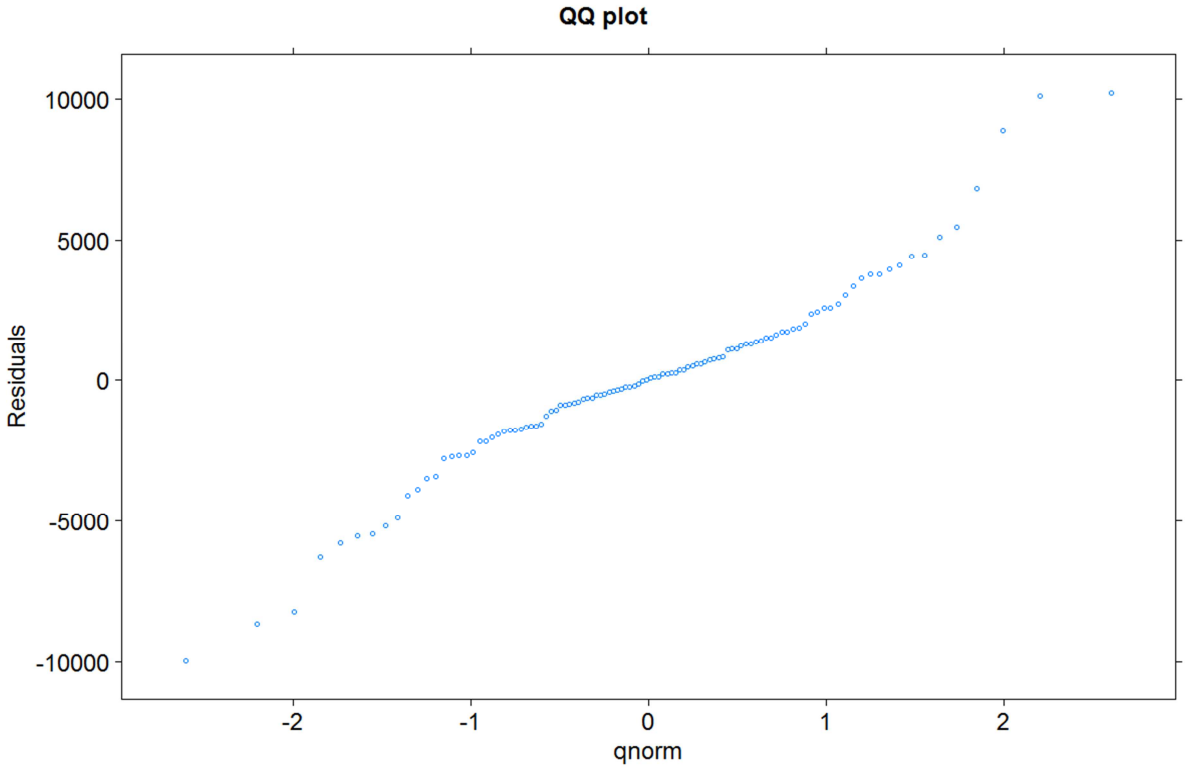


Figure 105: QQ Plot of fitted residuals.

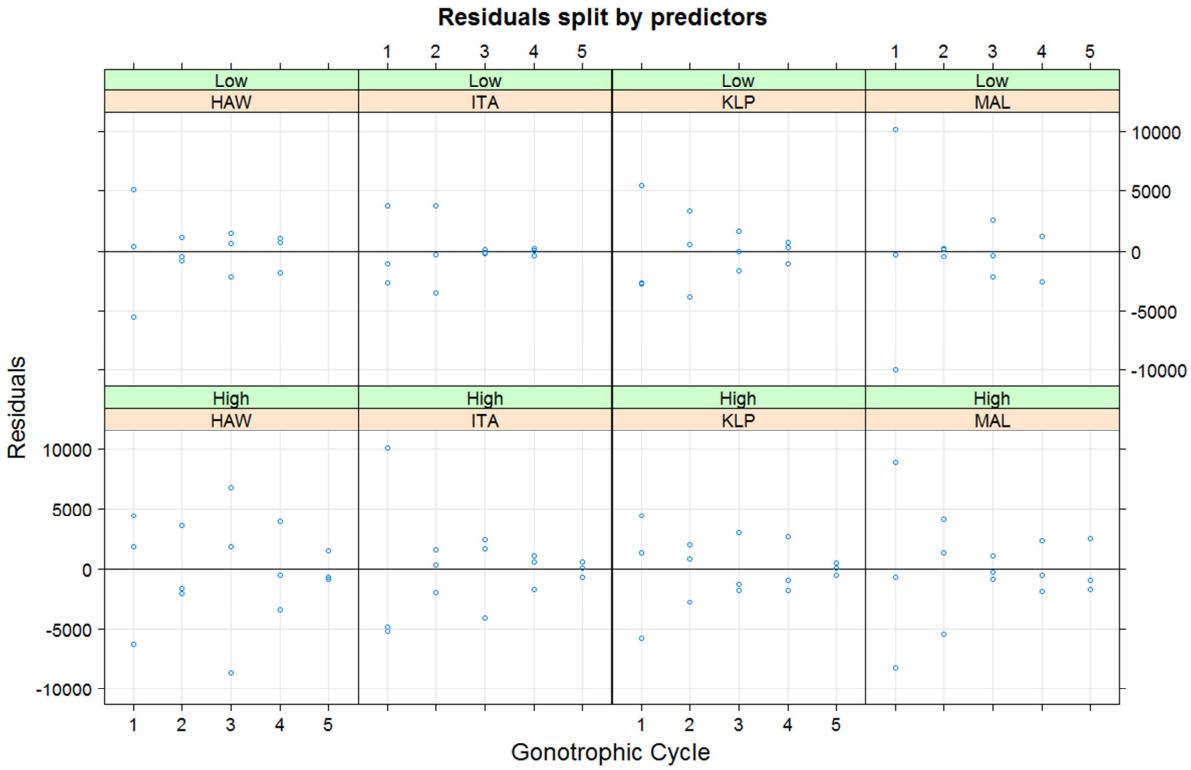


Figure 106: Residuals split by the fixed effect predictors (Line, GC and density).

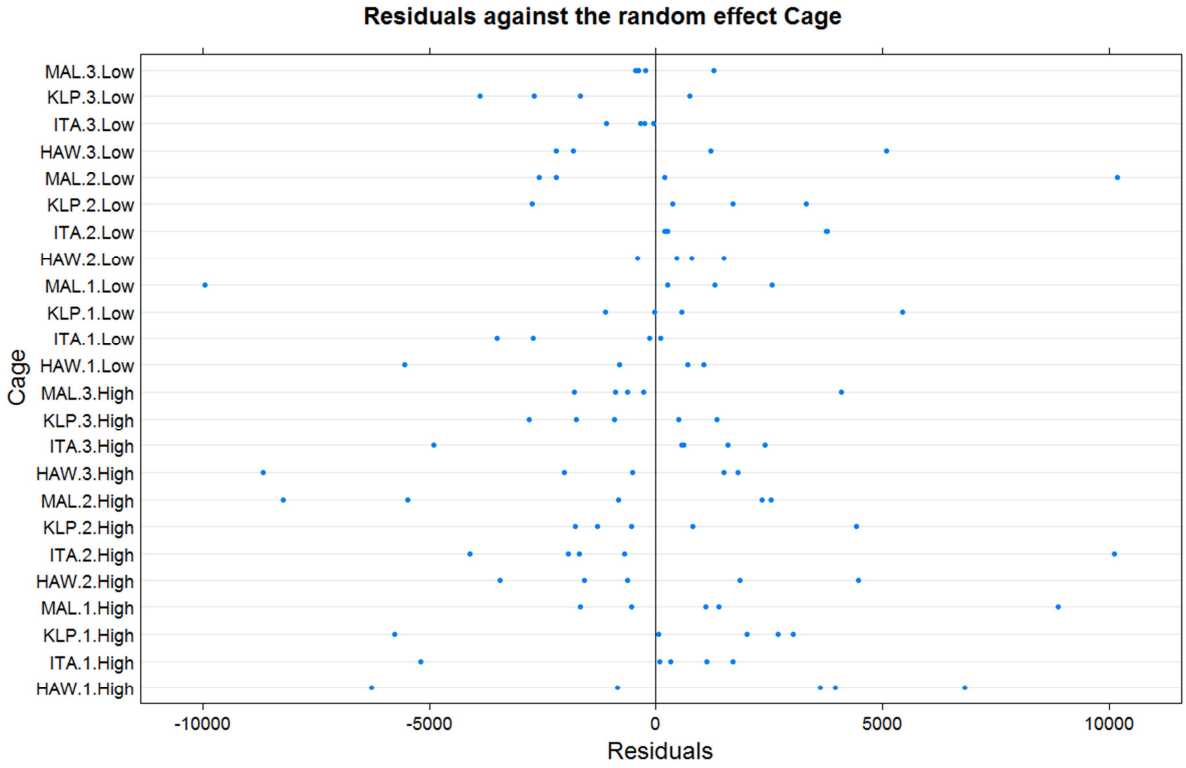


Figure 107: Residuals split by the random effect predictor (Cage). Cage is represented by the interaction of the Line, replicate and density.

Table 50: Simultaneous tests for general linear hypotheses of number of eggs per female between lines for the first and second gonotrophic cycle when reared at high density.

Comparison	Estimate	Std Error	Z value	P value
H.1 vs I1	-10345	3921	-2.638	0.0806 .
H.1 vs K1	9720	3921	2.479	0.1213
H.1 vs M1	-8941	3921	-2.280	0.1926
I.1 vs K1	20065	3921	5.117	<0.001 ***
I.1 vs M1	1404	3921	0.358	0.9997
K.1 vs M1	-18661	3921	-4.759	<0.001 ***
H.2 vs I2	6925	3921	1.766	0.4903
H.2 vs K2	10856	3921	2.769	0.0564 .
H.2 vs M2	9720	3921	-0.290	0.1213
I.2 vs K2	3931	3921	1.003	0.9340
I.2 vs M2	2795	3921	0.713	0.9878
K.2 vs M2	-1136	3921	-0.290	0.9999

Table 51: Simultaneous tests for general linear hypotheses of number of eggs per female between lines for the first and second gonotrophic cycle when reared at low density.

Comparison	Estimate	Std Error	Z value	p value
H.1 vs I1	11.99	3921.22	0.003	1.00000
H.1 vs K1	-7716.03	3921.22	-1.968	0.356
H.1 vs M1	-5680.68	3921.22	-1.449	0.712
I.1 vs K1	-7728.02	3921.22	-1.971	0.354
I.1 vs M1	-5692.66	3921.22	-1.452	0.710
I.1 vs M1	2035.36	3921.22	0.519	0.998
H.2 vs I2	1351.73	3921.22	-0.550	0.997
H.2 vs K2	-3883.73	3921.22	-0.990	0.938
H.2 vs M2	-2531.99	3921.22	-0.646	0.993
I.2 vs K2	1727.67	3921.22	-0.441	0.999
I.2 vs M2	-375.94	3921.22	-0.096	1.000
K.2 vs M2	1351.73	3921.22	0.345	1.000

Appendix H

Fitted dose response model to assess insecticide concentration on mortality rates from a WHO insecticide bioassay.

$$drm\left(Mortality \sim Dose, Int, data = ds, fct = LL.4\left(fixed = c(\ NA, 0{,}100, NA)\right)\right)$$

- drm: Dose response model
- Mortality: adjusted mortality values
- Dose: insecticide concentration
- Int: interaction term combining Insecticide and Line
- ds: data frame containing Mortality, Dose and Int
- LL.4: Log logistic four parameter model, with minimum mortality set to 0 and maximum to 100.

The fitted model was shown to be meaningful in representing the original data, as seen by the plot of fitted values for the predictors (Figure 108). A Tukey Anscombe plot of fitted vs residuals indicates normal distribution of the residuals (Figure 109). A QQ plot of the residuals indicate the errors are also normally distributed (Figure 110). Visualization of the residuals split by the predictors (insecticide and line) also demonstrates normally distributed residuals (Figure 111).

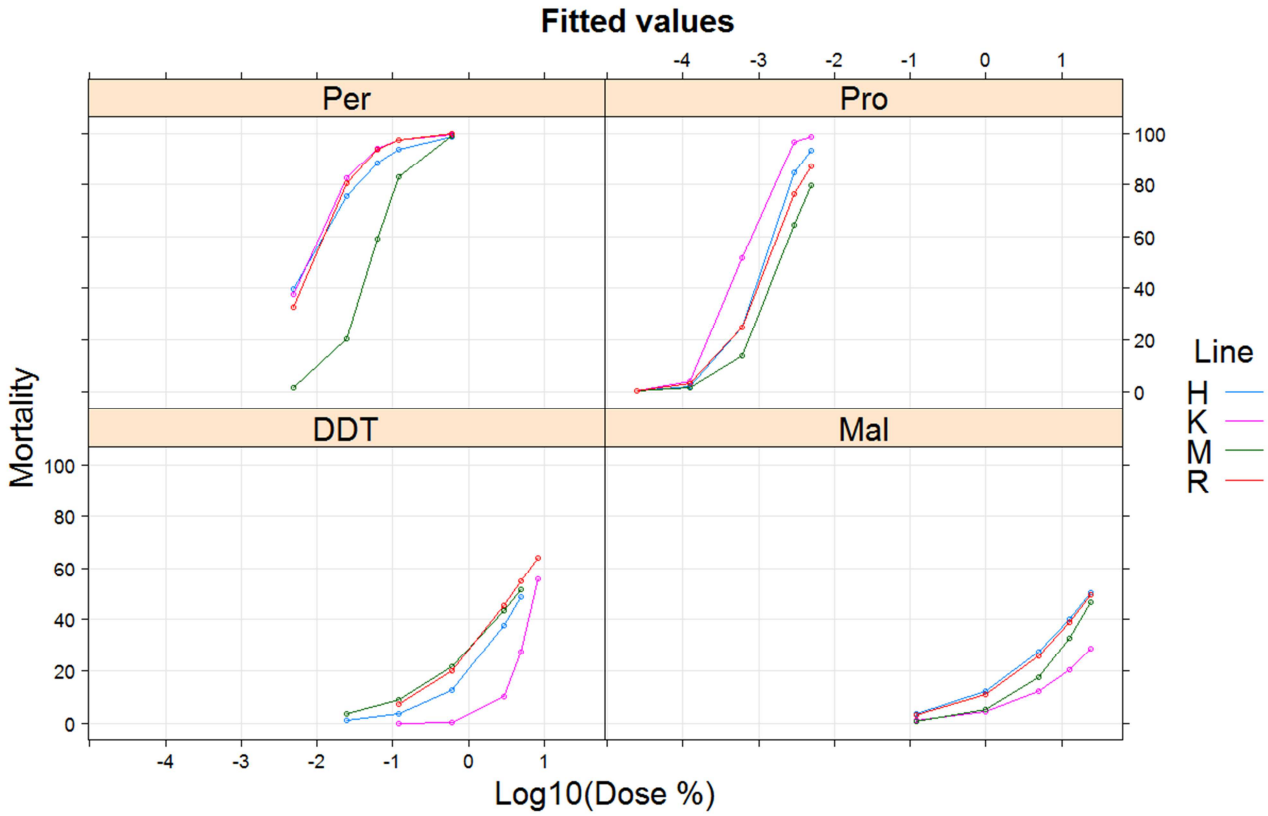


Figure 108: Fitted values of the dose response model for the predictors (log(concentration), line and insecticide).

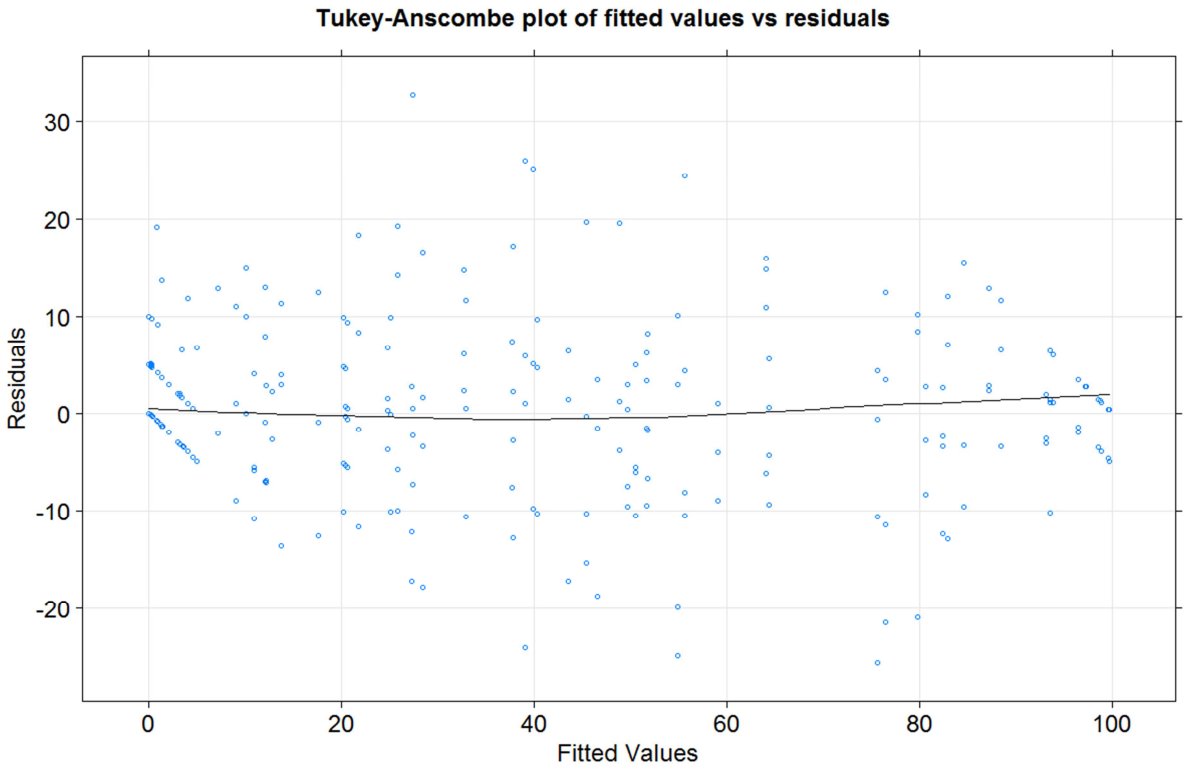


Figure 109: Tukey Anscombe plot of fitted values against residuals for the dose response model.

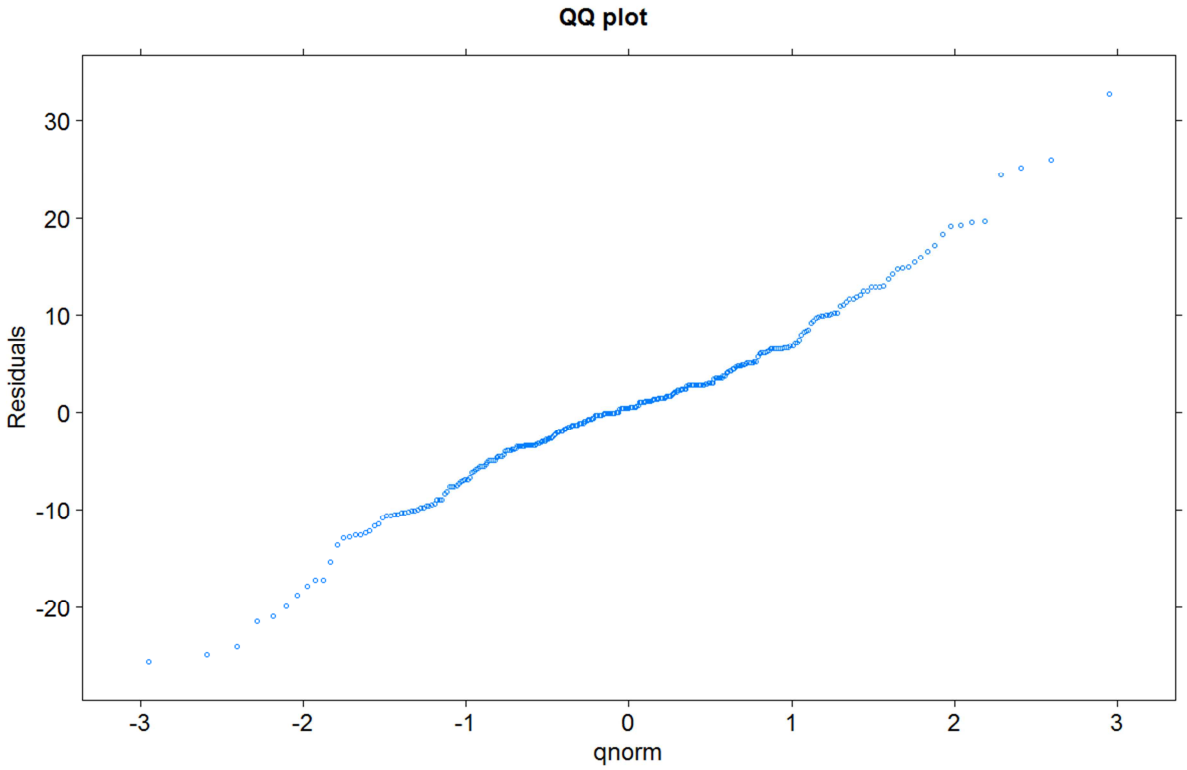


Figure 110: QQ Plot of fitted residuals.

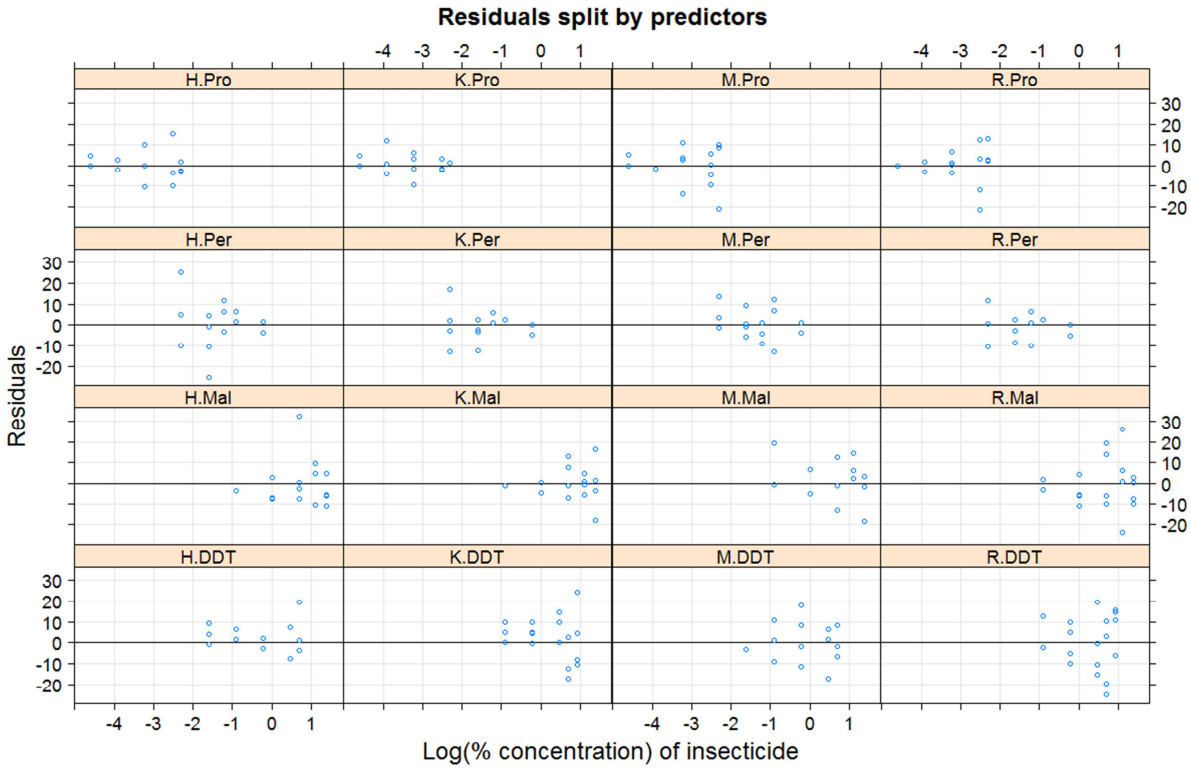


Figure 111: Residuals split by the interaction term of Line and insecticide.