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Generation and fitness of transgenic *Anopheles gambiae* and the impact of multiple feeding on anti-malarial properties of the vida3 transgene

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Abstract

Malaria, the leading cause of death due to vector-borne disease. is responsible for around 250 million cases and 1 million deaths annually, mostly in sub-Saharan Africa where *Anopheles gambiae* is the major vector.

Novel approaches to malaria control include theoretical use of transgenic mosquitoes to suppress or replace wild type populations. Generation of mosquitoes carrying either a conditional lethal gene (RIDL), or one encoding a molecule able to kill malaria parasites, is a key component of this strategy. Detailed genetic and physiological knowledge of transgenic mosquitoes is important for epidemiological models to accurately determine the outcome of putative field releases. This includes testing the potential for post-integration remobilisation of transgenes in the host genome and testing the reproductive fitness of transgenic mosquitoes in comparison to wild type.

Complex plasmids, designed to create both RIDL strains and strains within which *piggyBac* remobilisation could be tested, were injected into the Keele strain of *An. gambiae*. Ultimately, although microinjection techniques were optimised and efficiencies improved over time, generation of such transgenic strains was unsuccessful.

A previously established transgenic strain (EVida3), expressing the Vida3 peptide which is active against ookinete stages of *Plasmodium*, was subsequently investigated with respect to multiple feeding and relative fitness.

Multiple feeding experiments indicate that complex interactions take place between Vida3 and the natural immune system when mosquitoes are infected with the murine malaria *P. yoelii nigeriensis*. Vida3 production appears to impact on parasite intensity and mosquito fecundity in both positive and negative ways dependent on factors such as gonotrophic cycle and parasite-stage. Analysis of three life-table fitness parameters, comparing EVida3

hemizygotes to the KIL wild type laboratory strain, indicate no fitness cost associated with transgene presence or expression. Analysis of a further two parameters indicates a possible fitness cost associated with inbreeding rather than transgenesis alone.

List of Abbreviations

AAPP	Anopheline Anti-Platelet Protein
AMP	Anti-Microbial Peptide
CELIII	C-type Lectin III (from sea cucumber)
CLIPs	Clip-domain Serine Proteases
CSP	Circumsporozoite Protein
DCE	Dopachrome Conversion Enzyme
DDT	Dichloryldiphenyltrichloroethane
DHI	5,6 – dihydroxyindole
EU	Engineered Underdominance
GMO	Genetically Modified Organism
HE	Homing Endonuclease
iNOS	inducible Nitric Oxide Synthase
IRS	Indoor Residual Spraying
ITN	Insecticide-treated bednet
ITR	Inverted Terminal Repeat
Kdr	Knockdown resistance gene
MD	Meiotic Drive
Medea	Maternal-effect dominant embryonic arrest
MLA	Multi-Locus Assortment
NLS	Nuclear Localisation Signal

NO	Nitric Oxide
PABA	Para-aminobenzoic acid
PAH	Phenylalanine Hydroxylase
PAP	PPO-Activating Protease
PfEMP1	P. falciparum Erythrocyte Membrane Protein 1
PLA2	Phospholipase (from bee venom)
PO	Phenyloxidase
PPO	Prophenyloxidase
RIDL	Release of Insects with a Dominant Lethal
ROS	Reactive Oxidative Species
SIT	Sterile Insect Technique
TE	Transposable Element
TSS	Transgenic Sexing Strain
tTA	tetracycline-dependent Transcriptional Activator

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Chapter One

1. Introduction

1.1. Introduction to Malaria

Malaria, a disease caused by protozoan parasites of the genus *Plasmodium* and carried by anopheline mosquitoes, is one of the most important parasitic infections of man with around 50% of the world's population living in areas where malaria is transmitted (CDC website). On a worldwide scale there were 216 million cases of malaria in 2010 resulting in the deaths of 655 000 deaths. The majority of these were children under the age of 5 in sub-Saharan Africa (WHO World Malaria Report 2011). More recent findings suggest, however, that the malaria burden is larger than previously thought, especially with respect to adults (Murray *et al.*, 2012).

Malaria is also thought to be a major cause of anaemia in pregnant women in sub-Saharan Africa, especially among those women who are pregnant for the first time (Savage *et al.*, 2007). It has also been documented that these women are more susceptible to *Plasmodium falciparum* infection than those who have been pregnant before (Savage *et al.*, 2007). The consequences of pregnancy-associated malaria are not only the risk of maternal anaemia but also an increased incidence of low birth weight and infant mortality (Ndam and Deloron, 2007).

The morbidity and mortality caused by malaria are problematic, however the economic and social losses the disease causes within those countries in which it is endemic are also a major issue. Sachs and Malaney, 2002 review article published in Nature makes the remark "where malaria prospers most, human societies have prospered least", which seems to be a fair reflection on the situation. Average growth in per-capita GDP (Gross Domestic

Product) for countries without *P. falciparum* malaria between 1965 and 1990 was, on average, 2.3% per year whereas in those countries with such malaria infections the average was just 0.4% per year thus showing a substantial difference in economic growth (Gallup and Sachs, 2001). Malaria-endemic countries have also been shown to be poorer than their non-malaria-endemic counterparts (Gallup and Sachs, 2001), and as such can little afford the preventative measures used to control malaria infections among their citizens. It is a vicious cycle.

1.1.1. Species of parasite causing malaria infection

Malaria is caused by the apicomplexan protozoan *Plasmodium*, of which there were originally four recognised species that cause the disease in humans; *P. falciparum, P. vivax, P. malariae* and *P. ovale* (Greenwood *et al.*, 2005). Of these four species of malaria parasite, *P. falciparum* and *P. vivax* are the most prevalent in causing infection around the world (Mendis *et al.*, 2001).

Although infection with any of these four species causes malaria, the pathology of the disease varies slightly depending on the species responsible. The most severe disease and mortality is caused by *P. falciparum* which is responsible for the majority of infections in Africa (Greenwood *et al.*, 2005).

P. ovale and *P. vivax* are however notable due to their ability to form hypnozoites in the liver of the infected person. Hypnozoites are resting or quiescent stages of the parasite that can become re-activated many months after initial infection thus causing a relapse (Greenwood *et al.*, 2005).

Interestingly, a fifth species of the malaria parasite, which was thought to cause disease solely in simians (most common in two macaque species found in Southeast Asia), is now recognised as causing disease in humans (White, 2008). *Plasmodium knowlesi* has, in the

past, been misdiagnosed as *P. malariae* due to their morphological similarities. It is thus deemed useful to use molecular screen methods alongside traditional blood smears in areas of Southeast Asia where *P. knowlesi* infections are known to exist in order to determine the exact species of parasite causing the disease (Lee *et al.*, 2009).

1.1.2. Geographical Distribution of *Plasmodium* species

Of the five species of *Plasmodium* which cause human malaria infection, *P. vivax* is the most widespread. It is present not only in the tropics and sub-tropics, but also in some temperate areas (Martens *et al.*, 1999).

P. ovale, a similar parasite to *P. vivax*, replaces it in West Africa (Martens *et al.*, 1999).

P. falciparum is the most common species found in tropical areas and *P. malariae* is the least common of the four malaria parasites, being found mainly in tropical Africa (Martens *et al.*, 1999).

P. knowlesi is found to be most common in forested areas of Southeast Asia (White, 2008).

Limitations on the distribution of the human disease caused by the genus *Plasmodium* in general are due to climate and vector distribution. Temperature affects the distribution of the host mosquito itself and also affects replication of the parasite within the insect host (Martens *et al.*, 1999). For *P. falciparum* and *P. vivax*, the minimum temperature required for replication of the parasite within the mosquito host is approximately 18°C and 15°C, respectively (Patz and Reisen, 2001).

1.1.3. Life cycle and stages of *Plasmodium falciparum* with reference to pathology

The life cycle of *P. falciparum* involves two hosts. Namely a human host and an intermediate vector host, the anopheline mosquito. Its lifecycle is complicated and includes both asexual and sexual reproduction, as well as different stages within each host

species (Figure 1). Although the life cycle has been documented and explored for many years, some mechanisms are still not fully understood.

An infected mosquito, whilst taking a blood meal from a human, injects sporozoites from the salivary glands into the dermis (Kappe *et al.*, 2003). After entering the blood stream, sporozoites migrate rapidly to the liver where they leave the bloodstream and invade hepatocytes. It is here that they develop into the exo-erythrocytic forms and thus undergo the first round of asexual replication, in the human host, to produce vast numbers of first generation merozoites (Kappe *et al.*, 2003).

The journey from hepatocyte to sinusoid (blood vessel) is a 'dangerous' one for the parasite due to patrolling macrophages (Kupffer cells) within liver sinusoids. Sturm *et al.*, 2006, have shown, using a variety of techniques both *in vitro* and *in vivo*, that *P. berghei* parasites are capable of causing the detachment of host hepatocytes and the formation of membrane-bound vesicles called merosomes. The detached cells, and the merosomes which form from them, contain infective merozoites in large numbers. The authors conclude that it is these merosomes which ensure the safe release of such merozoites directly into live sinusoids thus protecting the parasite from being recognised by Kupffer cells of the host immune response (Sturm *et al.*, 2006).

The merozoite stage is briefly extra-cellular prior to invasion of erythrocytes, and is therefore of immunological importance (Bannister and Mitchell, 2003). It is the release of merozoites into the bloodstream that causes the classical fever symptomatic of a malarial infection. The merozoites of *P. falciparum* are probably the smallest of all the *Plasmodium* species. They are ovoid in shape and possess all the cellular machinery required not only for invasion of, and feeding on, red blood cells, but also for escaping what remains of the previous old red blood cell (Bannister and Mitchell, 2003). Due to its invasive nature, the merozoite has developed features or structures which aid in the

invasion of erythrocytes. It has, on its surface, clumps of filaments known as bristles, which capture an erythrocyte but which are cleaved upon entry into the host erythrocyte (Bannister and Mitchell, 2003). The *P. falciparum* merozoite has an low, flat-ended projection at one end known as the apical prominence. It is within this structure that dense granules, rhoptries and micronemes are found. These vesicles are bound by membranes and are thought to release their respective contents during invasion, by the merozoite, of the red blood cell, thus altering the shape and composition of the erythrocyte membrane (Bannister and Mitchell, 2003). Fowler *et al*. (1998) have also demonstrated that dinitroaniline drugs inhibit invasion of merozoites into red blood cells in assays by de-polymerising f-MAST (*falciparum* merozoite assemblage of subpellicular microtubules); thus indicating that microtubules may be implicated in *P. falciparum* merozoite invasion of red blood cells (Fowler *et al.*, 1998).

Stages in the anopheline mosquito host **Stages in the human host**

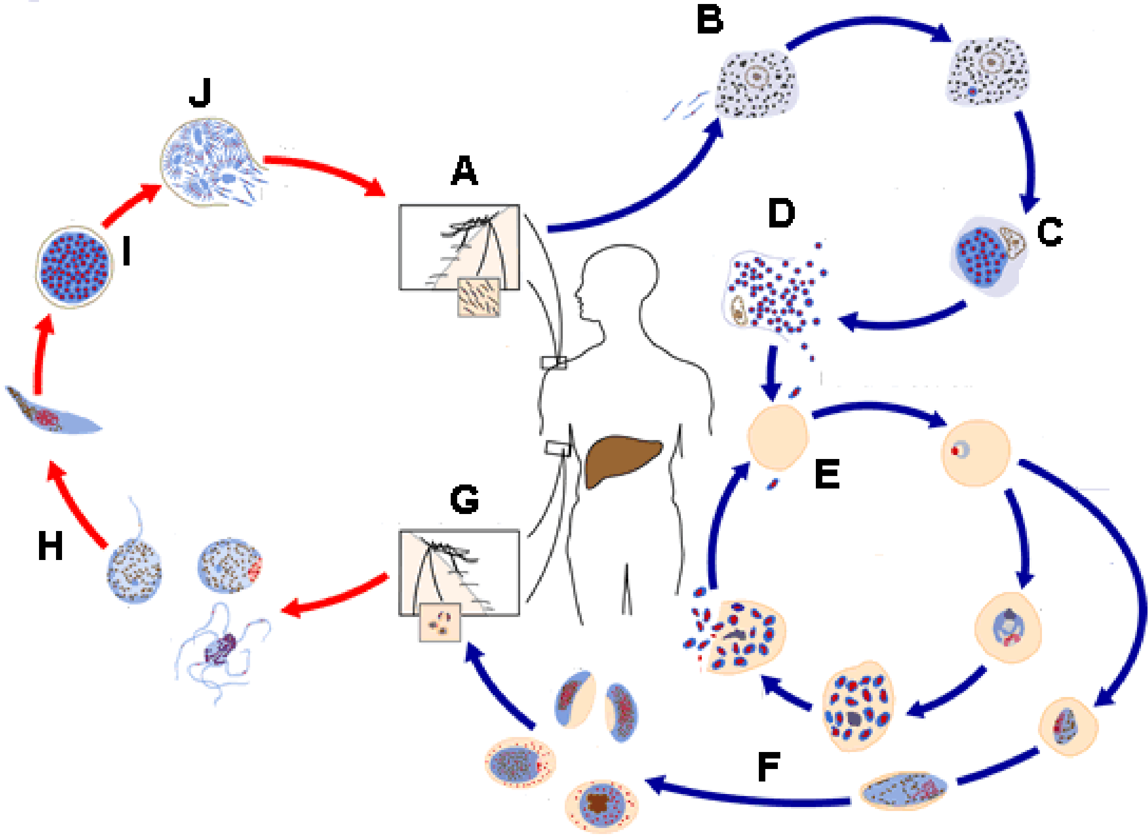


Figure 1.1 (opposing page): A diagrammatic representation of the lifecycle of *Plasmodium* species

The life cycle of *Plasmodium* is complicated and involves different stages and rounds of replication within each of it's two hosts.

A) The infected mosquito takes a blood meal; sporozoites are injected with the saliva into the dermis of the human host.

B) Sporozoites migrate to the liver where they invade hepatocytes.

C) In the hepatocytes the parasite undergoes several rounds of asexual reproduction or schizogony which produces vast numbers of merozoites.

D) The number of merozoites within the hepatocyte causes the cell to burst releasing the merozoites into the bloodstream

E) The merozoites invade red blood cells where they undergo morphological changes and become ring stages (early trophozoites) and trophozoites which are feeding stages, taking nourishment from the erythrocyte the parasite has invaded. The trophozoite stage become a schizont and undergoes further schizogony to form erythrocytic cycle merozoites that burst out of the red blood cell and are free to either invade other red blood cells to continue the erythrocytic cycle indefinitely, or continue the life cycle.

F) Some merozoites differentiate into gametocytes which are essentially male and female forms of the parasite.

G) These gametocytes are ingested by an anopheline mosquito when a blood meal is taken from an infected human host.

H) Various mosquito factors cause the micro- and macro-gametocytes to exit the ingested erythrocytes in the insects mid-gut. It is here that the gametocytes fuse to form a zygote.

I) The zygote transforms into a stage known as an ookinete and exits the remnants of the blood meal and enters the midgut epithelial cells where it migrates to the basal sub-epithelial space

J) The parasite is now known as an oocyst. It undergoes further rounds of asexual reproduction (known as sporogony) to produce thousands of sporozoites which, after bursting out of the oocyst, migrate through the haemolymph and into the mosquito salivary glands. From there they can be injected by the act of blood feeding into a human host, and thus the life cycle of *Plasmodium* begins again.

Figure adapted from http://www.dpd.cdc.gov/dpdx, accessed 19/09/11)

Once it has invaded the erythrocyte, the parasite becomes the flattened, ring-like shape, which has become a characteristic of malaria parasite infection in the human hosts' blood. The middle of this ring-stage parasite becomes thin and contains very few organelles. These are pushed towards the outer edge of the parasite and are surrounded in thick cytoplasm (Bannister and Mitchell, 2003). The ring-stage is a feeding stage (early trophozoite). It uses a small dense ring at its surface, called a cytostome, through which it feeds on the surrounding red blood cell, converting haemoglobin into inert haemozoin crystals that build up in the pigment vacuole of the parasite (Bannister and Mitchell, 2003). The surface area of the parasite relative to the erythrocyte, becomes greatly increased as the parasite feeds on the erythrocyte itself. The endoplasmic reticulum of the parasite becomes enlarged and the number of free ribosomes increases, indicating increased protein synthesis (Bannister and Mitchell, 2003).

It is at this point that bulges and clefts appear at the surface of the parasite. Indeed some of these protrusions extend deep into the cytosol of the red blood cell and may even come into contact with the membrane itself (Bannister and Mitchell, 2003). Trelka *et al*. (2000) have used transmission electron microscopy to detect the formation of electron-dense vesicles on the erythrocytic side of the parasite. These are similar to secretory vesicles in mammals and appear to bind with and fuse to the erythrocyte membrane. These vesicles could be responsible for the movement of parasite proteins into the cytosol of the red blood cell and could also be a precursor in knob structure formation, which occurs from the trophozoite stages through to the schizont stage of the parasite (Trelka *et al.*, 2000).

It has been documented that a protein known as PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) is exposed on the surface of infected red blood cells causing them to become 'sticky'. This occurs at around 16 hours into the asexual cycle (Newbold *et al.*, 1999). These surface 'knobs' are involved in mediating adhesion to blood vessel

endothelial cells and also can result in 'rosetting' whereby the infected red cell becomes bound to non-infected red blood cells (Newbold *et al.,* 1999). Both 'rosetting' and the binding of an infected erythrocyte to the blood vessel endothelium can result in blocking of blood vessels thus causing severe pathology in an infected person (Newbold *et al.,* 1999). Horrocks *et al*. (2005) have shown, using cytoadhesion assays, that both static and flow adhesion is reduced in all *P. falciparum* clones that do not possess 'knobs' on their surface, and that PfEMP1 displayed on the surface of such infected erythrocytes is reduced by 50% (Horrocks *et al.,* 2005).

Work undertaken by McMorran *et al.*, 2009, indicates that whilst platelets are responsible for major pathology associated with later stages of malarial infections caused by sequestering of infected red blood cells, they also form part of the hosts' protective response in early stages of red blood cell infection. This was indicated by work *in vivo* using *P. chaubaudi* within a mouse model, as well as *in vitro* whereby human platelets killed *P. falciparum* parasites cultured in erythrocytes (McMorran *et al.*, 2009). A review by Pleass, 2009, discusses implications of this finding and plausible mechanisms behind it.

After the trophozoite stages the parasite changes to become a form known as a schizont and undergoes asexual replication (schizogony). The nucleus divides four times to produce 16 nuclei and the individual merozoites are formed (Bannister and Mitchell, 2003). These are then released into the bloodstream, free to invade other red blood cells; again causing the classical fever which is typical of a malaria infection. This fever occurs with each round of merozoite release into the bloodstream, approximately every 48 hours in a *P. falciparum* infection and correlates with an immune response to the merozoites present in the bloodstream (Ramasamy, 1998). A rise in serum levels of tumour necrosis factor α (TNF-α) occurs 30 minutes before the onset of fever in *P. vivax* (*Pv*) malaria, which is thought to be stimulated by T cells and macrophages. This initiates the release of

interleukin 1 (IL-1), which is a fever-inducing cytokine (Ramasamy, 1998). This intra-erythrocytic cycle can continue indefinitely through rounds of multiplication within the bloodstream (Lampe *et al.,* 1999).

It is not, however, the merozoite form of *P. falciparum* (or indeed other *Plasmodium* species) that provides the link between vertebrate and invertebrate host. This link is provided by two specialist forms of the malaria parasite, the gametocytes. These stages are present in the peripheral blood of the human host and are taken up by the mosquito as it takes a blood meal (Lampe *et al.*, 1999). The environment that they are in alters significantly as their host changes. A temperature drop, a rise in pH and the presence of xanthurenic acid are what trigger the gametocytes to exit the red blood cells ingested by the mosquito (Billker *et al.*, 1998; Lampe *et al.*, 1999; Carter *et al.*, 2007). The female gametocyte exits the red blood cell as a round-shaped macrogamete. The male gametocyte undergoes three rounds of genome replication, which take place extremely quickly, and are shortly followed by nuclear division and axoneme assembly, after which eight motile microgametes are formed (Lampe *et al.*, 1999). Fertilisation takes place within the insect midgut, and a zygote is formed.

The mechanisms behind the decision for a merozoite to commit to sexual differentiation as opposed to the continuation of the erythrocytic cycle are not completely understood. Kariu *et al.*, 2006, have shown, by disruption of the gene coding for TPx-1 in *P. berghei*, that in the mouse host, the *TPx-1* gene is required for gametocyte development. It is most likely that gene expression regulates parasite differentiation to the gametocyte stages; however the trigger behind this 'decision' is not known in *P. berghei* or indeed *P. falciparum* (Kariu *et al.*, 2006).

It is now known that co-infection with different genotypes does not necessarily induce *P. chabaudi* to alter its gametocyte conversion rate, however this rate is increased in response

to increasingly hostile host conditions such as in the case of drug application (Babiker *et al.*, 2008).

It is reported that parasite virulence, according to genotype as opposed to species, also has an effect on gametocyte conversion rates, but it is not thought that coexisting in itself, within a host alongside another genotype, has an effect on conversion rates (Babiker *et al.*, 2008).

Furthermore, sex allocation of gametocytes within a *P. chabaudi* infection has been shown to correlate with factors such as gametocyte density, i.e. a male bias is seen when total gametocyte numbers are low (Babiker *et al.*, 2008).

Many observations have been recorded from experimental data looking at gametocyte development. Definite advances have been made to further our understanding of this subject. However, many of the mechanisms behind the observations remain poorly understood thus far.

Within 24 hours of the gametocytes being taken up by the mosquito during its blood meal the zygote transforms into a motile ookinete. The ookinete exits the remnants of the blood meal (bolus) and travels across the midgut epithelium to rest in the space between the midgut epithelium and the basal lamina, known as the basal subepithelial space (Carter *et al.*, 2007). The parasite, now known as the oocyst, within which several rounds of asexual reproduction (sporogony) occur, is responsible for the production of sporozoites that are released into the haemolymph when the mature oocyst bursts and migrate to the salivary glands of the mosquito (Kappe *et al.*, 2003). Upon arrival at the salivary gland, sporozoites attach to the gland cells, invade these cells and move through the cytoplasm exiting into the lumen of the gland where they await injection into the human host when the mosquito takes a blood meal (Kappe *et al.*, 2003). These sporozoites are the stage of *P. falciparum* infective to the human host, thus the life cycle becomes complete.

1.2. The anopheline mosquito: vector of malaria

Mosquitoes belonging to the genus *Anopheles* are the only vectors of human malaria. They belong to the subfamily Anophelinae which in turn is a member of the family Culicidae, or the 'true mosquitoes'. The Culicidae have many morphological features that enable them to be recognised as different from other members of the order Diptera. They are a relatively primitive family more closely related to midges (Ceratopogonidae) and crane flies (Tupilidae) than to houseflies (Muscidae) or blowflies (Calliphoridae) (Clements, 2000).

Mosquitoes are found in all parts of the world except those that are permanently frozen, with the tropics and sub-tropics being home to the most species as conditions in these areas favour development and survival of the mosquito (Clements, 2000).

Of the 430 species of *Anopheles* found worldwide, only around forty are important malaria vectors (Meek & Hill, 2007). It is interesting also to note that although there are a good number of species able to transmit malaria, it is unusual to find more than four different vector species in any particular geographic region, probably due to requirements in breeding and feeding habitats and their availability in a given location (Chandramoban, 2007). Figure 2 shows the distribution of 34 species of dominant vector over 260 endemic or potentially endemic regions (Kiszewski *et al.*, 2004). It can be seen from this figure that some countries have more than one dominant species of vector dependent on the geographical location within the country. This reflects the diversity of habitats within the country (Kiszewski *et al.*, 2004).

Figure 2 also demonstrates that the sibling species complex, *Anopheles gambiae sl*, is very commonly found in Afro-tropical regions and as such are responsible for a large proportion of malaria transmission in sub-Saharan Africa (Scott *et al.*, 2002). *Anopheles gambiae sensu stricto* is highly anthropophilic, which increases its potential as a malaria vector

(Scott *et al.*, 2002). *An. gambiae ss* and *An. funestus* are also both endophagic and tend to bite after 11pm, meaning they are more likely to come into regular contact with humans than other anophelines, thus increasing their competency as vectors (Githeko *et al.*, 1996). *An. albimanus*, the malaria vector in South and Central America, is largely exophagic and less likely to rest indoors, which means that outside workers and those who sleep outside due to the hot weather in these areas are subjected to being bitten by this species, hence malaria spreads between humans in these areas (Curtis and Lines, 2007).

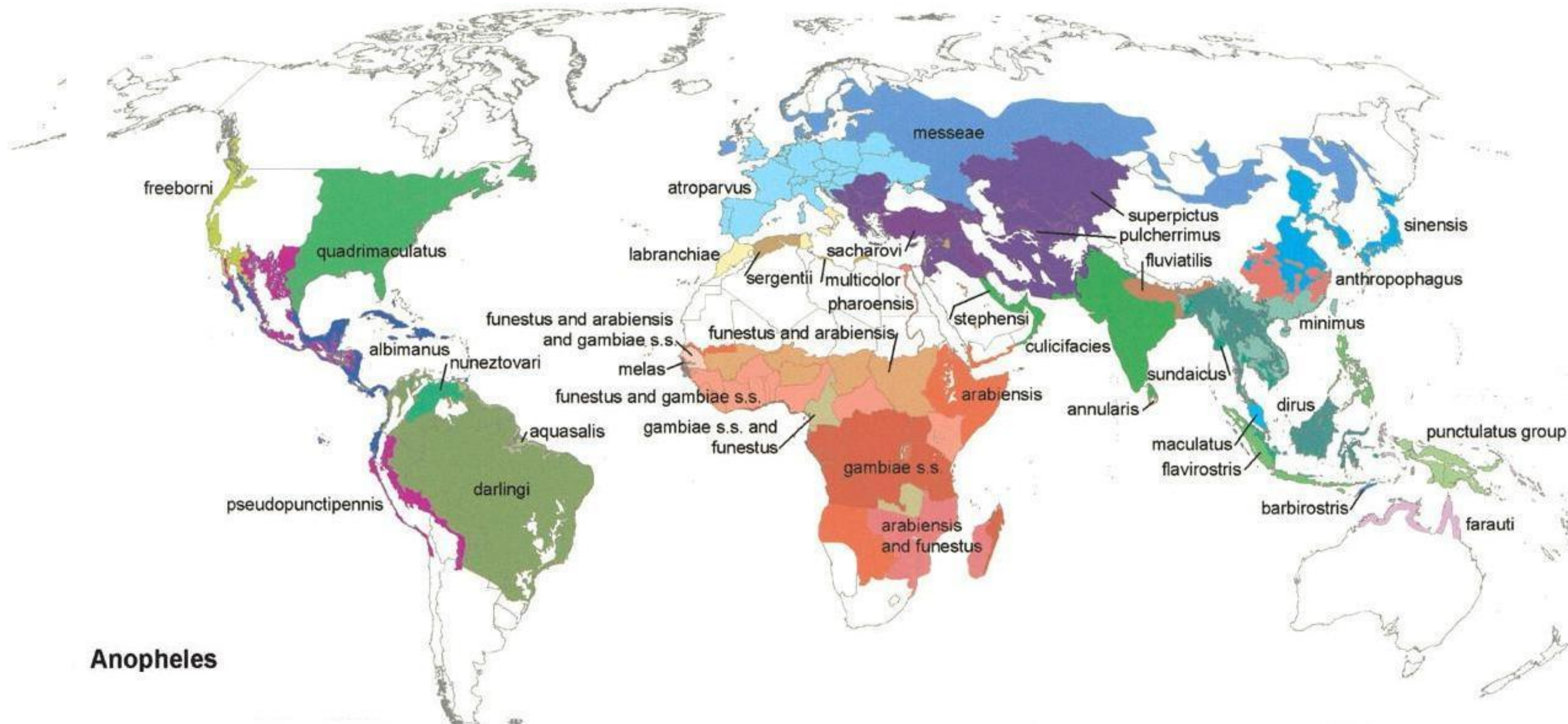

freeborni
quadrimaculatus
albimanus
nuneztovari
aquasalis
darlingi
pseudopunctipennis
atroparvus
labranchiae
sergentii
sacharovi
multicolor
pharoensis
funestus and arabiensis
and gambiae s.s.
funestus and arabiensis
melas
funestus and gambiae s.s.
gambiae s.s. and
funestus
gambiae s.s.
arabiensis
arabiensis
and funestus
messeae
superpictus
pulcherrimus
fluviatilis
stephensi
culicifacies
annularis
sundaicus
maculatus
flavirostris
dirus
minimus
anthropophagus
sinensis
punctulatus group
barbirostris
farauti
Anopheles
no vector
albimanus
annulans
anthropophagous
arabiensis
arabiensis & funestus
aquasalis
atroparvus
barbirostris
culcifacies
darlingi
dirus
farauti
flavirostris
fluviatilis
freeborni
funestus & arabiensis
funestus, arabiensis & gambiae ss
funestus & gambiae ss
gambiae ss
gambiae ss & funestus
labranchiae
maculatus
marajoara
melas
messeae
minimus
multicolor
nuneztovari
punctulatus group
pharoahensis
pseudopunctipennis
pulcherrimus
quadrimaculatus
sacharovi
sergentii
sinensis
stephensi
sundiacus
superpictus

Figure 1.2 (opposing page): The distribution of dominant or potentially important malaria vectors across the world (Robinson projection).

Dominant vectors were selected for by looking at longevity and feeding rate on humans. Keszewski *et al*. (2004) identified 260 endemic or potentially endemic regions which were infested by 34 species of dominant anopheline vector.

Figure adapted from Kiszewski *et al*., (2004).

The preferential biting habits of the different anopheline species in the various areas where malaria is endemic can have significant effects on the epidemiology of the disease. For example, Bockarie *et al.*, 1996, show that in Sierra Leone, parous females belonging to the species *An. punctulatus* bite later than nulliparous females. They also indicate that infection with the *Plasmodium* parasite in sporozoite form may alter biting behaviour depending on species of the mosquito (Bockarie *et al.*, 1996). It is useful to study these biting habits in order that preferences can be noted among the species in different locations. Such information could be critical in the implemented control of these vectors depending on the major vector present, amongst other factors.

The preferential breeding habitats of different species of *Anopheles* also impact on the epidemiology of the disease and need to be seriously considered when determining control strategies for a particular geographic region. The water used by the female anopheline on which to lay her desiccation-prone eggs can be permanent, such as a marsh, or temporary, such as a hoof print, puddle or water storage container (Service, 2004). The practical importance of permanent and semi-permanent habitats in controlling aquatic stages of *An. gambiae sl* is discussed with reference to their study in Mbita, Kenya in the paper by Fillinger *et al*. (2004). Their 20 month study shows that *An. gambiae sl*, identified using morphological characteristics, is found across many types of habitat whether they are man-made or natural, permanent or semi-permanent (Fillinger *et al.*, 2004). Therefore it is possible to suggest that their lack of preference in breeding habitat makes *An. gambiae sl* an undoubtedly good vector of malaria due to its ability to breed in any waterlogged habitat. If such habitats are man-made, this will certainly bring the mosquito into close proximity to humans, therefore increasing its competency as a vector. Fillinger *et al.* (2004) suggest that targeting the aquatic stages in areas such as Mbita would be necessary for sufficient malaria control programs.

1.2.1. Life cycle of the anopheline mosquito

Anopheline mosquitoes undergo a complete metamorphosis (holometabolism) during their life cycle as do other members of the Diptera. Female mosquitoes lay between 50 and 200 eggs at a time, and deposit them on the surface of clean oxygenated water. The anopheline egg is laid individually, (not in rafts like *Culex*) and its flattened upper surface is not covered with water whilst its lower part is submerged. It floats due to two air-filled extensions of exochorion on either side of the egg (Clements, 2002).

In tropical climates, eggs hatch within two to three days and aquatic stage larvae emerge. In temperate climates, development time of the mosquito takes longer. The larvae are filter feeders, feeding on micro-organisms, detritus and biofilm and, as with all mosquitoes, there are four larval instars (Clements, 2002; Service, 2004). During respiration, anopheline larvae utilise the pair of spiracles in the 8th abdominal segment only. The other nine pairs are responsible only for withdrawal of tracheae at ecdysis when the cuticle is shed (Clements, 2002). Anopheline larvae lie parallel to the waters' surface and are held to the surface membrane by a series of structures so the spiracles have direct contact with the air (Clements, 2002).

The larvae, dependent on food and temperature, usually pupate in tropical climates within 7 to 10 days after hatching. The pupae do not feed, however, they are mobile and need to respire, and do so by using a respiratory trumpet, within which short tracheal trunks connect the dorsal longitudinal tracheal trunks with the spiracles (Clements, 2002).

Adult anophelines emerge in a tropical climate within around 24 to 48 hours as a fully formed adult with the long legs, body and proboscis found in all mosquitoes (Clements, 2002). Male and female anophelines can be distinguished morphologically from each other. Males have plumose antennae and long clubbed palps, whereas females have pilose antennae and long simple palps (Service, 2004).

Both males and females of the *Anopheles* genus have the long proboscis associated with the Culicidae and both sexes feed on sugar from nectaries, rotting fruit and honeydew (Clements, 2002). It is only the female that requires a blood meal in order that she obtains enough protein so she can develop a batch of eggs (Clements, 2002). *An. gambiae* reportedly takes its first blood meal 12 hours after emerging, and will take multiple meals throughout its gonotrophic cycle (Briegel and Horler, 1993). The muscles at the base of the females' mandibles and maxillae, which are flattened and toothed, drive these mouthparts through skin tissue thus making a channel for the labrum and hypopharynx (Clements, 2002; Service, 2004). When taking a blood meal, saliva is pumped down the hypopharynx from the salivary glands and the blood is sucked up into the space formed by the relative positioning of the labrum and other piercing mouthparts and enters the midgut (Service, 2004). Around 70 possibly secreted proteins have been identified which may assist sugar and blood meals in *An. funestus* (Calvo *et al.*, 2007). Salivary proteins serve to prevent platelet aggregation, blood clotting and vasoconstriction as well as preventing some of the actions of the immune response (Calvo *et al.*, 2007). The combination of proteins evolved to do this clearly serve to ensure a blood meal can be taken from a host with minimal effort from the insect.

If a female mosquito has mated, it is the protein from a blood meal which will then enable a batch of oocytes to mature in the ovaries. The female will thus locate herself in a suitable environment where her eggs can be laid (Clements, 2002).

1.2.2. Life cycle of *Plasmodium* species within the anopheline vector

It is the adult female mosquito that is responsible for the transmission of *Plasmodium* species and it is within the adult that the malaria parasite undergoes developmental changes. It is the female mosquito's blood-feeding habit that allows uptake of the

gametocyte form of the parasite. This developmental stage of *Plasmodium* is the only stage infective to the anopheline mosquito host (Smith *et al.*, 2002). The trigger for the differentiation from the asexual reproductive cycle, which takes place in the blood of the vertebrate host, to the sexual reproductive stages remains unclear, but is thought to be partly a genetic response as loss of certain genes such as the *Pfg27* gene leads to a loss of the sexual phenotype in *P. falciparum* (Talman *et al.*, 2004). The *Pfs16* gene has also been shown to be vital for male microgamete exflagellation and therefore mosquito infectivity (Berry *et al.*, 2009). Differentiation is also thought to be partly triggered by a response to environmental factors whereby commitment to the sexual stage is increased when conditions in the host become unfavourable to the parasite (Talman *et al.*, 2004). However it is clear that this change is necessary in order to allow formation of male and female gametocytes and as such genetic recombination becomes possible which is a definite advantage for the parasite (Smith *et al.*, 2002). In the vertebrate host, sexually committed merozoites break out of red blood cells, invade new red blood cells and become trophozoites but instead of forming schizonts, they become gametocytes (Baton and Ranford-Cartwright, 2005). It has been shown that all merozoites from one schizont develop into gametocytes or continue the erythrocytic asexual cycle and all those that are committed to sexual development from one schizont become either male or female gametocytes (Talman *et al.*, 2004). As sexual development proceeds, the male and female forms of the parasite become increasingly dissimilar. Microgametes possess little synthetic apparatus and few cell organelles and assume a motile role lasting for only a short period of time. Conversely, macrogametes are packed with cellular organelles and apparatus and are capable of rapid stage-specific protein synthesis following fertilisation and are well adapted for survival within the hostile environment of the mosquito mid-gut (Baton and Ranford-Cartwright, 2005). The gametocytes within the vertebrates' red blood cells are

ingested by the mosquito during a blood feed and the change in environment triggers gametocytogenesis within the midgut of the female mosquito, whereby a drop in temperature, and rise in pH and the presence of a gametocyte-activating factor (xanthurenic acid) trigger the gametocytogenesis through calcium- and cGMP-dependent signalling pathways (Baton and Ranford-Cartwright, 2005; Muhia *et al.*, 2001).

Xanthurenic acid is part of the tryptophan oxidation pathway in mosquitoes, which protects the central nervous system from being over-stimulated by excitatory neurotoxins (Han *et al.*, 2007). The metabolites belonging to this pathway also influence the colour of compound eyes in mosquitoes with 3-hydroxykynurenine (3-HK) being the initial precursor for ommochrome production that contributes to pigment production in mosquito eyes (Han *et al.*, 2007). This pigmentation step occurs mainly in the pupal and adult stages of mosquito development and thus the conversion of 3-HK to the chemically stable compound xanthurenic acid is down-regulated in these stages to allow for transportation of some 3-HK to the compound eyes (Han *et al.*, 2007). Due to the production of xanthurenic acid being implicated in gametocytogenesis of *Plasmodium* spp., the tryptophan oxidation pathway and the proteins involved may provide a novel target for control of malaria especially given that parts of the pathway differ in mammals (Han *et al.*, 2007).

Macrogametes emerge from the red blood cells within the mosquito midgut and subsequently microgametes undergo exflagellation and produce eight product genomes that attach to axonemes rapidly assembled in the parasite cytoplasm (Baton and Ranford-Cartwright, 2005). Up to eight haploid microgametes break free and attach themselves to uninfected erythrocytes possibly enabling increased movement through the blood meal (Baton and Ranford-Cartwright, 2005). Fusion of micro- and macro-gametes forms diploid zygotes within the blood meal and endomeiotic replication follows producing a

single tetraploid zygote nucleus that is assumed to contain four haploid meiotic products (Baton and Ranford-Cartwright, 2005).

The sessile zygote gradually transforms into a banana-shaped motile ookinete present within the midgut of the mosquito (Barillas-Mury and Kumar, 2005). It is an extra-cellular stage of the parasite meaning that it is open to attack by gut digestive proteases and has therefore developed various defence strategies against such attack (Baton and Ranford-Cartwright, 2005). Two membranes, the outer zygote plasma membrane and the parasite plasma membrane, together are part of the Inner Membrane Complex (IMC) which forms the ookinete pellicle which is surrounded by a dense coat providing protection against digestive proteases (Baton and Ranford-Cartwright, 2005). The anterior portion of the IMC is capped by a rigid cone-shaped collar forming an apical prominence typical of the apicomplexan group of parasites when in their invasive stages (Baton and Ranford-Cartwright, 2005). This apical complex containing various cellular apparatus allows the parasite to penetrate and migrate through host tissues by the release of chitinases. These digest the peritrophic membrane surrounding the bolus (blood meal) and allow entry into the midgut epithelium (Barillas-Mury and Kumar, 2005; Baton and Ranford-Cartwright, 2005). This was demonstrated using *P. berghei* knockout parasites for the chitinase gene *PbCHT1* (Dessens *et al.*, 1999). This study showed that invasion of the parasite into *An. stephensi* midgut epithelium was reduced by 90% when compared to wild type parasites. The role of another key gene encoding membrane-attack ookinete protein was determined by Kadota *et al*. (2004). Parasites with null mutations of this gene lost infectivity to the midgut and were unable to rupture the cell membrane (Kadota *et al.*, 2004). This suggests a major role for this protein in invasion of the midgut by the *Plasmodium* ookinete. The ookinete must enter and traverse the midgut epithelial cells in order to reach the basal lamina. It has been observed that some ookinetes will cross a single cell in order to reach

the basal lamina, however in some cases the parasite performs extensive lateral movements crossing as many as six cells before the basal lamina is reached (Han and Barillas-Mury, 2002). Han *et al*. (2000) proposed a model of invasion they termed 'The Time Bomb Model'. It is based on observations of damage and repair systems occurring in the midgut at the time of parasite invasion whereby damage to the midgut epithelium is controlled so as to avoid contact between contents of the midgut and the haemolymph (Han and Barillas-Mury, 2002). Essentially, invaded epithelial cells are destined for destruction and, once the cascade of events leading to cell death is initiated, the parasite must leave the cellular compartment within a limited time window, before the 'bomb' detonates, to escape unharmed (Han and Barillas-Mury, 2002).

More recent studies indicate that it is not only insect cells invaded by *Plasmodium* species that undergo programmed cell death (PCD) (Arambage *et al.*, 2009). *P. berghei* and *P. falciarum* ookinetes show markers of apoptosis (similar to those shown by mammalian cells), expressed even in the absence of stressors. It is possible that PCD is used by the parasite to control its numbers in order to limit any host immune response (Al-Olayan *et al.*, 2002).

A study by Carter *et al* (2007) indicates the presence of an intermediate stage in the transformation between the ookinete form of *P. berghei* and the oocyst, which they have named a 'took' (transforming ookinete). They propose that the transformation, *in vitro*, of *P. berghei* ookinetes to young oocysts is not a time dependent transformation but is dependent on environmental stimuli. These stimuli, however, are not provided by basal lamina components (Carter *et al.*, 2007). It is thus proposed that transformation is initiated through bicarbonate stimulus and that full transformation requires nutrients and a suitable pH. This provides a better insight as to the requirements of the parasite system invaluable to further *in vitro* studies (Carter *et al.*, 2007).

Upon reaching the basal lamina, the ookinete is no longer motile and becomes surrounded by a thick extracellular capsule (Baton and Ranford-Cartwright, 2005). New basal lamina is secreted below the developing oocyst thus separating it from the midgut epithelium and within the oocyst itself, DNA-replicating machinery is up-regulated as is protein-making machinery, in order that sporogony can occur (Baton and Ranford-Cartwright, 2005).

The four presumed haploid products of meiosis that occurred in the zygote stage of the parasite within one nucleus now each undergo multiple rounds of endomitosis. Eventually the nucleus becomes lobular and divides into individual nuclei, followed by sporoblast formation after the oocyst cytoplasm becomes separated by clefts of subcapsular space (Baton and Ranford-Cartwright, 2005). During these rounds of asexual reproduction, the oocyst endures a massive increase in size of around 1000-fold and protrudes into the haemocoelic cavity of the mosquito. This size increase causes a tear in the basal lamina overlying the oocyst itself and as a result it becomes directly exposed to the haemocoel (Baton and Ranford-Cartwright, 2005).

A study by Aly and Matuschewski (2005) made use of targeted gene disruption and reverse genetic techniques to show that the gene encoding ECP1 (a cysteine protease) is responsible for *Plasmodium* sporozoite emergence from the oocyst. This indicates that oocyst rupture is an active developmental event, rather than a passive result of sheer numbers of sporozoites (Aly and Matuschewski, 2005).

Once the sporozoites have entered the mosquito haemolymph they begin their migration to the salivary glands in order that the *Plasmodium* life cycle can be completed. It has been suggested that sporozoites isolated from the haemolymph of a mosquito are less infectious than those found in salivary glands, which suggests that they undergo important changes as they migrate (Barillas-Mury and Kumar, 2005). A study by Myung *et al*. (2004) confirms previous findings that *Plasmodium* circumsporozoite protein (CSP) found on the surface of

sporozoites binds to salivary glands and not other haemolymph organs. A peptide from the N-terminal third of the CS protein is also shown, in the same study, to inhibit sporozoite invasion of salivary glands *in vivo* so together with previous evidence suggests CSP plays an important role in attachment and invasion of mosquito salivary glands (Myung *et al.*, 2004).

Engelmann *et al.*, 2009, identify that the *siap-1* gene is essential for sporozoite transmission from the mosquito to the mammalian host. In this study, targeted gene deletion experiments gave rise to *P. berghei* parasites which were able to form sporozoites but could not fully egress from oocysts and consequently were unable to establish an infection of *An. stephensi* salivary glands (Engelmann *et al.*, 2009).

The sporozoites enter the salivary gland cells by formation of a parasitophorous vacuole (PV) that dissolves once the parasite is internalised leaving the sporozoite in direct contact with the cytoplasm of the cell (Baton and Ranford-Cartwright, 2005). Once the sporozoites have reached the apical membrane of the cell the surrounding membranes disintegrate and the parasite is left exposed in the cavity of the salivary gland. Here they gather in high numbers in tightly packed bundles and are secreted into a bite wound when the mosquito feeds (Baton and Ranford-Cartwright, 2005).

1.3. Mosquito Immune Response to Infection

An immune response is mounted in an organism when its cells or tissues are invaded by 'foreign' matter, i.e. something not recognised as 'self'. When a pathogen enters the body of an organism it is recognised as a potential threat and is attacked by the immune system of this organism. The form of defence used by the immune system is dependent upon both the host and the attacking organism. Immune reactions within the host are complicated but it is necessary to understand, as much as possible, the interactions between host and the attacking organism if we are to in any way aid the immune system in fighting infection. Not only is this the case for the human immune system and its many diseases, but it also holds true when it comes to interactions between haematophagous insects, such as mosquitoes, and the parasitic diseases they transmit. Knowledge of the steps involved in immune processes when mosquitoes are infected with parasites may lead to the creation of opportunity to aid the mosquito in preventing parasite development and thus halt or at least decrease transmission to a human host (Yassine and Osta, 2010).

When anopheline mosquitoes are infected with the malaria parasite *Plasmodium* after taking a blood meal from an infected host, they mount an immune response against this invading parasite. This determines the outcome of the parasites' development within the mosquito host and ultimately whether the life cycle of *Plasmodium* can be completed and consequently infection passed to another mammalian host (Yassine and Osta, 2010).

The lifecycle of *Plasmodium* is such that it must cross the midgut epithelium. In doing so it encounters an immune response which is capable of vastly reducing parasite numbers (Yassine and Osta, 2010). The ookinete stage can be melanised in the basal labyrinth of the midgut or lysed in the cytosol of the midgut epithelial cells it is traversing. A genetic basis for these traits has been shown (Collins *et al.*, 1986; Vernick *et al.*, 1995).

A study by Vlachou *et al.*, 2005, has shown that of the transcripts analysed from half the genome of *An. gambiae*, 7.1% (650 genes in total) are differentially regulated upon invasion of the midgut by *P. berghei* 18 to 44 hours post-blood meal. This 7% was shown, by reverse genetic techniques, to include the most prominent regulators of actin and microtubule dynamics thus providing more evidence that actin cytoskeleton reorganisation occurs as a response to parasite invasion (Vlachou *et al.*, 2005). Further reverse genetic analysis also indicated that the lipid transporter RFABG plays a role in the development of both parasite and mosquito-egg development (Vlachou *et al.*, 2005). This study demonstrates the incredibly complex link between host response at a genetic level and parasite survival in the midgut. It further highlights the need for knowledge regarding the mosquito immune response to *Plasmodium* invasion should we wish to adapt this response with a view to creating refractory mosquitoes.

Lehmann *et al.*, 2009, looked at a variety of mosquito species including anthropophilic *An. gambiae*/*An. arabiensis* and zoophilic *An. quadriannulatus* to determine molecular evolution of immune genes by looking at polymorphism across these species. The authors suggest that purifying selection is the most common selection pressure acting on the immune genes in their study (*SP14D1, GNBP, defensin,* and *gambicin*), operating on all genes at the contemporary time scale, and indicate a presence of balancing selection on at least *gambicin* (Lehmann *et al.*, 2009). Their study also suggests that a diverse array of pathogens mediates balancing selection to maintain several alleles of immune genes and that positive selection may be associated with speciation following exposure of mosquito species to new pathogens (Lehmann *et al.*, 2009). Patterns of genetic variability were homogeneous across all mosquito species, whether anthropophilic or zoophilic, so consequently the conclusion was drawn that exposure to human pathogens does not mediate selection on immune genes (Lehmann *et al.*, 2009).

1.3.1. Melanisation

The *Plasmodium* ookinete traverses the midgut epithelium and becomes the oocyst, containing many sporozoites which are the infectious stage of the parasites (Collins *et al.*, 1986). Surrounding the ookinete stage in a capsule of an electron-dense melanin-type substance prevents development and thus causes death of the parasite (Collins *et al.*, 1986). The encapsulation reaction takes place in the refractory mosquito at about 16 hours post blood meal with the melanin-like substance appearing on the ookinete once it has traversed the gut wall as indicated by electron microscopy. These encapsulated parasites are located between the midgut epithelial cells and the basal lamina (Collins *et al.*, 1986). This space is permeated by haemolymph serum but is kept separate from mosquito haemocytes by the basal lamina (Volz *et al.*, 2006).

This melanisation response, part of the innate immune reaction to many invading organisms, is apparently unique to arthropods and involves at least 14 genes and a complex set of regulatory reactions within *An. gambiae* (Christensen *et al.*, 2005; Volz *et al.*, 2006). The encapsulation process involves three *Plasmodium* encapsulation genes, *Pen1, Pen2* and *Pen 3* of which *Pen1* is the main locus of these three mapped quantitative trait loci (Kumar *et al.*, 2003). Various experiments using micro arrays as well as physiological and morphological data have indicated that reactive oxygen species (ROS), levels of which are elevated in a refractory strain of *An. gambiae* especially after blood feeding, are involved in the melanisation pathway (Kumar *et al.*, 2003). It may be that the ROS themselves cause damage to the parasite thus triggering an immune response or increased levels of ROS directly stimulate an immune response. Both scenarios are possible though not confirmed, but the fact that ROS are involved in the encapsulation of ookinetes is likely (Kumar *et al.*, 2003).

Phenylalanine hydroxylase (PAH) catalyses the hydroxylation of phenylalanine to form tyrosine (Christensen *et al.*, 2005). Phenoloxidase (PO) then is able to catalyse the hydroxylation of the formed tyrosine molecule to produce dopa which is the start of the pathway leading to eumelanin production (Christensen *et al.*, 2005).

The proteolytic serine protease cascade responsible for the activation of PPO (prophenoloxidase) to PO is involved in the melanisation process (Volz *et al.*, 2006). Activation of the serine proteases responsible for converting PPO to the active PO is thought to be regulated by serpins (Christensen *et al.*, 2005).

Sequential activation of several serine proteases eventually leads to activation of PPO-activating protease (PAP) which cleaves PPO to the active molecule PO, ultimately leading to the production of eumelanin (Michel *et al.*, 2006). Conversion of PPO to PO is probably the rate-limiting step in eumelanin production (Michel *et al.*, 2006).

Michel *et al.*, 2006, report that the *An. gambiae* recombinant serpins (SRPNs), 1 and 2 are capable of binding and inhibiting a heterologous PAP (PAP3 from the moth *Manduca sexta*) *in vitro*. *In vivo*, a reduction of SRPN2 causes an increase of melanin laid down on introduced negatively charged sulphadex beads as well as causing a 90% loss of *P. berghei* ookinetes. This suggests that knockdown of SRPN2 causes a greater melanisation response, indicative that it is a down-regulator of a mosquito PAP (Michel *et al.*, 2006). Similar *in vivo* studies using a *P. falciparum* infection did not however result in an increase in melanisation of ookinetes which the authors explain by suggesting that melanisation is a consequence of, rather than the cause of, the killing of *P. berghei* ookinetes (Michel *et al.*, 2006).

SRPN2 seems to down-regulate immune responses across the insect group, as do many other SRPNs which play a role in the serine protease cascade. However, studies on SRPN6 in both *An. gambiae* and *An. stephensi* indicate a difference in the function of this

particular serpin between these closely related species, possibly due to interaction with other genes responsible for an antiparasitic immune response (Abraham *et al.*, 2005). RNAi experiments to knock down SRPN6 function from *An. stephensi* (AsSRPN6) lead to a significant increase in the number of oocysts present on the midgut thus meaning that the gene has a role, either direct, or indirect, in parasite killing as knock down increased vectorial capacity (Abraham *et al.*, 2005). On the other hand, knock down of the *An. gambiae* SRPN6 (AgSRPN6) did not have an effect on oocyst load in the midgut, but did have an effect on clearance of dead parasites indicating that its function lies with promoting lysis of dead parasites in the midgut (Abraham *et al.*, 2005).

The clip-domain serine proteases (CLIPs) are key components of this protease cascade with five subfamilies identified in mosquitoes, two of which are non-catalytic and three of which are catalytic (Yassine and Osta, 2010). CLIPB17 (catalytic) and CLIPA8 (non-catalytic) are positive regulators of the melanisation process in *An. gambiae* and have suggested roles in activation of PPO (Volz *et al.*, 2006). CLIPs acting as negative regulators in the melanisation process are all non-catalytic (Volz *et al.*, 2006).

Once dopa is formed from the hydroxylation of tyrosine, PO then catalyses the oxidation of dopa to dopaquinone (Christensen *et al.*, 2005). Dopachrome is formed spontaneously from dopaquinone which is, in the presence of dopachrome conversion enzyme (DCE), decarboxylated to form 5,6-dihydroxyindole (DHI) which subsequently undergoes oxidation to become indole-5,6-quinone (Christensen *et al.*, 2005). It is these indolequinones which polymerise to form brown eumelanin without the requirement of enzymes (Christensen *et al.*, 2005). These melanisation reactions, though complex, are specific against various pathogens including sympatric, but not allopatric, *Plasmodium* species, and can occur within minutes of exposure to haemolymph (Christensen *et al.*, 2005).

1.3.2. Lysis

Vernick *et al.*, 1995, reported that the laboratory infection of *An. gambiae* with the bird malaria parasite, *Plasmodium gallinaceum*, indicated a refractoriness to infection not involving parasite encapsulation. Parasite death was seen to be occurring, by use of ultrastructural studies, in the midgut epithelial cell cytosol within 27 hours of midgut invasion thus showing, by microscopy, a lack of oocyst development on the mosquito midgut (Vernick *et al.*, 1995). The same study indicated that, by crossing of susceptible and refractory lines, a simple genetic basis existed for refractoriness to infection due to parasite lysis in the epithelial cell cytosol (Vernick *et al.*, 1995; Yassine and Osta, 2010). Blandin *et al.*, 2004, knocked down the TEP1 gene (which codes for a haemocyte-specific complement-like protein) in *An. gambiae* mosquitoes by RNAi techniques. This caused the abolition of the refractory phenotype in a refractory strain of *An. gambiae* and caused an increase in the number of developing parasites in a susceptible strain of this mosquito species. The authors suggest that the TEP1-dependent killing of parasites is immediately followed by TEP1-independent lysis and/or melanisation of ookinetes (Blandin *et al.*, 2004). However, although it is known that TEP1 binds to *P. berghei* ookinetes egressing from the midgut epithelial cells on the basal side, the mechanism behind the killing of these parasites remains unclear (Blandin *et al.*, 2004). TEP1 is however both structurally and functionally similar to mammalian complement factor whilst having several distinct features, all of which require further investigation in order to understand the role that mosquito complement protein has in the immune defence against malaria parasites. However, there is now increasing evidence that TEP1 is almost certainly responsible for lysis of parasites in midgut epithelial cells (Vlachou and Kafatos, 2005; Volohonsky *et al.*, 2010). A leucine-rich immune factor (LRIF1), containing structural motifs similar to those found in immune related molecules such as Toll and Toll-like receptors, is also involved in

lysis of *Plasmodium* ookinetes. Moreover, by means of gene silencing techniques, LRIF1 is known to be an antagonist on development of ookinetes to oocysts (Osta *et al.*, 2004).

1.3.3. Nitric oxide and its role in immunity

Several cell types are capable of expressing an inducible nitric oxide sythase (iNOS) which converts L-arginine to citrulline and nitric oxide. The latter is toxic to a variety of pathogens including *Plasmodium* in both murine and human hosts (Mellouk *et al.*, 1994). *In vitro* studies with human hepatocytes have shown that gamma interferon and *P. falciparum* act together to increase the production of NO by 33% compared to the presence of *P. falciparum* alone (Mellouk *et al.*, 1994). The same studies indicate that NO synthesis plays a role in anti-malarial activity (Mellouk *et al.*, 1994). It seems that as well as playing a role in defence against the malarial parasite in both murines and humans, iNOS activity is known to limit *Plasmodium* development in *Anopheles stephensi* mosquitoes (Luckhart *et al.*, 1998). *An. stephensi* NOS levels were shown to be elevated 1 day post blood-meal, when *P. berghei* invasion of the midgut is maximal, and also later whereby enzyme activity would correlate with sporozoite release (Luckhart *et al.*, 1998). It is suggested that the midgut is the first line of defence against *Plasmodium* (and other invading organisms) with carcass induction of AsNOS acting as a signal-mediated secondary response to mediate those pathogens crossing the midgut barrier (Luckhart *et al.*, 1998). The same study also showed that mosquitoes fed an L-arginine supplemented diet had a 28% reduction in infection rates, and a significant reduction in infection when fed a thousand-fold dilution of the same supplement. This indicates that parasite killing is dependent on the NOS substrate, L-arginine (Luckhart *et al.*, 1998). However, supplements of the NOS inhibitor, L-NAME, significantly increased *Plasmodium* oocyst infections. When looked at together, the evidence from this study suggests strongly that nitric oxide is capable of

limiting *Plasmodium* in *An. stephensi* mosquitoes (Luckhart *et al.*, 1998). More recent studies both *in vitro* and *in vivo* (using *An. stephensi*) also indicate that NO has a direct effect on *Plasmodium berghei*, inducing apoptosis in the ookinete stage of the parasites development prior to invasion of midgut epithelial cells (Ali *et al.*, 2010).

1.3.4. Antimicrobial Peptides (AMPs)

Antimicrobial peptide production is hugely important in terms of the innate immune response mounted by both vertebrates and invertebrates to combat attack by various pathogens. In *Drosophila*, production of AMPs is regulated by either the *Toll* or *Imd* immune pathways, and involves various members of the NF-κB/Rel family of transcription factors which regulate expression of these AMPs in the fat body of the insect (Luna *et al.*, 2006). In the mosquito, *An. gambiae*, there are three families of AMPs reported. These are the defensins, cecropins and gambicins (Luna *et al.*, 2006). Molecules similar to defensins have been found in various members of the invertebrate group including the Mollusca and Arthropoda, as well as in the vertebrate group. However, there is little homology in terms of sequence or structure, only in terms of mode of action, between these two groups (Froy and Gurevitz, 2003). Cecropins have been found in some members of the phylum Nematoda and in various members of the Arthropoda (Luna *et al.*, 2006). Defensins and cecropins found in *Ae. aegypti* as well as *An. gambiae* are expressed in naïve mosquitoes in the thorax and abdominal tissues as well as in the anterior part of the midgut (Vizioli *et al.*, 2001a). Gambicin has also been found in *Ae. aegypti* and *Culex pipiens pipiens* (Luna *et al.*, 2006).

In *Anopheles*, the mature peptide gambicin, containing 61 amino acids including eight cysteines in four disulphide bridges, shows an expression pattern similar to that of the defensins and cecropins. In its mature form, it is active against the ookinete stage of the

Plasmodium parasite and is a novel AMP, not sharing homology with other database entries, which could possibly suggest that it evolved as to be active specifically against *Plasmodium* parasites (Vizioli *et al.*, 2001a).

Defensins in insects all contain six cysteine residues engaged in three disulphide bridges, with the *An. gambiae* defensin being similar in sequence to other insect peptides belonging to this family (Vizioli *et al.*, 2001b). Defensin RNA had been detected in various mosquito tissues but the mature peptide had only been isolated from haemolypmph of adult *Ae. aegypti* and larval *An. gambiae* prior to the study by Vizioli *et al.*, 2001b. In this study, MALDI-TOF-MS and RP-HPLC indicated the presence of mature defensin in adult *An. gambiae* hamolymph as well as in the midgut. An ELISA was used to quantify this amount (Vizioli *et al.*, 2001b). In a study by Dixit *et al.*, 2008, a novel defensin was identified and characterised after selective enrichment during extraction from *Plasmodium*-infected salivary glands of the *An. stephensi* mosquito. The exact nature and transcriptional activity of this protein is yet to be assessed (Dixit *et al.*, 2008) .

Cecropins were first isolated from the moth species, *Hyalophora cecropia*, and have since been identified in other members of the Lepidoptera and also from dipterans including *Drosophila*, and more recently, the mosquito group (Ekengren and Hultmark, 1999). The highly basic cecropin protein, usually around 3-5 kDa in size, is secreted into the haemolymph of the insect after it is induced upon infection, and is active as a broad-spectrum antibiotic against both Gram negative and Gram positive bacteria (Ekengren and Hultmark, 1999). *Drosophila* cecropins are also known to have antifungal properties (Ekengren and Hultmark, 1999). In a study by Sun *et al.*, 1999, three isoforms of cecropin were characterised from cDNA from the *Ae. albopictus* cell line, C7-10. These were shown to bear only 40% similarity to *Drosophila* cecropins when taking into consideration the overall sequence, with the N-terminus bearing a 71% homology in terms of amino acid

sequence when direct comparisons were made with this region of the mature peptide alone (Sun *et al.*, 1999). Lowenberger *et al.*, 1999, have shown that a cecropin isolated from the haemolymph of bacterially-challenged adult *Ae. aegypti*, whilst being effective against the same species of bacteria and filamentous fungi, is less active than its *Drosophila* counterpart. The authors suggest that differences between the two peptides can be explained by the lack of tryptophan residue and C terminus amidation in the *Ae. aegypti* cecropin A (Lowenberger *et al.*, 1999). It has been documented that cecropin RNA from the malaria vector *An. gambiae* is up-regulated in response to *Plasmodium* infection, and where expressed in the midgut, is seen solely in the anterior part (Vizioli *et al.*, 2000).

1.3.5. Infection and immune response in relation to mosquito fitness

It has been documented that infection with *Plasmodium* reduces the fecundity of the mosquito vector both in the lab and in the field even though a reduction in blood meal size is not seen (Hogg and Hurd, 1995a; Hogg *et al.*, 1996). Carwardine and Hurd, 1997, using *An. stephensi* mosquitoes infected with *P. yoelii nigeriensis*, show that the reduction in fecundity observed when comparing mosquitoes fed on infected blood with those fed on non-infected blood, is due to resorption of the follicles within the mosquito ovaries. In this study, assessment of follicular development using Christopher's stages indicated that follicular resorption takes longer than 12 hours post-blood meal to begin, and affects more follicles in infected mosquitoes than in their non-infected counterparts (Carwardine and Hurd, 1997).

Ahmed *et al.*, 2002, stimulated the humoral immune response of *An. gambiae* mosquitoes by a post-blood meal injection of LPS (lipopolysaccharide) from Gram-negative bacteria. Up-regulation of the immune response, possibly via the NF-κB/Rel pathway, was gauged by measurement of antimicrobial activity in the mosquito haemolymph against

Micrococcus luteus, and this immune stimulation was compared against two fitness parameters (Ahmed *et al.*, 2002). Egg production was measured by counting eggs laid plus eggs retained and accumulation of yolk protein (vitellin) was also measured (Ahmed *et al.*, 2002). The study indicates that the stimulation of the mosquito immune response leads to fewer resources being made available for reproduction thereby reducing fecundity of these mosquitoes (Ahmed *et al.*, 2002). A similar study showed that stimulation of AMPs by the method described by Ahmed *et al.*, 2002, as well as stimulation of the melanisation pathway by injection with Sephadex beads gave rise to caspase activity in follicular cells within the ovaries of *An. gambiae* (Ahmed and Hurd, 2006). Caspase activity is an indicator of apoptosis, and was seen to be up-regulated in *An. gambiae* infected with *P. yoelii nigeriensis* in a more pronounced manner than with injection of beads of LPS (Ahmed and Hurd, 2006). It can therefore be concluded that up-regulation of the immune response in *An. gambiae* by a variety of methods is costly in terms of reproductive fitness (Ahmed and Hurd, 2006). It is possible therefore that the lack of refractoriness to *Plasmodium* in field populations of anophelines is because the cost of immunity in terms of reproductive fitness would outweigh the benefits (Schwartz and Koella, 2001). Interestingly, *Ae. aegypti* injected with Sephadex beads also showed reduced fecundity associated with an increased melanisation response, but only when the beads were positively charged (Schwartz and Koella, 2004). No reduction in fecundity was observed with neutral beads even though the melanisation response was still strong, thus suggesting that re-allocation of reserves is not the underlying mechanism for fecundity-reduction, or at least the underlying mechanism is not at all simple (Schwartz and Koella, 2004). It has also been documented that dietary resources, namely blood and sugar, are a requirement for development of an active melanisation immune response, and as field populations are often malnourished, this may have an impact on released transgenic refractory mosquitoes

(Koella and Sorensen, 2002). Transgenic mosquitoes, refractory to *Plasmodium*, may struggle when released, in competing with field populations if their immune response to the infection is hindered by malnourishment. This may only prove to be problematic if expression of anti-*Plasmodium* effector genes is driven by immune-specific promoters which may have altered expression patterns at times of malnourishment. If driven by, for example, blood feed-specific gene promoters such as the carboxypeptidase promoter, then although it is effectively an immune response, expression of such a gene would not be affected by malnourishment in the same manner.

1.3.6. Multiple blood feeding habits

It has been noted that *An. gambiae* mosquitoes given an initial infected blood feed, upon receiving a second blood meal, show an increased sporozoite load in comparison to those not given a second blood meal (Beier *et al.*, 1989). It is suggested that the increase in sporozoite load is due to an increased stimulation of oocyst maturation (Beier *et al.*, 1989). Lopes *et al.*, 2007, suggest that this occurs due to a higher availability of nutrients for the parasite due to a second blood meal, and also may trigger a diversion of energy resources, within the mosquito, from an immune reaction against the parasite to digestion and protein production for egg development. The same study also showed that the oocyst burden was higher in those mosquitoes given a second blood meal when compared to those only given the initial infected feed, and that this effect was blocked when the second feed contained rat immune serum (Lopes *et al.*, 2007). Briegel and Horler, 1993, show that *Anopheles* mosquitoes will take more than one blood meal per gonotrophic cycle. A study by Nirmala *et al.*, 2005, supports this finding, and suggests that even when fully engorged, a female will seek blood every 24 hours. The authors suggest, after looking at the accumulation of mRNAs post-blood feed, that this multiple feeding behaviour can be used to an advantage

when creating transgenic mosquitoes whereby an effector molecule is linked to a blood-meal-specific promoter such as the carboxypeptidase promoter (Nirmala *et al.*, 2005). It is important to consider the biting behaviour of *An. gambiae* when looking to create transgenic mosquitoes which are effective against *Plasmodium*, especially where there is a possibility that blood meals taken after the initial infected feed may cause an increase in parasite burden within the mosquito, due to a divergence of resources from an immune response, as this may have an impact on an effector molecule. Further clarification will be required before any release of mosquitoes containing anti-*Plasmodium* effector molecules is deemed feasible.

Clearly, an understanding of insect immunity and host-parasite interactions is imperative, especially when considering future work aiming to control diseases such as malaria by the introduction of transgenic mosquitoes which express novel anti-*Plasmodium* peptides.

1.4. Vector control

Control of the vector is regarded as one of the most effective ways of preventing malaria transmission and as such has become one of the four implemented elements of the Global Malaria Control Strategy (WHO, 2006).

In order that control of the vector is successful, several factors must be considered in relation to the vector that is to be controlled. A knowledge of the local species of *Anopheles* responsible for malaria transmission in that area is vital, so it can be gauged as to their feeding and breeding habits which will have an impact on the best type of control method to be used (Walker and Lynch, 2007). Eradication of invading populations of *An. gambiae* has been possible even before the invention of insecticides, such as DDT, whereby extensive high profile political programs in places such as Egypt and Brazil were sustained by community involvement (often enforced by legislation) and were concerned with targeting larval populations requiring detailed knowledge of species breeding habitats (WHO, 2006).

The introduction of DDT meant that adult mosquitoes could be targeted either instead of, or as well as, the larval stages and Indoor Residual Spraying (IRS) with DDT was thought to be able to provide a good chance of eradication of malaria if continued until malaria infection died out (WHO, 2006). Certainly, this seemed to be the case from early reports in southern Europe, north America and Taiwan and as such IRS with DDT, and later with other insecticides, became the backbone to malaria control (WHO, 2006). However IRS relies on mosquitoes resting on walls indoors either before or after feeding and also relies on human shelters having walls and that these walls are accessible for spraying and are not likely to be re-plastered (WHO, 2006). The IRS strategy wasn't adhered to in terms of logistics, human resources and funding and as such the eradication effort, especially in Africa, was abandoned as it was no longer deemed possible (WHO, 2006).

It was decided that vector control was important in order to reduce transmission of malaria, thereby reducing the number of cases requiring treatment and easing pressure on health services (WHO, 2006). However it was also recognised that vector control can only be most successfully undertaken when measures are taken to properly plan the methods to be used in particular areas and ensure that they are implemented properly by making certain that costing is accurate and the plan is feasible given the biology of the vector in that area (WHO, 2006; (Walker and Lynch, 2007).

Insecticide-treated bed nets (ITNs) and IRS are now the more common methods of vector control directed at adult *Anopheles* populations as they are more applicable to different areas than larval based control programs which require a more in-depth knowledge of both the biology of the local vector and the geography of the local area (WHO, 2006).

Smith *et al.*, 2009, discuss the use of mathematical models to map malaria endemicity following the distribution of ITNs using *Plasmodium falciparum* parasite rate (*Pf*PR) measured in children over two and under ten years of age. Such models indicate that high levels of ITN ownership would alter malaria epidemiology, thus reliable models could prove useful as a tool when considering ITN distribution as a control measure across various parts of Africa (Smith *et al.*, 2009).

The insecticides used to treat bed nets are currently pyrethroids however there is evidence to suggest that primary malaria vectors such as *An. gambiae sl* and *An. funestus* have developed resistance across many parts of Africa over the past few years (N'Guessan *et al.*, 2003). *Culex quinquefasciatus*, the vector of lymphatic filariasis caused by *Wuchereria bancrofti* and *Brugia malayi*, also shows high levels of resistance to pyrethroids and as such it is concerning that as local inhabitants are aware the *C. quinquefasciatus* are not as highly affected by ITNs then they may deem them as pointless, although they may still offer protection against *Anopheles* spp (N'Guessan *et al.*, 2003).

Koella *et al.*, 2009, suggest that emergence of insecticide resistance to those used in the treatment of bed nets and for IRS has been exacerbated by legislation such as that put in place by WHO. Their Pesticide Evaluation Scheme for Laboratory Testing states that potential insecticides for ITNs and IRS must kill 80% of 2-5 day old female *Anopheles* within 24 hours of exposure (WHO, 2005). It is thought that this places great evolutionary pressure on mosquitoes which serves only to increase probability of emerging resistance amongst mosquito populations. This is because younger females have not yet completed a gonotrophic cycle and thus haven't been able to successfully reproduce and it is also thought that insecticides which have a delayed effect place less evolutionary pressure on insects (Koella *et al.*, 2009). The authors suggest ways of providing protection using an approach that would decrease the risk of resistance developing.

Two main mechanisms confer resistance to pyrethroids. The *kdr* (knock-down resistance) mutation involves alterations at the site of action in the sodium channel. Both DDT and pyrethroids act by altering the gate kinetics of the sodium channel causing them to remain open for prolonged periods of time and thus causing paralysis and therefore death (Davies *et al.*, 2007). Two base substitutions in the *kdr* gene confer resistance and each is found in different populations of *An. gambiae* in different parts of Africa (Davies *et al.*, 2007). In resistant West African *An. gambiae*, leucine is substituted for phenylalanine in the S6 hydrophobic segment of domain II of the sodium channel (Davies *et al.*, 2007). In Kenyan populations of *An. gambiae* the same leucine molecule is replaced with serine, which confers a decreased resistance to pyrethroids than in the west African *An. gambiae* populations but an increased resistance to DDT (Davies *et al.*, 2007). An increase of detoxification and/or metabolism through high levels of multi-function oxidase (MFO) and non-specific esterase (NSE) is the alternative mechanism of resistance to pyrethroids and DDT (N'Guessan *et al.*, 2003). Matambo *et al.* (2007) used *An. arabiensis* from the

Sennar region of Sudan in a resistance study whereby it was indicated that a colony selected for DDT resistance showed an elevated level of glutathione-S-transferase in both sexes and elevated levels of esterases in males only when compared to a colony which had not been subjected to selection for DDT resistance. In both cases the colonies were subjected to all four classes of commonly used insecticides. Where both mechanisms are functional, resistance can reach extremely high levels (Davies *et al.*, 2007).

Donnelly *et al.*, 2009, review work undertaken by various groups to monitor resistance among *Anopheles* populations in order to determine whether *kdr* genotyping does show a real correlation with a resistance phenotype. They conclude that *kdr* screening, at the present time, is the best molecular diagnostic tool for predicting sensitivity of mosquito populations to pyrethroids (Donnelly *et al.*, 2009). However, the authors do consider that other mechanisms conferring resistance, such as the metabolic resistance mechanism discussed above, may play a bigger role than previously thought, but is momentarily hard to screen for due to a lack of diagnostic tools (Donnelly *et al.*, 2009).

The malaria eradication programs of the 1950's and 1960's are thought to have been major contributors to the selection of pyrethroid and DDT resistance in *An. gambiae* populations (N'Guessan *et al.*, 2003). However it has been noted that insecticide use in agriculture is much more extensive than its usage in vector control with insecticide resistance levels being higher in mosquito populations found around urban vegetable growing areas in which insecticides against crop pests are used more extensively than in rice field areas where insecticide usage is much lower (N'Guessan *et al.*, 2003). The study by Matambo *et al.* (2007) indicates that selection pressure for resistance by DDT especially causes a high movement from susceptibility of a colony to resistance. This has particularly worrying consequences for control measures especially in The Sudan where the colonies of *An.*

arabiensis used in this study were from and indicates that the mosquito is highly adaptable when placed under selection pressure.

A study by N'Guessan *et al.* (2007) shows that an insecticide marketed for controlling agricultural pests and termites known as Chlorfenapyr has the potential to be used as part of a malaria control program. The study shows that strains of *An. gambiae* resistant to pyrethroids, by means of the *kdr* resistance gene mutation, and organophosphates, as a result of the $Acel^R$ insensitive acetylcholinesterase mechanism, gave no indication of cross resistance to Chlorfenapyr (N'Guessan *et al.*, 2003). A more recent eight-week study in Benin, West Africa, whereby M-form *An. gambiae* were tested for susceptibility to chlorfenapyr, indicated that although resistance to pyrethroids is well-documented in this area, chlorfenapyr had a distinct knockdown effect (N'Guessan *et al.*, 2009). However, this insectide reduced efficacy over time as it has only a short 'shelf life' of around four weeks. The authors suggest that, if longer-lasting formulations become available, this insecticide shows promise for malaria control programs, however selection pressure will be placed upon mosquito populations, and given that resistance can occur rapidly (Matambo *et al.*, 2007) it must be taken into consideration that it may not be long before populations of mosquitoes become resistant by some mechanism to this insecticide too (N'Guessan *et al.*, 2009). Other more sustainable methods of vector control must be given some serious thought.

The creation of genetically modified mosquitoes has been suggested as a way of controlling malaria by either of two general methods; population replacement or population suppression (Marrelli *et al.*, 2006). Sparagano and De Luna, 2008, discuss both methods of control with reference to population structure amongst disease systems which is, of course, a huge consideration when looking at these systems for vector and therefore disease control.

1.4.1. Population Suppression

A mechanism which causes a reduction in numbers of vector insects present within an area would thereby reduce the transmission of the malaria parasite. This could be achieved by techniques such as the Sterile Insect Technique (Knipling, 1959), or RIDL (Release of Insects with a Dominant Lethal) technology (Thomas *et al.*, 2000; Alphey, 2002) or a combination of both (Marrelli *et al.*, 2006).

1.4.1.1. Sterile Insect Technique (SIT)

Sterile Insect Technique (SIT) has been used with well documented success as a method of controlling pest insects and disease vectors. The technique involves release of a large number of irradiated sterile males across a wide geographical area, often following a program of insecticide use (Alphey, 2002). Over whole areas in the USA and Central America, SIT has been used with some success to control the New World Screwworm fly *Cochliomyia hominivorax*, and in Latin America SIT has been used to combat the Mediterranean fruit fly *Ceratitis capitata*, also a pest insect (Alphey, 2002). SIT has been previously used to suppress the population of *Glossina* species, the tsetse fly vector of African sleeping sickness (trypanosomiasis). In 1994, Zanzibar initiated a tsetse eradication program which was to make use of SIT on the island of Unguja, Zanzibar. Sufficient time for six generations of tsetse was allowed to elapse after the last recorded catch of male and female flies before release of sterile males was stopped. The area has since been considered free of the insect (Vreysen, 2001).

Although SIT has been deemed successful in several areas with a variety of insects, it does have several limitations as a control method. SIT requires mass rearing of insects, and therefore a huge space in which to keep them. Only the males are irradiated and released, thus the reared population needs sexing and males retaining which is a laborious and time

consuming process. Methods used are seldom completely effective in separating sexes (Alphey, 2002). The process of irradiating males is expensive, and poses a fitness cost to the insect, whereby it has been shown that irradiated *C. hominivorax* males are at a disadvantage when compared to wild type males of the same species (Mayer *et al.*, 1998). Thus mass release is required in order that the sheer number of these irradiated males outweighs the fitness cost and therefore means that they can compete with the wild type population for females. The laboratory-reared males have this decreased fitness due to their being smaller than their wild type relations and interruption of physiological processes, both caused by the irradiation process itself (Spradbery, 1994). This is partly demonstrated by Helinski and Knols, 2009, by collection of *An. arabiensis* sperm from male reproductive organs to determine both sperm quality and length as a measure of male ‘fitness’. This was done using non irradiated lab strain males, field-collected males (from the same region as the lab strain was originally collected), as well as pupal-stage radiated males and adult-stage irradiated males. Those males irradiated at pupal stage were seen to produce fewer and smaller sperm when compared to unirradiated and those irradiated at adult-stage (Helinski and Knols, 2009). This suggests that whilst natural variation exists, as seen in other species and indicated by the results from the males collected from wild populations, irradiating males does have an effect on sperm quality when done at certain life stages (Helinski and Knols, 2009).

Different methods of sterilising males have been used to generate flies to be used in SIT programs where population reduction was not necessarily the intended outcome (Benedict and Robinson, 2003). In the studies where release of males was intended for population reduction there have been varying levels of success, dependent on both the species of mosquito and the area in which the study took place (Benedict and Robinson, 2003). In Pensacola, Florida, USA, 4.6 million sterile male *Aedes aegypti* sterilised by using gamma

radiation were released between 1960 and 1961. Despite good ratios of released males to wild males, no reduction in the population was observed (Benedict and Robinson, 2003). In 1967 the population of *C. quinquefasciatus* in Okpo, Myanmar (Burma) was eradicated by release of 5000 sterile males daily for 9 weeks. In this case sterilisation was achieved by Cytoplasmic Incompatibility (CI) known to be caused by the presence of *Wolbachia* bacteria (Benedict and Robinson, 2003). In Burkina Faso, 240,000 sterile male *An. gambiae* were released over a period of 9 weeks. However, it was seen that male competitiveness was poor compared to wild type males, and no significant reduction in population numbers was observed (Davidson *et al.*, 1970). The more successful large-scale releases of mosquitoes (whereby population suppression, or in some cases eradication, was achieved) have tended to occur in populations which are isolated and therefore migration effects are not seen (Benedict and Robinson, 2003).

1.4.1.2. Release of Insects with a Dominant Lethal (RIDL)

Thomas *et al* (2000) describe a modification to SIT whereby transgene integration technology is utilised in order to create an insect which has a dominant gene incorporated into its genome causing lethality to insects at a specific life stage. RIDL is designed to be repressible using the tetracycline-repressible expression system as described by Alphey (2002) meaning that the action of the lethal gene in the homozygous insects can effectively be ‘switched off’ in order that the population can be reared in the laboratory under conditions used for wild type strains. When access to tetracycline is removed from the population this activates the lethal system, and causes lethality at the intended life stage (Figure 3). The RIDL system ensures that any released homozygous male mating with a wild type female passes on the lethal gene to all the offspring and due to the gene’s

dominance, the lethal phenotype is apparent in all female offspring thus reducing the insect population.

The RIDL system can be used, not only to create an insect line containing a gene which, when activated, causes life-stage-specific death, but can also use sex-specific splicing of a promoter in order that the lethal action of the system is active in only one sex (Thomas *et al.*, 2000, Gong *et al.*, 2005, Fu *et al.*, 2007, Morrison *et al.*, 2009).

In most cases the sex-specific lethality is targeted at females as it is often this sex which causes the damage in terms of both crop-pests (oviposition) and vector insects (biting). Consequently it is beneficial, should such strains be released as a population control method, that females do not survive to reproduce. With this system it also follows that any wild-type females mating with homozygous RIDL males have offspring which survive to breed only if they are male. These males will all carry a copy of the dominant gene and will subsequently pass on this gene to a proportion of the next generation following the usual Mendelian heritability patterns (Thomas *et al.*, 2000, Gong *et al.*, 2005, Fu *et al.*, 2007). In terms of RIDL as a direct modification of SIT, release of a huge number of males is reliant on efficient sexing mechanisms. Morrison *et al.*, 2009, present this lethal system in terms of the creation of a transgenic sexing strain (TSS) of Mediterranean Fruit Fly (*Ceratitis capitata*) which would allow for initial mass rearing and, once the colony is large enough and a release required, the production of males only. Following competition studies, the authors suggest that these TSS strains are competitive enough against wild-type strains to allow for further research prior to eventual release (Morrison *et al.*, 2009).

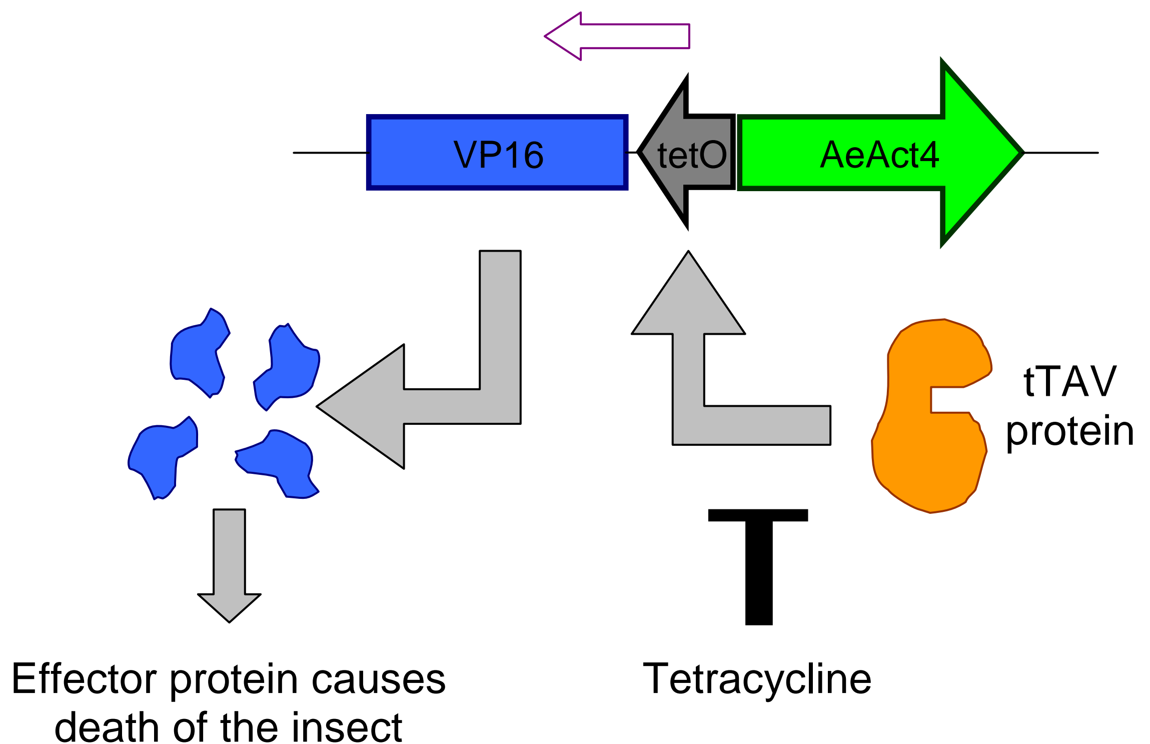


Figure 1.3: An illustration of the tetracycline-repressible expression system.

Expression of the tetracycline-repressible transcriptional activator (*tTAV*) is under the control of a promoter of choice (in this case the *Ae. aegypti Actin4* promoter which drives expression of Act4 primarily in female flight muscle). The translated tTAV protein binds to the tetO operator DNA sequence, resulting in activation of a minimal promoter. This leads to expression of an effector protein, which results in the flightlessness of the insect. In the system represented above, VP16 from the *Herpes simplex* virus is the effector protein which builds up in the flight muscle of the mosquito causing flightlessness and consequently, death. Presence of a low level of tetracycline prevents binding of tTAV to tetO by itself binding to the tTAV protein. Adapted from Alphey, 2002.

This system has certain advantages over traditional SIT in that it doesn't require facilities for sex separation or irradiation. RIDL males do not undergo irradiation therefore released RIDL males would be fitter in terms of sperm competition than released SIT males (Alphey, 2002). Other fitness costs associated with the irradiation step involved in SIT as discussed by Mayer *et al.* (1998) for the new world screwworm fly *C. hominivorax* would also be removed, however the RIDL system is not problem-free.

Mayer *et al.* (1998) discuss, with reference to SIT, the reasons why factory-reared *C. hominivorax* males are less competitive for females than wild type males. Some of the reasons are SIT-specific, for example due to irradiation effects, however some are applicable to any laboratory-reared species and would also pose a problem for the RIDL system. Handling of and dispersal of flies can cause damage to wings and legs, for example, thus reducing fitness. Laboratory reared strains are under very different natural selection pressures to those placed upon natural populations. As such, they may possess different behaviours not suitable to successful release into a wild population which may cause a reduction in competitive mating compared to the wild type males (Mayer *et al.*, 1998).

RIDL also poses a further problem which is irrelevant in SIT. Release of genetically modified organisms (GMOs) is always a cause for concern for both the public and governing bodies. A good example of this has been given by the ongoing arguments for and against release of genetically modified or altered crops as discussed by Dlugosch and Whitton (2008). This paper also highlights a possibility that transfer of the transgene to neighbouring plants via gene flow mechanisms is occurring in the absence of direct selection pressure favouring the transgene (Dlugosch and Whitton, 2008). Although this finding occurs in plants, it only serves to concern the scientific community as well as regulatory bodies about the action of releasing any GMOs including insects. It would be

wise, as suggested by Alphey (2000), to ensure that any RIDL gene introduced into an insect should not be linked with resistance to insecticide or antibiotic, therefore incurring no selective advantage under those selection pressures.

Stability of the transgene involved in the RIDL system is a major concern given that transformation of insects such as mosquitoes involves the use of transposable elements such as *minos, Hermes, mariner* and *piggyBac* (Lobo *et al.*, 2006; Alphey, 2002). The involvelment of mobile DNA elements poses a theoretical risk that a transgene may alter its location within a genome or even transfer horizontally to other individuals or species.

Knols *et al.*, 2007, discuss the implications of using genetically modified mosquitoes to control malaria. They suggest that the use of these transgenic mosquitoes, whilst being (theoretically at least) a promising control strategy, requires careful management in terms of not only overcoming difficulties with public perception of GMOs in general, but also ensuring proper consideration of release (Knols *et al.*, 2007).

1.4.2. Population Replacement

If a population of vector were to be replaced by insects of the same species but which were refractory to the malaria parasite then a reduction in transmission of malaria would also be noted. There are many issues with this method of reducing transmission of a disease, such as the release of GMOs which is part of an ongoing debate, stability of the transgene associated with refractoriness to the disease within the population of released insects (Sethuraman *et al.*, 2007; Scali *et al.*, 2007, Woods *et al.*, 2007, Franz *et al.*, 2009), as well as relative fitness of the transgenic insect in comparison to the wild type (Marrelli *et al.*, 2006).

Marelli *et al.* (2006) discuss the associated fitness costs related to the creation of transgenic mosquitoes and examine the issues which surround the need for a drive mechanism to push

the transgene through the population thus creating a refractory strain. The creation of transgenic mosquitoes has been made possible by fine tuning of transformation techniques, and the invention of site-specific transformation systems (Lobo *et al.*, 2002; Nimmo *et al.*, 2006). Fluorescent markers and appropriate promoters are now also known to work in mosquito species and so the creation of refractory lines of various different species of mosquito vectors to their respective diseases has been made possible (Horn *et al.*, 2003).

1.4.2.1. Effector genes and their promoters

The mobile ookinete stage of the parasite develops in the midgut and traverses the mid gut epithelium becoming an oocyst which then encounters the haemocoel of the mosquito. After a certain length of time, the oocyst bursts to release sporozoites which invade the salivary glands. The ideal transgenic anopheline mosquito would therefore express an appropriate effector gene expressed in the gut, haemocoel or salivary glands which would target the developing or migrating *Plasmodium* parasite (Jacobs-Lorena, 2003).

Several research groups have successfully created transgenic strains of mosquito that carry genes associated with refractoriness to a particular parasite. Jacobs-Lorena (2003) describes transformation of *An. stephensi*, a malaria vector, whereby the transgenic strain expresses a tetramer peptide known as SM1 (Salivary-binding and Midgut-binding protein 1). The protein was found by screening a phage display peptide library for phages that bind to midgut and salivary gland epithelia thus preventing parasites (*Plasmodium* species) binding to these surface due to their using a common receptor (Ghosh *et al.*, 2002). Expression of this peptide was linked to a carboxypeptidase promoter and infection studies with *P. berghei* showed a reduction in oocyst number of 80% when compared to the control (Ito *et al.*, 2002). Transformation of *An. stephensi* whereby the transgenic mosquito expresses bee venom phospholipase A2 (PLA2) under control of a

carboxypeptidase promoter from *An. gambiae* has also been undertaken (Jacobs-Lorena, 2003). Transgenic mosquitoes were challenged by a *P. berghei* infection and an 87% reduction in oocyst numbers was observed (Moreira *et al.*, 2002). Moreira *et al.* (2002) inhibited PLA2 enzyme activity and again looked at development of the parasite which continued to be impaired. Inactivity of the enzyme suggests that the enzyme acts on the parasite purely by its binding action to exposed membrane lipids. There is also further evidence to suggest it does not actually kill the parasite, but merely impedes its development Moreira *et al.*, 2002). The ideal situation would be that the expressed gene would encode a peptide which caused death of the parasite.

A study by Yoshida *et al.* (2007) demonstrates that transgenic *An. stephensi* expressing C-type lectin CEL-III isolated from the sea cucumber *Cucumaria echinata*, when challenged with a *P. berghei* and *P. falciparum* infection, show an impairment of development of both rodent and human parasites. Expression of the effector gene was driven by the *An. gambiae* carboxypeptidase promoter therefore the protein was localised to the midgut (Yoshida *et al.*, 2007). CEL-III was seen to haemolyse red blood cells of both humans and rats both *in vitro* and in the midgut of the transgenic *An. stephensi*. It is also known to induce apoptosis, thus causing destruction of the red blood cells before the gametocytes differentiate (Yoshida *et al.*, 2007). Although the protein was not seen to haemolyse mouse erythrocytes, there was still a reduction in oocyst numbers in comparison to the control, thus indicating that CEL-III may have a direct effect on the parasite, and may also possibly reduce fertilisation efficiency of the parasite (Yoshida *et al.*, 2007).

James (2003) reviews the use of effector genes that target the salivary gland stage of the malaria parasite. He makes the point clearly that various studies have shown that there is a substantial difference between regions of the anopheline salivary gland lobes. The sporozoites have been shown to prefer to invade the distal-lateral and medial lobes of the

female salivary glands (James, 2003). This has obvious implications for the inclusion of salivary gland targeting peptides expressed in a transgenic mosquito.

Catteruccia *et al.* (2005) expressed EGFP under the control of the ß2-tubulin promoter in the gonads of 3rd instar larvae (and later stages) of *An. stephensi* in order that efficient sex separation be possible either manually or by automated sorting machines thus creating a viable sexing strain which could be used for application in vector control programs. The fluorescent sperm produced by these transgenic mosquitoes was shown to be viable and the males were capable of mating normally (Catteruccia *et al.*, 2005). Following on from this work, Windbichler *et al.*, 2008, transformed *An. gambiae* with the EGFP marker expressed as a fusion protein with the homing endonuclease, I-Ppol, in the hope that this enzyme would behave as it does in *An. gambiae* cell lines and selectively cleave ribosomal rDNA repeats exclusive in *An. gambiae* to the centromeric region of the X chromosome thus rendering this chromosome unable to function properly and thus distorting the sex ratio with a male bias. The experiments showed that distortion of the sex ratio towards a male bias is possible though embryonic lethality occurred as well which in itself is an interesting observation, again in terms of mosquito control, but both areas require further study (Windbichler *et al.*, 2008).

For an effector gene to be successful it has to be expressed at the right time and in the right place to target the particular parasite stage. Several promoters have been discovered which can be linked to the effector gene to ensure it is transcribed at the correct time. Yoshida and Watanabe (2006) describe the discovery and use of a salivary gland-specific promoter driving expression of anopheline anti-platelet protein (AAPP). The authors constructed a cDNA expression library from mRNA collected from female *An. stephensi* at 6 hours post blood meal. After cloning a cDNA, sequence analysis showed that the gene encodes a homologue of an *Ae. aegypti* 30 kD allergen of unknown function. The sequence of the

cDNA was identical to the gene encoding *An. stephensi* GE-rich protein which inhibits platelet aggregation and was therefore termed AAPP (Yoshida and Watanabe, 2006). *An. stephensi* embryos were transformed with a vector containing the 1.7 kb AAPP promoter region, the DsRed reporter gene and the *Antryp1* polyadenylation signal. RT-PCR analysis showed that an abundance of DsRed mRNA was expressed specifically in salivary glands of female *An. stephensi* and not in male salivary glands. Maximal expression of the DsRed gene was found to be at 48 hours post blood meal which corresponded to maximum levels of the AAPP mRNA expression as confirmed by qRT-PCR analysis (Yoshida and Watanabe, 2006). The authors conclude that a robust salivary gland expression system is viable in *An. stephensi* mosquitoes to drive a salivary gland-specific effector gene.

Studies by Chen *et al.* (2007) indicated that the *An. gambiae* vitellogenin promoter can be used to drive EGFP marker expression in transgenic *An. stephensi*. As the major yolk precursor, vitellogenin gene transcription is up-regulated following a blood-meal, with expression localised to the fat body of a female thus making the promoters of such genes theoretically useful for targeting *Plasmodium* sporozoites traversing the haemolymph (Chen *et al.*, 2007b). Initial expression of vitellogenin genes occurs earlier than the appearance of sporozoites in the haemolymph of the female mosquito, however the authors suggest that, as *An. gambiae* and *An. stephensi* are known to take multiple blood meals, this would cause sufficient transcription for an effector to be present at the same time as haemolymph sporozoites (Chen *et al.*, 2007b). In this set of experiments, *An. stephensi* were injected with a construct whereby EGFP was expressed under control of the *An. gambiae* vitellogenin gene, but with the 3' UTR from the homologous mammalian gene (BGH) which may account for the longer-lasting presence of EGFP mRNA in the mosquito than was expected, though the actual mechanism behind this requires further investigation (Chen *et al.*, 2007b). As such, it remains unclear as to whether one can say

for definite that the vitellogenin promoter would ideal for driving expression of an effector molecule targeting sporozoites in the female mosquito haemolymph, so further investigation is required in order to answer this question (Chen *et al.*, 2007b).

A recent study by Franz *et al.* (2009) has highlighted a problem with using the carboxypeptidase promoter for driving expression (in the female mid-gut after a blood-meal) of an antiviral effector gene in *Aedes aegypti*. The mosquitoes were transformed using a mariner vector containing containing the *Aedes* carboxypeptidase promoter driving expression of inverted repeat (IR) sequences which would eventually form dsRNA from IR-RNA to trigger an RNAi response targeting Dengue virus serotype 2 (DENV-2) (Franz *et al.*, 2006). The initial studies by Franz *et al.* (2006) on the established Carb77 line, up until Generation 8, indicated, by use of Northern blot analysis, IFAs and plaque assays, that the vast majority of individuals belonging to this transgenic line showed high resistance levels to infection with DENV-2. Presence of siRNAs derived from the IR transgene were found to be present which is indicative that the mosquitoes were completely blocking virus transmission past the midgut stage by use of the RNAi mechanism (Franz *et al.*, 2006).

When testing the same Carb77 strain at later generations (G9-G17), Franz *et al.* (2009) showed that although the RNAi mechanism still provided the basis for the resistance phenotype, it was seen in fewer mosquitoes and had been completely lost by G17. The authors conclude that mutation in either the virus or the transgene was not the cause for phenotype loss, however, IR effector gene expression was undetectable in G17 mosquitoes using Northern blot and qRT-PCR techniques, though the transformation marker was still present (Franz *et al.*, 2009). Loss of expression may just be a phenomenon unique to this single situation, but nevertheless is indicative that serious consideration is required when

designing refactory mosquitoes as transgenic insects would only be thought of as a viable control method if the transgene were proved to be stable over time (Franz *et al.*, 2009).

It is possible to drive effector gene expression in the necessary environments to reduce parasite transmission at the insect stage. However there are clearly both ethical and scientific problems to be overcome before this method of control can be implemented. Jacobs-Lorena (2003) suggests the use of more than one promoter-effector gene targeting more than one life stage of the *Plasmodium* parasite in order to reduce the likelihood of the wild type *Plasmodium* overcoming the effector gene and thus becoming resistant.

1.4.3. Fitness costs associated with transgenic mosquitoes

Marelli *et al.* (2006) discuss the fitness costs involved in the creation of transgenic mosquitoes with special regard to the actual burden from the transgene product and insertional mutagenesis. These studies have shown that transgenic mosquitoes capable of reducing their parasite burden have a clear advantage over non-transgenics with the same background. This indicates that being able to kill the parasite is desirable in fitness terms but only when fed on mice infected with gametocyte-producing parasites (Marrelli *et al.*, 2007). This advantage may not transfer well to the field as infection rates are so low and the advantage may be outweighed. However many other studies looking into fitness in transgenic insects indicate there is a cost in reproductive fitness to transgenic insects possibly associated with inbreeding to a large degree and maintenance of homozygous lines (Marrelli *et al.*, 2006).

Studies by Li *et al.* (2008) tested three transgenic *An. stephensi* lines in terms of fitness loads of the inserted transgene as well as mating success and reproductive capacity. All three lines express the SM1 peptide under control of the vitellogenin promoter but have differing insertion sites with one line (VD9) containing a double insertion. In cage

experiments, whereby homozygous transgenic lines were competing with their non transgenic counterparts, the frequency of the transgene decreased over time (Li *et al.*, 2008). Further study suggested that this observation was due to reduced mating success and mating capacity of the transgenic lines, leading to the conclusion that a driving mechanism would be required to overcome the fitness cost associated with the transgenic mosquito if they were to be released for control purposes (Li *et al.*, 2008). This replacement method of population control will only work in the field if the transgenic mosquito can out-compete its wild type counterparts for resources. When considering the population suppression strategy using RIDL technology, whereby mass release of transgenic males is necessary then these released males must be able to compete effectively against their wild type counterparts for females in order for the strategy to have any chance of working. Mating competitiveness of transgenic *An. arabiensis* males was looked at by Howell and Benedict (2009). The transgenic strain expresses EGFP under control of the ß2-tubulin promoter as well as a DsRed transformation marker driven by the eye-specific 3xP3 promoter. Mating experiments were conducted in small cages and spermathecae examination as well as blood feeding/egg laying/screening determined the ability of heterozygous males to mate successfully when competing for genetically similar or dissimilar females with wild-type males (Howell and Benedict, 2009). These experiments show that the genetic background of neither males nor females has an effect on male survival or frequency of mating thus indicating that, for this species, with this insertion, there is no cost in terms of male competitiveness. Clearly this study does not provide conclusive proof that all transgenic insects are able to compete effectively with wild-type counterparts but does go someway to suggesting that larger scale field trials are worth doing (Howell and Benedict, 2009).

In summary, fitness of transgenic mosquitoes needs to be compared not only with non-transgenic laboratory populations but with out-bred populations which will resemble field populations more closely. Also, linking of the transgene to a drive mechanism may prove useful (Marrelli *et al.*, 2006).

1.4.4. Drive mechanisms

Sinkins and Gould (2006) review various selfish genetic elements, including transposons, meiotic drive genes, homing endonuclease genes and *Wolbachia*. All of these have a degree of potential for driving effector genes through a population of vector insect if they are more fully understood (Sinkins and Gould, 2006).

Chen *et al.* (2007) report a synthetic drive mechanism first described in the beetle *Tribolium castaneum* and termed *medea* (maternal effect dominant embryonic arrest). This triggers maternal-effect lethality of offspring that do not inherit the element-bearing chromosome from the maternal or paternal genome. The authors created a synthetic *medea*-like transgene to be used in the model organism *Drosophila*. A *P* element vector was used that contained a *bicoid* (maternal-germline specific gene expressed in early embryo development) promoter to drive expression of a polycistronic transcript encoding two microRNAs (miRNAs) designed to silence expression of *myd88*. The latter is a gene responsible for dorsal-ventral patterning of the early embryo. Females experiencing loss of function of *myd88* in the germline produce embryos that do not hatch even with a wild type allele present (Chen *et al.*, 2007a). The vector, known as medea^{myd88}, also coded for a zygotic 'antidote' gene whereby the embryo-specific bottleneck (*bnk*) promoter drives expression of a maternal miRNA-insensitive *myd88* transgene (Chen *et al.*, 2007a). The experiment showed that all surviving adult progeny carried the medea$^{myd88-1}$ synthetic element, suggesting that a single copy of *bcd*-driven miRNAs targeting *myd88* expression

was sufficient to induce maternal-effect lethality and a single copy of zygotic *bnk*-driven *myd88* expression was adequate for rescue (Chen *et al.*, 2007a). If a synthetic *medea* element was constructed for use in mosquitoes and linked to one or more effector genes, there is evidence to suggest, if introduced into a population at a high enough frequency, it would rapidly transform the population into element-bearing heterozygotes and homozygotes. However, these models do not take into account migration, non-random mating and genetic drift, which must be considered in terms of 'real' populations especially where species complexes such as *An. gambiae* are concerned (Chen *et al.*, 2007a). There is still a great deal to be discovered about these elements and their function in the mosquito. In addition, little is known about the genes and promoters used during early embryonic development in such species. However *medea* elements have definite possibilities as a genetic drive mechanism for anti-parasitic transgenes.

Rasgon (2009) suggests multi-locus assortment (MLA) as a method of transgene dispersal among mosquito populations. This involves release of mosquitoes carrying anti-parasite effector genes at multiple genomic locations. The study indicates that only four transgenes at different loci are required, and, depending on fitness cost of said transgenes, they can be maintained within the population by sequential releases of relatively small numbers whereby halted release would cause transgene elimination over time (Rasgon, 2009). However it is not likely that transgenes would be spread across wide geographical areas using this method of dispersal especially if there is an imposed fitness cost associated with carrying the transgenes themselves, and as such, MLA would be useful only in terms of dispersal within defined local areas (Rasgon, 2009). There are also limitations imposed by use of the mathematical models themselves as they assume parameters which are not necessarily a reflection on the real situation, and would need to be addressed as entirely separate issues (Rasgon, 2009).

Different gene drive systems have different properties. For example, Engineered Underdominance (EU) is able to drive its two associated transgenes to high and stable frequencies within a population but does require the release of a larger number of insects than other systems would, whilst Meiotic Drive (MD) and *Wolbachia* systems are unable to maintain high frequencies, but can drive the spread of the transgene from initial low frequencies (Huang *et al.*, 2007). It has thus been suggested that a combination of gene drive strategies can be utilised as an improved method of driving inserted transgenes across insect populations (Huang *et al.*, 2007). The authors use statistical modelling techniques to determine the combination of EU, MD and *Wolbachia* systems which could increase gene drive efficiency. The differing properties of each system indicates, via the models, that a combination of EU with either MD or *Wolbachia* may provide a plausible mechanism of transgene dispersal through insect populations and is certainly worthy of further thought in order that a combination strategy can be given a thorough assessment (Huang *et al.*, 2007).

1.5. Transformation using Transposable Genetic Elements

Transposable genetic elements are capable of promoting recombination reactions which result in the movement of the element from one site in the genome to another (O'Brochta and Atkinson, 1996). Class I transposable elements transpose using a mechanism similar to that involved in retroviral integration, by reverse transcription of an RNA intermediate. Class II appear to transpose directly from DNA to DNA using a cut and paste mechanism (O'Brochta and Atkinson, 1996; O'Brochta *et al.*, 2003). The *P* element has been used successfully for germline transformation of *Drosophila* with great success, and remains the transposon of choice in this insect (Rubin and Spradling, 1982;Venken and Bellen, 2007). It has however proved unsuccessful in transformation of non-drosophilid insects where class II transposable genetic elements, such as *mariner, minos, Hermes* and *piggyBac* are used for effective germline transformation (O'Brochta *et al.*, 2003).

Class II transposons such as those mentioned above are generally short to medium length sequences of less than 10kb, and encode the transposase enzyme which catalyses the transposition event through the inverted terminal repeat sequences at either end of the element (O'Brochta and Atkinson, 1996; Venken and Bellen, 2007). A schematic of a generalised transposon is shown in Figure 4.

Since the successful transformation of *Drosophila* using *P* elements in the early 1980s, progress has been made in order that it is now possible to transform several different insect species including vector species such as *Ae. aegypti, An. stephensi* and *An. gambiae* with various transposable elements (Grossman *et al.*, 2001; Catteruccia *et al.*, 2000; Coates *et al.*, 1998; Jasinskiene *et al.*, 1998).

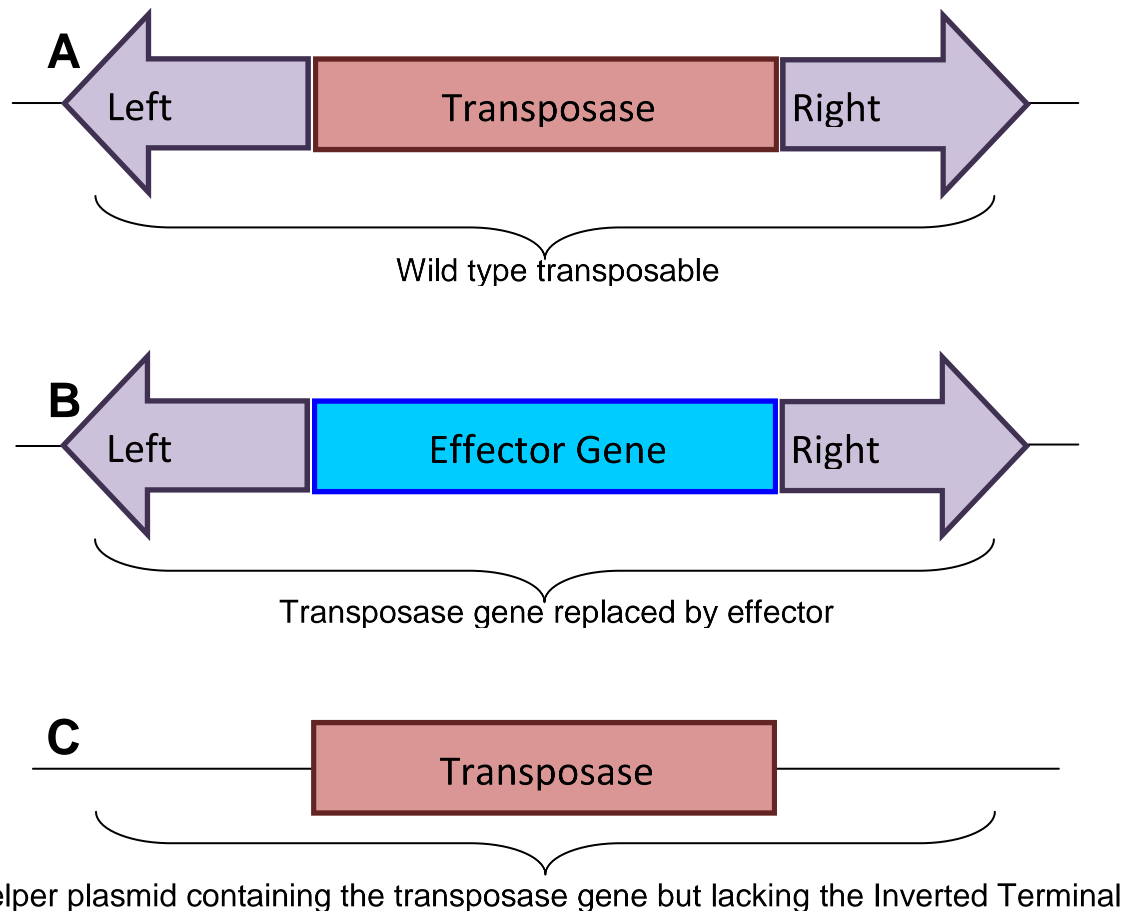


Figure 1.4: A generalised schematic of a Class II transposon showing the transposase gene flanked by inverted terminal repeat sequences

When using transposons (A) for transformation of insects the transposase gene is replaced by a transgene of choice, possibly an anti-malaria effector gene (B). The transformation vector is injected into the embryo with a helper plasmid (C) containing the transposase gene sequence, or transposase mRNA (Lobo *et al.*, 2006; Nimmo *et al.*, 2006).

The Class II transposon *piggyBac* is a 2.4kb long transposable element, originally isolated from baculovirus genomes from infected *Trichoplusia ni* (Lepidopteran) cells (Fraser *et al.*, 1995), which has been used successfully in transformation of many insects including mosquitoes (Grossman *et al.*, 2001; Kokoza *et al.*, 2001; Lobo *et al.*, 2002; Moreira *et al.*, 2002; Allen *et al.*, 2004; Sethuraman *et al.*, 2007). The element terminates in 13bp perfect inverted repeats and also contains internal asymmetrically located 19bp internal inverted repeats (Cary *et al.*, 1989). Its open reading frame (ORF), at 1783bp in length, encodes a protein consisting of 594 amino acids at a predicted mass of 68kDa. This protein, as with all transposons, is an enzyme which catalyses transposition of the *piggyBac* element itself at a specific TTAA target site (Fraser *et al.*, 1995).

Precise excision of *piggyBac* from the donor site is via a hairpin intermediate created at the donor ends and involves cleavage at the recognition site leaving TTAA overhangs on the ends of the donor DNA meaning a simple ligation reaction is all that is required for these ends to re-join (Mitra *et al.*, 2008). This hairpin intermediate leaves TTAA overhangs on the 5' ends of the excised linear transposon which base-pair with 5' TTAA single-strand gaps on the target-site DNA which again can be sealed by a ligation reaction (Mitra *et al.*, 2008). It is suggested that *piggyBac* is a member of the DDC recombinase superfamily due to the evidence collected from studies on its transposition mechanism (Mitra *et al.*, 2008).

Experiments to truncate the n and c-terminals, cause specific amino acid mutations and targeted gene deletions of the ORF coding for the transposase enzyme, demonstrate that a functional nuclear localisation signal (NLS) is present within c-terminal residues (Keith *et al.*, 2008). A NLS is necessary for the enzyme to be active in the presence of the nuclear envelope of a cell as this would require interaction with nuclear transport proteins allowing the transposase access to the nucleus of S2 cells (Keith *et al.*, 2008).

Lobo *et al* (2006) describe a protocol pertaining to high efficiency transformation of mosquitoes. This is based on micro-injection of plasmid DNA containing the required effector gene under transcriptional control of an appropriate associated promoter. The chosen transposon inverted terminal repeats flank the effector gene/promoter and marker sequences. The plasmid DNA requires injection with a 'helper plasmid' containing the transposase gene in order that the transposon can facilitate the jumping of the effecter gene into the genome of the mosquito (Lobo *et al.*, 2006). Injection of the plasmid DNA with transposase mRNA is also possible for successful integration into the genome of the target species (D. Nimmo, *personal communication*).

Transformation of mosquitoes using transposable elements has always been proved difficult, even with laboratories investing a large amount of time in fine-tuning techniques designed to improve efficiency. Transformation frequencies are often low, for example, transformation of *Ae. aegypti* with *piggyBac* is typically around 8% (Handler, 2002). Often, laboratories experience different transformation efficiencies with a relatively large range between individual experiments as illustrated by Lobo *et al* (2002) where efficiencies ranged between 0% and 13%. Catterucia *et al.* (2000) showed transformation efficiencies of 7% when using a *minos* element to transform *An. stephensi* and indicate that the first experiments yielded a much lower survival efficiency than later ones. Typically, those experiments showing higher transformation effiencies were the same as the later ones which gave a greater survival rate. Grossman *et al* (2001) also discuss poorer survival efficiencies near the beginning of their study, hypothesising that as they improved their protocol for microinjection and after-care of the embryos, they achieved a greater survival rate.

Even when transformation is successful there are still limiting factors when using transposons to facilitate insertion of novel DNA into a mosquito. There is a natural

limiting carrying capacity associated with transposons which limits the size of the effecter gene the element can insert into the host insects genome (Nimmo *et al.*, 2006). The transposition event is essentially random within the genome, and therefore can result in insertional mutagenesis. This involves disruption of the function of a gene required for normal function of the mosquito, for example a housekeeping gene. This results in death or severe deformation of the progeny of the G_0 survivors. It is also possible for there to be a position effect on the transgene itself due to its site within the genome causing low expression levels, and the gene not functioning as it should in order to be effective within the transgenic mosquitoes (Nimmo *et al.*, 2006).

Nimmo *et al.* (2006) discuss a site-specific system that proposes to overcome the three main problems associated with transposon-mediated transgene insertion into the mosquito germline. This system, from a *Streptomyces* bacteriophage, was shown to work in both *Eschericia coli* cells and *in vitro* assays using purified integrase to catalyse recombination of *attP* with *attB* sites to generate new products (*attL* and *attR*) which are junctions no longer recognised by integrase thus meaning that the system is unidirectional (Thorpe and Smith, 1998).

Studies using *Drosophila* S2 cells have indicated that recombination occurs at a rate of around 50%. In embryos, the recombination rate is around 80 to 100%, producing transgenic *Drosophila* in the range of 43 to 56% of fertile adults at *attP* sites previously inserted using *P element* (Groth *et al.*, 2004). Therefore this system is seen to be an improvement on previously used methods of genetically modifying the fly genome, generating a transgenic frequency of 8% (once optimised) as opposed to 2% using the transposon-mediated conventional method. The phiC31 system also produced a higher transformation rate than both the FLP and homologous recombination systems and requires

the generation of only one suitable *attP*-containing line which can then be used indefinitely for gene insertions using the phiC31 system (Groth *et al.*, 2004).

In *Drosophila*, this site-specific integrase system has been optimised whereby visual selection for precise *attP* targeting has been generated using a *white*-gene based reconstitution system, and it is demonstrated that chromosomal *attP* sites can be modified *in situ* thus increasing efficiency as docking sites (Bischof *et al.*, 2007).

In the case of *Ae. aegypti*, Nimmo et al. (2006) used the *piggyBac* transposon to incorporate an *attP* insertion site into the genome of this target species. The authors characterised genomic integration sites of this construct by inverse PCR, and four homozygote lines were used to test the site-specific phi C31 integration system by microinjection of integrase mRNA and a construct containing the *attB* site and a DsRed marker linked to an eye-specific promoter. Interaction between *attP* and *attB* sites is mediated by integrase and creates *attL* and *attR* junctions that are no longer recognised by integrase, therefore making the system unidirectional. Transformation efficiency for this second phase of the experiment averaged 23%. Southern blotting and PCR techniques were used to confirm site-specific integration at *attP* target sites at the molecular level (Nimmo *et al.*, 2006).

The site-specific integration system has, in theory, an unlimited carrying capacity, meaning that multiple transgenes can be incorporated into the genome of a mosquito targeting different life stages of the parasite. The system also overcomes the problems of insertional mutagenesis and position effects by careful selection of the *attP* target sites.

1.6. Stability and re-mobilisation of transposons

A major problem associated with the use of transposons to insert transgenes into the genome of mosquitoes is the potential for remobilisation. Further transposition events after the element has become incorporated into the genome may lead to a lack of stability of the associated transgene. This clearly has implications at the population level, and may cause concern were transgenic mosquitoes to be released. It would be impossible to accurately describe future phenotypes of descendents of released transgenic mosquitoes within a population, or model the behaviour of a transgene over a period of time in modified species if there was instability linked to the transgene itself due to its association with the transposon (O'Brochta *et al.*, 2003).

Several studies have been undertaken to assess the potential that remobilisation occurs after the initial insertion of the element into the genome. Catterucia *et al*. (2000) showed that it was possible to transform *An. stephensi* using the *minos* element. In 2001, Kokoza *et al* were able to transform *Ae. aegypti* using the *piggyBac* element. Although proving that successful germ-line integration of novel DNA using transposons is possible, these experiments did not test the stability of these integrations in successive generations. Since then, several studies have been undertaken to assess the re-mobility potential of various transposons in different insect species (O'Brochta *et al.*, 2003; Horn *et al.*, 2003; Lorenzen *et al.*, 2007; Scali *et al.*, 2007; Wilson *et al.*, 2003). These studies have indicated that different transposons do not behave in the same manner in any one particular organism. They also show that the same transposon may behave differently in another organism, even when considering related species. For example, *Hermes* transforms *Drosophila* with a frequency of over 50%, but transforms *Ae. aegypti* with a frequency of less than 10% (O'Brochta *et al.*, 2003). *Mariner* has been shown to remobilise efficiently in *D.*

mauritiana but very inefficiently in *D. melanogaster,* even though they are closely related species (O'Brochta *et al.*, 2003).

Remobilisation of the *Hermes* element is discussed by O'Brochta *et al* (2003). The remobilisation potential of the element in *Ae. aegypti* was assessed using transposable element (TE) display. In this technique genomic DNA from individuals is digested with restriction enzymes resulting in a junction fragment between the transposon and the wild-type DNA. The ends of the fragment are ligated to specific adapters, and a nested PCR reaction follows, whereby the second reaction uses an element-specific primer labelled with Cy5. The fragments are size fractionated by denaturing acrylamide gel electrophoresis and visualised on a phospho-imager (O'Brochta *et al.*, 2003). Darker bands (representing an abundance of product) of a different size to the expected fragment length indicate that remobilisation has occurred within the germline and the element has been inherited vertically. Weaker bands represent transposition events occurring in somatic cells of an individual (O'Brochta *et al.*, 2003). Well isolated bands can be excised, cloned and sequenced to confirm the presence of the *Hermes* terminal sequences and the unique flanking genomic DNA resulting from the transposition event. These techniques have demonstrated that remobilisation of the *Hermes* element occurs in the soma of *Ae. aegypti* however it has not been shown to remobilise in the germline of this species. It does however show remobilisation potential in the germline of *Drosophila* (O'Brochta *et al.*, 2003).

Similarly, Scali *et al.* (2007) have demonstrated that an X-linked *minos* element does not appear to remobilise within the germline of *An. stephensi* mosquitoes, but transposition and excision events are common within the somatic cells of males heterozygous for both the transposon and the transposase. The transposon *piggyBac* was used to create transgenic helper lines expressing the *minos* transposase gene. An ML10 donor line was created

using the *minos* element containing the EGFP gene under the control of the *D. melanogaster actin5C* promoter. Both lines were characterised using southern blot techniques (Scali *et al.*, 2007). Virgin homozygous ML10 females were crossed with homozygous helper line males. Male progeny were then crossed with virgin wild-type females and their progeny were assessed for remobilisation events using TE display (Scali *et al.*, 2007).

In *Ae. aegypti* it has been shown that the *Mos1 mariner* element is only rarely able to remobilise in somatic cells, and is even less likely to transpose in germline cells. This study was undertaken in a similar manner to that described above, using transposase-expressing helper strains crossed with donor strains containing a *Mos1* element flanking a marker/promoter sequence. TE display was again used to assess remobilisation events (Wilson *et al.*, 2003). The fact *Mos1* shows a low potential for remobilisation when compared to *Hermes* indicates that it may be a useful tool for introduction of stable transgenes within a population of *Ae. aegypti*. The authors do suggest however, that the lower remobilisation potential of this transposon may be due to the lack of critical sequences and/or sub-optimal spacing of the inverted terminal repeat sequences (Wilson *et al.*, 2003). More recent studies of the mariner transposon have demonstrated that transposition rates are temperature-dependent whereby higher temperatures cause a decreased rate possibly related to the transposase enzyme kinetic properties altering at higher temperatures (Sinzelle *et al.*, 2008). This may have an impact on not only initial integration events into host genomes using mariner, but also on remobilisation potential post-integration especially when it is considered that mosquitoes are responsive to ambient surrounding temperatures.

Horn *et al.* (2003) reported that the *piggyBac* element remobilises in the presence of transposase in *D. melanogaster* at a rate similar to the *P* element. This appears also to be

the case in the beetle *T. castaneum* as shown by Lorenzen *et al.* (2007) whereby the donor element containing an EGFP marker was remobilised in 84% of hybrid crosses. Sethuraman *et al.* (2007) have shown that, contrary to studies using other species, *piggyBac* is stable in the soma of *Ae. aegypti* and did not remobilise the reporter gene when the transposase source was provided by either a helper plasmid or by a transposase-expressing helper line. The authors have also shown no transposition events occurred in the germline of *Ae. aegypti* in the absence or presence of a transposase source. These data indicate that *piggyBac* may indeed be a good tool for integration of effector genes into *Ae. aegypti* as these integration events would be stable and show a very low propensity for remobilisation thus causing less concern about excision of, or movement of the gene within the genome of the species concerned. Vertical transmission may still be possible.

Future work

PiggyBac is commonly used in the transformation of many organisms (Handler, 2002). This includes the major African malaria vector *An. gambiae* (Grossman *et al.*, 2001). It would therefore be very beneficial to look at the potential for remobilisation of this transposon within *An. gambiae* as this insect species is the target for many studies regarding the creation of transgenic lines refractory to the malaria parasite.

1.7. Aims and Objectives

The overall aim of the work undertaken here was to create a transgenic line of *Anopheles gambiae* containing an effector gene which, in the absence of tetracycline, would be expressed, resulting in a female-specific flightless phenotype. Females of this line, when reared without tetracycline, are not able to mate or feed and thus the phenotype is akin to that of a line containing a dominant lethal gene. This transgenic line, once generated, was to be characterised and assessed for the remobilisation potential of the *piggyBac* transposable element used in the integration process. A further aim was to assess the fitness of a previously generated transgenic line, EVida3, containing an effector gene known to be active against *Plasmodium* parasites in the mosquito midgut. Therefore the objectives for the work undertaken here were as follows:

I. Creation of a transgenic RIDL strain of *Anopheles gambiae* using the double-ended *piggyBac* system (Dafa'alla *et al.*, 2006) to insert the RIDL gene into the genome of *An. gambiae* and utilisation of this system to test the remobilisation potential of *piggyBac* in *An. gambiae* as well as the application of RIDL technology in *An. gambiae*

II. Assessment of the effect of multiple blood meals on the anti-malarial properties of the Vida3 peptide within transgenic *An. gambiae*.

III. Initial laboratory-based fitness studies on the transgenic EVida3 line of *An. gambiae*.

Chapter Two

2. Microinjection of *Anopheles gambiae*

2.1. Introduction

The introduction of novel DNA into insects was documented in the model organism *Drosophila melanogaster* by Rubin & Spradling in 1982. This work showed that, by using the 3Kb transposon, *P element*, a stable, heritable integration of a known genetic sequence was not only possible, but was efficient and reproducible. The wild-type phenotype in the transformed strain was rescued thus indicating correct expression of the inserted gene (Rubin and Spradling, 1982). Initial introduction of the plasmid carrying the wild-type eye colour gene (*rosy* plasmid) was via microinjection into *Drosophila* embryos. It was co-injected with a plasmid containing an entire *P element* which would provide the necessary enzyme for the transposition of the *rosy*$^{+}$ allele into the fly genome (Rubin and Spradling, 1982). Since then, microinjection of constructs into insect embryos has become a standard technique for the introduction of novel DNA into many species (Coates *et al.*, 1998; Catteruccia *et al.*, 2000; Tamura *et al.*, 2000; Grossman *et al.*, 2001; Handler and Harrell, 2001; Concha *et al.*, 2011). Although the microinjection technique for germ line transformation of insects has been pioneered in *Drosophila*, the transfer of this technique to other species has many applications. It is possible to genetically modify insects of agricultural, veterinary and medical importance in order that they are less able to have a detrimental effect on their natural environment. It is estimated that the tsetse-borne disease, trypanosomiasis, which affects cattle and humans, costs Africa US$4.5 billion per year (Eisler *et al.*, 2003). The Medfly puts the production of €5.3 million per year of certain subtropical and fresh fruit at risk in Madeira alone (IAEA, 2005). The human cost

of the disease malaria in 2008, was estimated at 1 million deaths with more than 250 million people contracting the disease thus having a marked effect, not only on human life itself but quality of human life for those who could not work due to their becoming ill (WHO, 2010). Control of pest insects is therefore of significant importance and is a well documented control strategy for diseases such as malaria (WHO, 2010). Due to increasing insecticide resistance in populations of vector insects within endemic areas, the emphasis on vector control strategies has been shifted away from insecticide use and towards alternatives such as genetic modification of the vector. The long term goal for the use of these genetically modified insects would be their mass release with a view to eventually suppressing the vector population, or to replacing the vector insect with a modified alternative with impaired vectorial capacity (Marrelli *et al.*, 2006; Sparagano and De Luna, 2008).

Mosquito lines have been generated which express peptides active against the stages of *Plasmodium* present within the mosquito. This has been demonstrated for avian malaria (Jasinskiene *et al.*, 2007), as well as rodent model systems (Ito *et al.*, 2002; Moreira *et al.*, 2002; Yoshida *et al.*, 2007), and now, for the human malaria parasite, *P. falciparum* (Meredith *et al.*, 2011, Isaacs *et al.*, 2011). Interrupting the *Plasmodium* life cycle whilst within the vector could thus result in a total block in transmission of the parasite and therefore control of the disease. Although (at least partially) refractory transgenic strains of *An. gambiae* and *An. stephensi*, the two major vectors of malaria exist, very stringent field tests would have to be completed before release can even be contemplated. Fitness costs may be unavoidable when creating a transgenic strain so there may also be a requirement for the refractory gene to be linked to a drive mechanism in order that this gene is spread through a mosquito population (Sinkins and Gould, 2006; Marshall, 2009). Several selfish genetic elements which occur naturally, such as homing endonucleases,

meiotic drive genes and transposable elements themselves have been proposed as a gene drive mechanism as well as *Wolbachia* which, although not a selfish genetic element, is capable of driving itself through insect populations (Nolan *et al.*, 2011; Sinkins and Godfray, 2004; Sinkins and Gould, 2006; Chen *et al.*, 2007a). Offspring produced by females containing MEDEA (maternal-effect dominant embryonic arrest) selfish genetic elements which do not inherit these elements are inviable. Studies suggest that use of MEDEA elements could help drive a transgene through an insect population (Guevara-Souza and Vallejo, 2011). Use of MEDEA elements is reviewed by Chen *et al.* (2010). However, none of these mechanisms have proved to be a conclusive answer thus far to the problem of driving transgenes through vector populations.

The problem of driving transgenes through populations is also a major factor when considering the population suppression strategy as a method of malaria control. SIT has long been used as a species-specific method of controlling insect populations with documented success targeting the New World Screwworm, *Cochliomyia hominivorax*, in the Southern states of America, Mexico and parts of South America as well as Libya (Lindquist *et al.*, 1993; Wyass and Society for Tropical Veterinary, 2000). This method of control involves the mass-release of irradiated sterile males which mate with the indigenous female population thus reducing their reproductive capabilities and, after sufficient release, leads to suppression, and possible eradication of the target species. Not only is this method of control species-specific, but it is not detrimental to the environment in the same way as insecticides. A successful reduction in populations of Mediterranean fruit fly (Medfly), *Ceratitis capitata*, in Latin America has been documented using SIT. However, irradiation has fitness impacts, lowering mating competitiveness and reducing lifespan. This has lead to estimated 4-10 fold fitness costs in Medfly (Shelly *et al.*, 1994; Lance *et al.*, 2000). The problems associated with conferred fitness costs due to irradiation

as well as those brought about by mass rearing and sex-sorting has meant that improvements to SIT have been a necessary research focus. This has led to the development of molecular genetic techniques for insect population suppression such as Release of Insects with a Dominant Lethal, commonly known as RIDL (Thomas *et al.*, 2000; Alphey, 2002; Alphey *et al.*, 2008). A repressible genetic sexing system which, under certain conditions, would cause female-specific lethality could be used simply as a mechanism by which insects could be sexed in mass rearing facilities. Alternatively, such a mechanism could itself be used as a method of population suppression (Alphey, 2002). SIT requires the sorting of sexes in order to discard females which are not part of the release programme. Original sex-sorting methods are expensive, as twice as many insects are reared than necessary before sorting can take place, and time consuming which again incurs an economic cost to the procedure (Alphey, 2002). Accuracy rates of 100% are often not achievable which can have implications further along in the programme as release of females is undesirable (Alphey, 2002; Nolan *et al.*, 2011). Introduction, into target insects, of a dominant lethal gene which affects only females and at a purposefully chosen development stage provides a method of sex-sorting with 100% accuracy. A repressible system ensures that the transgenic strain can be reared on a mass scale prior to release but then can be activated when only males are required (Alphey, 2002). For example, in the RIDL system, the gene is lethal only in the presence of tetracycline so under normal field conditions this system minimises any environmental hazard due to accidental release from a laboratory setting (Thomas *et al.*, 2000). Alphey (2002) proposes that this repressible lethal system could not only be used as a genetic sexing mechanism as part of an SIT approach but could itself replace SIT as mass release of a strain of insect carrying a dominant lethal gene would achieve a similar outcome to that of an SIT program. Eggs produced as a result of SIT males breeding with wild type females are non-

viable. With a RIDL system, progeny produced as a result of RIDL males breeding with wild type females will possess a copy of the lethal gene. This will be switched on in females causing lethality prior to them being able to bite, in the case of mosquitoes, and before they can breed, and males receiving a copy of the gene will pass it on to a quarter of their offspring. Mass release of RIDL strains would therefore incorporate the benefits of a SIT program in terms of vector control but would have the added advantage of reducing the cost of irradiation and avoiding the issue of fitness costs associated with irradiation, though genetic modification may bring its own fitness costs (Alphey, 2002).

A female-specific lethal system was demonstrated in *Drosophila* using the pro-apoptotic gene *head involution defective* (*hid*) which causes lethality when expressed ectopically (Heinrich and Scott, 2000). Expression of this gene was controlled by a tetracycline-mediated switch (tTA), which in turn was regulated by a female-specific enhancer from the *Drosophila yolk protein 1* (*yp1*) gene (Heinrich and Scott, 2000). This whole system is inactive in the presence of tetracycline, which was fed to the flies via supplemented yeast-based medium. Moreover, lethality was demonstrated in females only, when tetracycline was removed from the diet (Heinrich and Scott, 2000). This system, illustrated in Figure 1.3, echoes that demonstrated by Thomas *et al.* (2000) whereby *Drosophila* were genetically modified to express a cytotoxic gene product under the control of tTA. When reared on tetracycline-containing medium, the sex ratio of flies produced was unbiased, and all flies appeared to be suffering no detrimental effect. Upon removal of tetracycline from the diet however, no female progeny were produced (Thomas *et al.*, 2000). The authors also demonstrated that this tetracycline-repressible system can be used to cause lethality in females by using it to drive expression of a gene product which itself is toxic only to females (Thomas *et al.*, 2000). Since the original studies in *Drosophila*, the tetracycline-repressible system is now considered a feasible alternative to standard SIT and

has been demonstrated in both pest and vector insects (Alphey, 2002; Alphey *et al.*, 2008). Use of this technology has lead to the creation of genetic sexing strains in pest insects such as *C. capitata*, a previous SIT target, which have been shown to be sexually competitive (Morrison *et al.*, 2009). The pink bollworm, *Pectinophora gossypiella*, a destructive pest of economic importance within the cotton industry has also been a previous SIT target species. A bollworm strain carrying a lethal gene has been developed which shows larval stage mortality rates of up to 92% (Simmons *et al.*, 2007). Field trial data for this RIDL strain of pink bollworm is currently awaiting publication (Derric Nimmo, personal communication). The yellow fever mosquito, *Aedes aegypti*, has also been the target of RIDL strain development. A bi-sex RIDL strain of *Ae. aegypti* carrying a dominant lethal gene causes mosquito progeny to die at late larval/early pupal stage (Phuc *et al.*, 2007). Thus mass release of males of this strain would suppress the native mosquito population by causing progeny resulting from their mating with native females to die before producing offspring, or indeed becoming a biting nuisance and disease vector. The study showed a 95-97% reduction in survival to adulthood of the RIDL strain when compared to a wild type strain (Phuc *et al.*, 2007). A dominant, female-specific, late-acting RIDL strain of *Ae. aegypti* has also been constructed, which gives a flightless phenotype in adult female mosquitoes (Fu *et al.*, 2010). This strain is not therefore directly lethal to the female, but reduces their capacity to find and attract mates, and lessens the ability to find a host and consequently a blood meal, thereby rendering females of this strain effectively sterile (Fu *et al.*, 2010). This technology relies on the natural alternative splicing of the *Actin4* gene in male and female mosquitoes. In *Ae. aegypti* mosquitoes Actin4 is predominantly expressed in the indirect flight muscles (IFM) of female pupae (Munoz *et al.*, 2004). The alternative splicing of this gene results in males having a low-abundance transcript which contains 244 extra nucleotides in the 5` UTR compared to that seen in females. This extra

sequence contains several start and stop codons which may be responsible for premature initiation or termination of translation which ultimately reduces the amount of functional Actin4 protein present in male pupae (Fu *et al.*, 2010). As illustrated in Figure 2.1, replacement of the *Actin4* gene with a tTAV gene whose expression is driven by the AeAct-4 promoter allows for a transgene to be engineered whereby translation of a detrimental protein can not only be controlled by presence of tetracycline, but can be differentially expressed in males and females. Tetracycline is known to readily cross lipid bi-layers and enter cells by diffusion and without use of specific protein channels. This process, albeit slow, means that the drug can penetrate most parts of the body and thus is an appropriate choice for use as a gene switch (Berens and Hillen, 2003; Lycett *et al.*, 2004).

The anopheline mosquitoes, vectors of malaria, are a possible target for RIDL technology, and several candidate genes have been proposed which would cause sterility or female lethality in anophelines (Nolan *et al.*, 2011). Combining RIDL technology shown to be effective in *Ae. aegypti* with proven transformation techniques in anophelines may be the first step towards providing an integrated malaria control programme.

A major consideration in the engineering of any genetically modified organisms is the stability of the insertion. This is of particular concern for organisms which would be released into the natural environment. Several studies have been undertaken which have assessed the re-mobility of transposons in different insect species (Horn *et al.*, 2003; O'Brochta *et al.*, 2003; Wilson *et al.*, 2003; Lorenzen *et al.*, 2007; Scali *et al.*, 2007). These studies show that each transposon has a different re-mobility potential which also seems to depend on which organism they are in. Further transposition events after the transposable element has become incorporated into the genome may lead to a lack of stability of the associated transgene. This has clear implications at the population level. It

would be impossible to accurately describe future phenotypes of descendents of released transgenic mosquitoes within a population, or model the behaviour of a transgene over a period of time in modified species if there was instability linked to the transgene itself due to its association with the transposon (O'Brochta *et al.*, 2003). Injection of constructs, such as OX3688 (Figure 2.3) and OX4356 (Figure 2.8), designed to assess mobility of the *piggyBac* transposon in *An. gambiae*, would provide a useful assessment of the re-mobility potential of this transposon. This would be beneficial in downstream applications of transgenic insects where novel effector genes had been inserted using the *piggyBac* transposon, which is the element of choice in many laboratories (Handler, 2002).

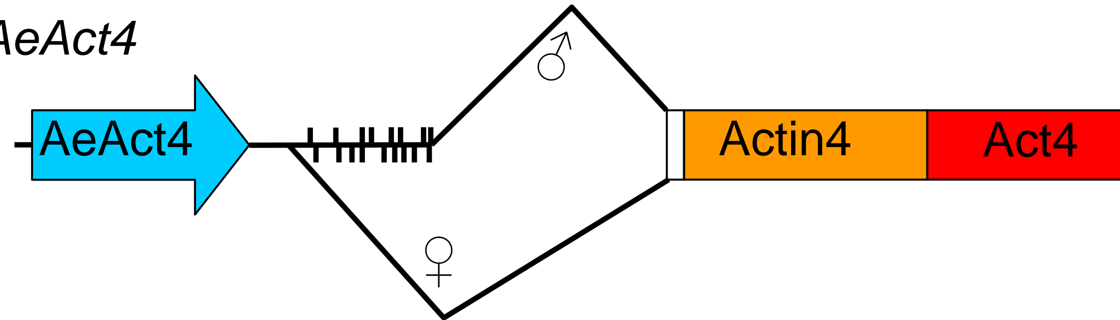
The microinjection technique used to insert novel DNA into mosquito embryos is itself both challenging and demanding in terms of skill and time. Unsurprisingly, microinjection of embryos has a detrimental effect on survival, as the injection itself is quite traumatic. The associated handling of embryos both pre-injection and post-injection also contribute to lower survival rates than expected from non-injected embryos. In mosquitoes, especially *An. gambiae*, which can be difficult to rear under laboratory conditions, optimisation of the whole microinjection technique is imperative to increase the likelihood of creating transgenic lines. Lobo *et al.*, 2006 outline an optimised protocol for the microinjection of mosquito embryos in an attempt to minimise the number of steps causing increased mortality. It is this protocol which forms the basis of the microinjection of *An. gambiae* used in this chapter, though adjustments were made to maximise survival within this laboratory. A summary of plasmids used for injections can be seen in Table 2.1.

Plasmid number (size)	**Transformation promoter/marker**	**Effector gene**	**Effector gene promoter**	**Brief description**
OX3081 4813bp	N/A	*piggyBac* transposase	*T7*	*in vitro* transcription of *piggyBac* transposase mRNA
OX3497 7719bp	N/A	*Mariner* transposase	*T7*	*in vitro* transcription of *Mariner* transposase mRNA
OX3688 18500bp	•Hr5-IE1 DsRed2 •3xP3 DsRed2 •3xP3 AmCyan	RIDL gene (flightless phenotype in females)	*Ae. aegypti Actin4*	•RIDL gene flanked by 2 entire *piggyBac* sequences •Insertion of entire plasmid results in larval fluorescence phenotype of red/blue eyes & red body
OX4244 13908bp	•Hr5-IE1 DsRed2	RIDL gene (flightless phenotype in females)	*An. gambiae Actin4*	•RIDL gene within single *piggyBac* cassette
OX4356 13492bp	•3xP3 AmCyan	*piggyBac* transposase	*An. gambiae nanos*	•Effector gene contained within *Mariner* cassette •Entire *piggyBac* sequence separates a fluorescent marker from its promoter •Transposition of *piggyBac* within line results in change in fluorescence phenotype

Table 2.1: A summary of plasmids used for injections into *An. gambiae*

Promoter 5`UTR Intron CDS 3`UTR

A) Native *AeAct4*



B) OX3688

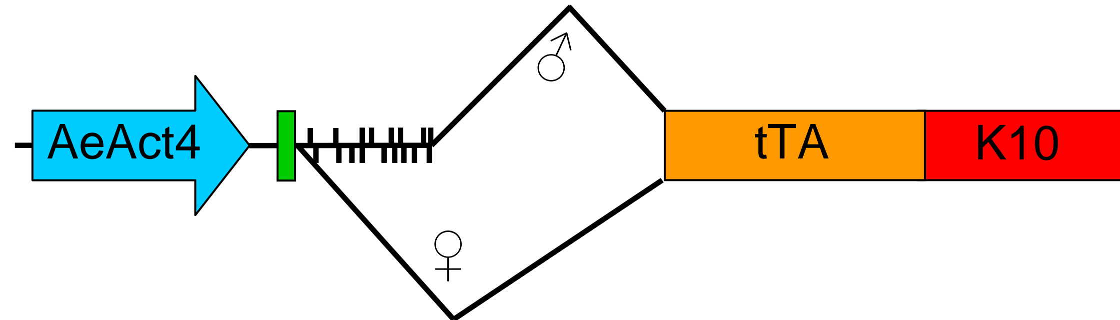


Figure 2.1 (opposing page): Native *Aedes aegypti Actin4* gene splicing and gene splicing in transgenic flightless constructs (adapted from Fu *et al.*, 2010).

A) Native *Actin4* gene; B) OX3688 (OX4244). Coding sequences (CD) and 3` UTR sequences are indicated (K10 represents the *Drosophila* gene *fs(1)K10*). Short lines above and below the thicker horizontal line represent potential start (ATG) and stop codons respectively.

Alternative splicing pathways results in the production of transcripts of differing lengths in males (826bp) and females (583bp). Multiple ATGs at the 5` end are seen in male splice variants of *AeAct-4* whereas in females, the first ATG is that of the *Ae-Act4* coding region. In its native form, *Actin4* expression is seen mainly in the indirect flight muscles (IFM) of female pupae, with only low-abundance transcripts seen in the male. In the transgenic construct an additional ATG was engineered adjacent to the 5` end of the male-specific exon (indicated in green). In females this ATG is in frame with *tTA* but in males it causes a frame shift to occur and thus introduces stop codons. This in turn results in the prevention of functional tTA production in males therefore the detrimental VP16 protein is not produced even if the *AeAct-4* promoter is active. This is because *tTA* drives expression of VP16, a protein from the human *Herpes simplex* virus (Fu *et al.*, 2010).

2.2. Methods and Materials

2.2.1. Rearing of *Anopheles gambiae* populations

2.2.1.1. Oviposition and traying out

Mosquitoes of the 'Keele' strain were allowed to lay eggs in a polystyrene oviposition pot placed inside a cage of adults offered a blood meal 48 hours previously. Eggs were allowed to hatch in the distilled water contained within the pot. Once hatched, around 150 L_1 larvae were counted and transferred into plastic trays (35cm x 25cm) using a disposable Pasteur pipette in 1 litre of distilled water (Elga Water Systems, High Wycombe, UK). Three drops of Liquifry (Interpret Ltd., Dorking, UK) were added to each tray.

2.2.1.2. Larval maintenance and feeding

After the initial feed of Liquifry, larvae were sustained on a diet of Tetramin fish flakes (Melle, Germany) until pupation at around 7 – 12 days after hatching. Pupae were hand picked daily using a disposable Pasteur pipette (with the tip cut off) or with a flask linked to a pump. Pupae were then placed in a weigh-boat in the bottom of a perspex cage (Talking Plastics, Fareham, UK) and allowed to emerge.

2.2.1.3. Adult maintenance and blood feeding regimes

All adults had *ad libitum* access to 10% glucose solution (containing Penicillin/Streptomycin at a concentration of 0.28%) and distilled water. All stages of mosquito populations were kept in a purpose-built room maintained at 28°C with a relative humidity of around 75-80% with a 12h:12h cycle of light and dark. The glucose solution was removed from adult cages around 12 to 24 hours prior to a blood feed being offered.

Distilled water was removed from cages in the morning of the feeding day. Blood was offered to cages of adults using a Hemotek membrane blood feeding system (Discovery Workshops, Accrington, UK). Defibrinated horse blood (TCS Biosciences, Buckingham, UK) was contained within metal 3ml reservoirs sealed on one side by plastic plugs, and on the side in contact with the adult mosquitoes, by a layer of trimmed sausage skin (Plant and Wilson, Newcastle-under-Lyme, UK) covered by a layer of pre-stretched Parafilm (Pechiney Plastic Packing, Chicago, USA). The reservoirs were attached to heated blocks and placed on top of the cage of mosquitoes so females could easily probe through the mesh and gain access to blood. The assembled feeders were left on the cage for 20 to 30 minutes.

2.2.2. Preparation of plasmid DNA constructs used for injection – General DNA techniques

2.2.2.1. Transformation of plasmid DNA constructs into competent cells

Manufacturer's protocol was followed exactly for electroporation of plasmid DNA into One Shot TOP10 Electro-competent *E. coli* (Invitrogen, Paisley, UK). Electroporation of the vector into the competent cells followed the following settings: cuvette gap: 0.2cm; voltage: 1.8kV; capacitance: 25µF (Bio Rad Micropulser Electroporator, Hertforshire, UK).

Manufacturer's protocol was followed exactly for transforming XL10-Gold ultracompetent cells (Agilent Technologies, Santa Clara, USA) when transforming cells following a ligation reaction. Changes were made to the protocol when transforming from minipreps – see Appendix I.

With both transformation procedures, cells were allowed to recover in the supplied medium at 37°C for 1 hour with vigorous shaking. Cells were then plated onto LB-agar plates containing the required antibiotic and incubated overnight at 37°C.

2.2.2.2. Picking overnight bacterial colonies

Antibiotics were added to LB broth to a concentration of either 50µg/ml (ampicillin) or 100µg/ml (kanamycin). 3ml of this sterile LB broth was added to a 15ml Falcon tube, and a sterile 200µl tip used to pick an individual colony which was inserted into the broth in order to inoculate it. Each tube was labelled and placed in a shaking incubator for 12-16 hours (overnight) at 37°C. Once removed from the incubator, tubes were kept at 4°C until required for further applications.

2.2.2.3. Colony PCR

A PCR master mix was made up containing 1.25U *Taq* polymerase (Promega UK, Southampton, UK), buffer (Promega UK, Southampton, UK), 0.2mM dNTPs (Promega UK, Southampton, UK) and water as well as specific forward and reverse primers each at a concentration of 10µM. 10µl of PCR mastermix was added to each well of a 96 well PCR plate. 100µl of LB broth containing ampicillin (50µg/ml) was added to each well of a 96 well flat-bottom tissue culture plate.

Individual colonies were picked using a 2µl pipette and tips. The end of the pipette tip was used to stir in the LB broth in a well of the tissue culture plate. The pipette tip was then transferred to the corresponding well of the PCR plate and the mix pipetted up and down several times. The tip was then discarded and the procedure repeated for an appropriate number of colonies. Once inoculated, the tissue culture plate was covered and placed in a

shaking incubator for 2 hours at 37°C. The PCR plate was placed in the thermocycler at the desired program, and once finished, 5µl of PCR product from each well was run on a 1.5% agarose gel. The gel was then visualised using a SynGene Gel Documentation System (Cambridge, UK).

2.2.2.4. Minipreps

Plasmids were purified from inoculated bacterial broths using the GeneJET Plasmid Miniprep Kit (Fermentas) following the manufacturers instructions. Cells were centrifuged to form a pellet, then lysed. Bacterial cell debris and chromosomal DNA is discarded whereas plasmid DNA remains adsorbed onto the GeneJET spin column. This was then washed prior to eluting in GeneJET elution buffer. The DNA in solution was then stored at -20°C until required for further applications.

2.2.2.5. Endotoxin-free Maxipreps

The Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Crawley, UK) was used to purify plasmid DNA following manufacturers instructions. Cells were pelleted following centrifugation of the overnight bacterial culture in LB broth. Addition of an alkaline solution lyses cells within the pellet. Plasmid DNA is then able to bind to the Qiagen anion-exchange resin under appropriate low salt and pH conditions. A medium-salt wash removes impurities such as RNA, proteins and dyes A high-salt buffer is then used to elute plasmid DNA which is then precipitated using isopropanol, then re-dissolved in endotoxin-free water.

2.2.2.6. Rapid Phenol Preps

Phenol preps were used for rapid evaluation of plasmid DNA. In a 1.5ml micro-centrifuge tube, 30µl of overnight culture LB broth was added followed by 30µl of phenol:chloroform (1:1 ratio) and 12µl of 6 x Orange G loading dye. The tube was vortexed vigorously for 30 seconds prior to being spun at >13000g for 5 minutes. 20µl of the top aqueous phase was loaded into a 1% TAE-agarose gel and compared to a negative control for evaluation.

2.2.2.7. Glycerol Stocks

Glycerol stocks of bacterial cultures were made for long term storage of all plasmids. 850µl of bacterial overnight culture was placed in a fully-labelled 2ml screw-lid cryo-vial. 150µl of sterile 50% glycerol was then added to the culture and the solution vortexed then stored at -80°C.

2.2.2.8. Restriction digests

Plasmid DNA was cut with restriction enzymes (NEB UK Ltd, Herts, UK; Promega UK, Southampton, UK) in order to help confirm presence of the correct plasmid within a Miniprep or Endofree Maxiprep or to cut plasmid DNA prior to downstream applications. Restriction digests were also undertaken to linearise plasmids containing the coding region for *piggyBac* transposase prior to *in vitro* transcription of the mRNA. Restriction enzymes used were dependent on the plasmid. Enzymes were used in the presence of enzyme-specific buffer and BSA to cut DNA. Enzyme and DNA were added to an appropriate microcentrifuge tube in amounts suggested by the manufacturer. DNA was digested at the temperature specified by the manufacturer in a heat block for the recommended time and the digests terminated following manufacturers' protocols.

2.2.2.9. TAE-Agarose gel electrophoresis

In order to visualise DNA, samples were fractionated using TAE-agarose gel electrophoresis. Agarose concentrations used were appropriate to the fragments of DNA to be separated. Agarose was added to TAE in a glass bottle and the solution warmed in the microwave oven until the agarose was fully dissolved. The solution was left to cool until it was possible to handle the glassware without gloves at which point ethidium bromide was added to a concentration of 0.5µg/ml. The solution was then poured into a gel tank fitted with a comb. Gels were run at 70v for 40min to 1.5hours depending on their size after which they were visualised using a SynGene Gel Documentation System (Cambridge, UK).

2.2.3. Microinjection of *Anopheles gambiae* embryos

2.2.3.1. Egg laying for microinjection

An oviposition vessel comprising of a 30mm Petri dish base filled with moist cotton wool and topped with a 30mm Whatman filter paper was placed inside a cage of 4-6 day old adult mosquitoes 2-3 days post blood-meal. To prevent oviposition on any other surface, water was provided to the cage used for laying for microinjection by means of a damp cotton wool ball placed on top of the cage. The mosquitoes were given access to the oviposition site for between 10 and 20 minutes before it was removed so that embryos could be lined up prior to microinjection.

2.2.3.2. Pulling microinjection needles

Filamented aluminosilicate glass capillaries (Intracel Ltd, Royston, UK) were pulled into two symmetrical microinjection needles using a Sutter Micropipette Puller, model P-2000

(Sutter Instrument, Nevato, USA). The capillaries were 10cm in length with an outer diameter of 1mm and inner diameter of 0.64mm. They were pulled on the following two-stage program: Heat: 445; Fil: 120; Vel: 50; Del: 200; Pul: 140 and subsequently Heat: 95; Fil: 235; Vel: 235; Del: 235; Pul: 235.

2.2.3.3. Lining up of embryos prior to microinjection

Embryos were left on the filter paper oviposition surface for around 20 minutes to allow time for the chorion to harden slightly before manipulation. During this period of time they were kept in insectary conditions; 28°C and around 75% relative humidity. Subsequently, the filter paper was viewed under a dissecting microscope (Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland) and aligned using a mounted needle so that they were in the same plane for microinjection (posterior end to the top and the dorsal surface resting downward) taking care to cause minimum disruption to the floats either side of the individual embryos. Once manipulated and aligned the embryos were transferred to a cover slip using double-sided sticky tape (Sellotape, Henkel Consumer Adhesives, Winsford, UK) so that the dorsal surface was pointing up and posterior ends of all embryos were to one edge of the cover slip. Desiccation of embryos was performed by eye under the microscope and embryos were covered by a layer of water-saturated Halocarbon oil (700 series) (Sigma-Aldrich, Dorset, UK) as soon as the embryos were at the appropriate stage of desiccation. Lining up and desiccation of embryos was undertaken at room temperature.

2.2.3.4. Microinjection of embryos

The cover slip on which the embryos were aligned was transferred to a Leica M125 dissecting microscope (Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland) and secured on a glass mount on the microscope stage. The posterior regions of the embryos were all facing in the direction of the micro-manipulator. The injection needle was mounted on a Narishige MN-151 joystick manipulator (Narishige International Ltd, London, UK), itself mounted directly onto the microscope base. It was connected to an Eppendorf FemtoJet Express (Eppendorf UK Ltd, Cambridge, UK) which had pressure supplied by a Jun-Air Condor MDR-2 compressed air pump (Gast group, Redditch, UK). Injection was triggered by the left-click of a mouse button whilst the facility for clearing the needle was via the right-click of a mouse button.

Previously pulled needles were backfilled using an Eppendorf Microloader pipette tip (Eppendorf, UK Ltd, Cambridge, UK) with injection mix. The assembled injection mix was cleared by centrifugation at >13000g for 5min at 4°C prior to backfilling.

Embryos were pierced with the needle using the micromanipulator joystick and the DNA/RNA mix injected into the embryo using the mouse button. Use of the FemtoJet allowed for easy control of the quantities of injection mix introduced into each embryo and the micromanipulator allowed for precise needle placement. The aim was to enter the clear space at the posterior of each desiccated embryo and insert the injection mix here to restore the turgidity of each embryo, whilst also piercing the inner membrane allowing the injection mix contact with the syncytial mass whilst attempting to minimise injection trauma as much as possible. The injection process was repeated for all embryos in the line. Embryos not injected for any reason were killed immediately whilst still on the cover slip. The cover slip was then placed at an angle into a small well filled with distilled water to

allow the halocarbon oil to drain off and to soften the double-sided sticky tape. These wells were returned to the insectary to allow the embryos to recover.

2.2.3.5. Recovery and husbandry of injected embryos

Embryos were left immersed in distilled water attached to the cover slip for a minimum of 1 hour. The cover slip was removed from the water and placed face-up on to a moist 90mm piece of Whatman filter paper. Using a Leica M125 microscope and a mounted needle, each embryo was individually and carefully transferred from the cover slip to the filter paper. The embryos were then rinsed into small polystyrene oviposition pots lined with filter paper containing distilled water and left in insectary conditions until hatching. From 48 hours post-injection, hatched larvae were transferred to individual wells of a 12-well flat-bottomed tissue culture plate at a density of 1 larva per well. Wells were filled with pre-conditioned water containing Liquifry (one drop per litre). Each larva was fed a small individual flake of Tetramin fish flake from its second day in the culture plate. Each day the old flake, and remnants of it, were removed and fresh flake added. When possible, cloudy water was removed and replaced with clean distilled water. Upon reaching pupation, pupae were picked, sexed using a Leica M125 microscope, and placed in a weigh boat within an appropriate cage ready to be backcrossed with the host strain.

2.2.3.6. Screening for transient expression of fluorescent markers in G_0 larvae

Larvae were screened at the L_2 or L_3 stage for transient expression of the fluorescent marker on the injected plasmid. Water was removed from each well containing a larva using a plastic pasteur pipette so each larva had only a minimum amount of water within the well. This meant that screening had to be done relatively quickly and only one plate at

a time. Larvae were starved of food from the day before screening to minimise auto-fluorescence from food debris. Individual larvae were screened for fluorescence using a Leica MZ FLIII microscope with the appropriate filter sets (Chroma Technology Corp., Rockingham, USA). ECFP filter: exciter D436/20x; barrier D480/40m and DsRed filter: exciter HQ545/30x; emitter HQ620/60m. Presence or absence of transient expression in each larva was recorded, distilled water added to the wells containing larvae, and the larvae returned to the insectary after screening.

2.2.3.7. Backcrossing of surviving G_0 adults

Male and female G_0 pupae were kept in separate cages until eclosion. Emerged adults were then allowed to become sexually mature, a process taking around 2-3 days. At this time female G_0 adults were mated in small groups with a surplus of sexually mature adult wild-type host-strain males in order that all females were mated. Male G_0 adults were mated in groups of no more than 4 with around 6 mature adult wild-type host-strain females per male in order that all males were given a chance to mate at least once. All back-cross cages were allowed 2-3 days to mate and were then blood-fed. An oviposition pot was placed in the cage 48 hours post blood-meal. G_1 larvae were trayed out in suitably-sized vessels according to the number of larvae hatched. Their maintenance followed the usual husbandry routine.

2.2.3.8. Screening G_1 larvae for fluorescence

Larvae were screened for fluorescence as described previously. Preparation of L_2/L_3 larvae did differ due to larvae being maintained together and not individually. Larvae were chilled on ice in weigh boats with a minimal amount of water. Once chilled, the weigh

boat was placed within a petri-dish containing ice to maintain the chilling during screening. The weigh boat had parallel lines drawn on its underside to facilitate screening in terms of ensuring all larvae were looked at. Any larvae found to be fluorescing were hooked from the weigh boat using a mounted needle. Once screened, larvae were returned to normal insectary conditions.

2.2.3.9. *in vitro* transcription of mRNA

A plasmid containing the coding region of the required transposase gene was linearised using a restriction enzyme. This linearised template was used in the mMessage mMachine T7 Ultra kit (Ambion, Texas, USA) (following manufacturers instructions with some adaptations – see Appendix I) to generate transposase mRNA to be used in downstream applications. The mRNA transcribed was then further processed using the MEGAclear Kit (Ambion, Texas, USA) (following manufacturers instruction with some adaptations – see Appendix I) in order to generate clean mRNA for use in injections. Quality and quantity of the mRNA was checked using gel electrophoresis and NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

2.2.3.10. Preparation of injection mix

Prior to use, mRNA solutions were centrifuged at >13000g for 5 minutes at 4°C. Injection mixes contained a final concentration of 700ng/µl of mRNA and 300ng/µl of plasmid DNA. Injection buffer (10x) and molecular grade water were added to make up the injection mix to 30µl. The injection mix was divided equally between three 0.2ml micro centrifuge tubes and stored at -80°C until required. When needed, a tube containing injection mix was placed on ice and allowed to defrost. Once thawed, it was centrifuged at

>13000g for ten minutes at 4°C to allow any debris to settle at the bottom of the tube. It was then placed back on ice ready to be loaded into the needle.

2.2.4. Making a single-ended *piggyBac* flightless RIDL construct from a single-ended *piggyBac* vector and flightless RIDL insert

2.2.4.1. Restriction digests of vector (OX3982) and insert (OX4077)

A digest of plasmid OX3982 (Oxitec Ltd.) was carried out with *Pac1* (NEB) to linearise the plasmid (Fig. 2.2A). A double digest of plasmid OX4077 (Oxitec Ltd.) was carried out with *Pac*1 (NEB) and *Nde*1 (NEB) in order to linearise the plasmid and to fragment the un-required band (Fig. 2.2B). This was to ensure that the required band could be excised cleanly from the agarose gel. The digested fragments were then purified prior to a further restriction digest.

2.2.4.2. Purification of DNA following restriction digests

DNA was purified following restriction digests using the Qiagen MinElute Reaction Clean Up Kit. The manufacturer's instructions were followed with the exception that spin columns from the Qiagen PCR Clean Up Kit were used when fragments of greater than 4Kb in size were present. This kit allows clean up of DNA from aqueous solutions. In the presence of high salt solution, up to 5µg of DNA is absorbed to silica-membrane in the spin column whereas contaminants pass through. These impurities are discarded and DNA is eluted in 30µl molecular grade water (a change to the manufacturer's protocol which suggests 10µl).

2.2.4.3. Restriction digests of OX3982 and OX4077

A digest of OX3982 and OX4077 was carried out with *Srf*1 (NEB) according to manufacturer's directions (Fig. 2.2A). This cut OX3982 creating a band of 9Kb and one of 5.5Kb. The latter contained the required sequence. *Srf*1 was also used to cut OX4077 to create two bands of 4.6Kb and one of 8.35Kb (Fig. 2.2B). Again, the latter contained the required sequence. The entire of the digests with both plasmids were run on a 0.7% TAE-agarose gel. The required DNA fragments were then excised and placed in a 1.5ml micro-centrifuge tube and stored at -20°C prior to gel purification.

2.2.4.4. Extraction of DNA fragments from agarose gel

Once a DNA band had been visualised on a TAE-agarose gel and identified as the required fragment, it was excised using a scalpel blade, excess agarose removed and the DNA purified using a Qiagen Qiaex II Gel Purification Kit for purification of DNA fragments from 40bp to 50Kb. Manufacturer's guidelines were followed. Addition of a high salt buffer solubilises the agarose at 50°C and causes dissociation of the DNA binding proteins from the DNA fragments. The DNA fragments adsorb in the presence of high salt to the Qiaex II particles in the spin column whilst impurities including the solubilised agarose remain in the supernatant. After further wash steps with an ethanol-containing buffer, DNA fragments are eluted in a low salt medium. A sample of the extracted, purified, eluted DNA was run on a 0.8% agarose gel to ensure presence of the expected-length fragments of DNA.

2.2.4.5. Ligation of DNA fragments

The blunt ends of vector and insert were ligated using the NEB Quick Ligation Kit following the manufacturer's protocol. Various ratios of vector: insert were used to optimise ligation conditions. 1µl of Quick T4 DNA Ligase was added to the solution of insert, vector and 2 X Quick Ligation buffer at a total volume of 20µl. After leaving the ligation reaction for 5min at room temperature, the reactions were placed on ice prior to transformation of plasmid into chemically-competent cells.

2.2.4.6. Colony PCR

Colony PCR was carried out for 96 colonies transformed previously, using the general technique described above. The primers used to amplify a known sequence within the plasmid were #100 (GGGCGCGGCGATCGCGTTTAAACGGATCCGCGCT) and #TD137 (GGCGGGTACCGGAGCTTGATAACATTATACCTAAACCC) which, when used together would give a DNA fragment with an expected length of 1049bp. The following cycling conditions were used: 1 cycle of 94°C for 2min; 30 cycles of 94°C for 30sec, 55°C for 30sec, 72°C for 1min; then an extension step of 72°C for 7min. 5µl of PCR products were run on a 1% TAE-agarose gel. Four of the colonies generated fragments of the expected size and 15µl of the relevant cultures were used to inoculate 3ml of LB (Amp) broth which was then placed in a shaking incubator for 16 hours (overnight).

2.2.4.7. Diagnostic restriction digests

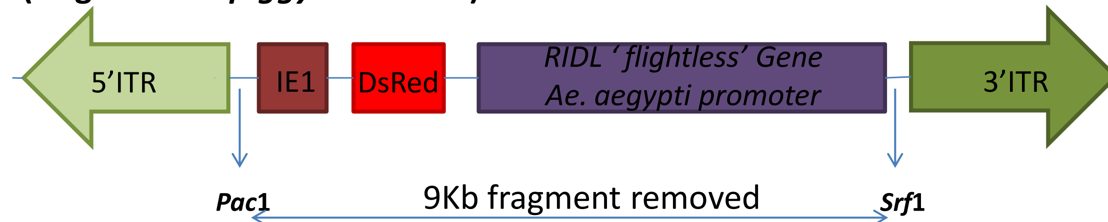
Minipreps from the four inoculated cultures were digested using the restriction enzymes *Kpn*1 and *Nde*1 (NEB). This double digest was expected to cut the new plasmid into fragments of the following lengths; 8.5Kb, 2.9Kb and 2.5Kb. The digested samples were

visualised on a 1% TAE-agarose gel. Three of the four minipreps from individual colonies were shown to contain the correct plasmid and could be sent for sequencing (GATC Biotech, London) using the primers Diag4-pB3 (ATGAGACGAGAGTAAGGGGTCCGTC) and Diag4-pB5 (CCAGAGCGATACAGAAGAAGC).

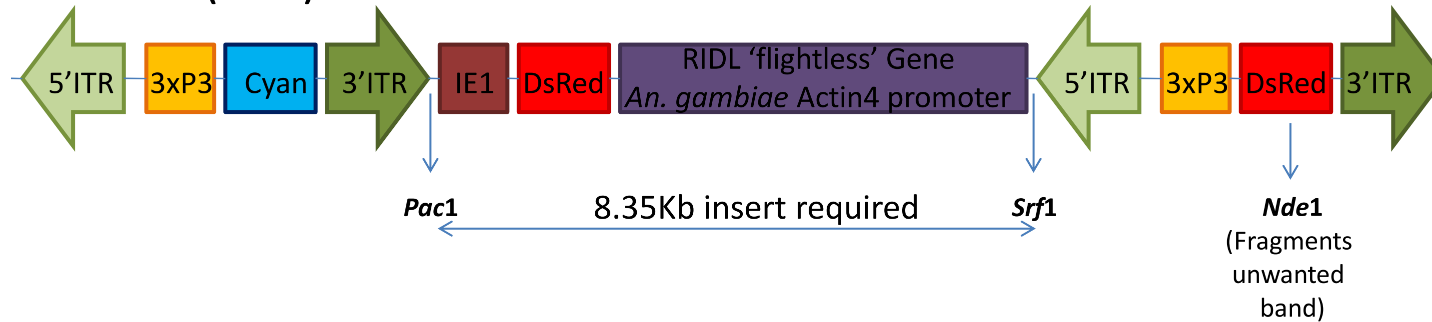
2.2.4.8. Endo-free maxiprep of new plasmid #4244

Sequencing of all three minipreps indicated that the plasmid had been correctly constructed and an endo-free maxiprep was made in order that the plasmid could be used for microinjection of *An. gambiae* embryos. The plasmid was assigned the number OX4244 (pB-Attp-Hr5IE1-DR-tet014AngaA4pi-ubittAv2-FRTlox). Glycerol stocks were also made and stored at -80°C.

A: OX3982 (single ended *piggyBac* vector)



B: OX4077 (insert)



C: OX4244 (single ended *piggyBac* vector containing correct RIDL gene insert)

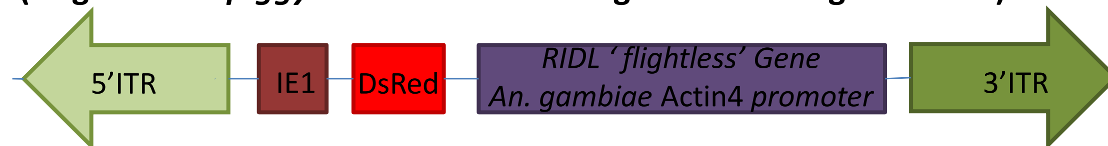


Figure 2.2 (opposing page): Schematic of construction of a single ended *piggyBac* flightless RIDL plasmid from a single ended *piggyBac* vector and flightless RIDL insert.

A) Single ended *piggyBac* vector containing a RIDL gene not appropriate for injection into *An. gambiae* (OX3982). This plasmid was digested with *Pac*1 to linearise the plasmid. After DNA purification a further digest with *Srf*1 was carried out. The required 5.5Kb fragment was excised from the agarose gel and purified.

B) Plasmid containing a flightless RIDL insert under control of the *An. gambiae* Actin4 promoter (OX4077). This plasmid was double digested with *Pac*1 and *Nde*1 to linearise the plasmid and fragment the un-required band. Plasmid DNA was purified and a further digest with *Srf*1 was undertaken to remove the 8.35Kb insert from the original plasmid. Required DNA fragments were excised from the gel and purified.

C) Successful ligation of the 2 purified blunt-ended fragments created the new OX4244 single ended *piggyBac* vector containing a flightless RIDL gene whose expression is driven by the *An. gambiae* promoter.

2.3. Results

2.3.1. Microinjection of *An. gambiae* with OX3688

Preblastoderm embryos of *An. gambiae* (Keele) were co-injected with OX3688 (Oxitec Ltd., Figure 2.3) at 300ng/µl and *piggyBac* messenger RNA at 700ng/µl in 1 x injection buffer to generate a mosquito line carrying the RIDL flightless gene, under control of the *Ae. aegypti Actin4* gene, between two independent *piggyBac* cassettes.

Prior to injection, *in vitro* transcription of *piggyBac* mRNA was performed using plasmid OX3081 (Figure 2.4). The plasmid was linearised prior to *in vitro* transcription using the restriction enzyme *Xba*1. The mRNA generated was then visualised on a TAE agarose gel to check its quality as seen in Figure 2.5.

Over a 6 month period a total of 6 rounds of injections were undertaken. A total of 6468 Keele embryos were injected, of which 293 hatched (4.5%), 210 pupated (71.7%) and 203 survived (96.7%) to adulthood (G_0). Groups of 1-3 G_0 males were backcrossed to virgin Keele females. Table 2.2 shows the breakdown of survival rates for each round of injections with this construct co-injected with *piggyBac* mRNA. Groups of 5-10 G_0 females were backcrossed to virgin Keele males. All progeny resulting from the backcrossed G_0s were screened for fluorescence and G_1 transformants identified by DsRed2 expression all over the body of the larvae (Hr5IE1), or by eye-specific expression (3xP3) of either ECFP or DsRed2. This construct (OX3688) is unusual in that integration could occur in several different ways thus fluorescence patterns could differ between integration events.

G_1 transformants were identified from progeny generated as a result of backcrossing G_0 adults from injection round 3. Relatively small numbers of G_0 adults generated from this injection round meant that after the first gonotrophic cycle, adults from this round were

pooled to promote feeding and oviposition. This meant that, as the transformed mosquitoes were identified as arising from the second gonotrophic cycle, they could not be traced back to a narrower group of G_0s. No further transformants were identified from subsequent blood feeds therefore it can be assumed that the integration event occurred in a mosquito that had since died. The presence of only 12 fluorescent G_1 larvae all with the same fluorescence profile suggests that there was just one single integration event (Figure 2.6). The transformed larvae only showed expression of ECFP under control of the eye-specific 3xP3 promoter. Visualising the larvae under the DsRed2 filter did not show fluorescence profiles due to expression of DsRed2 under control of either 3xP3 or Hr5IE1 promoters. This indicates that integration of construct OX3688 was only partial, that is to say, only one *piggyBac* end was integrated into the mosquito genome and thus the RIDL gene and the second *piggyBac* cassette was not included in the integration event.

Injection Round	**Number of injected embryos**	**Larvae (% of number injected)**	**Adults entered into backcrosses (% of larvae)**	**Number of transgenic G_1 resulting from backcrosses**
1	629	35 (5.6)	29 (82.9)	0
2	1983	50 (2.5)	35 (70.0)	0
3	970	27 (2.8)	18 (66.7)	12
4	1573	31 (2.0)	19 (61.3)	0
5	807	16 (2.0)	8 (50.0)	0
6	274	134 (48.9)	94 (70.2)	0

Table 2.2: Total number of injections, survival rates and G_0 adult numbers generated by each round of injections into *An. gambiae* (Keele) using OX3688. Numbers of G_1 transformants are also identified.

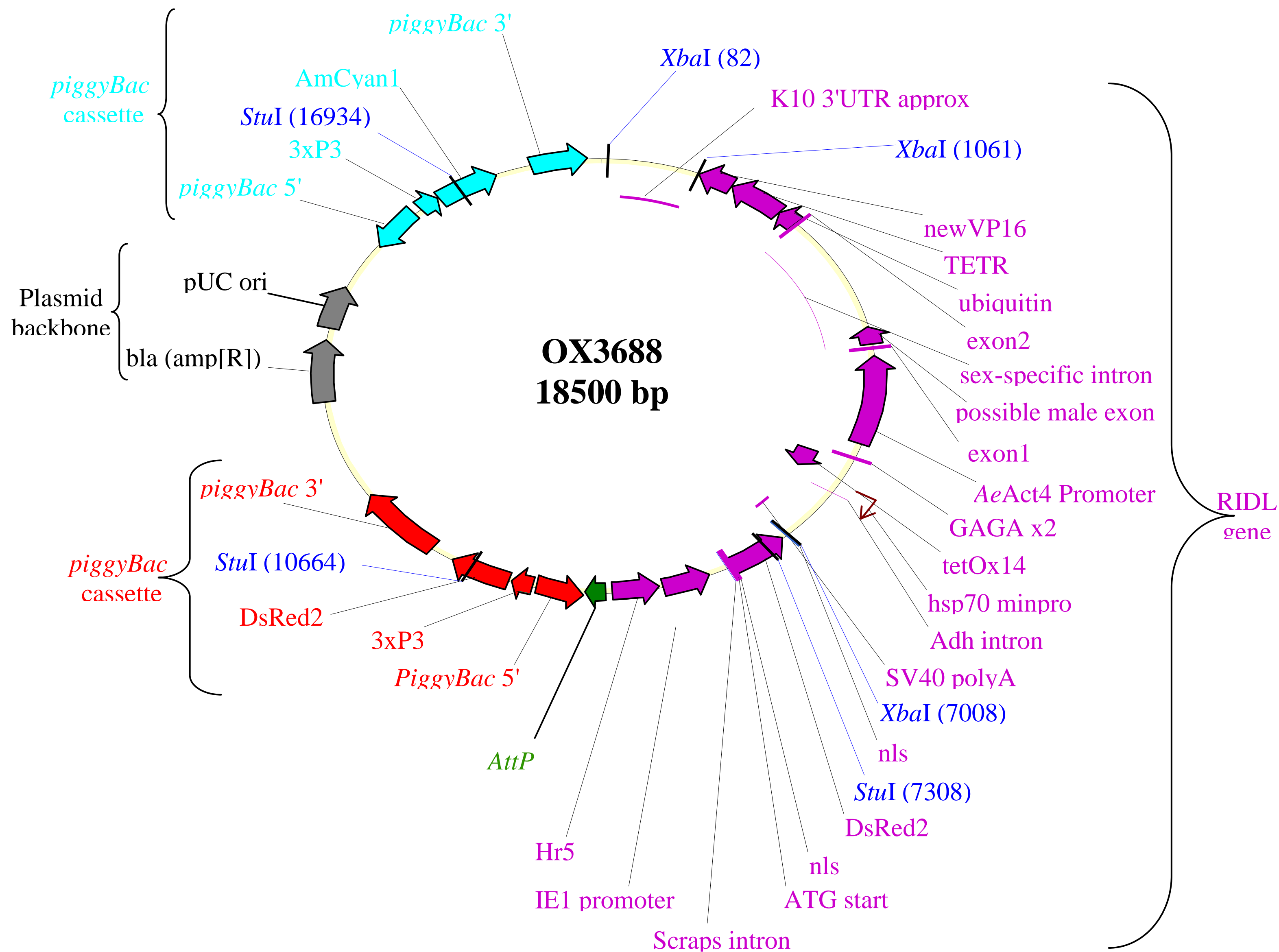

piggyBac cassette
piggyBac 3'
AmCyan1
StuI (16934)
3xP3
piggyBac 5'
XbaI (82)
K10 3'UTR approx
XbaI (1061)
newVP16
TETR
ubiquitin
exon2
sex-specific intron
possible male exon
exon1
AeAct4 Promoter
GAGA x2
tetOx14
hsp70 minpro
Adh intron
SV40 polyA
XbaI (7008)
nls
StuI (7308)
DsRed2
nls
ATG start
Scraps intron
IE1 promoter
Hr5
AttP
RIDL gene
OX3688
18500 bp
Plasmid backbone
pUC ori
bla (amp[R])
piggyBac cassette
piggyBac 3'
StuI (10664)
DsRed2
3xP3
PiggyBac 5'

Figure 2.3 (opposing page): Plasmid OX3688 containing two complete *piggyBac* cassettes and flightless RIDL gene (18.5Kb)

This plasmid was provided by Oxitec Ltd. and contains a RIDL gene with a tetracycline switch, flanked by two entire *piggyBac* cassettes (Dafa'alla *et al.*, 2006). In the absence of tetracycline, the integrated RIDL gene causes a build up of the protein VP16 (from the *Herpes simplex* virus) in flight muscles of the female mosquito causing death of female mosquitoes only. In this construct, the tetracycline-dependent transcription activator (tTAV) is driven by the *Ae. aegypti Actin4* promoter. The *attP* site is a short sequence of 60bp that ensures site-specific integration of any Phase 2 plasmid that carries an *attB* site.

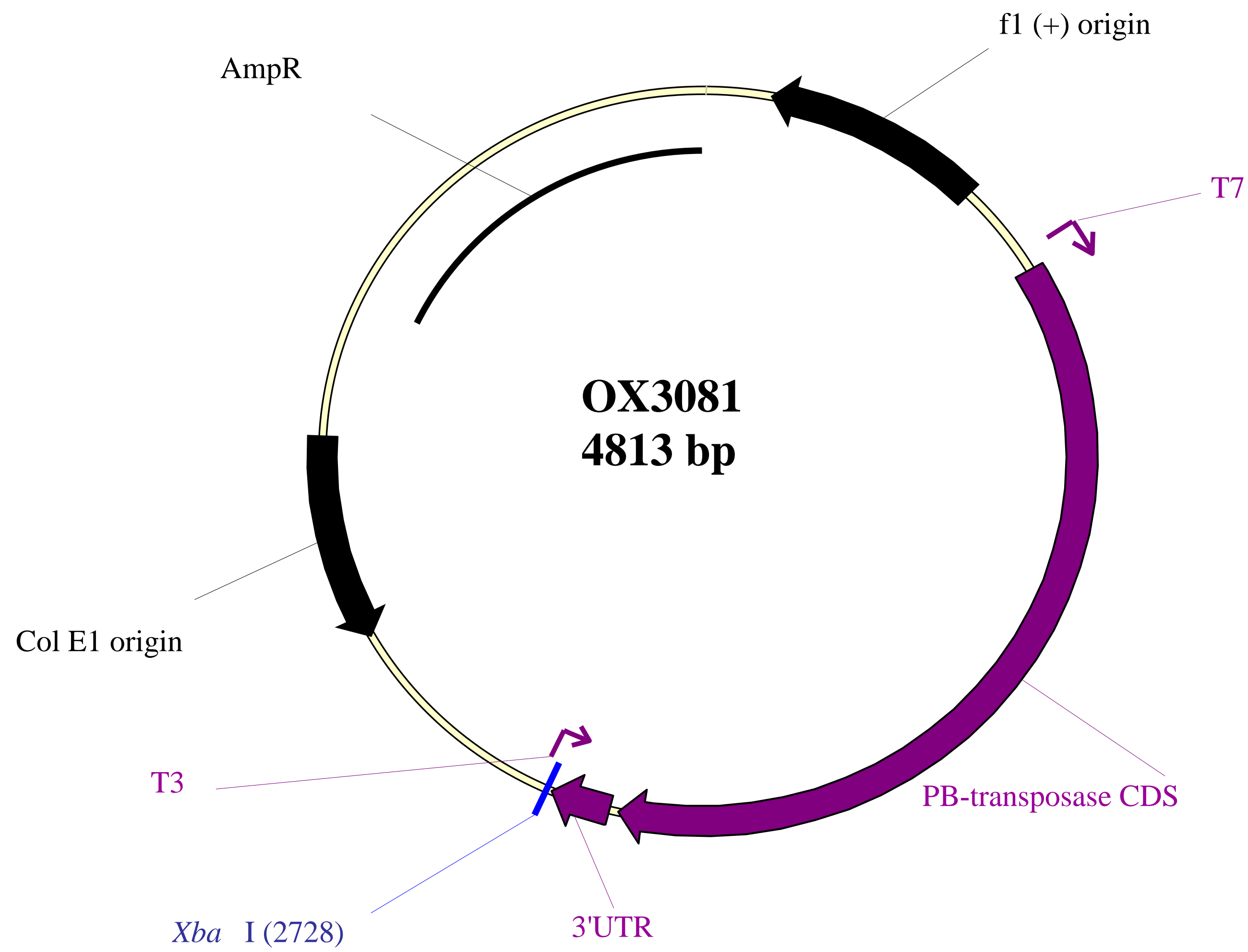

f1 (+) origin
AmpR
T7
OX3081
4813 bp
Col E1 origin
T3
PB-transposase CDS
Xba I (2728)
3'UTR

Figure 2.4 (opposing page): Plasmid OX3081 (4.8Kb).

This plasmid is used for *in vitro* transcription of *piggyBac* transposase mRNA that is co-injected with the transformation vector OX3688 (piggyBac transposase coding sequence under the control of the T7 promoter and the 3` UTR from the *DmVasa* gene). After linearization with *Xba*1, a T7 polymerase reaction is performed, which transcribes the plasmid DNA downstream of the T7 bacteriophage promoter, followed by mRNA preparation for microinjection.

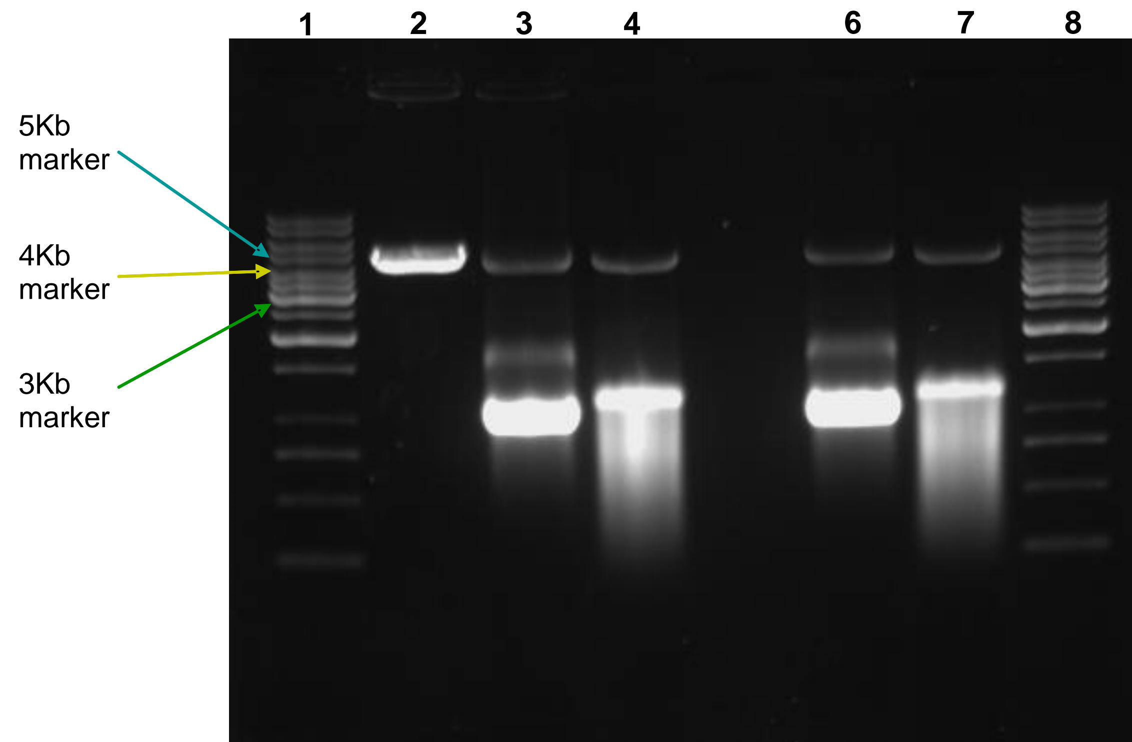

1
2
3
4
6
7
8
5Kb marker
4Kb marker
3Kb marker

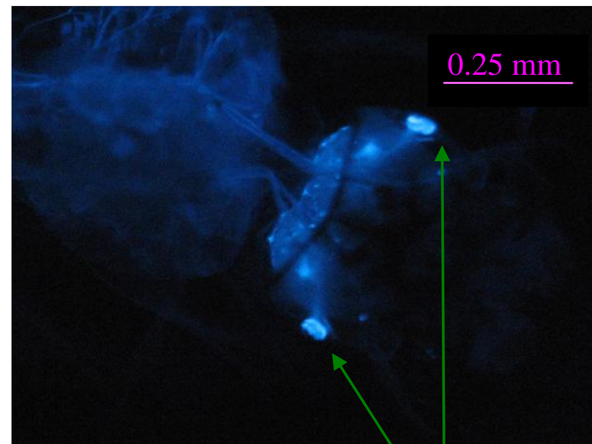
Figure 2.5 (opposing page): Visualisation of *in vitro* transcribed *piggyBac* mRNA on a native TAE gel to assess quality and quantity for microinjection

A 1% agarose RNA gel run for 50 minutes in TAE buffer at 70V. Lanes 1 and 8 show the 1Kb GeneRuler DNA ladder (Fermentas). Lane 2 is a sample of linearised plasmid, OX3081, cut with *Xba*1 prior to the T7 transcription reaction taking place. This shows clearly that the DNA is both linear and of the correct size (4.8Kb). Lanes 3 and 6 are samples of mRNA after the transcription reaction has taken place, from two different batches of the reaction. The gel shows that the quantity of mRNA transcribed is consistent and clear bands with little smearing indicate that the mRNA is of good quality and that samples are not contaminated with RNases. Lanes 4 and 7 are samples of corresponding mRNA after clean-up with the MEGAclear Kit (Ambion). There is a smaller amount of mRNA in these samples in comparison to the amount of RNA prior to clean-up, but there is a clear definition to the RNA bands and the amount is consistent between the two batches, thus indicating that the mRNA is injection quality and can therefore be aliquoted into RNase-free microcentrifuge tubes and stored at -80ºC ready for use in subsequent microinjections.

1A

1B

2A



2B

Figure 2.6 (opposing page): *An. gambiae* larva expressing the AmCyan marker under the control of the 3xP3 promoter.

This expression profile indicates that a partial integration of OX3688 has occurred whereby only a single *piggyBac* cassette has integrated into the genome. 1A and 2A are bright field images of the same larva at different magnifications (x20 and x80 respectively). 1B and 2B are images at the same magnifications as their corresponding pictures but as seen under the Cy GFP 31044v2 filter set (Leica MZFLIII). 1B and 2B are typical 3xP3 profiles, with the arrows indicating eye (E) AmCyan expression.

2.3.2. Microinjection of *An. gambiae* with OX4244

Preblastoderm embryos of *An. gambiae* (Keele) were co-injected with OX4244 (Oxitec Ltd., Figure 2.7) at 300ng/µl and *piggyBac* messenger RNA at 700ng/µl in 1 x injection buffer to generate a mosquito line carrying the RIDL flightless gene under control of the *An. gambiae Actin4* promoter, contained within a single *piggyBac* cassette.

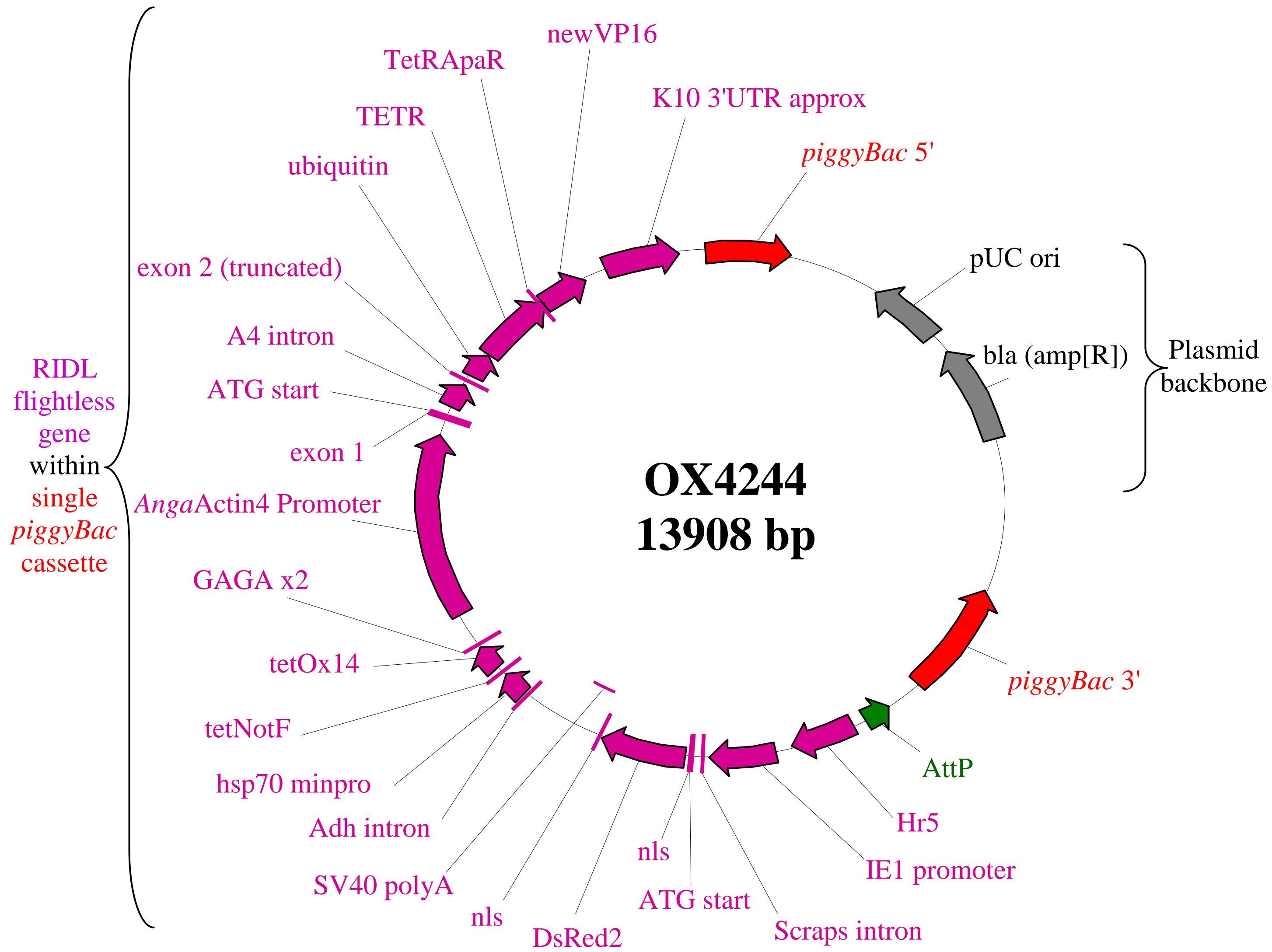
Prior to injection, *in vitro* transcription of *piggyBac* mRNA was performed using plasmid OX3081 (Figure 2.4). This mRNA was then visualised on a TAE agarose gel to check its quality as seen in Figure 2.5.

Over a 6 month period a total of 6 rounds of injections were undertaken. A total of 4858 Keele embryos were injected, of which 314 hatched (6.5%), 26 larvae (13.5%) showed transient expression and 131 survived (40.5%) to adulthood (G_0). Groups of 1-3 G_0 males were backcrossed to virgin Keele females. Table 2.3 shows the breakdown of survival rates for each round of injections with this construct co-injected with *piggyBac* mRNA. Groups of 5-10 G_0 females were backcrossed to virgin Keele males. All G_0 larvae were screened for transient expression of DsRed2. All progeny resulting from the backcrossed G_0s were screened for presence of DsRed2 fluorescence driven by the Hr5IE1 enhancer-promoter.

Although transient expression was seen in 8.3% of G_0 larvae, no G_1 progeny resulting from back crosses were seen to exhibit a fluorescence profile.

Injection Round	**Number of injected embryos**	**Larvae (% of number injected)**	**Number of transients (% of larvae)**	**Adults entered into backcrosses (% of larvae)**
1	767	72 (9.4)	0	14 (19.4)
2	479	39 (8.1)	0	15 (38.5)
3	1391	109 (7.8)	0	63 (57.8)
4	524	27 (5.2)	5 (16.7)	16 (59.3)
5	1485	58 (3.9)	18 (30.9)	20 (34.5)
6	212	9 (4.7)	3 (33.3)	3 (33.3)

Table 2.3: Total number of injections, survival rates and G_0 adult numbers generated by each round of injections into *An. gambiae* (Keele) using OX4244. Numbers of G_0 transients are also identified.



OX4244
13908 bp
newVP16
TetRApaR
TETR
K10 3'UTR approx
piggyBac 5'
ubiquitin
pUC ori
exon 2 (truncated)
A4 intron
bla (amp[R])
Plasmid backbone
RIDL flightless gene within single piggyBac cassette
ATG start
exon 1
AngaActin4 Promoter
GAGA x2
tetOx14
tetNotF
hsp70 minpro
Adh intron
SV40 polyA
nls
DsRed2
nls
ATG start
Scraps intron
IE1 promoter
Hr5
AttP
piggyBac 3'

Figure 2.7 (opposing page): Plasmid OX4244 containing the flightless RIDL gene within a single *piggyBac* cassette (13.9Kb)

This plasmid contains a RIDL gene with a tetracycline switch, contained within a single *piggyBac* cassette. In the absence of tetracycline, the integrated RIDL gene causes a build up of the protein VP16 (from the *Herpes simplex* virus) in flight muscles of the female mosquito causing death of female mosquitoes only. In this construct, tTAV is driven by the *An. gambiae Actin4* promoter. The *attP* site is a short sequence of 60bp that ensures site-specific integration of any Phase 2 plasmid that carries an *attB* site.

2.3.3. Microinjection of *An. gambiae* with OX4356

Preblastoderm embryos of *An. gambiae* (Keele) were co-injected with OX4356 (Oxitec Ltd., Figure 2.8) at 300ng/µl and *mariner* messenger RNA at 700ng/µl in 1 x injection buffer to generate a mosquito line carrying a promoter-marker sequence separated by a whole *piggyBac* cassette, contained within a single *mariner* cassette.

Prior to injection, *in vitro* transcription of *mariner* mRNA was performed using plasmid OX3947 (Figure 2.9). The plasmid was linearised prior to *in vitro* transcription using the restriction enzyme *Bam*H1.

Over a 5 day period a single round of injections was undertaken. A total of 3344 Keele embryos were injected, of which 162 hatched (4.8%), 39 larvae (24.1%) showed transient expression and 43 survived (26.5%) to adulthood (G_0). Groups of 1-3 G_0 males were backcrossed to virgin Keele females. Table 2.4 shows the breakdown of survival rates for injections with this construct co-injected with *mariner* mRNA. Groups of 5-10 G_0 females were backcrossed to virgin Keele males. All G_0 larvae were screened for transient expression of DsRed2. All G_1 progeny were screened for DsRed2 fluorescence driven by the Hr5IE1 enhancer-promoter.

Although transient expression was seen in 24.1% of G_0 larvae, no G_1 progeny were seen to exhibit a fluorescence profile.

Number of injected embryos	**Larvae (% of number injected)**	**Number of transients (% of larvae)**	**Adults entered into backcrosses (% of larvae)**
3344	162 (4.8)	39 (24.1)	43 (26.5)

Table 2.4: Total number of injections, survival rates and G_0 adult numbers generated by the round of injections into *An. gambiae* (Keele) using OX4356. Numbers of G_0 larvae exhibiting transient expression are also identified.

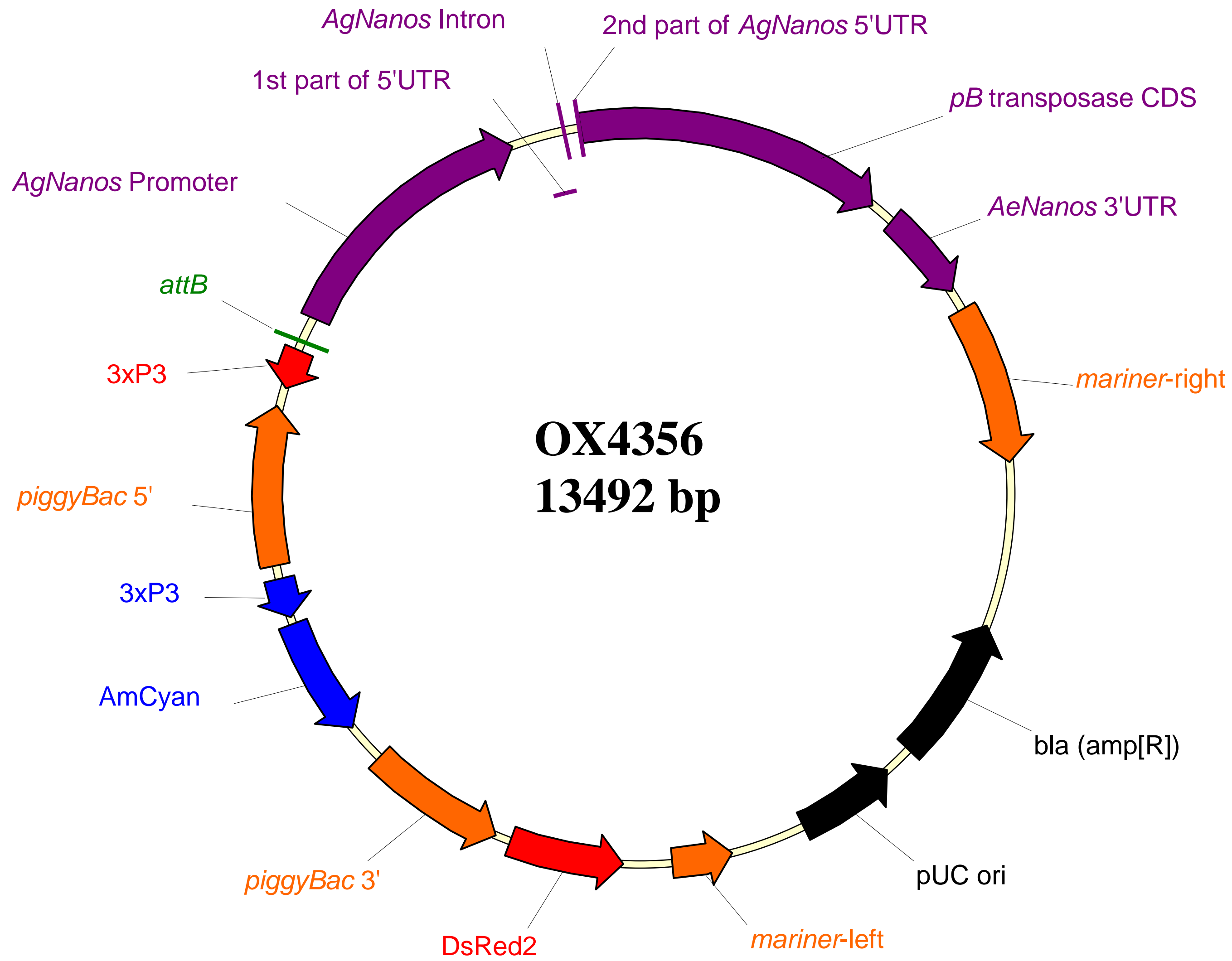

AgNanos Intron
2nd part of AgNanos 5'UTR
1st part of 5'UTR
pB transposase CDS
AgNanos Promoter
AeNanos 3'UTR
attB
3xP3
mariner-right
OX4356
13492 bp
piggyBac 5'
3xP3
AmCyan
bla (amp[R])
pUC ori
piggyBac 3'
DsRed2
mariner-left

Figure 2.8 (opposing page): *piggyBac* mobilisation assessment plasmid OX4356 (13.5Kb)

This plasmid was constructed with the intent of using it to assess mobility of the *piggyBac* transposon. A *mariner* transposon is used to insert the plasmid into the genome of the mosquito. Transformants are identified as showing eye-specific expression of the AmCyan fluorophore only. The *piggyBac* transposase gene is also included in the plasmid sequence, driven by the *An. gambiae nanos* promoter. Remobilisation of the *piggyBac* element within the construct, due to active transposase expression at early-stage embryonic development, would result in a second fluorescence profile (eye-specific DsRed2) being generated in progeny of the mosquitoes it was mobilised in, as the3xP3 promoter and DsRed2 marker sequences would then be joined. Construction of OX4356 by Oliver St John, Oxitec Ltd.

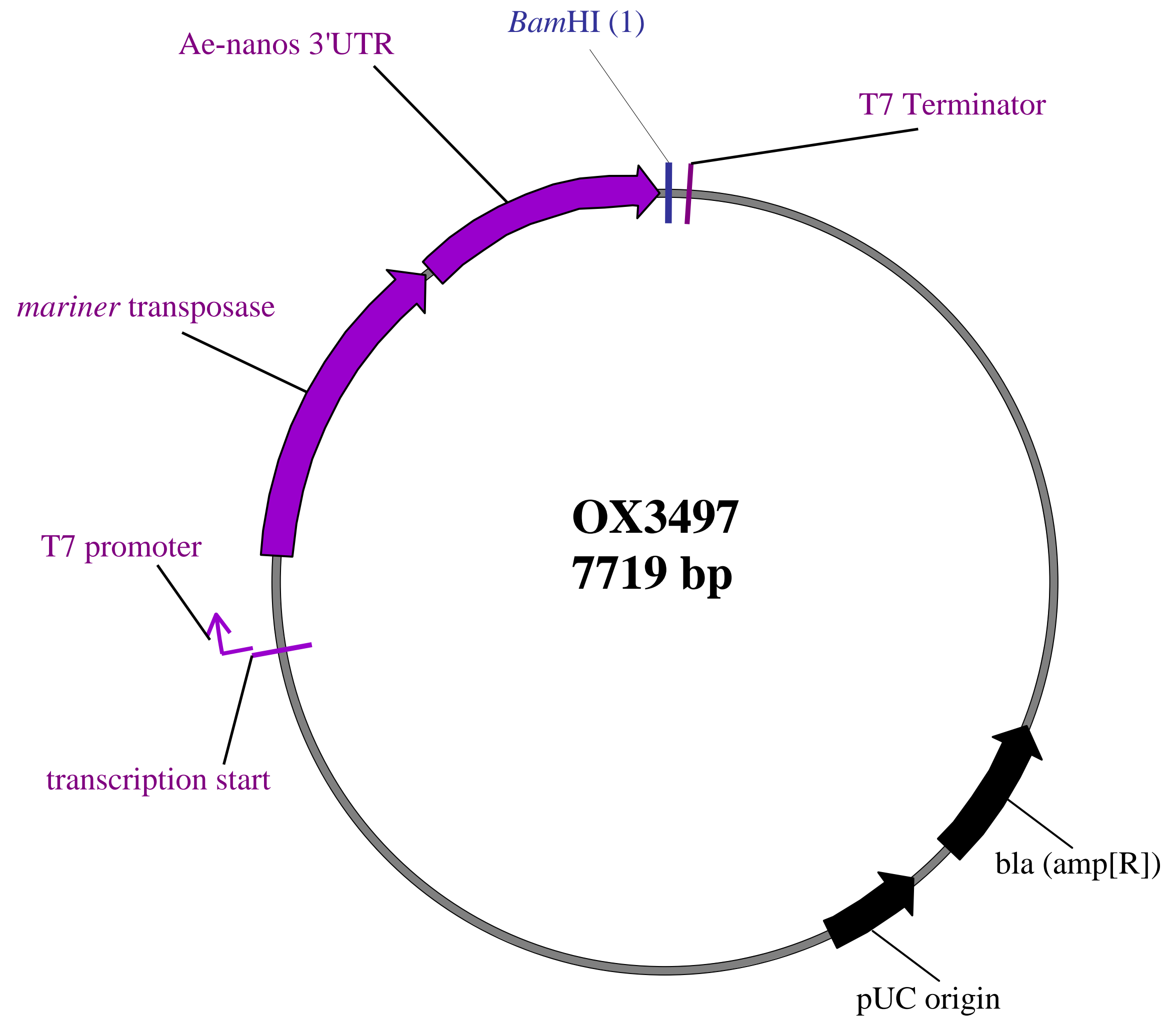

Ae-nanos 3'UTR
*Bam*HI (1)
T7 Terminator
mariner transposase
T7 promoter
transcription start
OX3497
7719 bp
bla (amp[R])
pUC origin

Figure 2.9 (opposing page): Plasmid OX3947 (7.7Kb).

This plasmid is used for *in vitro* transcription of *mariner* transposase mRNA that is co-injected with the transformation vector OX4356. After linearization with *Bam*HI, a T7 polymerase reaction is performed, which transcribes the plasmid DNA downstream of the T7 bacteriophage promoter, followed by mRNA preparation for microinjection.

2.3.4. Effect of Tetracycline on hatch rates, larval survival and pupation rates of *An. gambiae* (Keele)

Mosquitoes injected with a RIDL construct containing a tetracycline switch are raised in water containing tetracycline to prevent activation of the RIDL gene. In order to assess the effect of being reared in tetracycline, hatch rates, pupation rates and mortality rates were calculated from populations of *An. gambiae* reared in two concentrations of tetracycline, 10μg/ml and 30μg/ml.

The average hatch rate is higher in the control group than in either experimental group (Table 2.5 and Figure 2.10A). Data was tested for normality and analysed using ANOVA (with Tukey's post-test) showing that this difference was not significant ($p = 0.40$; d.f. = 2).

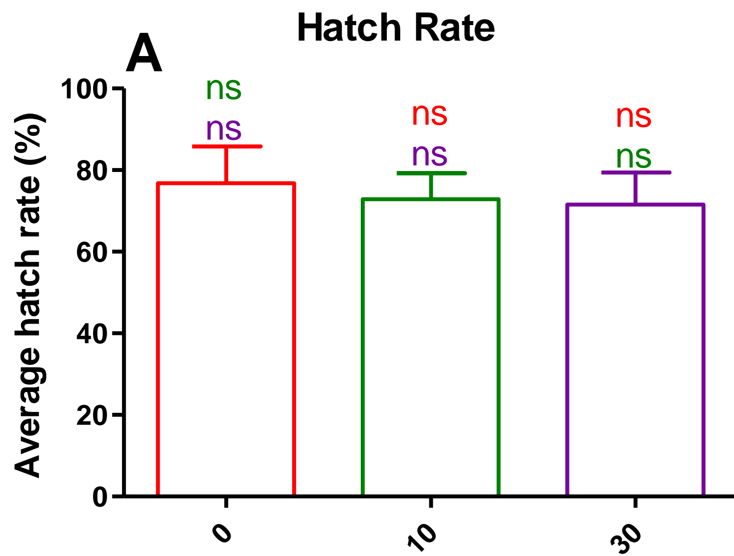
Pupation began on day 8 (post-hatching) for the control and experimental groups, and for all groups pupation peaked on day 10 as shown by Figure 2.11. A two way ANOVA confirms that the day does have an impact on pupae numbers which was as expected and is the case for all groups; $p < 0.0001$ (d.f. = 5). However, there is no significant difference in the pupation rate each day (proportionally to the number of larvae at the start of the experiment), $p = 0.58$ (d.f. = 1) between experimental groups and the control. The average pupation rate is higher in the control group than the two experimental groups (Figure 2.10B). Data was tested for normality and analysed using ANOVA (with Tukey's post-test). This analysis indicated that this difference was not significant ($p = 0.42$; d.f. = 2).

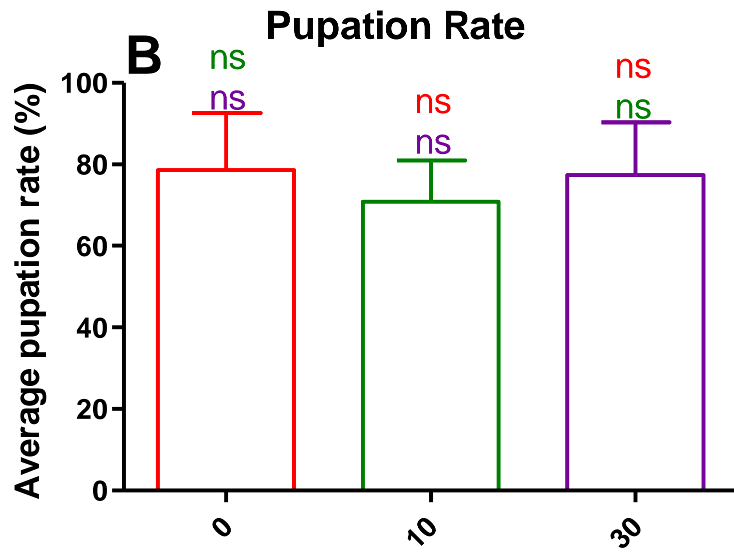
The mortality rate for both experimental groups is higher than in the control group (Figure 2.10C). After testing for normality, data was analysed using ANOVA (with Tukey's post

test). The difference in mortality between groups was found to be significant ($p = 0.0057$; d.f. = 2). Tukey's post test indicates that the significant difference in mortality rates lies between the control group and the group reared at 10μg/ml tetracycline ($p < 0.05$). There is no significant difference when comparing any other pairs of groups.

Tetracycline concentration	Av. hatch rate	Av. pupation rate	Av. mortality rate
0 mg/ml (control)	0.77	0.79	0.20
10 mg/ml	0.73	0.71	0.36
30 mg/ml	0.72	0.77	0.27

Table 2.5: Average hatch rate, pupation rate and mortality rate of *An. gambiae* reared in water containing different concentrations of tetracycline.


Hatch Rate
A
Average hatch rate (%)
100
80
60
40
20
0
ns
ns
ns
ns
ns
ns
0
10
30


Pupation Rate
B
Average pupation rate (%)
100
80
60
40
20
0
ns
ns
ns
ns
ns
ns
0
10
30

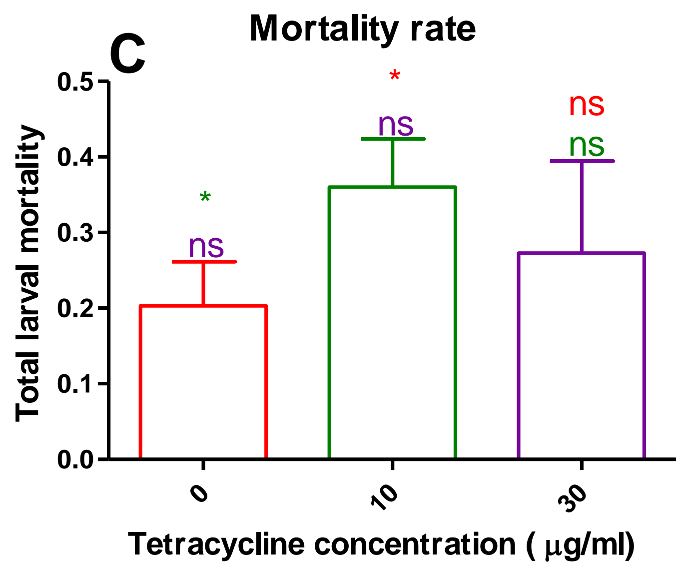

Mortality rate
C
Total larval mortality
0.5
0.4
0.3
0.2
0.1
0.0
*
ns
*
ns
ns
ns
0
10
30
Tetracycline concentration (μg/ml)

Figure 2.10 (opposing page): Effect of tetracycline on larval development and growth.

L_1 larvae were allowed to hatch and larvae were then maintained at a density of 0.2 larvae/ml in water or water supplemented with tetracycline. Bars represent means and standard deviations are indicated. A) Effect of tetracycline on hatch rates in *An. gambiae* (Keele). There is no significant difference between control and experimental groups; B) Effect of tetracycline on pupation rates in *An. gambiae* (Keele). There is no significant difference between control and experimental groups; C) Effect of tetracycline on mosquito mortality rates. There is no significant difference between the control group and those mosquitoes reared at 30µg/ml tetracycline, but there is a significant difference between the control group and those mosquitoes reared at 10µg/ml ($p < 0.05$). Significant levels are indicated above bars in the colour of the bar the significance level corresponds with (*; $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; ns, no significant difference)

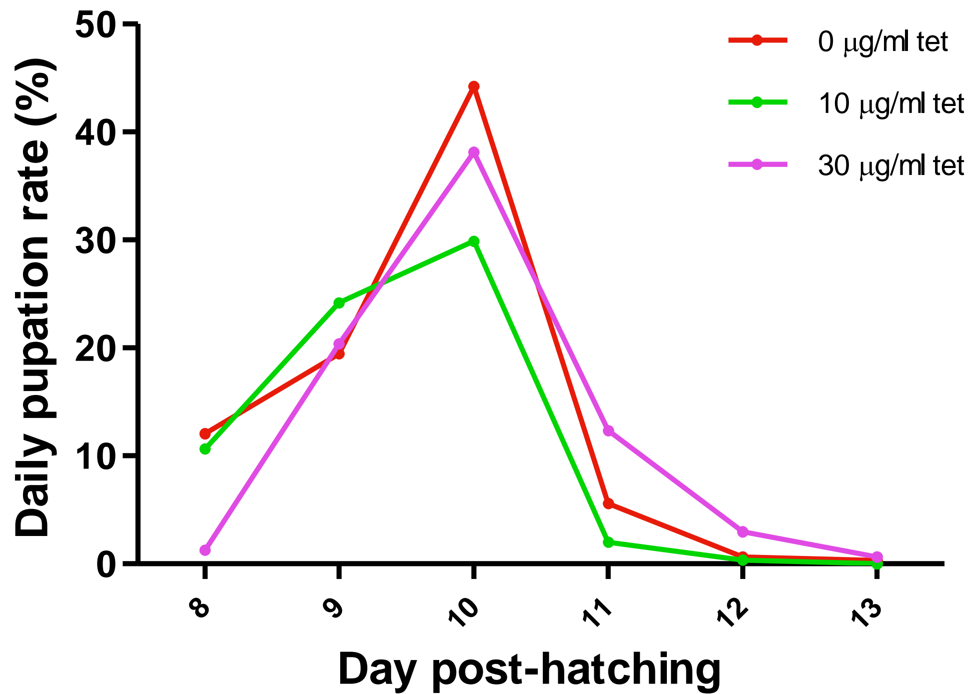


Figure 2.11: Effect of tetracycline on daily pupation rates of *An. gambiae* (Keele). Control groups were set up whereby larvae were reared in distilled water only. Two experimental groups contained larvae reared in water containing either 10µg/ml or 30µg/ml of tetracycline respectively. Pupation rates relative to the initial numbers of L_1 larvae (those that had successfully hatched) were calculated per day from absolute pupae numbers. Statistical analysis (ANOVA) confirms that there is no significant difference between experimental groups and the control ($p = 0.58$).

2.4. Discussion

2.4.1. Larval development in tetracycline

Tetracycline is known to cross cell membranes easily, without use of cross-membrane channels (Lycett *et al.*, 2004). It can therefore access every cell within the developing larvae which makes it very useful as a 'switch' for the RIDL system. When rearing transgenic mosquitoes containing a tet-off switch, they must, in order for the system to have the desired effect, be subjected to a large enough concentration of tetracycline throughout the larval rearing process. However, there is also a possibility that a tetracycline concentration at too high a level may itself have a detrimental effect on mosquito development. As *An. gambiae* are notoriously difficult to transform and subsequently maintain as transgenics, concern was raised that rearing larvae in tetracycline may have a deleterious effect due to direct toxicity of tetracycline to larval cells. *Ae. aegypti* mosquitoes injected with RIDL constructs are reared in water containing tetracycline at a concentration of 30μg/ml (Fu *et al.*, 2010). As *An. gambiae* are well known for being less robust in terms of rearing than *Ae. aegypti*, it was sensible to assess survival/pupation rates of *An. gambiae* at different tetracycline concentrations in order to maximise survival of injection survivors and further generations. It was expected that rearing larvae in water containing tetracycline would have some detrimental effect on pupation rates and overall mortality, with a greater effect at the higher concentration of tetracycline. However, as is clear from Figures 2.10 and 2.11, there is no difference in pupation rates between control groups and those reared on tetracycline at either 10 or 30 μg/ml. Hatch rates also remain unaffected by presence of tetracycline (Figure 2.10A) but there is a significant difference in mortality rates (survival to adulthood) between experimental and control groups. Unexpectedly, there is a greater detrimental effect at

10μg/ml of tetracycline than at 30μg/ml (Figure 2.10C). This suggests that it is not the mere presence of tetracycline which has a toxic effect on the larvae. A direct toxic effect may only be clear at higher concentrations of tetracycline than the ones used here. Mosquitoes contain, within their mid-guts at both larval and adult stages, a range of microorganisms which are thought to aid the digestion process (Rani *et al.*, 2009; Gusmao *et al.*, 2010). *Ae. aegypti* are known to host a dense population of rod-shaped Gram-negative bacteria within the midgut. As a consequence of this, it is suggested that these bacteria share a nutritional interdependence with the host (Gusmao *et al.*, 2010). Studies looking at the effect that natural gut flora have on insects often use tetracycline to clear mosquito guts of bacteria (Gaio Ade *et al.*, 2011). Rani *et al.* (2009) demonstrate the rich diversity of gut micro-flora in lab strain and field-caught *An. stephensi* mosquitoes. A more diverse flora was observed in field-caught mosquitoes, which is consistent with the hypothesis that their diet is more varied and certainly much less sterile than mosquitoes bred and reared in a laboratory setting. This again suggests that gut microorganisms are a necessary resource for aiding the digestive process (Rani *et al.*, 2009). It is also possible that gut flora are partially responsible for vectorial capacities of vector insects, by maintaining parasites such as *Plasmodium spp*. at a level which is less detrimental to both the host and the parasite itself, allowing transmission of the parasite (Rani *et al.*, 2009). Certainly it has been documented that a reduction in mid-gut microflora increases infection rates of *P. falciparum* in experimentally infected *An. gambiae* (Beier *et al.*, 1994). Gaio *et al.* (2011) demonstrate that reducing gut bacteria by feeding antibiotics (including tetracycline) to adult *Ae. aegypti* mosquitoes affects the digestion of blood proteins. Lysis of red blood cells was slower in mosquitoes with reduced gut bacteria, thus they had fewer protein products available to them after a blood meal which impacted on fecundity (Gaio Ade *et al.*, 2011). This evidence suggests that consistent exposure to antibiotics such as

tetracycline could have a detrimental effect on mosquitoes which may not be a result of direct toxicity. Results from this study suggest that a lower level of antibiotic is more harmful than a higher level. This was unexpected. Reasons for this are unclear but it is possible that the higher level of tetracycline caused a decrease in all bacteria present in the mosquito as well as those in the water. This could mean that larvae, although having fewer beneficial gut bacteria, were also subjected to fewer harmful bacteria and therefore any deleterious effects were counterbalanced. However, larvae subjected to a lower level antibiotic concentration may have had their gut flora partially diminished thus having an effect on digestion and ability to utilise food resources whilst larval water also had only a partial reduction in flora thus allowing any harmful bacteria present to have a greater effect. The insectary where the experiment was undertaken is used routinely for rearing RIDL strains of *Ae. aegypti* with a view to mass rearing. Therefore a higher level of antibiotic in larval-rearing water would have a greater effect on reducing these bacteria than a lower level of antibiotic. It would thus be expected that those larvae reared at lower concentrations of tetracycline would come into contact with a greater number of harmful bacteria which may account for the greater mortality rates seen in this cohort. The data produced from this set of experiments provided the basis for the decision to rear G_0 mosquitoes and subsequent generations, from injections with the RIDL construct, at a tetracycline concentration of 30µg/ml.

If RIDL technology is to be used in terms of mass rearing more studies are required to examine the consequences of rearing mosquitoes in water containing antibiotics. Use of antibiotics in the laboratory may also impact these mosquitoes when they are released, as a reduction in gut flora may impact on fitness of mosquitoes in the field, especially when they come into contact with a much less sterile diet.

2.4.2. Microinjection of Anopheles gambiae

Microinjection of *An. gambiae* embryos is a challenging technique whereby successful transformation is not efficient. *An. gambiae* is not an easy organism to work with, and there are many steps in the transformation procedure which could be assigned as factors influencing the efficiency of this technique. Initial injections were into embryos that had been desiccated for a set amount of time once attached to the cover slip. It was found that there was a great range of variability in desiccation even when timing was so accurate. This was especially the case between egg papers and between injection days, and was thus attributed to a differing level of humidity in the local environment. Injections from Round 1 of those done using OX4244 were into embryos which underwent not timed desiccation, but rather desiccation by eye. This resulted in a much smaller range in the variability of the desiccation of the embryos between days and egg papers and consequently went some way to standardising this step of the procedure therefore improving the microinjection protocol. *An. gambiae* embryos are not resistant to desiccation in the same way as *Ae. aegypti* embryos, and consequently need to be restored to full turgor during the injection process. Not injecting enough mix increases the risk of mortality prior to hatching due to over-desiccation, but conversely, injection of too much mix can cause the embryo to burst which again, will increase mortality rates at the embryonic stages. The dichotomy presented here probably contributed to the very low hatch rates attained in the initial rounds of injection with OX3688 (Table 2.1). The hatch rate in the final round of injections into *An. gambiae* with OX3688 was almost 20 times higher than in several of the previous injection rounds. It is likely that, with practice, more embryos were injected with sufficient mix to ensure they had reached a suitable internal pressure without being overfilled. However it is not likely that this, alone or even coupled with a very pure DNA/mRNA mix, would result in such a dramatic increase in survival of G_0s. It is worth

noting however, that immediately prior to round 6 of injections with OX3688, following advice from Dr D. Nimmo, the programme used to pull needles for microinjection was altered, thus making the needles both longer and much finer. Finer needles were more prone to blunting and blocking, and therefore had to be changed more frequently, but were thought to be less damaging to the embryos themselves. Fewer injections overall were undertaken in this round as the newer needles were more flexible than previous ones used. It is therefore logical to suggest that finer needles and injection of fewer embryos with more care taken over each injection, may account for the sharp increase in survival to larval stages.

Initial rounds of injection were into the periplasmic space at the posterior end of the embryo which became more pronounced after desiccation. The inner membrane surrounding the syncitial mass was not pierced. Physiologically, injection into the periplasmic space causes less disruption to the embryo and consequently survival rates were expected to be higher. It was also suggested that transgenics previously produced by members of the research group had been created via injection into this space between membranes. As a result of this, all rounds of injection of construct OX3688 were into the periplasmic space (Table 2.1). This did give rise to transformed individuals but the technique was not at all efficient. It was suggested that the site of injection may play a big role in altering transformation efficiency. Injection of a small amount of DNA/mRNA mix into the syncitial mass itself with a very fine needle was thought to increase the rate of transient expression seen in surviving G_0 larvae. This was an indicator that sufficient DNA had entered the embryo which in turn would increase transformation efficiencies. The initial 3 rounds of injection with OX4244 were performed in exactly the same manner as with OX3688, with the site of injection being the periplasmic space. It was thought that the smaller size of OX4244 would improve transformation efficiencies without the need to

alter the site of injection. It has been documented that transformation efficiencies of *mariner*-like transposons decrease as the size of the transposon increases (Geurts *et al.*, 2003). Certainly OX4244 and OX4356 are approximately 5Kb shorter than OX3688. When transforming *Drosophila melanogaster* with *piggyBac*, it was shown that an increase in transposon size by one third caused a decrease in transformation efficiency of two-thirds (Handler and Harrell, 1999). However, after 3 rounds of injection with the smaller OX4244 construct into the same site it was decided that it was worth injecting directly into the syncitial mass. Rounds 4-6 of injection with OX4244 were performed with a very fine needle into the syncitial mass of each embryo. A small amount of injection mix was placed into the cell mass at the very posterior end, the needle retracted slightly and the embryo filled back to full internal pressure by injecting more mix into the periplasmic space. Piercing of the inner membrane and subsequent injection of foreign DNA/mRNA into the embryo had a detrimental effect of survival (Table 2.2) as expected. However, the rate of transient expression in G_0 surviving L_1 larvae increased indicating that plasmid DNA was staying within posterior cells. With practice, survival rate when injecting into the syncitial mass also increased as indicated by hatch rates for round 6 when injecting OX4244 (Table 2.2) and round 1 when injecting OX4356 (Table 2.3). Injections with this construct were also into the syncitial mass.

It is not possible to determine whether the transgenic larvae which resulted from a partial integration of OX3688 came from a G_0 exhibiting transient expression as at the time these injections took place, screening for transient expression in G_0 larvae was not undertaken as a matter of course. Anecdotal evidence from other groups routinely transforming mosquitoes suggests that increased transient expression rates are a good indicator for increased transformation rates regardless of the size of construct. However, even when

transient expression rates were increased by altering the site of injection, transformation with OX4244 and OX4356 was not achieved.

It is possible that transformation efficiency into *An. gambiae* with such large and complicated plasmids is so low that the numbers of embryos injected were just not sufficient to produce enough G_0 adults which had plasmid DNA integrated into germline cells, and which were fertile and could consequently successfully mate with wild type mosquitoes to produce viable and possible transgenic offspring. Transformation efficiencies using a *piggyBac* vector vary between mosquito species and are worked out as the proportion of integration events per fertile injection survivor. Often a 50% fertility rate is assumed amongst injection survivors. In *Ae. aegypti*, efficiency ranges between 4% and 11% (Kokoza *et al.*, 2001; Lobo *et al.*, 2002; Nimmo *et al.*, 2006) and in *An. stephensi* it ranges between 4% and 12% (Ito *et al.*, 2002; Moreira *et al.*, 2002). For both *Ae. albopictus* and *An. gambiae* (both species known to be more technically demanding), transformation efficiency is around 2-3% (Grossman *et al.*, 2001; Labbe *et al.*, 2010) which is considerably less than has been achieved with other species. In only one documented case has a much higher transformation efficiency been achieved with *An. gambiae* of 3-18% (Lombardo *et al.*, 2005). The authors suggest that the increased efficiency is due to improvement and modification to the original paper by Lobo *et al.* (2006) which outlines the procedure for germ-line transformation of mosquitoes. The authors also suggest that time of injection as well as temperature, both in relation to the embryo may play a part. But it is not possible to extrapolate an exact reason for the increased transformation efficiency seen with *An. gambiae* in the work performed by Lombardo *et al.* (2009) compared with previous studies and the injections carried out here. At each life stage after microinjection, no matter how carefully eggs and larvae are cared for, loss is inevitable. Increasing numbers at each step of the whole protocol increases the

likelihood of getting transformants. Working alone limits the number of injections possible in a finite amount of time which is not ideal in terms of effectiveness. It also means that G_0 injection survivors hatch out over an extended period of time and thus pupate over an extended period of time. As a result it is necessary to have wild type strains (for back-crossing) pupating over the same time frame in order to have adult mosquitoes mating and feeding at the optimum time points. Maintaining injections to generate enough numbers to maximise the potential for transgenesis whilst caring for G_0 injection survivors and sustaining wild type populations at optimal levels for back-crossing proved to be quite difficult. However, having small populations of back-crossed G_0 adults at different stages due to injections being spread over several days meant that these populations were fed on different days rather than all together as they were when injections were undertaken with the help of others. This therefore meant that G_1 offspring were laid and hatched over a longer period of time and not simultaneously thus screening of G_1 larvae was less intense which meant fewer long periods of time spent screening for fluorescence, but instead it had to be managed alongside screening G_0 larvae for transient expression, sexing G_0 pupae ready for back-crossing, as well as setting up back-crossing and dealing with maintenance of wild type mosquitoes for back-crossing. It could be possible that working alone could reduce the chance of seeing transformants. The injections using OX3688 were undertaken with a second person lining up embryos and the overall rate of survival of G_0s from larval stage to adult stage is 69.3%. This decreases to 41.7% when injections with the similar OX4244 construct were undertaken alone. However, injections with OX4356 were also performed with the help of a second person lining up embryos and the rate of survival to adult stage is even lower, at 26.5%. It was expected that, due to their being reared in water containing tetracycline to ensure that the RIDL gene remained ‘switched off’, survival of G_0 survivors to adulthood would, in fact, be reduced when the RIDL constructs were

injected in comparison to non RIDL constructs. However, experiments to determine the effect of tetracycline on wild-type larvae showed that rearing larvae in water containing tetracycline at a concentration of 30mg/ml had no effect on any parameters measured including mortality rates. Therefore it can be expected that rearing in tetracycline at this concentration would have no effect on rate of survival. Consequently, seeing a lower survival rate when injecting the non-RIDL construct OX4356 is not surprising. This therefore, points to other factors causing an effect on survival rates and possibly, as a result of this, an effect on transformation efficiency.

2.4.3. Microinjection of RIDL constructs

There is evidence to suggest that *piggyBac* will remobilise easily once inserted in the genome of a host insect (Horn *et al.*, 2003; Handler, 2004; Lorenzen *et al.*, 2007). Although this is not necessarily the case for all insect species. For example, there is experimental evidence which strongly suggests that *piggyBac* will not remobilise within the genome of *Ae. aegypti* (Sethuraman *et al.*, 2007). Little is known about its potential for remobilisation within *An. gambiae*. Post-integration instability could compromise its ability as a transformation vector as it would be less than ideal for an effector gene to be able to move around the genome. Movement around the genome could compromise the effectiveness of this gene due to position effects, or could compromise the fitness of the host (Handler, 2004). Dafa'alla *et al.* (2006) describe a method for transposon-free insertion of an effector gene in the Medfly, *Ceratitis capitata*. This involves injection with a construct whereby the effector gene and marker is flanked by two complete *piggyBac* ITR sequences each containing fluorescent marker genes. Upon integration of the whole cassette, three markers can be visualised in the transformed insects using fluorescence microscopy. Once the line is established, injection of embryos with a transposase source

causes remobilisation of either of the independent *piggyBac* ends, and upon repetition of this can result in removal of both *piggyBac* sequences leaving a transposon-free effector gene and its marker (Dafa'alla *et al.*, 2006). The same principle was applied in the case of injection with OX3688 whereby the RIDL gene is flanked by two independent *piggyBac* cassettes. Integration of both *piggyBac* cassettes as well as the effector gene was expected to occur as this transposon does not always use the closest pair of terminal sequences (Li *et al.*, 2001). However, a transposition event may be more likely when transposons are shorter (Berg and Spradling, 1991). Indeed, as indicated by the fluorescence profile of the transgenic larvae produced by injection with OX3688 (Fig. 2.6) which displayed only expression of AmCyan under control of the 3xP3 promoter, only one shorter *piggyBac* cassette was integrated into the genome of *An. gambiae* throughout the whole set of injections. Changes to the injection protocol to ensure maximal survival of embryos, as well as other factors, as discussed above, may increase the likelihood of obtaining transgenic mosquitoes using double ended constructs, and indeed large constructs in general

2.4.4. Microinjection of piggyBac remobilisation construct

As previously discussed, it is known that *piggyBac* is capable of remobilising once in the genome of several host species (Horn *et al.*, 2003; Handler, 2004; Lorenzen *et al.*, 2007). If large scale release programs of transgenic insects are to take place, stability of transgenes must be assessed, in terms of mass rearing and subsequent release so that realistic models can be produced. Understanding the stability of insertions is necessary to ensure effectiveness of control programs can be maintained (Handler, 2004). Relatively little is known about the stability of transposon-mediated transgene-insertions with no studies having been undertaken to address the issue of transgene stability within large-scale

applications and over extended generations for any insect species including *Drosophila* (Schetelig *et al.*, 2011). The *piggyBac* transposon is thought to remain immobilised once in the germ-line cells of *Ae. aegypti* mosquitoes, even in the presence of a viable transposase source (Sethuraman *et al.*, 2007). The transposon *Mariner* did show some propensity for germ-line remobilisation but this was incredibly rare, which, unlike with *piggyBac*, mirrored that of previous work looking at post-integration stability of *Mariner* in Drosophilds (Wilson *et al.*, 2003). *Hermes* was also shown to transpose somatically in *Ae. aegypti* cells but no germ-line remobilisation events were detected (O'Brochta *et al.*, 2003). In the malaria vector *An. stephensi*, the transposon *Minos* was shown to be stable with no germ-line remobilisation events detected (Scali *et al.*, 2007). Currently, little is known about the stability of transposon-mediated insertions in the major malaria vector *An. gambiae*. Microinjection of OX4356 with *piggyBac* was undertaken as the initial step of addressing this issue. The construct was designed to be inserted into the genome using the *Mariner* (*Mos1*) TE. Insertion of the plasmid would then be verified using fluorescence microscopy whereby expression of AmCyan under control of the eye-specific promoter 3xP3 would indicate the integration of the entire sequence (Fig. 2.8). This would include a gene for the production of an endogenous *piggyBac* transposase source under the control of the *An. gambiae nanos* promoter, responsible for embryonic pattern formation. The nanos mRNAs accumulate in oocytes and in the posterior of early developing embryos (Calvo *et al.*, 2007). The tight control and placement of the mRNAs makes *nanos* an ideal promoter for a transposase source as it would then be located in the posterior of the early embryo. This endogenous *piggyBac* transposase source would be present in all mosquitoes within the transgenic line and should allow the entire *piggyBac* element also integrated within the plasmid to remobilise. Elick *et al*. (1996) report, using genetically tagged elements, that the *piggyBac* transposon is capable of precise excision and insertion from

donor plasmid to target sites in a Lepidopteran cell line. These precise events are catalysed by the transposase enzyme coded for by the transposon itself (Elick *et al.*, 1996). Grossman *et al*. (2000) followed these studies by showing that *piggyBac* is capable of precise excision and insertion, only in the presence of an exogenous transposase, in *An. gambiae* Mos55 cell lines. Indeed, *piggyBac* is consistent in showing precise excision, leaving the target TTAA site intact upon excision (Fraser *et al.*, 1995). It inserts at a specific sequence in the host genome (TTAA) and in fact has a sequence which bears little in the way of similarity to other superfamilies of transposons (Sarkar *et al.*, 2003). This precision means that *piggyBac* is ideal as a transformation vector. In the case of a transgenic strain containing the OX4356 construct, precise excision of *piggyBac* would leave the TTAA site intact and would cause the 3xP3 promoter sequence and DsRed2 marker sequence to become aligned thus altering the fluorescence profile of any mosquito in which *piggyBac* remobilised. If remobilisation were to occur in somatic tissue, the mosquito would have patches of DsRed expression in these tissues. In mosquitoes whereby germline remobilisation had occurred, the expression profile of DsRed would match exactly that of the original transgenic AmCyan marker. This would allow visualisation of *piggyBac* movement post-integration and could be done over many generations. Any movement seen visually could then be verified by molecular methods. A limiting factor with this approach to assessing the potential of *piggyBac* to remobilise once integrated into the genome of *An. gambiae* lies in the initial creation of the transgenic line. As clear from the results of this experiment, OX4356 did not readily integrate upon microinjection of around 3300 embryos. This could be due to factors specific to the microinjection technique rather than the construct itself, or could be due to the large size of the construct. There is also the possibility that transposase itself is toxic to the developing embryo. As the promoter controlling expression of transposase is nanos, an early-onset

gene, the transposase would be translated early in embryonic development and if toxic to *An. gambiae*, could cause a higher likelihood of mortality in any transformed embryos. Therefore *An. gambiae* could have been transformed in this experiment but death of the embryo prior to hatching or very soon after hatching, and consequently before screening, would mean that any transgenics were not seen. If this is the case, then it would be difficult to create a transposase-driver line of *An. gambiae*, and would make assessing stability of *piggyBac* difficult via this route.

It has been demonstrated that transformation of the malaria vector *An. gambiae* is possible, not only with transformation markers by themselves (Grossman *et al.*, 2001), but also with antimalarial peptides (Meredith *et al.*, 2011). However, the creation of viable transgenic strains of any insect, especially *An. gambiae*, is challenging for many reasons. Although transgenic strains of *Anopheles* exist which have exhibited anti-malarial properties (Ito *et al.*, 2002; Yoshida *et al.*, 2007; Meredith *et al.*, 2011), there is much to be learned about the long-term stability of transposable elements, and consequently the transgenes, if these transgenic mosquitoes are to be reared in a mass scale with a view to release. If future studies prove the *piggyBac* element to be mobile within the germline post-integration into the *An. gambiae* genome, it may be necessary to use molecular technology which would render the TE immobile. This could be by complete removal of the TE by utilising the transposons' own propensity for remobilisation (Dafa'alla *et al.*, 2006) or by disruption of the ITR sequences which are recognised and bound by the transposase thus facilitating excision and movement of the TE. This could be achieved by the inclusion of *loxP* and FRT recognition sites in the plasmid integrated into the genome of the mosquito. Subsequent exposure to the required recombinases would thus cause removal of undesirable portions of the integrated DNA sequences leaving only the effector gene, whether it be a conditional lethal gene or one encoding an antimicrobial peptide (Handler,

2004). The use of site-specific genetic technologies to create stable transgenic insect strains is discussed by Schetelig *et al*. (2011). The authors conclude that maintenance of stable integrations of any transgene and reduction in the likelihood of horizontal gene transfer is of utmost importance when considering any release program (Schetelig *et al.*, 2011). The microinjection technique, certainly with *An. gambiae*, is challenging with much more information required to ensure its reliability and efficiency. However, regardless of the effector gene, much more needs to be learned about stability within the germ line and long-term effects of the effector on the mosquito itself regarding reproductive fitness.

Chapter Three

3. Effects of multiple feeding on the antimalarial properties of Vida3 peptide within transgenic *An. gambiae*

3.1. Introduction

It has been speculated that the use of transgenic insects involved in a large-scale release program could be the future of control initiatives to combat various diseases of both medical and veterinary importance, and to target agricultural pests. Traditional control methods, certainly for the control of vector insect species such as mosquitoes, involve the use of insecticides. Resistance to these compounds has become widespread and has proved to be one of the most rapid cases of evolutionary adaptation documented (Hemingway *et al.*, 2004). Pyrethroids are commonly used for indoor residual spraying and for impregnating bednets as they have a low toxicity to humans. It is thought that pyrethroid resistance in *An. albimanus* developed in just 3 years (Read *et al.*, 2009). The ability of mosquito populations to become resistant so quickly, also reflected in M-form *An. gambiae* populations of West Africa where pyrethroid resistance is now significant, affecting control measures which were once effective (N'Guessan *et al.*, 2009) means that other methods of mosquito control must be considered.

There are two broad methods of controlling vector-borne disease using genetically modified insects – population replacement and population suppression. Both methods involve the alteration of DNA within the host species. Population suppression using the Sterile Insect Technique (SIT) has been carried out with great success targeting several

insect species. The agricultural pest causing myiasis, *Cochliomyia hominivorax* (New World screwworm fly) was eliminated from parts of Southern and Central America (Krafsur *et al.*, 1987). Success has also been seen when targeting the crop pest, *Ceratitis capitata* (Mediterranean fruit fly) (Robinson, 2002) and the sleeping sickness vector, *Glossina austeni* (tsetse fly) (Vreysen *et al.*, 2000). Similar attempts were made to suppress the population of *An. albimanus* in El Salvador (Lofgren *et al.*, 1974). This effort was successful when targeting a small 15km^2 area but less so when a larger area was targeted on the Pacific coast. A previous attempt at population suppression of *An. albimanus* on the Pacific coast was unsuccessful due to immigration (Benedict and Robinson, 2003). Conventional SIT does come at a cost in terms of male reproductive fitness, which is reduced by exposure to radiation, and female contamination as both sexes have to be reared to adulthood then sex-sorted (Thomas *et al.*, 2000). In fact radiation and its effect on fitness of released males is a major concern with this technology (Andreasen and Curtis, 2005). Release of Insects with a Dominant Lethal (RIDL) is a newer molecular-based technology which is, in effect, an upgrade of SIT involving integration of a dominant lethal gene into the genome of the insect using microinjection techniques. This gene is under control of a tetracycline 'switch' thus causing its effect to be conditional. This conditional lethal effect is life-stage and sex-specific (Thomas *et al.*, 2000). Using RIDL technology negates the use of radiation to compromise viable egg production, and reduces costly mechanical sex-sorting methods by use of genetic sex-sorting mechanisms. In fact, a transgenic sexing strain of *An. stephensi* has already been developed which would be highly beneficial in terms of mass rearing and release of males only (Catteruccia *et al.*, 2005). The exploitation of sex-specific splicing in *C. capitata* (medfly) has lead to the production of strains of this insect which have non-viable females, when tetracycline is removed from the system (Fu *et al.*, 2007). This technology has been extended to

mosquitoes, leading to the development of *Ae. aegypti* strains which exhibit a flightless phenotype only in females. This has the same effect as an entirely lethal gene, as the inability to mate and blood feed successfully leads indirectly to population suppression of the species (Fu *et al.*, 2010). The long-term aim concerning production of such a strain is mass rearing with a view to large-scale release in field trial situations to assess male fitness outside of the laboratory setting. As with SIT, male fitness is key in using RIDL as part of an integrated control program. Although sexing strains exist there are currently no documented strains of *Anopheles* in existence which possess integrated RIDL genes under control of a tetracycline switch, thus suitable for mass rearing. However, a strain of *An. gambiae* which demonstrates early embryonic lethality and sex-ratio biasing via exploitation of homing endonuclease genes has been created (Windbichler *et al.*, 2008). Although RIDL technology offers some solutions to the problems caused by SIT, it is by no means fool-proof and there are many issues which still remain to be addressed, especially in terms of released-male fitness (Nolan *et al.*, 2011).

The population replacement control approach also involves the integration of novel DNA into target species. Genes encoding natural or synthetic antimicrobial peptides (AMPs) are inserted into the genome of the target species with the aim of reducing its vectorial capacity for the parasite in question. *Ae. aegypti, An. stephensi* and *An. gambiae* are amongst the mosquito species successfully transformed with genes coding for molecules that impair pathogen development (Ito *et al.*, 2002; Moreira *et al.*, 2002; Kim *et al.*, 2004; Abraham *et al.*, 2005; Jasinskiene *et al.*, 2007; Yoshida *et al.*, 2007; Isaacs *et al.*, 2011; Meredith *et al.*, 2011). These peptides target specific stages within the parasite lifecycle by using gene promoters to drive expression of the peptide at a specific time point and within a particular tissue, thus increasing efficacy of the effector molecule. The mid-gut specific carboxypeptidase promoter has been used to drive expression of many effector

molecules in several different mosquito species with the aim of targeting parasites shortly after the blood meal has been taken (Coutinho-Abreu *et al.*, 2009). Transgenic strains of *An. stephensi* expressing PLA2 (bee venom phospholipase) and SM1 peptides in the midgut have been shown to have a reduced vectorial capacity for transmission of the rodent malaria, *P. berghei*. Although mosquitoes expressing PLA2 were less able to pass on malaria infections than wild type counterparts, they were also significantly less fit (Moreira *et al.*, 2002; Moreira *et al.*, 2004). Reduced fitness was not a problem with those mosquitoes expressing the SM1 tetramer however they were shown only to have a reduced vectorial capacity with regards to *P. berghei* (Ito *et al.*, 2002). *An. stephensi* mosquitoes expressing CELIII (haemolytic C-type lectin from sea cumber) have been documented as the first anophelines to show a reduction in vectorial capacity across different *Plasmodium* species, possibly as the molecule targets red blood cells as well as the parasite itself. The authors suggest anecdotally that no fitness cost to the mosquito is incurred but this is yet to be tested (Yoshida *et al.*, 2007).

The vitellogenin promoter has also been used to express effector molecules in mosquitoes to target stages present in the fat-body. Reports by Shin *et al*. (2003) suggest that transgenic *Ae. aegypti* expressing either Cecropin A or Defensin A under control of this promoter are more likely to survive injection with the pathogenic bacteria *Enterobacter cloacae* (Shin *et al.*, 2003). This promoter was then used in *An. stephensi* to drive expression of a DsRed reporter gene thus providing the initial indicator that it can function very well as a promoter, causing accumulation of reporter gene products in the fat-body of the insect (Chen *et al.*, 2007b). It has also been demonstrated that a salivary gland-specific promoter (anopheline antiplatelet protein, AAPP) is able to drive expression of the DsRed reporter gene (Yoshida and Watanabe, 2006). This was shown, once again, in *An. stephensi* mosquitoes, as a candidate promoter to drive a transgene which could target the

Plasmodium parasite at the sporozoite stage in the salivary glands. Expressing such a peptide alongside a peptide specifically produced in the midgut in a double-barrelled attack on the parasite may be even more effective. In the attempt to block malaria transmission in anophelines, the major malaria vector has not been forgotten. The *Ae. aegypti* carboxypeptidase promoter has been used to drive expression of the naturally occurring AMP, Cecropin A, in the midgut of *An. gambiae*. Challenge of these mosquitoes with *P. berghei* saw a 60% decrease in oocysts in transgenic mosquitoes compared to their wild type counterparts (Kim *et al.*, 2004).

More recently, transgenic *An. gambiae* have been created which express a synthetic peptide known as Vida3 in the midgut, using the *An. gambiae* carboxypeptidase promoter sequence (Meredith *et al.*, 2011). The data show a consistent reduction in parasite intensity of the mouse malaria *P. yoelii nigeriensis* by around 85%, and also indicate a knockdown effect on the human malaria *P. falciparum*, though this result was less consistent (Meredith *et al.*, 2011). Vida3, a 14 amino acid synthetic peptide, was selected for use as an effector molecule as it was designed to be effective against all sporogonic stages of the murine malaria parasites, *P. berghei* and *P. yoelii* (Meredith *et al.*, 2011). In comparison to other similar short hybrid peptides which had different expected secondary structures, Vida3 exhibited the highest level activity against *P. berghei* and *P. yoelii* sporogonic stages in culture, and showed the highest knockdown of *P. berghei* when injected and orally administered to infected *An. gambiae* mosquitoes (Arrighi *et al.*, 2002). The difference in effectiveness of these peptides may be due to their secondary structure, and the exact mode of action against parasites may be different to that assigned to many other larger AMPs (>20 amino acids). The smaller size of Vida3 is responsible for a loss of ability to span parasite membranes to form pores, and consequently an alternative mode of action is proposed as a mechanism by which a membrane is targeted (Arrighi *et al.*,

2002). The sequence coding for the Vida3 peptide was integrated into the genome of *An. gambiae* by means of the two-phase site-specific phiC31 integration system. It is the first reported integration of an effector gene using the unidirectional *Streptomyces* phiC31 site-specific system in the malaria vector, *An. gambiae* (Meredith *et al.*, 2011). The first step of this two-phase process involves transposon-mediated insertion of an *attP* recognition sequence into the host genome, alongside a promoter-marker sequence combination, allowing identification of transformed individuals. This *attP* site is crucial in the second stage of the two-phase process, whereby the phase one lines are injected with plasmids containing an *attB* recognition sequence which, catalysed by phage phiC31 integrase, interact with the *attP* site and recombine to give *attL* and *attR* sequences, no longer recognised by integrase. The plasmid injected, as well as the *attB* site and the effector gene with tissue-specific promoter, also contains a second promoter-marker sequence in order that individuals transformed with the phase two construct can be uniquely identified. Any phase two transgenic insect displays two fluorescence profiles. This system was described as a tool for precise transformation in *Drosophila* (Groth *et al.*, 2004), and was subsequently described in *Ae. aegypti* as providing a means for increased efficiency of transformation (Nimmo *et al.*, 2006). However, Meredith *et al.* (2011) did not see increased efficiency when using this system for integration of novel DNA, but speculate that there may be an impact on efficiency caused by size of plasmid. Nevertheless, the phiC31 integration system demonstrates an ability to transform *An. gambiae* with an effector molecule which exhibits an anti-malarial phenotype when expressed in the mid gut of mosquitoes. Not only that, but phase one lines generated and characterised could be used in the future to generate further phase two lines also showing an anti-malarial phenotype whereby Vida3, or other effector, is expressed in a different tissue within the mosquito thus targeting different stages of *Plasmodium*.

Interestingly, Arrighi *et al*. (2002) report that Vida3 is active against oocysts, which develop under the basal lamina in a suspected immune-privileged site, protected from AMPs by a capsule made up of basal lamina components. This suggests that Vida3, when injected into the haemolymph, is small enough to be able to come into contact with the developing oocyst (Arrighi *et al.*, 2002). As the EVida3 *An. gambiae* strain expresses the peptide under control of the carboxypeptidase promoter, expression should be limited to the midgut of the mosquito, as demonstrated by RT-PCR of Vida3 expression in midgut and carcass from EVida3 strain (Meredith *et al.*, 2011) as the signal peptide which directs the transport of the protein is also included. It is not, therefore expected that this peptide, in this strain, would show activity against any other parasite stage other than ookinetes in the midgut. However, due to its small size, and clear ability to access oocysts under the basal lamina, it was speculated that it may be able to cross the midgut epithelium and have a detrimental effect on developing oocysts. It was also hypothesised that a second blood meal may boost the immune response of an infected mosquito as it provides more resources to fight an infection, but also, in the case of the EVida3 strain, provides a second boost of Vida3 peptide. This of course, would only have an effect if the midgut stages of the parasite were still in the midgut at the time of the second blood meal, or indeed, if the peptide could cross the midgut epithelium to target oocysts in the same manner as when injected into the haemolymph (Arrighi *et al.*, 2002). It has previously been documented that anopheline mosquitoes will take multiple blood meals within one gonotrophic cycle (Briegel and Horler, 1993; Nirmala *et al.*, 2005), and certainly within the time taken for an infection of *Plasmodium* to become infective, at the sporozoite stage (Lopes *et al.*, 2007). Furthermore, there is evidence to suggest that infection with *Plasmodium* increases the volume of blood taken by a sporozoite-infected mosquito, as well as increased probing and the likelihood that more hosts will be fed upon (Koella *et al.*, 1998). Certainly blood

feeding habits of mosquitoes are important topics to research in order to create accurate mathematical models. This is especially true when considering the effect of novel anti-malarial peptides against *Plasmodium* when mosquitoes expressing these peptides obtain further resources from multiple blood feeds. Consequently a study was designed whereby the transgenic EVida3 strain of *An. gambiae* expressing the Vida3 peptide was offered a second naïve blood meal in order to ascertain the effect this had on the intensity of *P. yoelii nigeriensis* infection within the mosquitoes by counting live oocysts within the midgut. The transgenic line EVida3 would be compared directly to the phase one transgenic line E as a control to ensure the only difference between the two lines was the production of Vida3 peptide. This meant that the experiment would look at the effect of the second blood feed on parasite intensity with respect to production of Vida3 peptide in the mosquito midgut. Meredith *et al*. (2011) had already demonstrated the efficacy of the Vida3 peptide in challenge experiments with *P. yoelii nigeriensis* against EVida3 transgenic line. Providing a second blood feed was not expected to have a significant effect on prevalence of infection with *P. yoelii nigeriensis* due to the timing of the second feed, and because it has already been demonstrated that although a considerable knockdown effect of the parasite occurs due to presence of the Vida3 peptide, it does not prevent all ookinetes invading the mosquito midgut epithelial cells (Meredith *et al.*, 2011). This is an interesting topic in terms of insects transformed with genes coding for novel immune peptides as there is documented evidence to suggest a reproductive-fitness cost associated with mounting an immune response in wild type *An. gambiae* (Ahmed *et al.*, 2002; Hurd *et al.*, 2005; Ahmed and Hurd, 2006).

In order to better relate the effect of any enhanced immune response to production of Vida3, an experiment to more accurately determine how much Vida3 peptide is produced by the transgenic mosquito was designed. A dose-response curve approach was taken with

the idea of feeding Vida3 peptide to the phase one host strain (line E), used to generate the EVida3 phase two strain, at varying concentrations. The mean knockdown effect of oocyst burden could then be established for each peptide concentration against a control whereby no peptide was fed alongside the infected blood meal. EVida3 mosquitoes fed on the same infected blood could then be dissected, knockdown effect determined and the dose-dependent curve from the E line data used to determine an approximate level at which Vida3 is produced in the transgenic line.

Ethics Statement

All animals were handled in strict accordance with good animal practice as defined by the UK Animal (Scientific Procedures) Act 1986 and approved by the UK Home Office, licence number PPL 40/2997. Work was also approved by the University of Keele Animal care and Ethical Review Committee.

3.2. Materials and Methods

3.2.1. Multiple feed experiment comparing An. gambiae transgenic lines E and EVida3 when challenged with *Plasmodium yoelii nigeriensis*

3.2.1.1. Maintenance of *P. yoelii nigeriensis*

Infection with *P. yoelii nigeriensis* in male T0 mice was established using cryopreserved infected mouse blood. Subsequent passage of blood to a fresh mouse ensured better synchrony of infection. Mice were reared under controlled conditions at a temperature of 18°C with a 14:10 light:dark cycle. Thin smears of tail blood were Giemsa-stained in order to establish parasitaemia of each mouse used. Exflagellation rates, and thus infectivity, within each mouse were estimated by counting the mean number of exflagellating gametocytes in 10µl of tail-blood over 12 fields of view. All experiments involving mice were carried out by trained personnel with appropriate Home Office licences.

3.2.1.2. Setting up lines prior to infected feed

Two weeks prior to the feeding date, 8 trays of 200 newly hatched L_1 stage larvae for both the E and EVida3 lines were set up. These were maintained following the standard protocol. For each line, pupae were collected every day and placed into a small cage containing 10% glucose solution with 0.05% PABA (para-aminobenzoic acid) and left to emerge. The following morning adults were removed from the cage and placed in a separate cage containing 10% glucose with 0.05% PABA so all adults were of a known age.

Around 150 adult females from each line of around 3-4 days old were transferred to a large pot to ensure ease of blood-feeding. Females were transferred to these pots 2 days prior to

the feed to allow them to settle. Mosquitoes were starved of sugar overnight to maximise blood-feeding. Water was removed from cages on the morning of the infected feed.

3.2.1.3. Giving an infected blood-feed

A T0 mouse infected with *P. yoelii nigeriensis*, shown to be exflagellating, was anaesthetised and placed over both pots of mosquitoes so both lines could feed simultaneously from the same mouse. Records were made of parasitaemia, exflagellation rates and which line got the head/tail of the mouse. The feeding was supervised at all times and the mouse was kept warm throughout the feeding procedure. The mosquitoes were given ample time to feed without causing distress to the mouse. Once the majority of mosquitoes had been fed, each pot was given 10% glucose with 0.05% PABA.

3.2.1.4. Removal of non-blood-fed mosquitoes

The morning after the blood feed was offered, both lines of mosquitoes were knocked down by chilling. This ensured that engorged mosquitoes could be separated from those which had not fed. Consequently it was certain that, at this point in the experiment, all remaining mosquitoes had taken no less than one blood meal. Engorged mosquitoes for each line were split into two equally sized cohorts and placed into small cages containing 10% glucose with 0.05% PABA.

3.2.1.5. Multiple blood meals

One cage of each line was allowed to lay 2 days post blood-meal. At the same time as the oviposition vessel was placed in these cages, the sugar was removed. The following day (3 days after initial feed) the mosquitoes in these two cages were offered a blood meal using

the Hemotek feeding system following the usual protocol. Gravid females who had not laid eggs were not removed. The mosquitoes were given sufficient time to feed without being disturbed. The following morning non-engorged mosquitoes were removed, after chilling, to ensure that all mosquitoes in those cages had received no less than two blood-meals.

3.2.1.6. Post-blood meal maintenance of lines E and EVida3

Non-engorged mosquitoes were removed, after chilling, from the cages which were offered the second blood meal to ensure that all mosquitoes in those cages had received no less than two blood-meals. Mosquitoes in all cohorts were subsequently not given an opportunity to oviposit. All cages were maintained on 10% glucose with 0.05% PABA *ad libitum* for the remainder of the experiment, and this was checked twice daily to ensure its availability.

3.2.1.7. Mid-gut dissections

At 6 and 7 days post infected feed, 50 surviving mosquitoes from each cohort were dissected and mid-guts examined for presence of *P. yoelii nigeriensis* oocysts in order that prevalence and intensity of infection be determined. The four populations were randomised by a colleague not involved in the experiment so that dissections be carried out ‘blind’. Prior to dissection, mosquitoes were immobilised by chilling for approximately 20 minutes. Dissections were carried out by 2-4 colleagues, each dissecting a similar number of mosquitoes to minimise operator-error. Each mosquito was transferred to PBS on a labelled slide and the mid-gut dissected out of the abdomen using forceps. The mid-gut was transferred to PBS on a separate labelled slide, covered with a cover slip and viewed

on a light microscope at a magnification of x100. The number of oocysts infecting the mid-gut was recorded as was the number of melanised ookinetes. Ovaries were also dissected out and egg number counted and recorded alongside oocyst number/melanised ookinete number for each mosquito in every population. Carcasses were stored in 70% alcohol so wing length could also be measured to gain a mean wing length per population to rule out significant size difference between cohorts.

3.2.2. Vida3 dose-dependent curve to assess levels of expressed Vida3 peptide in *An. gambiae* EVida3 transgenic line

3.2.2.1. Maintenance of *P. yoelii nigeriensis*

As described in section 3.2.1.1.

3.2.2.2. Setting up lines prior to infected feed

Two weeks prior to the feeding date, 12 trays of 200 newly hatched L_1 stage larvae for the E line, and 3 trays of EVida3 line were set up. These were maintained following the standard protocol. For each line, pupae were collected every day and placed into a small cage containing 10% glucose solution with 0.05% PABA and left to emerge. The following morning adults were removed from the cage and placed in a separate cage containing 10% glucose with 0.05% PABA so all adults were of a known age.

Two days prior to the infected feed 80 adult females between 4 and 7 days old were transferred to each of 5 small Perspex cages for the E line. At the same time 80 adult females, also aged between 4 and 7 days old, from the EVida3 line were transferred to a single Perspex cage. All cages contained 10% glucose solution with 0.05% PABA which was removed at least 4 hours before giving the infected feed.

3.2.2.3. Vida3 dimer peptide solution

2mg of Vida3 dimer peptide (Molecular Weight of 3.875kDa) was dissolved in Molecular Grade Water (Sigma) to yield a stock solution with a concentration of 2mM which was stored at -20°C until required. This stock solution was used to create four different concentrations of peptide in 50µl of water. This was then diluted in 450µl of *P. yoelii nigeriensis*-infected mouse blood which was fed to four cohorts of the E line. The fifth cohort of the E line was fed on 50µl of molecular grade water alone, mixed with 450µl infected mouse blood. The EVida3 line was also fed on 450µl infected blood mixed with 50µl water containing no peptide. All 50µl aliquots of peptide and water controls were kept on ice until blood was collected and was ready to be mixed with peptide and placed in the Hemotek feeding system.

3.2.2.4. Feeding *An. gambiae* transgenic lines on *P. yoelii nigeriensis*-infected mouse blood

In order that each cohort of mosquitoes could be statistically compared in terms of prevalence and intensity of infection, populations had to be fed on homogeneous blood. Feeding 6 cages of mosquitoes requires 3 infected mice so, once anaesthetised, each mouse underwent a cardiac puncture and blood was pooled in a 15ml falcon tube kept on ice at 4°C until ready to be transferred to a membrane feeder. 450µl infected mouse blood was mixed, by pipetting, with 50µl peptide solution (or water controls) and transferred to a 1.5ml reservoir. The blood was contained within the reservoir by a single layer of Parafilm. The Hemotek feeding system maintained the blood at 37°C whilst each of the six cages of mosquitoes were simultaneously fed on the blood-peptide (or control) mix. The 5

E line populations were fed on a water control (0µM Vida3), and final peptide concentrations of 25µM, 50µM, 100µM and 200µM respectively. The population of EVida3 were fed on a water control (0µM Vida3).

3.2.2.5. Post-blood meal maintenance of lines E and EVida3

As described in section 3.2.1.6.

3.2.2.6. Mid-gut dissections

As described in section 3.2.1.7.

3.3. Results

3.3.1. Effects of multiple feeding on infection levels in *An. gambiae* transgenic lines E and EVida3 when challenged with *Plasmodium yoelii nigeriensis*

Parasite challenge experiments were designed to test the efficacy of the Vida3 peptide expressed in the mosquito midgut upon ingestion of an infected blood meal, and to establish any further action on the parasite if a second, naïve blood meal was taken. Four separate and independent challenge experiments with *Plasmodium yoelii nigeriensis* were undertaken over a period of 3 months (Table 3.1). In all experiments, the initial infected blood feed was given to both transgenic and control mosquitoes with both groups feeding on the same mouse to ensure a comparable initial infection between cohorts. The effect of taking one or two blood meals was measured by counting oocyst and melanised oocysts on the midgut of infected mosquitoes to determine intensity and prevalence of infection (Table 3.2). Melanised parasites were included in the counts as they were frequently observed, and as they had traversed the midgut epithelium, were seen as invading parasites. Any non-blood fed mosquitoes were removed from the study, therefore leaving only those which had taken a blood meal. In addition to this, only those mosquitoes that had eggs present in the ovaries upon dissection were included in the study.

3.3.1.1. Female wing lengths

No difference in wing lengths between the homozygous strains of E and EVida3 was observed (p = 0.4524). This indicates that a similar size blood meal was taken by each group, as wing length is a good measure of body size (Fig 3.1).

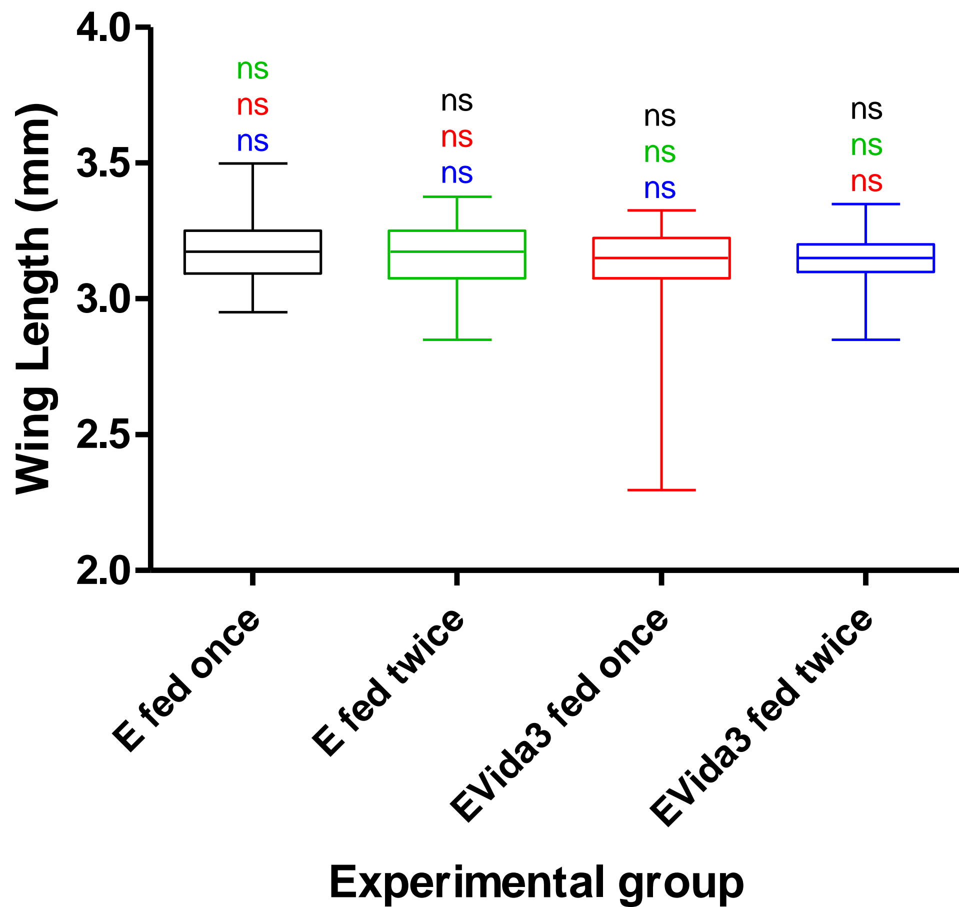


Figure 3.1: Wing lengths of female mosquitoes dissected as part of *P. yoelii nigeriensis* infection challenge

Wing lengths are well documented as a good representation of body size. Mosquitoes with similar size bodies are expected to take a blood meal of a similar size. Data were found not to follow a normal distribution in one of the four experimental groups. Therefore non-parametric analysis was performed. Vertical lines denote the maximum and minimum values, horizontal lines mark the median and the interquartile range of the data is boxed. A Kruskall-Wallis test was used to confirm that there were no significant differences in wing lengths between the groups (p = 0.4524). Statistical difference is shown above boxplots in the colour of the boxplot to which the significance level corresponds (ns, not significant).

3.3.1.2. Prevalence of Plasmodium yoelii nigeriensis infection

In all experiments prevalence was lower in line Evida3 fed once compared to line E line fed once (Table 3.2). When looking at groups receiving two blood meals, experiments 2 and 3 show a reduction in prevalence in line EVida3 compared to line E. However, experiment 1 indicates that prevalence is unaffected by the line, and experiment 4 shows the opposite result to that seen in experiments 2 and 3 where the highest prevalence is observed in line EVida3 (Table 3.2). The pooled data indicates the general trend for higher prevalence in E when compared to EVida3 when mosquitoes receive either one (88% compared to 68%) or two (85% compared to 79%) feeds (Table 3.2). Fig. 3.2A shows differences in prevalence between lines E and EVida3 in all 4 independent experiments, and pooled data. The data shown is representative of all members of line E and line EVida3 regardless of feeding status. This difference is significant for both experiment 2 ($p = 0.0014$) and for the pooled data ($p = 0.0010$). When taking into account only line E, prevalence decreases when a second blood meal is given, in all but experiment 2. The pooled data again reflects the general trend showing a decrease in prevalence from 88% to 85% when these mosquitoes take a second, naïve, blood meal post-infection. Interestingly and conversely, with the exception of experiment 3, there is an increase in prevalence when EVida3 mosquitoes obtain a second blood meal at 3 days post-infection (Fig. 3.2C). The pooled data shows an overall increase in prevalence from 68% to 79% when EVida3 are given a second blood meal (Table 3.2). Although general trends exist when comparing prevalence in line E fed once to line E fed twice, and when comparing prevalence in line EVida3 fed once to the same line fed twice, none of these differences are statistically significant for either strain (Fig. 3.2).

Experiment	Parasitaemia (exflagellation) (mouse)	Strain (times fed)	n	Median parasite intensity (mosquito)	Interquartile range (mosquito)
1	13% (0.6)	E (once)	24	4.5	0.25-13.5
		E (twice)	19	2	0-7
		EVida3 (once)	36	1	0-4
		EVida3 (twice)	31	3	0-8
2	12% (1.5)	E (once)	37	32	12.5-120.5
		E (twice)	34	43.5	8-122.5
		EVida3 (once)	46	6	0-22.25
		EVida3 (twice)	33	11	1.5-40
3	10% (1.5)	E (once)	5	5	4.5-67.5
		E (twice)	6	13.5	1.5-46.5
		EVida3 (once)	12	10	4-13.5
		EVida3 (twice)	10	2.0	0-44
4	9% (1.0)	E (once)	41	67	18.5-217.5
		E (twice)	40	76.5	10-206.5
		EVida3 (once)	42	29.5	3.75-44.5
		EVida3 (twice)	38	23	7.75-60.25
1-4		E (once)	107	27	5-110
		E (twice)	99	28	3-114
		EVida3 (once)	136	6	0-23
		EVida3 (twice)	112	9	1-35.75

Table 3.1 (opposing page): Details of *P. yoelii nigeriensis* infection experiments with E and EVida3 transgenic lines

Parasitaemia and exflagellation rates within infected mice are given, along with numbers of mosquitoes dissected. Median parasite intensity and interquartile ranges are also shown. Data was tested for normality using D'Agostino and Pearson omnibus normality test which found all data not be non-normally distributed ($p < 0.01$ in all cases). Parameters are given for experiments 1-4 as well as for pooled data.

Experiment	Strain (times fed)	n	Prevalence of infection[a]	Mean infection intensity ± SD[b]	p^c (significance)
1	E (once)	24	0.75	11.25 ± 16.38	0.0722 (ns)
	E (twice)	19	0.68	5.53 ± 8.003	
	EVida3 (once)	36	0.53	2.67 ± 3.505	
	EVida3 (twice)	31	0.68	5.00 ± 6.501	
2	E (once)	37	0.92	65.08 ± 76.57	<0.0001 (***)
	E (twice)	34	0.97	71.35 ± 72.73	
	EVida3 (once)	46	0.72	15.85 ± 25.01	
	EVida3 (twice)	33	0.79	36.58 ± 64.91	
3	E (once)	5	1.00	29.80 ± 45.69	
	E (twice)	6	0.83	25.17 ± 33.86	
	EVida3 (once)	12	0.83	10.75 ± 8.75	
	EVida3 (twice)	10	0.70	23.30 ± 36.19	
4	E (once)	41	0.93	124.00 ± 130.7	0.0012 (**)
	E (twice)	40	0.85	121.80 ± 146.6	
	EVida3 (once)	42	0.81	37.36 ± 51.29	
	EVida3 (twice)	38	0.89	43.66 ± 55.79	
1-4	E (once)	107	0.88	73.93 ± 102.7	<0.0001 (***)
	E (twice)	99	0.85	76.29 ± 111.3	
	EVida3 (once)	136	0.68	18.55 ± 34.67	
	EVida3 (twice)	112	0.79	29.05 ± 51.22	

Table 3.2 (opposing page): Oocyst prevalence[a] and mean intensity[b] of *P. yoelii nigeriensis* infection in *An. gambiae* E and EVida3 lines fed either once or twice

[a]Prevalence reflects the percentage of mosquitoes within which parasites had invaded midgut epithelial cells; [b]Mean intensity of infection represents the mean number of invading parasites within the midgut of the infected mosquito; [c]A Kruskal-Wallis test was used to evaluate the statistical significance of the mean intensity of infection data. This was followed by a Dunns multiple comparison test to establish which pairs of infection intensities showed statistically significant differences. Statistical analysis of experiment 3 is not given as *n* is too low. This experiment is, however, included in the pooled data.

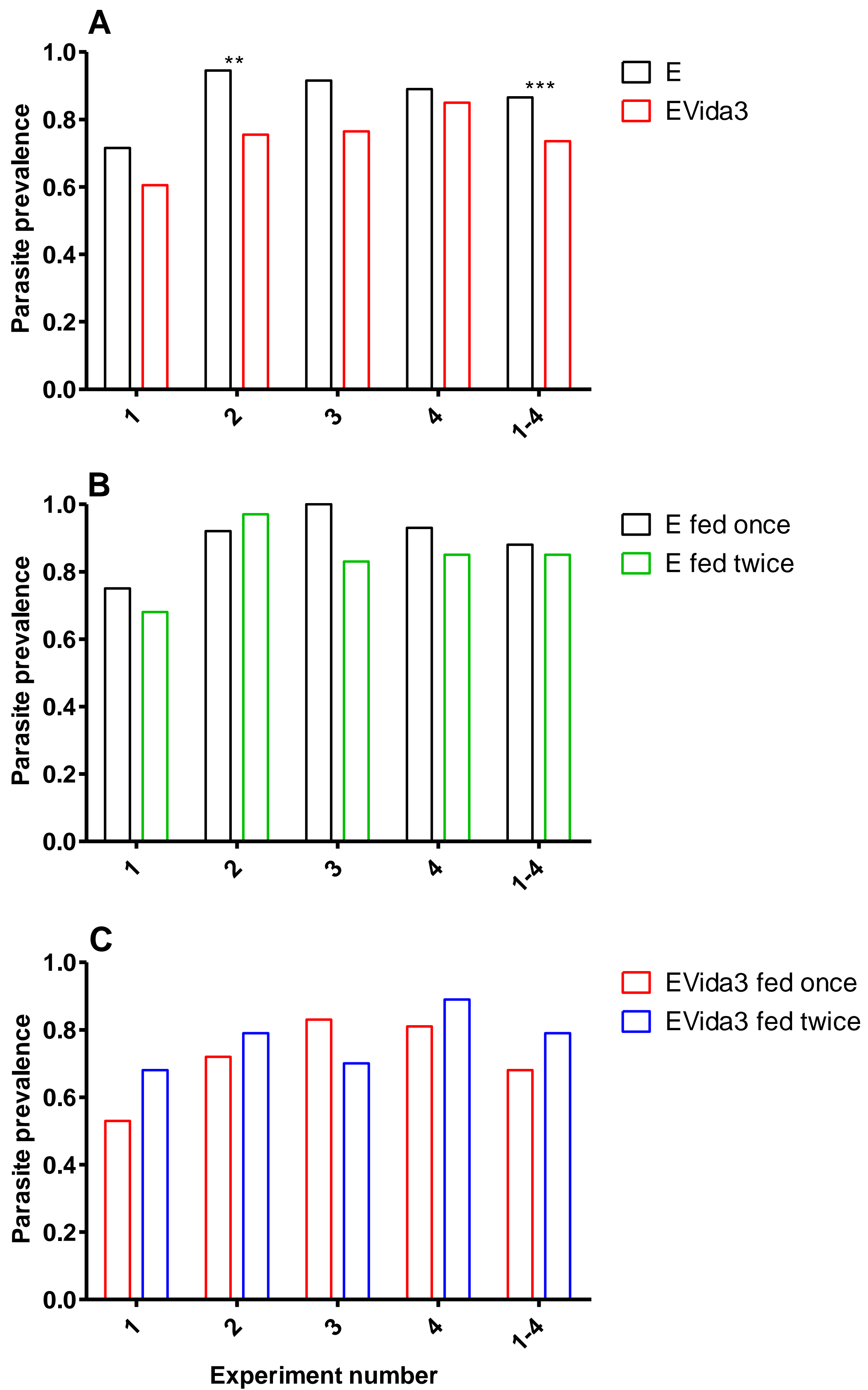

A
Parasite prevalence
1.0
0.8
0.6
0.4
0.2
0.0
**
***
1
2
3
4
1-4
E
EVida3
B
E fed once
E fed twice
C
EVida3 fed once
EVida3 fed twice
Experiment number

Figure 3.2 (opposing page): Prevalence of infection with *P. yoelii nigeriensis* in transgenic lines E and EVida3

(A) Histograms show prevalence for lines E and EVida3 in experiments 1–4 and the pooled data. There is a trend to reduced prevalence in EVida3 although significant differences are only seen in Experiment 2 ($p = 0.0014$) and in the pooled data ($p = 0.0010$). (B) Histograms show prevalence for line E, fed either once or twice in experiments 1-4 and pooled data. There is a general trend towards reduced prevalence in line E fed twice but none of these differences are significant. (C) Histograms show prevalence for line EVida3 fed either once or twice, in experiments 1-4 and pooled data. There is a general trend towards increased prevalence in EVida3 fed twice but none of these differences are significant. Data was analysed by chi squared goodness of fit, with a Fishers Exact test.

Statistical differences are indicated above bars according to significance level (**, $p < 0.001$; ***, $p < 0.0001$)

3.3.1.3. Links between mouse and mosquito infections

Overall a general inverse correlation between parasitaemia in the murine host and mosquito parasite intensity is apparent. Mean intensity of parasite infection increases from an average of 6.1 in experiment 1, where the parasitaemia in the murine host was 13%, to 81.7 in experiment 4 where parasitaemia was 9%. The best infection rate (when taking into account all groups dissected) is found in the experiment which started with the lowest mammalian host parasitaemia (9%) and the lowest exflagellation rate (1.0).

3.3.1.4. Intensity of *Plasmodium yoelii nigeriensis* infection

Intensity of infection is variable over all four experiments; therefore separate boxplots are shown for each experiment as well as the pooled data (Fig. 3.3). Statistical analysis was not performed on data generated by experiment 3 as numbers were too low, but this experiment was included in the pooled data. A comparison of E with EVida3 when lines were fed only once shows a 75% decrease in oocyst burden on mosquito midguts when the Vida3 peptide is expressed ($p < 0.0001$). This is reflected by comparing those groups receiving a second blood meal 3 days post-infection. In this case the knockdown of *P. yoelii nigeriensis* is less marked but a decrease in mean intensity of infection of 63% is seen ($p = 0.0006$). When comparing the effects of the second blood meal on mean intensity of infection in line E, experiments 1, 3 and 4 show a decrease in parasite burden of 51%, 15% and 2% respectively in E line fed twice compared with the control group fed only once. Experiment 2 however indicates a 9% increase in mean intensity of infection when E line received a second blood meal. When the data from all four experiments is pooled, a small increase in parasite burden of 4% is seen in those groups of E line fed twice in comparison to those fed once, a non-significant result ($p = 0.9331$). A similar overall pattern is seen when comparing the effects of a second blood meal in EVida3

transgenic line. However, experiments 1 to 4 all show an increase in mean intensity of infection, of 47%, 57%, 54% and 14% respectively, when EVida3 receives a second blood meal. Pooling of data results in a mean intensity of infection which is 1.5 times higher in those EVida3 cohorts fed twice than in the control group of EVida3 fed once, though the result is not significant ($p = 0.0720$). Percentage differences in parasite burden between compared cohorts as well as *p* values from statistical analyses, from pooled data, are seen in Table 3.3.

3.3.1.5. Numbers of melanised parasites within mosquito midguts

Parasite intensity took into account numbers of oocysts and number of melanised parasites present on mosquito midguts as they are both representative of invading parasites. Numbers of melanised parasites were also recorded independently of oocyst number for each mosquito. Taking into account the pooled data, there is no significant difference in number of melanised parasites between line E fed once compared to E fed twice ($p = 0.7468$) or between EVida3 fed once and EVida3 fed twice ($p = 0.2300$). However, there is a significant difference between line E fed once and EVida3 fed once ($p < 0.0001$) and between line E fed twice and EVida3 fed twice ($p = 0.0011$). Differences between cohorts are illustrated in Fig. 3.4.

3.3.1.7 Numbers of oocysts within parasite midguts

The number of live oocycts were recorded for each mosquito midgut, as well as melanised ookinetes. Numbers of live oocysts were generally low with the majority of mosquitoes in each experimental group having no oocycts present at all. In each cohort of mosquites there was at least one individual which had a markedly high intensity of infection with live

parasites. In three of these cohorts (E fed once, E fed twice and EVida3 fed twice) these individuals contained over 300 oocysts. Taking into account the pooled data, there is no significant difference when comparing any pair of experimental groups ($p = 0.4470$). Graphical representation of the data can be seen in Fig. 3.5.

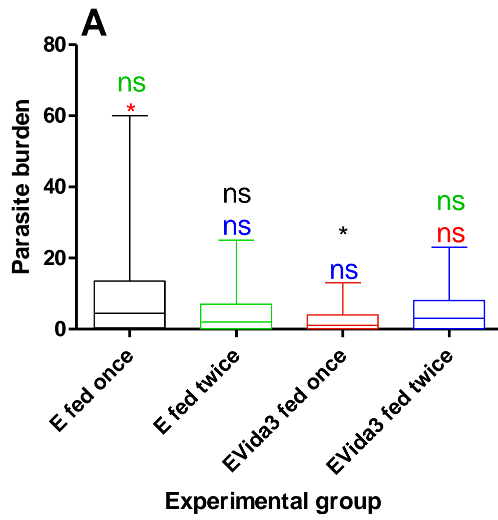
3.3.1.7 Numbers of eggs present in ovaries

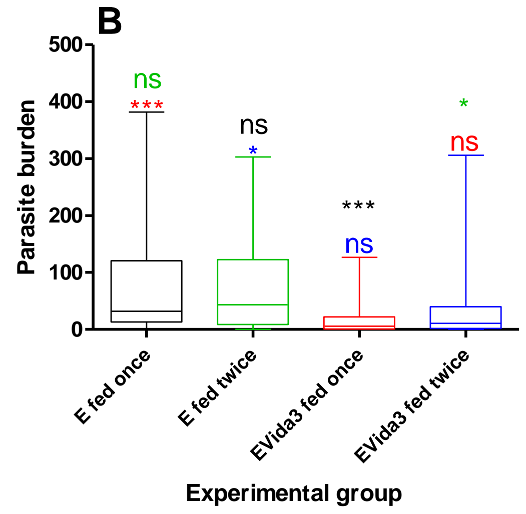
Egg numbers were also recorded upon dissection of mosquitoes. Statistical analyses of data using Mann-Whitney test to compare pairs of cohorts showed that there is no significant difference in egg number per female between lines E and EVida3 fed twice ($p = 0.7392$). There is a significant difference, however, in egg number per female between lines E and EVida3 fed once ($p = 0.0182$). Within transgenic lines, there is a highly significant difference in egg production between those cohorts fed once and those fed twice (E, $p < 0.0001$; EVida3, $p < 0.0001$). Differences between cohorts are illustrated in Fig. 3.6.

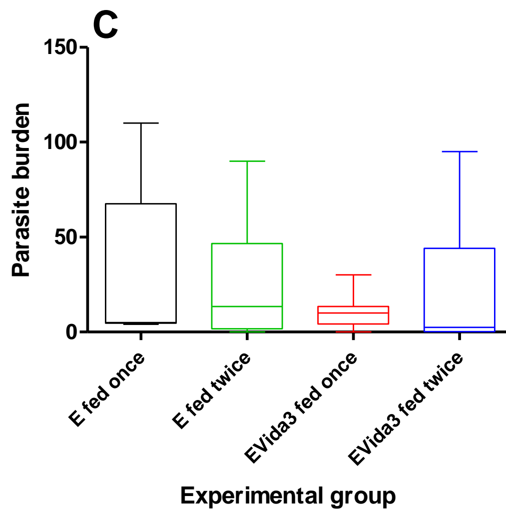
Cohorts compared[d]	Percentage difference[e] in mean intensity of infection	p value (significance)
E (C) (fed once) vs. EVida3 (fed once)	75% ↓	$p < 0.0001$ (***)
E (C) (fed twice) vs. EVida3 (fed twice)	62% ↓	$p = 0.0005$ (***)
E (C) (fed once) vs. E (fed twice)	3% ↑	$p = 0.8578$ ns
EVida3 (C) (fed once) vs. EVida3 (fed twice)	36% ↑	$p = 0.1029$ ns

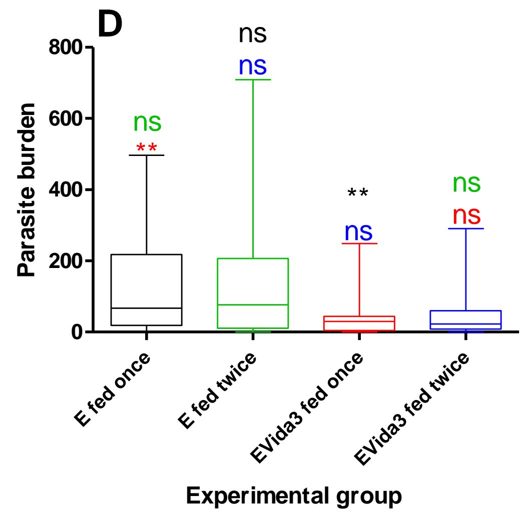
Table 3.3: Percentage differences[e] in mean infection intensity and *p* values for compared cohorts[d] of transgenic lines using the pooled data set

[d]Where cohorts are compared, the control group is denoted as (C); [e]An increase compared to the control is denoted with ↑ and a decrease with ↓. A Mann-Whitney test was used to evaluate pairs of data for statistical analysis and a 95% confidence limit was used to indicate significance.


A
Parasite burden
Experimental group
E fed once
E fed twice
EVida3 fed once
EVida3 fed twice
ns
*
ns
ns
*
ns
ns
ns


B
Parasite burden
Experimental group
E fed once
E fed twice
EVida3 fed once
EVida3 fed twice
ns
***
ns
*
***
ns
*
ns


C
Parasite burden
Experimental group
E fed once
E fed twice
EVida3 fed once
EVida3 fed twice


D
Parasite burden
Experimental group
E fed once
E fed twice
EVida3 fed once
EVida3 fed twice
ns
**
ns
ns
**
ns
ns
ns

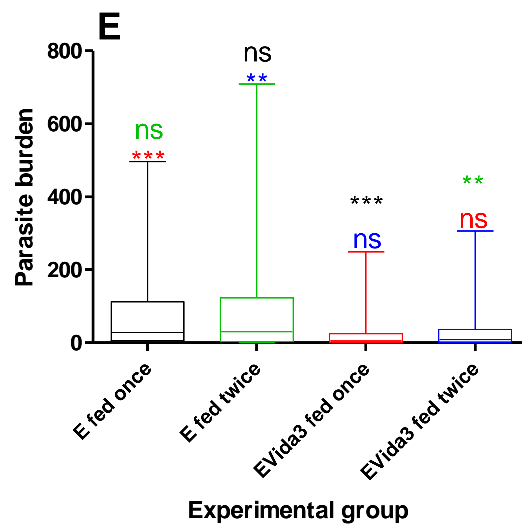

E
Parasite burden
Experimental group
E fed once
E fed twice
EVida3 fed once
EVida3 fed twice
ns
***
ns
**
***
ns
**
ns

Figure 3.3 (opposing page): Parasite intensity of *P. yoelii nigeriensis* in E and EVida3 transgenic lines after receiving either one or two blood meals

Boxplots show parasite intensity for lines E and EVida3 from four independent experiments (A-D) and the pooled data (E). Oocysts and melanised ookinetes were scored as invading parasites. Vertical lines denote the maximum and minimum values, horizontal lines mark the median and the interquartile range of the data is boxed.

Non-parametric statistical analysis (Kruskal-Wallis test) was carried out to ascertain significant differences in parasite intensity between lines. Dunns multiple comparison post test was performed to analyse differences between pairs of cohorts. Experiment 3 was not tested independently for statistical differences as numbers were too low in all cohorts, but this data is included in pooled data. Statistical difference is shown above boxplots in the colour of the boxplot to which the significance level corresponds (***, $p < 0.0001$; **, $p < 0.001$; $p < 0.05$; ns, not significant).

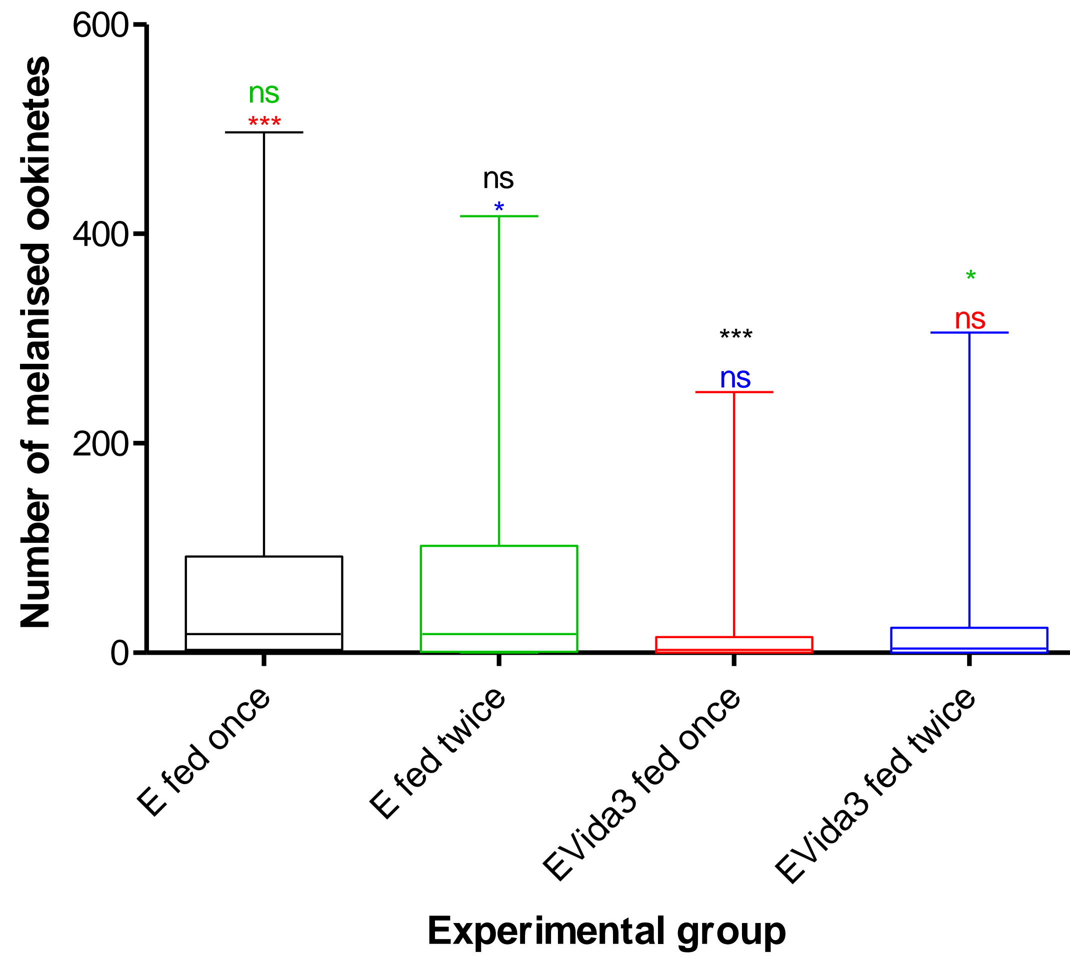

Number of melanised ookinetes
600
400
200
0
ns
***
ns
*
*
ns
***
ns
E fed once
E fed twice
EVida3 fed once
EVida3 fed twice
Experimental group

Figure 3.4 (opposing page): Number of melanised parasites per mosquito midgut in transgenic lines and EVida3 after receiving either one or two blood meals

The boxplot shows the number of melanised parasites for lines E and EVida3 from pooled data. Vertical lines denote the maximum and minimum values, horizontal lines mark the median and the interquartile range of the data is boxed. Data were found not to follow a normal distribution and therefore a Mann-Whitney test was used to analyse pairs of data. There was no significant difference between line E fed once and line E fed twice, or between line EVida3 fed once and EVida3 fed twice (p = 0.7468 and p = 0.2300 respectively). However there is a significant difference in number of melanised parasites between line E fed once and EVida3 fed once ($p < 0.0001$) and also between E fed twice and EVida3 fed twice (p = 0.0011). Statistical difference is shown above boxplots in the colour of the boxplot to which the significance level corresponds (***, $p < 0.0001$; *, $p < 0.05$; ns, not significant).

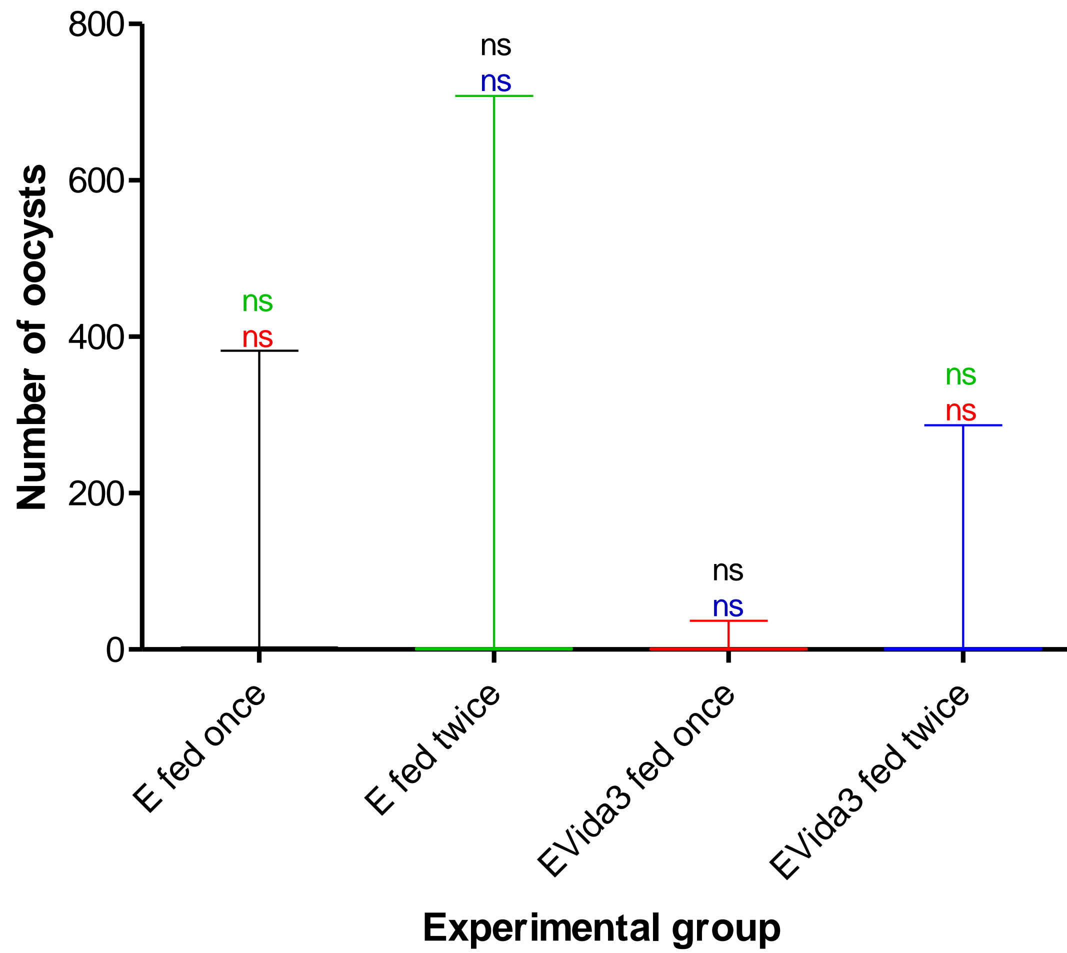
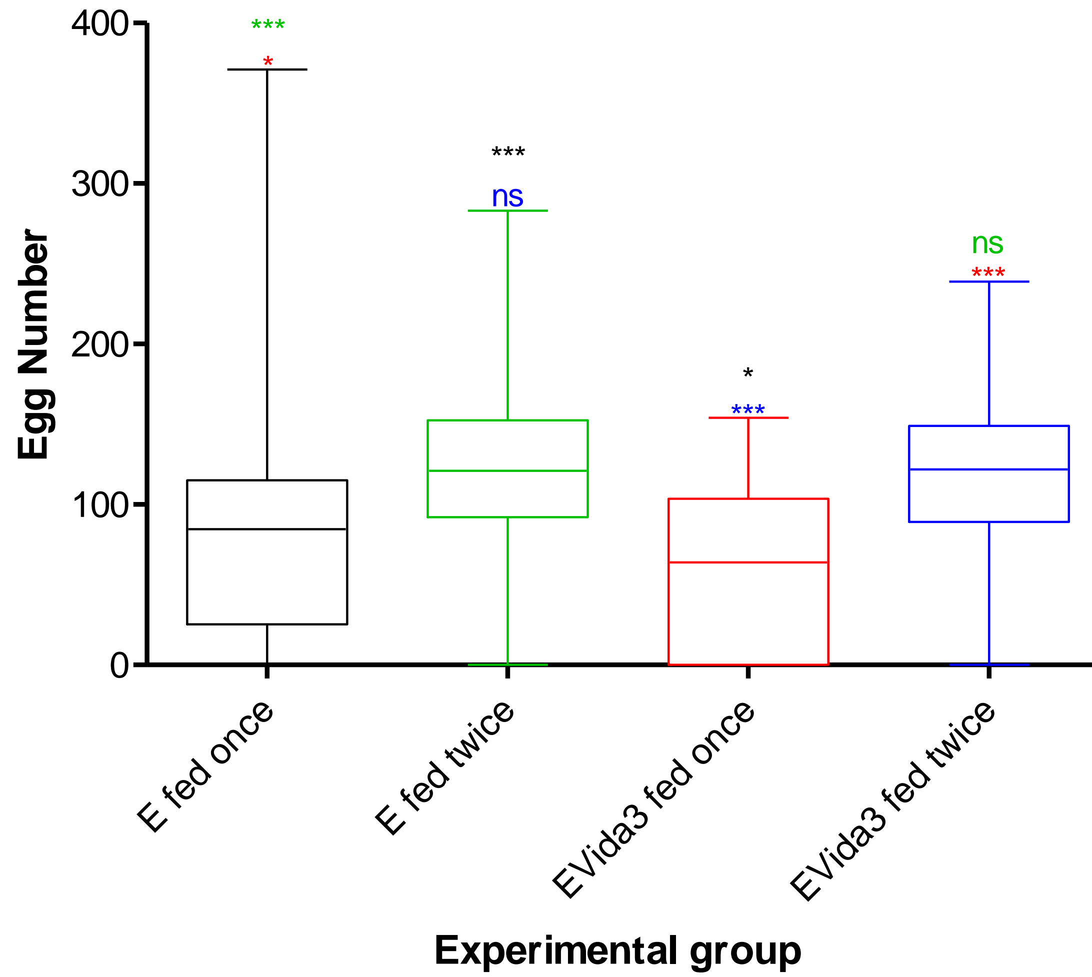

Number of oocysts
800
600
400
200
0
ns
ns
ns
ns
ns
ns
ns
ns
E fed once
E fed twice
EVida3 fed once
EVida3 fed twice
Experimental group

Figure 3.5 (opposing page): Number of oocysts per mosquito midgut in transgenic lines and EVida3 after receiving either one or two blood meals

The boxplot shows the number of oocysts for lines E and EVida3 from pooled data. Vertical lines denote the maximum and minimum values, horizontal lines mark the median and the interquartile range of the data is boxed. Data were found not to follow a normal distribution and therefore a Kruskal-Wallis test was used to analyse data. There was no significant difference between experimental groups analysed (p = 0.4470) Statistical difference is shown above boxplots in the colour of the boxplot to which the significance level corresponds (ns, not significant).



Egg Number
400
300
200
100
0
***
*
***
ns
*
***
ns
***
E fed once
E fed twice
EVida3 fed once
EVida3 fed twice
Experimental group

Figure 3.6 (opposing page): Egg numbers per mosquito in transgenic lines E and EVida3 after receiving either one or two blood meals

The boxplot shows number of eggs per female for lines E and EVida3 from pooled data. Vertical lines denote the maximum and minimum values, horizontal lines mark the median and the interquartile range of the data is boxed. Data were found not to follow a normal distribution and therefore Mann-Whitney tests were used to analyse pairs of data. There was no significant difference between lines E and EVida3 when fed twice ($p = 0.7392$). There is, however, a significant difference between lines E and EVida3 when fed only once ($p = 0.0182$). Within transgenic lines there is a very highly significant difference in egg production between those groups fed once and those fed twice (E, $p < 0.001$; EVida3, $p < 0.0001$). Statistical difference is shown above boxplots in the colour of the boxplot to which the significance level corresponds (***, $p < 0.0001$; *, $p < 0.05$; ns, not significant).

3.3.2. Vida3 dose-dependent curve to assess levels of expressed Vida3 peptide in *An. gambiae* EVida3 transgenic line

Mosquitoes fed on homogeneous *P. yoelii nigeriensis* infected blood via a membrane feeder. The only difference between cohorts was the concentration of Vida3 peptide added to the blood. The control group of E was fed water instead of peptide, as was the experimental cohort of line EVida3. At 7 days post infection, mosquitoes were dissected in order that parasite intensity on the midguts could be determined. This was repeated over several occasions with the intention of attaining results from at least 3 separate independent repeated experiments. On all occasions, although both parasitaemia and exflagellation rates of male gametocytes obtained from the murine host was indicative of a good infection, dissection of mosquitoes showed no infection to be present in any of the cohorts.

3.4. Discussion

3.4.1. Effects of multiple feeding on infection levels in *An. gambiae* transgenic lines E and EVida3 when challenged with *Plasmodium yoelii nigeriensis*

3.4.1.1. Effect of a second blood meal and the Vida3 peptide on parasite prevalence

Prevalence of infection with *P. yoelii nigeriensis*, which is the percentage of dissected mosquitoes showing evidence of a parasite burden on their midguts, is seen to be higher in transgenic line E than in EVida3 (p = 0.0010), regardless of whether one or two blood meals are taken. It is unlikely that this reduction in prevalence is due to EVida3 taking a smaller blood meal, and consequently ingesting fewer gametocytes as the wing length data shows there to be no significant difference between transgenic lines. Wing length is a good indicator of body size (Hurd *et al.*, 1995), and a positive, direct correlation between body size and blood meal size is known (Briegel, 1990). This fact, combined with the removal of any mosquitoes not fully engorged from the study, means that it is reasonable to assume that within each challenge experiment, both transgenic lines take a similar size blood meal, thus ingesting a similar number of gametocytes. Consequently blood meal size is not likely to a factor affecting parasite prevalence. The Vida3 peptide within the midgut of EVida3 is expected to have an immediate effect, upon ingestion of the blood meal, on the ookinete stage of the parasite as indicated by initial feeding studies (Arrighi *et al.*, 2002). Thus it could be extrapolated from this, that a reduction in the prevalence of infection was expected within EVida3 when compared to E. This is because a greater proportion of EVida3 mosquitoes are able to clear the infection prior to the ookinete invading the midgut epithelial cells. It may be expected therefore, that a subsequent blood meal may cause reduction in prevalence for both strains, in comparison to those receiving

only one blood meal as the mosquitoes have greater access to resources and mounting an immune response is costly to the mosquito (Ahmed *et al.*, 2002). Inclusion of a wild type strain, for example, KIL, when undertaking these experiments may have been useful in order to determine the effect of a second blood meal on infection with *Plasmodium*, whilst removing any confounding factors presented by transgenesis. A reduction in prevalence is seen in line E where a second blood meal is taken. It is not, however, a significant difference (p = 0.6733). This suggests that taking a second blood meal does not influence prevalence of infection, certainly not when the blood meal is taken 3 days post infection. This may be due to the timing itself of the blood meal or may be influenced as well by the immune status of the blood meal which, for this experiment, is naïve. It would be expected that EVida3 would also show a reduction in prevalence for the same reasons as E when given a second blood meal. In fact it would be reasonable to suggest that an even more marked reduction in prevalence may be possible in EVida3 if the second wave of translation of Vida3 peptide were detrimental to the parasite. For example, if the peptide were able to have an effect on the oocysts themselves due to its small size conferring an ability to cross the midgut epithelium. The peptide itself is expressed only on the midgut lumen side due to presence of a signal peptide which is cleaved from the protein after transportation so an ability to cross the midgut lumen would be necessary for Vida3 to have an effect on oocysts. However in the case of EVida3 there is, in fact, an increase in prevalence of infection in the cohorts fed a second time. Though again, this is not a significant difference (p = 0.0958). This makes it very unlikely that the Vida3 peptide is able to access the parasite once it has entered the midgut epithelial cells.

3.4.1.2. Inclusion of melanised parasites in intensity data

Intensity of infection was measured as the number of oocysts present on the midgut of infected mosquitoes as well as the number of melanised ookinetes present within the epithelium. The genetic background of the transgenic lines is *An gambiae* (KIL) which is a wild type laboratory strain originally unable to melanise *P. yoelii nigeriensis* ookinetes (Hurd *et al.*, 2005). However previous infection studies have since suggested that contamination of this wild type strain with another wild type strain kept in the same insectary has occurred prior to it being used as the background strain for transgenic lines. In these infection studies, a melanisation response was frequently observed (Meredith *et al.*, 2011). It is likely that the contaminating strain was *An. gambiae* (G3) as it has a documented melanisation trait (Hurd *et al.*, 2005). However, this issue was not investigated further. The ideal when undertaking infection studies is a comparison of numbers of viable oocysts only which means that the melanising trait within a host strain is considered to be a confounding factor. For the purposes of this experiment, the main target of which was to assess the effect of multiple feeding on infection levels, melanised ookinetes have been included in parasite counts on midguts of mosquitoes due to them being deemed successful as invading parasites. It is worth bearing in mind that some melanised ookinetes may never have been able to give rise to viable oocysts, even though they have invaded midgut epithelium cells, due to damage by the Vida3 peptide (or other immune peptide), prior to invasion of these cells, but there is no way of assessing this.

3.4.1.3. Action of the Vida3 peptide *in vivo*

A reduction in mean intensity of infection of 75% and 63%, for those mosquitoes taking one or two blood meals respectively, was found when comparing EVida3 to E ($p < 0.0001$ and $p = 0.0006$). Along with the prevalence data this indicates that the Vida3 peptide is

responsible for knockdown of *P. yoelii nigeriensis* at the midgut ookinete stage within the mosquito host and further corroborates studies undertaken by Meredith *et al.* (2011) where the mean reduction in parasite burden was 79% - a value similar to the one attained here for the mosquito groups receiving one blood meal. The Vida3 peptide clearly causes a highly significant reduction in numbers of invading parasites. It is possible to speculate that this is due to its structure – large numbers of ß-pleated sheets and coils, but no α-helices – since other peptides in initial studies showed a less marked effect on the parasite (Arrighi *et al.*, 2002). Structural differences altering hydrophobicity and charge distribution between peptides may result in differing modes of action and ability to act against a particular parasite. This may have been the reason for the increased knockdown ability of Vida3 in comparison to other synthetic peptides in the original AMP study undertaken by Arrighi *et al*. (2002). The authors speculate that action on the parasite membrane, due to the small size of Vida3, is less likely to be caused by formation of pores but is instead by means of other proposed mechanisms stated in previous studies (Arrighi *et al.*, 2002). In EVida3, the effector peptide is expressed under control of the carboxypeptidase promoter and is therefore present in the midgut, shortly after the blood meal is taken, from where it can exert its effect on the parasite. There is a 16 hour time frame post-infected feed, within which Vida3 can remain active against the parasites (Ahmed and Hurd, 2006). After this, the parasite invades the midgut epithelial cells and is no longer accessible to Vida3. Proteases within the mosquito midgut are not active at high levels until 24 hours post blood meal, and consequently will not cause significant degradation of Vida3 until well after ookinetes have exited the midgut, therefore its ability to target the parasite is not compromised in this way (Arrighi *et al.*, 2002).

3.4.1.4. Effect of the second blood meal on infection intensity in lines E and EVida3 with respect to increased resources

The main aim of this experiment was to test the effect of a second blood meal on infection intensity in both line E and line Evida3. When considering pooled data for line E, there is an increase in parasite intensity of only 4% when the mosquitoes took a second (naive) blood meal at 3 days post-infection. This result is not significant (p = 0.9331) and therefore the conclusion can be drawn that taking a second blood meal has no effect on *P. yoelii nigeriensis* infection intensity in line E. When considering line EVida3, there was also an increase in parasite intensity of 35% when the line is fed twice - a much greater increase than seen with line E. Although this result is once again not significant (p = 0.0720), it is quite close to the 0.05 significance level which suggests that a larger sample size may be required to test the hypothesis further for more conclusive results. An increase in mean parasite intensity for both transgenic lines is slightly surprising as a second blood meal theoretically provides more resources for an increased immune response against the parasite. The second blood meal at 3 days post-infection, even though it causes a second wave of anti-malarial peptide to be released into the midgut, clearly has no further detrimental effect on the parasite than that caused by the peptide present with the initial infected blood meal. If the slight increase in mean parasite intensity in both transgenic lines is a true trend, then this could suggest that a factor affecting both lines in the same manner is responsible for this increase rather than it being an effect of the Vida3 transgene itself. It would therefore be feasible that a second blood meal at this stage of development within the mosquito could provide a higher relative increase in resources for the parasite than for its host. A further explanation could be that the non-significant increase in intensity is just a random artefact of this experiment, and the availability of a second blood meal is incidental to parasite intensity. The more marked increase in mean parasite

intensity in EVida3, which is close to being significant, could then be deemed as being due to the presence of the transgene within this line. The mosquito uses more resources from the second blood meal than its line E counterparts in the production of the Vida3 peptide, which at this stage of the parasites' life cycle is ineffective. Therefore resources are less available to be put towards an immune response against the midgut stages of *P. yoelii nigeriensis* which manifests as an increase in parasite burden on dissection at 7 days post-infection. It is interesting to note that a challenge study using *P. yoelii yoelii* infected *An. stephensi* mosquitoes indicated an increase in infection intensity when mosquitoes were given a second (naïve) blood meal 3 days post infection when compared to mosquitoes only receiving the original infected feed (Lopes *et al.*, 2007). The authors suggest that the second blood meal provides a higher availability of nutrients leading to faster maturation of oocysts. The authors also give a different hypothesis in that the need for digestion of the second blood meal and/or requirement for further egg production could lead to a decreased immune response as the mosquito directs resources towards the other processes (Lopes *et al.*, 2007). This then leads to increased oocyst formation as it is more important to the mosquito to ensure further reproductive success than to prevent oocysts forming on the midgut. In the experiment undertaken here, the increased numbers of eggs produced by those mosquitoes in their second gonotrophic cycle (Fig. 3.5) would appear to partly corroborate this hypothesis as they seem to be putting more resources into the second egg batch than the first. A study by Rono *et al*. (2010) also indicates a close link between egg production and mosquito immune response to infection with *Plasmodium*. This is discussed in more detail in section 3.4.1.8.

3.4.1.5. Effect of the second blood meal on the melanisation pathway

The *An. gambiae* (KIL) host strain used here appears to have a propensity for melanising midgut parasites. As these encapsulated parasites are seen to be invading parasites, they were included in the intensity data. Production of melanin to encapsulate parasites uses up host resources, as does production of the Vida3 peptide. A second blood meal, at day 3 post-infection may provide mosquitoes with a second boost of resources which would enable them to boost their immune response such that they become efficient at melanising these invading stages so they are less likely to develop viable oocysts. This would result in the parasite intensity data being made up of mainly melanised parasites and fewer oocysts in those mosquitoes fed twice in comparison to those fed once in both transgenic lines. This experiment actually indicates that there is no significant difference between the number of melanised parasites seen when comparing the cohorts fed once to those fed twice with either line E ($p = 0.7468$) or line EVida3 ($p = 0.2300$) (Fig. 3.4). This suggests that the resources provided by the second blood meal have no effect on the melanisation pathway.

3.4.1.6. Differences in parasite intensity between lines E and EVida3

The reduction in mean parasite intensity observed in this experiment for EVida3 in comparison to E is clearly due to an effect of the Vida3 peptide on the parasite stages present within the midgut bolus. Due to this, the number of melanised parasites in the midgut epithelial cells should be reduced in EVida3 fed once when compared to the number of melanised parasites in line E fed once as there are fewer ookinete stages to invoke the encapsulation pathway. Although the second blood meal does not appear to influence melanin production in either line, production of the Vida3 peptide in line EVida3, when receiving the second blood meal, may divert resources away from melanin production to favour Vida3 production, thus causing a further decrease in the number of

melanised parasites observed in this line when compared to its line E counterparts also fed twice. This may go some way to explaining the significant reduction in melanised parasites seen in this experiment when comparing line E fed twice with line EVida3 fed twice ($p = 0.0011$) (Fig 3.4). It could be said that if the production of Vida3 peptide takes away resources from other immune defence mechanisms, such as the melanisation response, then any invading ookinetes are thus more likely to develop into viable oocysts. As a consequence, the life cycle of the parasite is able to continue, rendering these mosquitoes more effective vectors, as it only takes one viable oocyst to release sporozoites capable of invading salivary glands for that mosquito to become an efficient vector. Only by ensuring that any active novel antimalarial peptide expressed in the midgut of the mosquito provides 100% knockdown of the ookinete so it cannot invade epithelial cells will a totally compromised vector be created. Another solution would be to ensure a zero-sporozoite phenotype whereby the peptide is active against the sporozoite stage in the haemolymph or salivary gland of the mosquito prior to it becoming infective. Arrighi *et al*. (2002) report that the Vida3 peptide is indeed capable of knocking down stages of mouse malaria other than the ookinete. Thus an ‘attack’ of the parasite can be multistage and at more than one compartment to ensure an effective reduction of vectorial capacity. From this experiment it is impossible to know which, if any, oocysts are viable. To determine this, larger cohorts of mosquitoes would be required in order that some individually be dissected at later stages to establish presence/absence of sporozoite-stage parasites in the salivary glands. Alternatively, mosquito infectivity to mice could be gauged. Oocyst numbers in general were low in all sets of experiments, possibly due to the propensity of the background KIL strain to melanise invading ookinetes, so it is difficult to compare infection levels based on oocyst data alone. It could also be the case that line EVida3, although less melanisation occurs, perhaps due to a diversion of resources to

production of Vida3, still produces many fewer viable oocysts due to damage caused to ookinetes in the blood meal bolus. This can only really be gauged by assessing the number of sporozoites. So although the parasite is able to invade the epithelial cells, the damage is such that oocysts seen under the basal lamina will not produce sporozoites capable of infecting salivary glands of further mammalian hosts. Analysis of oocyst data alone indicates that, although there is a trend towards fewer oocycts being present in line EVida3 when compared to line E, these differences are not statistically different and thus conclusions cannot be drawn about decreased infectivity in the EVida3 strain. It does appear when analysing total numbers of invading parasites that there is certainly a knockdown effect from the Vida3 peptide, however, it is worth bearing in mind that only a single viable oocyst is required for a mosquito to become infective. It is possible that there is a parasite self-limiting factor which determines infection intensity in general on the host mosquito midgut. This would ensure that infections do not become so heavy as to endanger the life of the host, which would of course mean that the parasite could not be passed on to the mammalian host in order to continue its life cycle. If this is the case, it is possible that a second blood meal, at this time frame, would not increase numbers of oocyts present on the midgut, but would instead increase the sizes of the already present live oocyts. Further experiments would be necessary to look at oocyst size on midguts of mosquitoes fed twice in comparison to those only receiving a single blood meal.

At this stage it seems impossible to create real links between effects of a second blood meal and the effect on *Plasmodium* infection. This is partly because of several confounding factors which are as a result of the creation of the transgenic line in a melanising strain. The mosquito immune system is complicated, and the introduction of genes encoding novel AMPs only serves to make the system more complicated, increasing the number of unknown interactions. More work is necessary to ensure that links between

immune response, feeding and other parameters such as egg laying (fecundity) are mapped so it is possible to create accurate models for release.

3.4.1.7. Differences in fecundity between gonotrophic cycles in lines E and EVida3

Invasion of the midgut by the ookinete is associated with a massive immune response initiated in the midgut but also active systemically, which is thought to lead to apoptosis in ovaries at the same time, thus causing a possible reduction in reproductive fitness of the mosquito (Hurd *et al.*, 2005; Ahmed and Hurd, 2006). It is plausible, therefore, that a second blood feed taken by the mosquito within one gonotrophic cycle would increase the number of eggs that mosquito produced due to a further nutrient boost delivered by the blood meal. Briegel *et al.* (1993) have shown that a multiple blood feeding strategy in anophelines does indeed increase fecundity in non infected mosquitoes compared to those which receive only one blood meal. Extrapolating from this evidence leads to the possibility that were infected mosquitoes to engage in this multiple feeding strategy within a gonotrophic cycle, they too would also increase fecundity when compared to infected mosquitoes taking only a single blood meal per cycle. If resources were stretched due to the increased immune response directed at midgut-invading ookinetes, it is possible that subsequent blood meals could help provide these resources at the right time thus allowing for a reduction of apoptosis in the ovaries and increasing fecundity, that is if apoptosis here is linked to a reduction in nutrients. In the case of lines such as EVida3, the subsequent blood meals, if within the 16 hours post-infected feed when ookinetes are still present in the midgut, would provide a further wave of peptide production and may reduce the infection further. The timing of the second blood feed in this experiment meant that this was not a factor as the second feed was within the time frame (7 days) within which it

takes *P. yoelii nigeriensis* to traverse the epithelial cells of the midgut and develop to the oocyst stage, but it was not given to the mosquitoes within the initial gonotrophic cycle. It is harder, therefore to compare fecundity as an effect of the second blood meal as that would be a comparison between gonotrophic cycles. However, it is still possible to compare the effect of the second blood meal on fecundity between the transgenic lines and therefore ascertain an effect of the transgene on fecundity when mosquitoes are infected with *Plasmodium*. Incidentally, it has been documented that the first gonotrophic cycle of *An. gambiae* is not likely to be the most productive in terms of egg numbers per female. *An. gambiae* from Amani, Tanganyika (East Africa) reared at temperatures close to that maintained in our insectaries, were seen to have a peak egg production at 7 days post-eclosion which stayed at this level for several days before dropping off. This corresponded with the 3rd gonotrophic cycle (Armstrong and Bransby-Williams, 1961). Furthermore, *An. gambiae* from Suakoko (Liberia) showed a peak in mean egg production on the 4th gonotrophic cycle (Scholte *et al.*, 2006). These data indicate that fecundity is likely to increase with the second gonotrophic cycle of *An. gambiae* when compared to the first. For both transgenic lines investigated here, there is a significant difference in fecundity between the cohorts fed once and those fed twice ($p < 0.0001$ in both cases), with those fed twice showing the highest rates of fecundity (Fig. 3.5). This is in line with previous studies on wild type mosquitoes and indicates that infection with *Plasmodium* ookinetes within the midgut does not totally compromise egg production in either line. In some laboratories, mosquitoes receive only one feed which selects for those which produce most eggs during the first gonotrophic cycle. This would mean that comparisons with other studies could not be drawn. However, in our laboratory, mosquitoes are fed more than once in their life time, and as such, such selection pressures are not imposed. Ahmed & Hurd (2006) suggest that egg production (when infected with *Plasmodium*) during the first

gonotrophic cycle is compromised due to the immune response, instigated by invasion of the midgut epithelial cells, diverting resources away from oogenesis. It is likely that this phenomenon was observed in both lines in this experiment during the first gonotrophic cycle resulting in a lower egg production. The second blood feed was non-infective and consequently only provided resources and would not have initiated any further substantial up-regulation of the immune response in the mosquito host caused by introduction of further parasites. A review by Cirimotich *et al.* (2011) suggests that the antibacterial immune response of *An. gambiae* is at least partially responsible for immunity to *Plasmodium*. As such, it is possible that even a naïve blood feed as given to these cohorts of mosquitoes was enough to generate an immune response due to an increase in foreign bacteria present in the midgut. If this is indeed the case, then there would continue to be a possible diversion of nutrients from oogenesis and egg maturation toward an immune response and as such, a second feed would have no effect on egg development. It is perhaps worth noting that where a second blood feed was given, it was with horse blood treated so as to be free from growing bacteria. This would perhaps, provide fewer immune 'agonists' than would a blood feed from a mammalian host. It is worth noting at this stage, that the mosquitoes from all cohorts were maintained outside of blood feeding on sugar not containing antibiotics. This means that their natural gut bacteria would still be intact. It has been suggested that the natural bacterial load in the anopheline gut is associated with lower *Plasmodium* infections (Dong *et al.*, 2009). In this case, disturbing the natural load was not deemed useful, even if it would have increased the likelihood of infection with *Plasmoidium*. This was because a clear view, more representative of that which occurs naturally, was required, especially when analysing the effect of further blood meals. More knowledge of the interactions between natural gut bacteria, immune response in the mosquito and infection with parasitic diseases is necessary to ensure a full picture when

considering generating insects refactory to human disease (Cirimotich *et al.*, 2011). If the second feed did provide extra resources which were available to be used for egg development, rather than the immune response, the mosquito would be able to generate more eggs from this gonotrophic cycle at a level more inline with non-infected mosquitoes giving a more pronounced difference in egg production between cycles than is expected from non-infected wild type mosquitoes. To verify this, a similar study would have to be undertaken with non-infected mosquitoes as a control. A study by Hogg and Hurd (1995) however, indicates that infection with *Plasmodium* oocysts, which is the stage of the parasite present during the second gonotrophioc cycle, also results in a reduction in fecundity.

3.4.1.8. Differences in fecundity between lines E and EVida3

A comparison of the egg production between transgenic lines is quite interesting. There is a small, yet significant difference in egg production between line E and Evida3 (p = 0.0182) when both lines received only the initial infected blood meal with E producing more eggs than EVida3. Given the reduction in intensity of infection and parasite prevalence in the EVida3 line fed once, it seemed more likely that the immune response initiated by parasite entry into the midgut of these mosquitoes would be reduced compared to their line E counterparts. The expectation was that EVida3 would show higher fecundity than E when given only the initial challenge feed. Clearly this is not the case, and as the only difference between these two transgenic lines is the production of the Vida3 peptide itself (and its associated fluorescent marker) it seems likely that it is this which is behind the decreased fecundity of EVida3. It is feasible that Vida3 production is costly to the mosquito and resources ploughed into production of this peptide, as well as up-regulation of the immune response as the parasite enters the midgut, leave fewer available resources for egg production within the first gonotrophic cycle when *P. yoelii*

nigeriensis is present. There is no significant difference in egg production between line E and line EVida3 ($p = 0.7392$), both of which had received a second blood meal, and both of which produced more eggs than their cohorts which had only received the initial infected blood meal (Fig. 3.5). This suggests that production of a second wave of Vida3 peptide has no effect on egg production after the first gonotrophic cycle, certainly when mosquitoes do not receive a second challenge from a parasite. It is well documented that there are fitness costs associated with transgene insertion, and fecundity is often used as a parameter with which to measure fitness (Marrelli *et al.*, 2006; Amenya *et al.*, 2010). It has also been shown that some mosquitoes expressing genes which incur refractoriness to malaria parasites are 'fitter' than wild type mosquitoes when challenged with malaria parasites (Marrelli *et al.*, 2007). It is therefore necessary to determine fitness costs associated with the Vida3 peptide in the EVida3 transgenic line, not just in terms of fecundity, but in terms of several measurable parameters connected with evolutionary fitness of organisms including longevity as well as analysis of transgene frequency across several generations.

The interactions between the mosquito immune system and the parasite are complicated, and are made more so by having a novel antimicrobial peptide expressed which adds a further depth to the complex interactions seen in infected mosquitoes. Not only is there a possible complication added by the AMP, but the sheer presence of a transgene may exert fitness costs on the mosquito as well. Rono *et al*. (2011) demonstrate an interaction between immunity and reproduction and highlight the requirement for further exploration of the trade-off between the two processes. Their studies suggest that Lipophorin and Vitellogenin (both nutrient-transport proteins) are required, not only for oogenesis within the mosquito, but also for *Plasmodium* survival in the mosquito host. However, there is evidence to suggest an interaction with the antiparasitic factor TEP1. Knockdown of

Lipophorin and Vitellogenin by RNAi causes a reduction in the maturation of oocytes, as well as an increase in effieciency of TEP1 binding to the surface of *Plasmodium* ookinetes in the mosquito midgut. The authors therefore propose that Lipophorin is required for oogenesis and normal expression of Vitellogenin after an infectious blood meal. They also suggest that Vitellogenin also contributes to oogenesis as well as having a negative impact on TEP1 binding to ookinetes therefore indicating that the negative impact of lipophorin on ookinete survival is indirect (Rono *et al.*, 2011). The study also implicates NF-κB factors REL1 and REL2 as factors limiting Vitellogenin expression after an infectious blood meal (Rono *et al.*, 2011).

Clearly more studies are required before the complexities of the interactions between parasite and host are understood well enough to provide accurate models, should such transgenic lines be taken to the field.

3.4.2. Vida3 dose-dependent curve to assess levels of expressed Vida3 peptide in *An. gambiae* EVida3 transgenic line

Meredith *et al.* (2011) demonstrate that the transgenic line EVida3 shows midgut-specific expression of the synthetic Vida3 peptide which is able to significantly knockdown the mouse malaria *P. yoelii nigeriensis* in challenge studies. This evidence is corroborated further by the multiple feeding experiments detailed here. To date there is no data available on the quantity of Vida3 produced by this transgenic line upon ingestion of a blood meal. Thus an experiment was designed which would generate a dose-dependent curve by means of which a quantitative assessment of the amount of Vida3 produced could be achieved. Thus several different cohorts of the transgenic phase one line E (from which line EVida3 was generated) were fed mouse blood infected with *P. yoelii nigeriensis*. Each cohort had a different concentration of the Vida3 peptide mixed with the mouse

blood, ranging from 0μM to 200μM. A cohort of EVida3 was fed simultaneously on the same mouse blood mixed with a water control (Vida3 peptide at 0μM). The knockdown effect on the parasite by line E could then be charted with respect to the amount of Vida3 peptide fed. The dose-response curve generated could then be used to work out the amount of Vida3 produced by line EVida3 by determining the knockdown effect. This was to be repeated at least 3 times to ensure that results were comparable even with different murine/mosquito infection levels. The major consideration therefore, with this experiment compared to the previous challenge experiment was how to generate a statistically comparable infection in all 6 cohorts of mosquitoes. For this experiment to work, all mosquitoes had to be fed on the same blood in order that the curve generated was able to be used in the manner intended. As there were 6 different cohorts of mosquitoes to feed, it was impossible to use the blood from only one mouse as 0.5ml of blood was required per cage and a single mouse will only give between 1 and 1.5ml. Direct feeding from mice was not possible because Vida3 peptide had to be added to the blood (or water for proper controls) so feeding via a membrane was necessary. Due to this, and because several mice were required, it was decided to use three mice and pool blood from them. Aliquots of this pooled, and therefore homogeneous, blood were then taken and peptide, or water, added and the whole aliquot fed to the relevant cohort of mosquitoes. This ensured all mosquitoes were subjected to the same infection intensity which meant that groups would be comparable as knockdown effect of each group, in relation to the water control, was the measured parameter. The experiment was started on several separate occasions, and in each case the mosquitoes fed well from the membrane feeder. This was obvious not only from observations, but upon dissection where over 99% were found to be gravid. However at 7 days post-infection with *P. yoelii nigeriensis*, no oocysts were found on the midguts of the mosquitoes from any of the cohorts, and no evidence of melanisation was present.

Previous work by Arrighi *et al*. (2002) had shown that challenge of anophelines with murine malaria was possible when feeding AMPs with infected blood via a membrane feeder, so the challenge was then to determine a protocol which ensured that the mosquitoes in this dose-response curve were infected with *P. yoelii nigeriensis* thus allowing for reproducible data. In the previous experiment looking at the effect of multiple feeding, it was clear that mice with a parasitaemia of around 10% gave good infections. Anecdotal evidence had suggested that, when giving an infected feed via a membrane, a higher parasitaemia in the mice from which blood was obtained would be more likely to initiate better infection rates in the mosquitoes. As a consequence of this, initially the mice selected for this experiment had parasitaemias of around 20 to 25%. Smears of blood from each mouse showed exflagellation events though rates were not always measured. Smears of pooled blood were not looked at for exflagellation events as it was thought that it would take too long to observe this prior to using the blood and afterwards, with any remaining blood, would not give an accurate portrayal of the rate in the membrane feeders. After initial experiments did not result in mosquitoes with midgut infections, it was suggested that it would be perhaps more beneficial to use mice exhibiting a lower parasitaemia of around 5-8% as used for *P. berghei* infection studies (Arrighi *et al.*, 2002). This change to the protocol was not implemented as any infections previously achieved with anophelines in this laboratory with *P. yoelii nigeriensis* fed via membrane feeder have been attained with higher parasitaemias. As *P. berghei* and *P. yoelii* are different to handle in the laboratory, it was decided that other parameters would be altered as a matter of priority over parasitaemias. Deliberately for infection experiments, blood was not collected from mice directly infected from the cryovial as the infection can sometimes be erratic. This settles after passaging which gives a more stable parasitaemia which is useful when pooling blood from mice. It also means that the infection is more predictable thus making

it possible to gauge when it will be possible to start the experiment in terms of parasitaemia being at the right level at a suitable time. This has a consequence for mosquito husbandry which can influence the reproducibility of the experiment. Having a stable, predictable infection in mice means that age of mosquitoes can be tailored to maximise feeding potential, and will be comparable between replicates. Although the protocol used for infected membrane feeds for the 2002 Vida3 peptide study (Arrighi *et al.*) used blood from a mouse directly infected with *P. berghei* directly from the cryovial, this was not viable in terms of this experiment where reproducibility was paramount. Feeding mosquitoes of the same age each time and therefore having a predictable infection was key to this. Thus, after personal communication with Romanico Arrighi, it was decided that a change to the dose-dependent curve experimental protocol would be implemented whereby infected mice would not be considered for use once 4 passages had been attained. Infections on the midgut of mosquitoes were not observed after this change in protocol was implemented.

Infections were possible with the same mosquito-mouse-parasite system when feeding mosquitoes of the same lines directly on an infected mouse for the multiple feed experiments. This evidence, combined with sporadic infections having been achieved in the past when feeding via a membrane feeder suggested that the reason for the lack of infections observed in the dissected mosquitoes was not associated with the mouse infection. It therefore seemed more likely that the problem lay between collection, storage, pooling and transfer of blood. The initial step in collection of the blood is the cardiac puncture. This was performed by a skilled technician, taking around 1 minute per mouse. It was strongly believed that this step could not be optimised further. In the original protocol, once collected from the mouse, blood was transferred to a falcon tube kept in a 37°C water bath to maintain a constant temperature prior to delivery to the mosquitoes. Once blood was collected and transferred to a Falcon tube, addition of peptide (or water

control) and transfer to the membrane feeder was done as quickly and efficiently as possible. This was to reduce the likelihood of the parasites becoming less viable which is more probable the longer they are out of the natural host. No infections were seen in the mosquitoes when following this protocol so after personal communication with R. Arrighi, it was decided to make an alteration to the part of the protocol involving temporary storage of collected blood prior to addition of peptide and transfer to the membrane feeder. Instead of storing collected blood at 37°C, blood was stored at 4°C in a Falcon tube kept on ice. The microcentrifuge tubes containing the peptide solutions, and the water controls, were also stored on ice to maintain the lower temperature until the blood-peptide mix was transferred to the membrane feeder. The membrane feeder would then heat the mix back up to 37°C in order that the mosquitoes would feed. Despite the initial lower temperature, observations showed that mosquitoes were as quick to feed as they had been when the blood-peptide mix was maintained at 37°C. The small quantity of blood (50µl) transferred to the membrane feeder probably allowed the increase in temperature to take place very rapidly. Pooling and storage of infected mouse blood at 4°C prior to transfer to the membrane feeder followed the protocol used with similar infection studies undertaken previously with Vida3 and *P. berghei* (Arrighi *et al.*, 2002). It was thought that the lower storage temperature would slow down or halt any infection-related reactions which take place within the blood, and these would then be resumed once in the membrane feeder as temperature was brought back up to 37°C. It was thought more likely that this temperature decrease would therefore mimic more closely a direct feed from the mouse as it reduced the likelihood of aberrant reactions taking place in the blood outside of the host prior to feeding the mosquitoes which may have had an adverse effect on the parasite thus compromising the infection. This adapted protocol was used for three dose-dependent curve experiments, and in all of these, no parasites were detected on dissected mosquito

midguts. Running concurrently with these experiments, other groups were performing similar infection studies using *P. yoelii nigeriensis* to challenge *An. stephensi* mosquitoes. Upon dissection of these mosquitoes, they were also found not to be infected (V. Carter, *Pers. Comm.*).

3.4.3. Other methods for quantifying *in vivo* production of the Vida3 peptide

Meredith *et al*. (2011) conclusively demonstrated, by use of RT-PCR, that Vida3 is present exclusively in the midgut of the EVida3 transgenic line. Western blotting to further corroborate this data was not possible. Other laboratories have also struggled to obtain Western blots for midgut-expressed proteins (A. James & M. Jacobs-Lorena, *Pers. Comm.*) Moreira *et al.* (2002) did achieve it with the bee venom phospholipase PLA2, however this protein is bound to the midgut epithelial cells and not free in the lumen. There are of course a large number of total proteins present in the midgut when a blood meal is taken which may impede Western blot results. Presence of Vida3 peptide in the midgut of blood fed mosquitoes could have been shown by immuno-staining, using anti HA antibodies with a FITC/IgG conjugate. However, raising the Vida3-specific antibody would be expensive and time-consuming. Not only that, but the fluorescent conjugate would likely be larger than the Vida3 peptide itself. Nevertheless, Western blotting or immuno-staining would not have achieved a quantification of the Vida3 peptide present in the midgut which was the aim for the dose-dependent curve experiment. Using a Bradford's protein assay would allow for quantification of peptides and is both fast and accurate. However it is non-specific thus requiring the purification of Vida3 from the large amounts of proteins present in the blood meal bolus. This was not achievable in the time frame given. Quantifying the amount of Vida3 expressed by the EVida3 transgenic line has still not been possible. Knowing such information would be useful in terms of looking at resource requirements

for mosquitoes expressing novel peptide especially if such transgenic insects are to be trialled in the field.

Chapter Four

4. Fitness assessments of transgenic *Anopheles gambiae* expressing the Vida3 peptide

4.1. Introduction

It has already been demonstrated by several different laboratories that the creation of mosquitoes which are impaired in their capacity to transmit disease is possible (Ito *et al.*, 2002; Moreira *et al.*, 2002; Fu *et al.*, 2010; Isaacs *et al.*, 2011; Meredith *et al.*, 2011). Impaired vectorial capacity is achieved, either by insertion of an effector gene which hinders the pathogen's ability to survive within the insect host, or by insertion of a dominant lethal gene causing death of the mosquito prior to the pathogen completing its life cycle and reaching its infective stage. For transgenic technology to be a viable part of an integrated control strategy to reduce incidence of diseases such as Dengue, Yellow Fever (*Aedes spp*.) and Malaria (*Anopheles spp*.), then it relies on the genetically modified mosquito being able to successfully compete with wild type field mosquitoes for required resources. Genetically modified mosquitoes therefore have to exhibit minimal fitness costs associated with the transgene. Such imposed fitness costs would ultimately impair the released mosquitoes' ability to reproduce. Effective inheritance of the transgene by subsequent generations and its integration into field populations thus causing population replacement or suppression would lead to achieving the ultimate goal of disease control.

Creation of genetically modified mosquitoes by use of transposon-mediated transgenesis can impair fitness of the resulting transformed mosquito lines due to position effects, or as a direct negative effect caused by the gene products expressed in the transgenic line

(Marrelli *et al.*, 2006). When plasmids are microinjected into mosquito embryos it is necessary to be able to detect transformed individuals. It is usual to include a fluorescent marker gene as well as the effector gene in injected plasmids which means that the transgenic phenotype can be visualised in larval stages (Labbe *et al.*, 2010; Meredith *et al.*, 2011). Not only does this mean that the host genome must incorporate a much larger amount of DNA but it also means that the transgenic lines express the marker gene product as well as the intended effector gene product. Fluorescent marker genes are often expressed in a tissue-specific manner, for example linked to the eye-specific 3xP3 promoter. Even with simple promoter-marker combinations, there are reports of adverse fitness effects, in the laboratory, on the transgenic lines which have this DNA incorporated into their genome (Catteruccia *et al.*, 2003), however these reports are not widely reported in all generated lines (Moreira *et al.*, 2004). The mosquito lines transformed for the purpose of RIDL not only express fluorescent markers, but also a repressible transcription transactivator protein (tTA) which allows for the control of the whole system as explained in section 2.1 (Fu *et al.*, 2010). This protein can be ubiquitously expressed and as a consequence the mere presence and accumulation of this foreign protein may have a toxic effect on the cells. Indeed, this protein can itself be used as the effector in dominant lethal systems (Fu *et al.*, 2007; Phuc *et al.*, 2007). The RIDL system is designed to selectively kill mosquitoes of specific sex and life-stages by expression of toxic proteins, for example VP16 in the indirect flight muscles of females (Fu *et al.*, 2010). In the RIDL system, leaky expression of the VP16 protein (or indeed tTA protein) may result in males also suffering from effects of accumulation of these toxic products, which may manifest as a non-lethal phenotype, but with an overall detrimental effect on the reproductive fitness of the transgenic line. Indeed, evidence from Fu *et al.* (2010) shows that the alternative splicing pathway is not able to prevent tTA expression in some males. This may have implications

on the use of RIDL in the field as this requires transgenic males to mate with wild type females. If the male is unable to successfully compete with wild type males for mates then this technology becomes useless. Expression of antimicrobial peptides (AMPs), or peptides and/or proteins shown to be active against *Plasmodium* can be linked to tissue-specific promoters as these peptides are designed to target a particular life stage of the parasite for which the mosquito is the vector (Ito *et al.*, 2002; Moreira *et al.*, 2002; Meredith *et al.*, 2011). Contrary to the RIDL system, these peptides are not designed to be in any way detrimental to the mosquito itself however this does not automatically rule out any negative fitness effect. Such transgenic lines would be used in the field as part of a population replacement strategy. Although this would involve a males-only release, there is emphasis on both sexes being able to compete effectively for mates and being fit in terms of reproductive capability. This system is a little different to RIDL where much more emphasis is on reproductive fitness of males since females die due to the dominant lethal gene effect. Interestingly, Moreira *et al*. (2004) report a difference between two AMPs (both with their expression under the control of the carboxypeptidase promoter) in their effect on fitness of transgenic *An. stephensi*. This indicates that the choice of effector protein itself can have a considerable and possibly unpredictable effect on the fitness of transgenic lines. In a review of fitness costs imposed by transgenesis, Marelli *et al.* (2006) suggest that the reason for the decreased fitness in the PLA2 line (Moreira *et al.*, 2002) when compared to wild type is due to damage caused to midgut epithelial cells. This does not occur with the SM1 line (Ito *et al.*, 2002) which shows no difference in fitness when compared to wild type *An. stephensi*. This evidence reiterates that the difference in action of AMPs may have an effect on fitness.

Fitness costs can be imposed on transgenic lines, not only due to the presence of foreign proteins within host tissues, but also as a direct effect of the integration of DNA constructs

into the host genome. These position effects can cause insertional mutagenesis by disrupting native gene function in the transgenic line. A project (*Drosophila* Gene Disruption Project – GDP) has been developed which collects mutant strains of *Drosophila* created using the integration of transposons to disrupt gene function. Single transposon insertions associated with different genes allow for analysis of gene function (Bellen *et al.*, 2011). The *P* element was originally used in *Drosophila* to disrupt genes by targeting open reading frames (Spradling *et al.*, 1995). It is now known that the *P* element exhibits a strong tendency to integrate within promoter regions whereas it seems that *piggyBac* has evolved to, on the whole, avoid disruption due to transposition in potentially deleterious regions of host DNA (Bellen *et al.*, 2011). Both transposons, along with *Minos* tend not to transpose within *Polycomb*-regulated regions of DNA, but whereas *P* and *piggyBac* display preferences for certain areas outside the *Polycomb*-regulated regions, *Minos* is effectively random in its insertion sites (Bellen *et al.*, 2011). Most integrations of transposons do not cause any interruption to native genes as insertion occurs outside coding regions, so minimising fitness costs. However, disruption of a functional gene, for example one encoding an essential gene product, would incur severe fitness costs (Marrelli *et al.*, 2006). Insertional mutagenesis is not highly reported in mosquitoes, with data from published studies focusing on *Drosophil*a. This indicates that, in the case of most genes, the effect is largely recessive and therefore will only be seen in homozygotes as the wild type allele present in hemizygotes would be enough to rescue the deleterious genotype (Anholt *et al.*, 1996; Lyman *et al.*, 1996). In their review, Marrelli *et al.* (2006) conclude that insertional mutagenesis is unlikely to provide a transgenic line whereby homozygotes for the insertion have a fitness advantage over hemizygotes or wild type counterparts. However, in the creation of many lines, one can choose the line which exhibits a phenotype closest, in terms of fitness, to the wild type. Even so, when it comes to field releases, fitness costs

may have to be counteracted by release of the transgenic line in huge numbers, or by the addition of a gene drive mechanism which effectively gives the transgenic line a selective advantage (Marrelli *et al.*, 2006).

It is known that inbreeding can have a detrimental effect on fitness of any organism due to accumulation of deleterious alleles, though this is also dependent on parameters such as population size, growth rate and history (Theodorou and Couvet, 2006). Transgenic lines come from very few individuals, and often only a single mosquito, crossed to one or few wild type mosquitoes (depending on whether they are male or female), thus causing a genetic bottleneck and the fixation deleterious alleles which can impact on fitness of the line (Catteruccia *et al.*, 2003). A study comparing the effects of genetic modification and inbreeding on larval competition and adult energy reserves in *Ae. aegypti* indicates that the genetically modified strain fared the least well with limited larval food (Koenraadt *et al.*, 2010). The inbred strain also did worse under stress conditions, based on a composite index of survival, development rate and size, than the wild type strain thus demonstrating that inbreeding is likely to have a negative effect on fitness in mosquitoes (Koenraadt *et al.*, 2010).

Since transgenesis of mosquitoes has been established as a plausible method of control, several studies on fitness have been undertaken to help determine how transgenesis affects mosquito biology (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004; Amenya *et al.*, 2010; Koenraadt *et al.*, 2010; Bargielowski *et al.*, 2011). Irvin *et al.* (2004) and Catteruccia *et al.* (2003) assessed the fitness cost associated with insertion of plasmids containing fluorescent markers in homozygous *Ae. aegypti* and *An. stephensi* respectively. Catteruccia *et al.* (2003) assessed competition between transgenic and wild type strains of *An. stephensi*, assuming Hardy-Weinberg principles so that allele frequencies could be measured at each generation. Four independent transgenic lines possessing different

combinations of genes and markers and different insertion sites were assessed. A sharp decrease in transgene allele frequency over a few generations was observed in each line and the transgene became extinct in between 4 and 16 generations (Catteruccia *et al.*, 2003). Two lines created using the same construct showed a pattern of transgene persistence which was not significantly different from each other. This was particularly interesting as one insertion lay within the coding region of a gene similar to the *D. melanogaster* chaoptin precursor gene whereas the other insertion was outside of any open reading frames. The authors speculate that the gene product is non-essential in *An. stephensi* but disruption of the gene was likely to be selectively disadvantageous (Catteruccia *et al.*, 2003). The third line used within the study contained the same fluorescent marker as the lines mentioned above but within a different construct. Two repeats of the experiment using this line both showed an initial sharp drop in transgene frequency but eventual loss of the transgene allele was possibly due to random genetic drift (Catteruccia *et al.*, 2003). This analysis indicated that there was an excess of hemizygotes when experiments were undertaken with the fourth transgenic line containing a different marker gene to the previous three lines analysed. Again, eventual loss of the transgene allele was seen (Catteruccia *et al.*, 2003). Measuring fitness parameters such as longevity, feeding rate, larval development time and fecundity was not part of this study, however the authors do say that homozygous lines propagated for more than 40 generations showed no differences to the wild type when these parameters were assessed (Catteruccia *et al.*, 2003). Irvin *et al.* (2004) measured such parameters in their fitness study, looking at the effect of transgenesis on *Ae. aegypti* using three different transgenic lines. One line contained a simple EGFP marker cassette (termed EGFP), and the other two contained the marker along with a transposase gene encoding transposase for either the *Hermes* or *Mos1* transposable elements, termed autoHermes and pBacMOS respectively (Irvin *et al.*, 2004).

All transformed strains showed a significant reduction in fecundity and longevity when compared to the wild type strain. Interestingly autoHermes and *pBac*MOS strains showed the longest post-oviposition development times but the shortest pre-oviposition development time and fared the least well in terms of adult longevity. The EGFP strain was the least fecund of all strains (Irvin *et al.*, 2004). This indicates that transgenesis does not have a uniform effect across all fitness parameters, therefore a collective fitness assessment is a more accurate measure of fitness of transgenic lines. Across all parameters measured, Irvin *et al.* (2004) showed that transgenic strains fared significantly less well than wild type strain. However, they were keen to point out that all three strains were developed for use within the laboratory setting, and further work on those developed for possible release in the field would have to be assessed in terms of fitness separately and, depending on findings in the laboratory, in conditions more closely resembling those in the field (Irvin *et al.*, 2004).

Since these initial studies assessing fitness in strains of mosquitoes transformed purely for the laboratory, more studies have been undertaken which assess similar parameters in strains transformed for purposes of controlling disease (Moreira *et al.*, 2004; Li *et al.*, 2008; Amenya *et al.*, 2010; Koenraadt *et al.*, 2010; Bargielowski *et al.*, 2011). Moreira *et al.* (2004) set up cage experiments with equal numbers of (hemizygous) transgenic and wild type *An. stephensi* in order to determine transgene allele frequencies over 6 generations. The two separate transgenic lines they used express either SM1 or PLA2 into the midgut under the control of the carboxypeptidase promoter. Both of these peptides have been shown to inhibit *Plasmodium* development in these transgenic lines (Ito *et al.*, 2002 and Moreira *et al.*, 2002 respectively). It was expected, for both lines, that within the F_1 generation, the fluorescent phenotype would be at a frequency of 0.5 and all subsequent allele frequencies would follow the Hardy-Weinberg principle at each generation. No

consistent deviation from expected values was seen with the SM1 line. However deviation from expected frequency values was seen from the F_2 generation and all succeeding generations in the PLA2 line, with a sharp decrease in transgene allele frequency (Moreira *et al.*, 2004). Lifespan, fecundity and fertility were all measured and compared to a wild type control for each hemizygous transgenic line. No significant differences were observed with the SM1 line, but the PLA2 line showed a significant reduction in fecundity compared to the wild type, even when the decrease in blood meal consumption was taken into account (Moreira *et al.*, 2004). The authors suggested that differences in fitness observed may have been due to the difference in action of the two peptides, as SM1 is present in the midgut lumen whereas PLA2 attaches to epithelial cells and is known to cause damage (Moreira *et al.*, 2004). Transgenic *An. stephensi* lines expressing the SM1 peptide into the haemolymph, under control of the vitellogenin promoter, have also been the subject of fitness studies. Two of the lines carry a single insertion (VD35 and VD26) and one carries a double insertion of the transgene (VD9). Interestingly each line was tested in the homozygous state and all three lines exhibited some fitness costs, seemingly associated with carrying the transgene, and particularly in relation to fecundity, larval developmental time and reproductive longevity (Li *et al.*, 2008). Several other fitness parameters were also measured, such as adult survivorship, egg hatchability, larvae-to-pupae viability, and mating success. No significant differences between wild type and any of the transgenic lines was observed when measuring these parameters (Li *et al.*, 2008). In further studies, similar cage experiments to those undertaken by Moreira *et al.* (2004) were used to assess allele frequencies across 6 generations. For each transgenic line, transgene allele frequency was significantly lower by generation 6 than would be expected if the population were subject to Hardy-Weinberg principles (Li *et al.*, 2008).

Site-specific transgene integration has been reported as a stable and efficient way of introducing transgenes into a known site within the genome (Nimmo *et al.*, 2006) and can go some way to negating fitness effects caused by position effects and/or inbreeding depression and fixation of deleterious alleles associated with this (Amenya *et al.*, 2010). Seven fitness parameters were measured in four (hemizygous) transgenic lines of *An. stephensi* carrying a phiC31 *attP* docking site and fluorescent marker and results recorded for both males and females where possible. There were six instances where the transgenic lines significantly outperformed the wild type strain, but as was the case where the effects of transgenesis had significant negative effects on measured parameters, the results were not consistent across traits or lines (Amenya *et al.*, 2010). This lack of uniformity between fitness parameters means that it cannot be concluded that the transgene has an unequivocal impact on fitness and suggests that these lines are suitable for use as phase one docking strains for site-specific integration of effector genes (Amenya *et al.*, 2010). A transgene is thought to be stable within a population if it does not have the potential to move around the genome between generations. Further research is required to assess stability of the transgene within populations and to assess competitiveness of transgenic males compared to the wild type as these parameters seem to play a large role in fitness (Li *et al.*, 2008). Koenraadt *et al.* (2010) compared fitness of wild type, inbred and transgenic strains of *Aedes aegypti*. This was designed to test the principle that genetic modification has an effect on fitness due to the presence of the transgene itself, rather than inbreeding depression. The transgenic strain used in the study expressed a simple marker gene (EGFP) and was used in its homozygous state. Larval competition between strains and adult energy reserves were assessed, as well as several other parameters to create an overall index of performance (Koenraadt *et al.*, 2010). The transgenic line was found to fare least well in comparison to wild type for all measured parameters with most

differences exhibiting statistical significance. The transgenics also displayed lower survivorship and the longest development times of the three strains with the effect on development most pronounced at high-diet conditions but the effect on survival clearest when under more stress, i.e. low-diet therefore high competition (Koenraadt *et al.*, 2010). The authors suggest that there could be an interaction between parameters, therefore high competition for food resources increases larval mortality meaning fewer and more infrequent interactions between remaining larvae which in turn leads to larger and consequently more fecund adults and no eventual fitness cost (Koenraadt *et al.*, 2010). The index of performance found the wild type strain to have an overall competitive advantage at the larval stage and it exhibited overall increased fitness when compared to the inbred strain, which in turn was more fit than the transgenic strain. This suggests that the initial hypothesis was correct – genetic modification has an effect on fitness, and this effect is additional to that due to inbreeding alone (Koenraadt *et al.*, 2010). The competition element tested in this study is important as it mimics more closely the situation in the field. Bargielowski *et al.* (2011) report on the fitness of a RIDL strain of *Ae. aegypti* considered ready for field release. This strain (OX513A) contains a late-acting dominant lethal gene and a DsRed2 fluorescent marker gene and originated from the Rockefeller strain. It was subsequently outcrossed to a Mexican strain for 5 generations so that 97-99% of the genome corresponds to this strain. To minimise inbreeding effects, 44 independent homozygous females were mated with homozygous males and pooled to create the homozygous OX513A line as used for this study (Bargielowski *et al.*, 2011). Several parameters indicative of fitness were measured, each at different larval densities. A significant difference between wild type control and transgenic line was seen for all parameters measured but the effect density had on either the control or the transgenic line was not uniform across all measured parameters (Bargielowski *et al.*, 2011). Adult

longevity and wing length were the most greatly influenced by density, with the effect more pronounced in transgenic adults and males exhibiting the most severe density effect. The author comments that the shorter larval development time seen in the transgenic line is useful in terms of mass rearing, but speculates that this may be a cause of the smaller adult size observed in this line which may impact on fitness due to an effect on reproductive success (Bargielowski *et al.*, 2011). This is a major consideration in a line proposed for field release and indeed competition studies looking at male competitiveness compared to the wild type strain would go some way to addressing the issue. It is worth noting that the wild type strain used as the control for this study was not the Mexican strain, but the Rockefeller strain. The differences seen between the control and OX513A lines may therefore be, at least in part, due to genetic differences between the lines. However, it is reasonable to suggest that transgene products may be held to account for the apparent overall reduction in fitness observed in the transgenic line when compared to the wild type, with the build up of foreign gene products in mosquito cells having a toxic effect (Bargielowski *et al.*, 2011).

From various fitness studies undertaken, it is evident that loss of transgenic alleles within a laboratory population may be due to fixation of deleterious alleles during establishment of homozygous lines, or be directly associated with or insertion point within the genome or indeed with expression of the transgene itself. This points to site-specific integration as a way of combating this issue as it provides a method of assessing multiple phase one docking strains in terms of fitness and picking the one which more closely matches the relevant wild type strain. Even so, further assessment of fitness, once the effector gene was inserted, would have to be undertaken prior to release in the field to determine any effect caused by the effector gene itself. What is striking from looking at data generated from numerous fitness studies, is that the effect of genetic modification (and associated

inbreeding) on the fitness of mosquito lines is not consistent. This makes it even more important and relevant to ascertain fitness costs of any line which may have the potential to be used in the field as part of a control strategy.

The study undertaken here is designed to look at several fitness parameters in the transgenic *An. gambiae* line EVida3 (Meredith *et al.*, 2011). This is an important step in increasing our understanding of this line and its potential performance. Due to the known fitness costs of inbreeding, the study was undertaken using hemizygotes generated by outcrossing the homozygous line with the KIL laboratory strain. Hemizygotes carrying the Vida3 gene were generated via reciprocal crosses with the KIL line and the homozygous EVida3 line. This was so that a comparison could be made between those inheriting the Vida3 gene via the maternal route and those inheriting it via the paternal route. These hemizygous lines were named EV3M and EV3P, respectively, for the purposes of the fitness studies. KIL is the background strain for the transgenic line and was used as the wild type control for the fitness experiments.

4.2. Materials and Methods

4.2.1. Making EVida3 transgenic line homozygous using a combination of husbandry, screening and molecular methods

4.2.1.1. Enrichment of the EVida3 transgenic line

Once it was established that the EVida3 transgenic line was not homozygous, extra trays were put up for the line and were screened using the fluorescence microscope for the DsRed marker gene under control of the 3xP3 promoter. Larvae at the L_3/L_4 stages were screened and any negatives removed to ensure that any remaining larvae possessed at least one copy of the marker and consequently the Vida3 transgene. A new cage was set up for the screened pupae and once adults had emerged and were old enough to have mated they were offered a blood meal. This procedure was repeated for 5 generations so the population was enriched to ensure relatively high numbers of homozygotes.

4.2.1.2. Setting up small populations to generate homozygous lines

Small populations of 3 male and 3 female mosquitoes (1 day old) were set up in pots and given enough time to mate (around 3-5 days). Adult mosquitoes came from the main enriched population and were selected as larvae based on their perceived fluorescence intensity. All populations had access to sugar and water *ad libitum*. When satisfied that populations had mated, a blood meal was offered. Each population was then given the opportunity to lay in suitable oviposition vessels. Once the populations had laid, these larvae were reared as independent populations. After laying, parent mosquitoes were then knocked down and all 6 used to prepare genomic DNA for further downstream analysis. Any females that had not laid eggs were excluded.

4.2.1.3. Genomic preparations of each small potential homozygous line

Genomic DNA was prepared from each group of 6 adults which had laid eggs following removal of the heads using the Puregene Cell and Tissue Kit (Gentra Systems) following the protocol given, but with quantities of solutions adapted for use with multiple mosquitoes (See appendices for details). The genomic DNA was used for downstream applications to determine presence/absence of the Vida3 transgene.

4.2.1.4. PCR to determine phase one integration site

Two separate PCR reactions were undertaken for genomic DNA representative of each individual possible homozygous population. One universal forward primer (TD_UN-E_FWD 5` – CCATCCCCAAAAAAATGAACTGAAA – 3`) was paired with either a wild type-specific reverse primer (TD_WT-E_REV 5` – TCCCTCTTATAAGTAAGGGTTGC – 3`) or a transgene-specific reverse primer (TD_TG-E_REV 5` – GCAGACTATCTTTCTAGGGTTAAA – 3`). The universal forward primer anneals 140bp upstream of the insertion point and when paired with the wild-type-specific primer, which anneals 10bp upstream of the insertion point, produces a fragment, in wild-type KIL, of 172bp in length. When paired with the phase one-specific primer, which itself anneals to the left ITR of the *piggyBac* transposon and spans the insertion site, it produces a fragment, in transgenic mosquitoes, of 166bp in length. The PCR reactions were run simultaneously due to the similar lengths of the amplicons generated. For each reaction, 200-400ng of genomic template was amplified with the relevant primers (2µM) using the Promega *GoTaq* system containing 1x buffer and 1.5µM $MgCl_2$. Cycling parameters (MJ Research PTC-200) were 95°C for 3 minutes then 30 cycles (95°C, 15 seconds; 55°C, 30 seconds; 72°C, 30seconds) followed by a final

extension step of 72°C for 10 minutes. PCR products were identified using a 1.8% TAE agarose gel with HyperLadder IV (Bioline).

4.2.1.5. PCR to determine integrity of *attP* recognition sequence

Genomic templates from all possible homozygous lines were tested for presence of *attP*, and thus an unoccupied target site. This was identified using the primers attR-F-new and attL-F-new in reactions containing 200-400ng of genomic template in the presence of 1 x buffer (Promega), 1.5µM $MgCl_2$ (Promega) and *GoTaq* DNA polymerase (Promega). Existence of the unoccupied target site resulted in a DNA fragment, 391bp in length. Cycling parameters (MJ Research PTC-200) were 94°C for 1 minute then 30 cycles (94°C, 30 seconds; 58°C, 30 seconds; 72°C, 30 seconds) followed by a final extension step of 72°C for 10 minutes. PCR products were analysed using a 1.8% TAE agarose gel with HyperLadder IV (Bioline)

4.2.1.6. PCR to confirm presence of an occupied site by amplification of *attL* and *attR*

Genomic DNA indicative, by means of previous PCR analysis, of originating from homozygous populations were used as templates for PCR analysis to confirm the presence of occupied sites. The primers attL-F-new-2 and attR-F-new-2 were used to amplify *attL* and the primer pair, attR-forward and attR-reverse, were used to amplify *attR*. 200-400ng of genomic DNA template was amplified with the relevant primer pair using *GoTaq* DNA polymerase (Promega) in the presence of 1 x buffer and 1.5µM $MgCl_2$. Presence of *attL* and *attR* sites resulted in expected DNA fragments of 301bp and 224bp in length, respectively. Cycling parameters (MJ Research PTC-200) were 94°C for 1 minute then 30

cycles (94°C, 30 seconds; 58°C, 30 seconds; 72°C, 30 seconds) followed by a final extension step of 72°C for 10 minutes. PCR products were analysed using a 1.8% TAE agarose gel with HyperLadder IV (Bioline).

4.2.1.7. Building up the homozygous EVida3 line

Once the PCR assessment of genomic DNA had been completed, it was then apparent which of the original small populations contained only males and females homozygous for the Vida3 transgene. No wild-type PCR products were amplified and no *attP* (unoccupied site) amplification was detected whereas *attL* and *attR* were indeed present within this population. Thus, further care was taken to maintain only this population. Progeny from the adults from the original cross were maintained as larvae in suitable containers until pupation when they were transferred, in weigh boats, to a small cage and given approximately 5 days to emerge and mate. A blood feed was then offered and 48 hours later a suitable oviposition site was placed in the cage. Once a further generation had been laid, the population was again offered a blood meal. This was repeated a total of four times as after this few adult mosquitoes remained alive. The G_1 larvae produced as a result of these blood meals were maintained in suitable containers, again until pupation, when they were transferred to a further small cage and allowed to emerge and mate. This generation were then offered multiple opportunities for blood-feeding and consequently, egg-laying. This process was repeated until a robust colony of standard size was built up.

4.2.2. Larval development rate and survivorship

4.2.2.1. Creating comparable homozygous KIL and hemizygous Evida3 lines

Trays of KIL and EVida3 lines were set up with equal numbers of larvae and reared in the standard manner until pupation occurred. Pupae were picked and sexed from both wild-type KIL and homozygous EVida3 lines. 250 female KIL pupae were placed into each of two large cages. Into one of these cages 150 male KIL pupae were also placed, and into the other cage, 150 male EVida3 pupae were placed. A third cage was set up with 250 female EVida3 pupae and 150 male KIL pupae. This ensured that all homozygous and hemizygous progeny resulting from these crosses would be comparable. Pupae placed in all cages were allowed to emerge and pupal pots were removed from cages. Adults were maintained on water and 10% glucose solution available *ad infinitum*. Sugar was removed from cages approximately 18-24 hours prior to a blood feed. A blood meal was offered to all cages when adult mosquitoes were approximately 4-7 days old by means of a Hemotek feeding system following standard protocol. Oviposition pots, all of the same size and containing the same water volume, were placed into the three cages 2 days post blood-meal. Larvae produced as a result of this feed were used for the larval development rate and survivorship study. Comparisons were to be made using KIL against hemizygous EVida3 lines from reciprocal crosses named EV3P and EV3M. This was to ascertain any differences between hemizygous progeny with a paternally-inherited transgene and those with a transgene inherited maternally.

4.2.2.2. Randomised experiment for determining larval development rate and survivorship across three different lines

Exactly 30 L_1 stage larvae were counted out into ice-cream tubs (18.5cm length x 10cm width x 4.5cm height) and 150ml of distilled water containing liquifry at a concentration of 3 drops per litre added. For each line created, 15 repeats were set up. Tubs were placed on trays so one tub of each larval-type was present per tray, and these trays were placed in the same section of the insectary in order that each line had equal numbers of repeats per row and column so as to minimise placement effects. All repeats were fed exactly the same quantity of fine fish flake at the same time each day. Water levels were kept constant (150ml) by addition of more ddH_2O when necessary due to effects of evaporation within the insectary.

4.2.2.3. Daily larval counts and sexing of pupae

Larvae in all tubs were counted daily and counts recorded. This was done meticulously and any dead larvae visible in the tubs were removed. Once larvae began pupating, pupae were picked from tubs, sexed and numbers of males and females recorded for each tub and therefore each line. Pupae from tubs were amalgamated per day within their lines and placed in cages whilst keeping sexes separated. Pupal mortality was recorded by counting the number of dead pupae or those which did not give rise to fully emerged adults. Once emerged, adults from each sex were transferred to another cage to minimise the chances of them drowning in the pupal pots. The experiment finished when all mosquitoes had emerged as adults.

4.2.3. Adult Longevity

4.2.3.1. Creating comparable homozygous KIL and hemizygous Evida3 lines

Lines were created following the same protocol as specified in section 4.2.2.1.

4.2.3.2. Standardised rearing of mosquitoes to generate adults of equivalent age and size

Larvae at L_1 stage were counted into trays at a density of 0.2 larvae per ml (standard density). This density was upheld for each tray used and for each line used for the experiment. Distilled water containing 3 drops of liquifry per litre was added to the trays to create the correct rearing density. A strict feeding regime was followed in order that all larval trays received the same amount of food per larva per tray. Trays were fed morning and afternoon to encourage synchronous pupation. This standardised procedure ensured that all adult mosquitoes used in the longevity study would be comparable.

4.2.3.3. Set-up of adult longevity study

Pupae were picked from standardised trays, sexed and placed in weigh boats within small cages until emergence. Once emerged, adults were removed from these cages and placed in clear plastic pots (10.5cm height x 11cm diameter) with access, *ad libitum*, to a sugar cube and distilled water. Ten adult males of the same age were placed in each of ten pots per line, and the same was set up for emerged female mosquitoes. Pots were randomly distributed in the insectary. Each day sugar and water levels were maintained, and dead mosquitoes removed and counted/recorded for each pot within each line. The experiment reached the end when all mosquitoes in all pots were dead.

4.3. Results

4.3.1. Larval mortality

Larval death was recorded for each mosquito line each day until every larva had either pupated or died. Fifteen replicates were set up per line, each containing an initial 30 larvae so larval death was recorded as number of deaths per replicate and an average taken over fifteen replicates. Three independent experiments were undertaken. Results were slightly variable between the three experiments though statistical analysis showed no statistical difference between lines within any experiment. The control line (KIL) exhibited the most variation between experiments with the median larval mortality ranging from 1 larval death to 4 per 30 larvae. Mean larval mortality of KIL, EV3P and EV3M mosquito lines was 2.69 (9%), 0.96 (3.2%) and 1.67 (5.6%) respectively (Table 4.1) when using pooled data for analysis. Data were tested using the D'Agostino Pearson omnibus normality test and found to be non-normally distributed, thus further analysis to determine differences between lines was performed using a Kruskal-Wallis test followed by Dunns multiple comparison post test. There was no significant difference between lines for larval mortality ($p = 0.368$ for pooled data, Table 4.2) as illustrated by Fig. 4.1A-D.

4.3.2. Sex ratio

As with larval death, 15 replicates per mosquito line were set up, each containing an initial 30 larvae. Larval rearing was standardised across all replicates and between each of the three independent experiments. The sex of all larvae surviving to pupation was recorded and ratios determined from this data for each experiment as illustrated by Fig. 4.2A-D. Ratios from pooled data for KIL, EV3P and EV3M lines are 51.3♂:48.7♀, 49.5♂:50.5♀ and 50.2♂:49.8♀ respectively (Table 4.1) as illustrated by Fig. 4.2D. Data was analysed

using a chi-squared contingency test to determine if the mosquito line had an effect on sex ratios (Table 4.3). There was found to be no significant difference in sex ratio between lines (p = 0.673 for pooled data, Table 4.2). A very slight male bias was universal across all experiments in the KIL line. In two of three experiments, EV3M line also exhibited a slight male bias. However, in two of three experiments EV3P displayed a slight bias towards females. In both cases it was experiment 3 which showed the small anomalies in bias.

4.3.3. Age at pupation

For 3 independent experiments, 15 replicates per line, containing 30 larvae in each, were set up with larvae reared uniformly across all replicates, lines and experiments. Mosquito age at pupation was recorded for every mosquito reaching this life stage. Age at pupation was recorded separately for male and female mosquitoes. The mean age at pupation of male KIL, EV3P and EV3M lines is 7.66, 7.59 and 8.32 days respectively (Table 4.1). Mean age at pupation of female KIL and EV3M lines is 7.76 days, and for females of the EV3P line is 7.72 days (Table 4.1).

Analysis of male and female data using the D'Agostino Pearson omnibus normality test showed that both sets of data were non-normally distributed. Subsequent analysis was therefore undertaken using the Kruskal-Wallis test followed by Dunns multiple comparison test to further determine differences between lines. When considering data pooled from all three independent experiments, there was found to be no significant difference between lines for age at pupation of females (p = 0.367; Table 4.2) as illustrated by Fig. 4.3D1. All three experiments indicate independently that there is no significant difference in age at pupation of females between the KIL and EV3P lines (Figs. 4.3A1, B1 and C1). However, when looking at these 3 experiments and making comparisons between the two remaining

combinations of lines (KIL vs. EV3M and EV3P vs. EV3M), it appears that there are significant differences between them, of varying levels, even though pooling the data results in seeing no significant difference. This is because the differences are not uniform over all three experiments. This is illustrated in Figs. 2.3A1, B1 and C1.

When considering the pooled data for male age at pupation, statistical analyses shows a significant difference in age when comparing males belonging to the EV3M line with both the control (KIL) line and the other hemizygous transgenic (EV3P) line ($p < 0.0001$ in both cases; Table 4.2). This is illustrated by Fig. 4.3D2. These differences are a true reflection of all three independent experiments which show the exact same significance levels for each pair of mosquito lines as shown in Figs. 4.2A2, B2 and C2.

4.3.4. Wing length

Mosquitoes involved in the wing length study were reared following a strictly uniform regime to ensure that any differences observed were due to genetic differences and not due to environmental differences. Wing lengths were measured and recorded for both males and females from each of the three lines involved in the fitness studies. The average wing length of male KIL, EV3P and EV3M mosquito lines are 2.95, 2.96 and 2.95mm respectively (Table 4.1). Male wing lengths were shown to follow a normal distribution as indicated by the D'Agostino Pearson omnibus normality test. Consequently, data was analysed by ANOVA followed by Tukey's post test to compare pairs of data. Male wing lengths were found to show no significant difference between lines ($p = 0.590$; Table 4.2) as illustrated by Fig. 4.4A. The average wing length of female KIL, EV3P and EV3M mosquitoes are 3.11, 3.16 and 3.16mm respectively (Table 4.1). As indicated by the D'Agostino Pearson omnibus normality test, female wing lengths were shown to be non-normally distributed. Consequently, data was analysed by a Kruskal-Wallis test followed

by Dunns post test to compare pairs of data. Female wing lengths were found to show no significant difference between lines ($p = 0.112$; Table 4.2) as illustrated by Fig. 4.4B.

4.3.5. Longevity

Longevity was recorded for 100 male and 100 female mosquitoes per line. These were split into 10 replicates each containing 10 newly eclosed adult male or female mosquitoes making a total of 200 mosquitoes per line for each of 3 independent experiments. Every day, each replicate was checked for mosquito mortality in order that adult longevity, when maintained on sugar/water only, for each line could be mapped. This continued until all mosquitoes were dead. Mean longevity of male mosquitoes for the KIL, EV3P and EV3M lines is 29.5, 33.16 and 26.24 days respectively (Table 4.1). For female mosquitoes, average longevity is 28.57, 34.32 and 30.37 days for the KIL, EV3P and EV3M lines respectively (Table 4.1). Data was analysed using the log-rank (Mantel-Cox) test in order to determine significant differences in longevity between lines in both males and females. Analysis of pooled data for both female and male mosquitoes indicated a highly significant difference in longevity when comparing all the lines (Table 4.2) as illustrated in Figs. 4.5A and 4.5E respectively. Data was then analysed in pairs of lines to determine where the major differences in longevity lay. When considering longevity in females, there was no significant difference observed between the control (KIL) line and the EV3M line ($p = 0.462$; Table 4.2) and is illustrated by Fig. 4.5C. However, highly significant differences were observed in female longevity between the control (KIL) line and the EV3P line ($p < 0.0001$; Table 4.2) and between the two hemizygous lines ($p < 0.0001$; Table 4.2). This is shown by Figs. 4.5B and 4.5D respectively. When considering longevity in males, all pairs of mosquito lines exhibit significant differences between each other albeit to varying degrees. Comparison of the control (KIL) line with the EV3P line and with the EV3M line

indicates significant differences in longevity between these pairs of lines ($p = 0.004$ and $p = 0.001$ respectively; Table 4.2) which is illustrated by Figs. 4.5F and 4.5G respectively. Comparison of the two hemizygous transgenic lines also points to a difference in longevity at a higher significance level ($p < 0.0001$; Table 4.2) which can also be seen in Fig. 4.5H. Data from the three independent experiments can be seen in Appendix III.

Longevity of males in comparison to females were also analysed using pooled data from 3 experiments (Fig. 4.6). Longevity of sexes was not significantly different for the control (KIL) line (Fig. 4.6A) or for the hemizygous transgenic line EV3P (Fig. 4.6B). A significant difference in longevity was observed between males and females from the hemizygous EV3M line (Fig. 4.6C) whereby females were longer lived than males ($p = 0.0023$).

Fitness Parameter	KIL		EV3P		EV3M	
Mean larval mortality[a]	2.69 ± 0.284 (9.0%)		0.96 ± 0.159 (3.2%)		1.67 ± 0.185 (5.6%)	
Mean sex ratios (male: female)	51.3**:** 48.7 (n = 1229)		49.5**:** 50.5 (n = 1307)		50.2**:** 49.8 (n = 1275)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Mean age at pupation[b] (days)	7.66 ± 0.032	7.76 ± 0.036	7.59 ± 0.027	7.72 ± 0.028	8.32 ± 0.043	7.76 ± 0.033
Mean wing length[b] (mm)	2.95 ± 0.016	3.11 ± 0.018	2.96 ± 0.015	3.16 ± 0.016	2.95 ± 0.021	3.16 ± 0.022
Mean adult longevity[c] (days)	29.5 ± 0.830	28.57 ± 0.754	33.16 ± 0.740	34.32 ± 0.793	26.24 ± 0.755	30.37 ± 0.654

Table 4.1: Comparison of mean fitness parameters between the control line and hemizygous transgenic lines for pooled data from 3 independent experiments.

Mean values ± SEM is given where applicable.

[a]Larval mortality is given as the mean number of larvae dying before reaching pupation where n = 30 per replicate, of which there were 15 per line, within one experiment. Percentage mortality is shown in brackets.

[b]Data generated where n = 30 as above.

[c]Data generated where n = 10 per replicate, of which there were 10 per line, within one experiment.

All data was tested to see if it followed a normal distribution and following this, the appropriate statistical analyses were performed.

Fitness Parameter	Probability (significance)		KIL (control) vs EV3P		KIL (control) vs EV3M		EV3P vs EV3M	
Mean larval mortality[a]	$p = 0.368$ (ns)		ns		ns		ns	
Mean sex ratios (male: female)[b]	$p = 0.673$ (ns)							
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Mean age at pupation[a] **(days)**	$p < 0.0001$ (***)	$p = 0.367$ (ns)	ns	ns	$p < 0.0001$ (***)	ns	$p < 0.0001$ (***)	ns
Mean wing length (mm) male[c]**; female**[a]	$p = 0.590$ (ns)	$p = 0.112$ (ns)	ns	ns	ns	ns	ns	ns
Mean adult longevity[d] **(days)**	$p < 0.0001$ (***)	$p < 0.0001$ (***)	$p = 0.004$ (*)	$p < 0.0001$ (***)	$p = 0.001$ (*)	$p = 0.462$ (ns)	$p < 0.0001$ (***)	$p < 0.0001$ (***)

Table 4.2 (opposing page): Statistical differences between control (KIL) and transgenic lines for five fitness parameters from pooled data from 3 independent experiments

Where appropriate, data for each fitness parameter was tested using the D'Agostino & Pearson omnibus normality test and subsequent statistical analyses were carried out accordingly (ns denotes *p* values which are not significant; ***, ** and * denote *p* values which are statistically significant at the <0.0001, <0.001 and <0.05 levels respectively).

[a]Non-normally distributed data was analysed using a Kruskal-Wallis test followed by a Dunns multiple comparison post test. Where Dunns post test indicated significance between lines, a Mann-Whitney test was used to compare pairs of lines to get more exact significance levels.

[b]Data was analysed using a chi-squared contingency test.

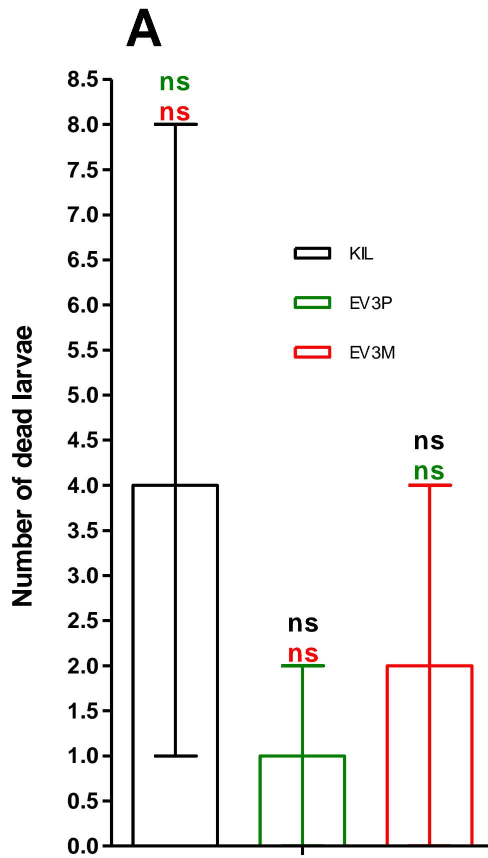
[c]Data following a normal distribution was analysed using ANOVA followed by Tukeys multiple comparison post test.

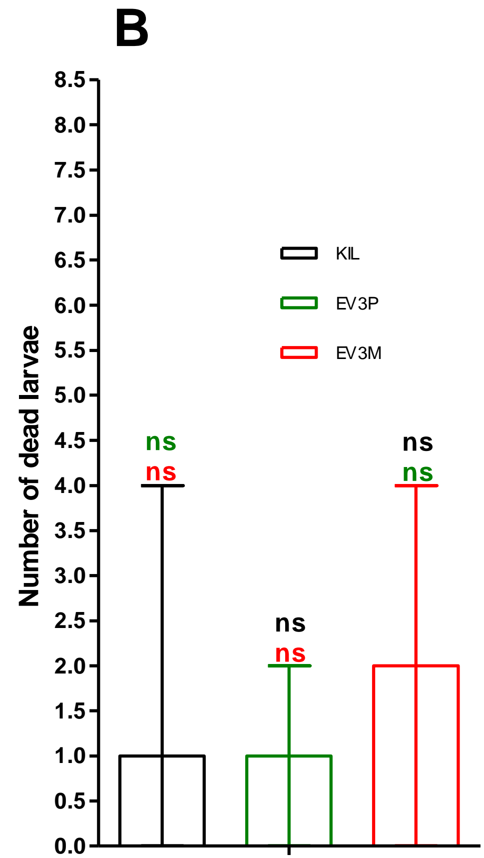
[d]Kaplan-Meier survival curves were analysed using the log-rank (Mantel-Cox) test.

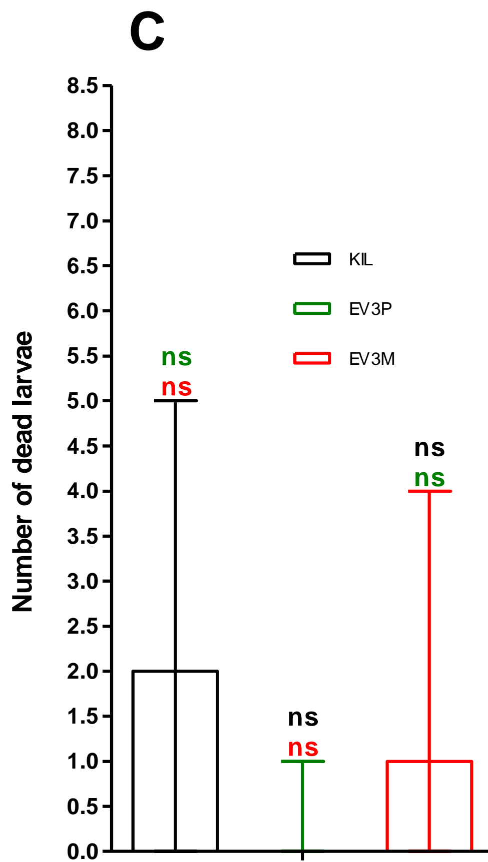
	Male	**Female**	**Totals**
KIL	630	599	**1229**
EV3P	647	660	**1307**
EV3M	640	635	**1275**
Totals	**1917**	**1894**	**3821**

Table 4.3: Contingency table showing numbers of male and female pupae for control and transgenic lines from pooled data from 3 independent experiments

Data was taken from each of 3 independent experiments. For each experiment, n = 30 larvae for each of 15 replicates per line. Larval mortality meant that less than 450 larvae per line for each experiment reached pupation but all which did were included in the data. Chi-squared analysis was performed on the pooled data (2 degrees of freedom) to determine if the sex bias was different between lines. $\chi^2 = 0.792$; $p = 0.673$ therefore there is no difference in sex bias between the three lines.


A
Number of dead larvae
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
KIL
EV3P
EV3M
ns
ns
ns
ns
ns
ns


B
Number of dead larvae
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
KIL
EV3P
EV3M
ns
ns
ns
ns
ns
ns


C
Number of dead larvae
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
KIL
EV3P
EV3M
ns
ns
ns
ns
ns
ns

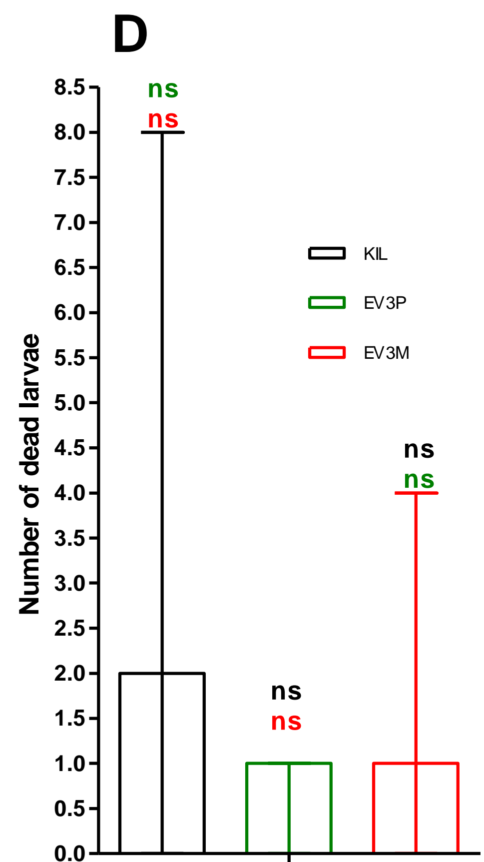

D
Number of dead larvae
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
KIL
EV3P
EV3M
ns
ns
ns
ns
ns
ns

Figure 4.1 (opposing page): Median larval death per replicate where 'n' = 30 for each replicate for the KIL control line and two hemizygous transgenic lines

A) Data from experiment 1; **B**) Data from experiment 2; **C**) Data from experiment 3; **D**) Data pooled from 3 independent experiments

Each replicate initially contained 30 larvae; 15 replicates were included in each of three independent experiments. Larval death was counted when any larva did not achieve pupation due to mortality.

Data were not normally distributed so all bars are representative of the median with the interquartile range indicated above and below the median.

Statistical significance is shown above bars in the colour of the bar to which the significance level corresponds (ns, not significant).

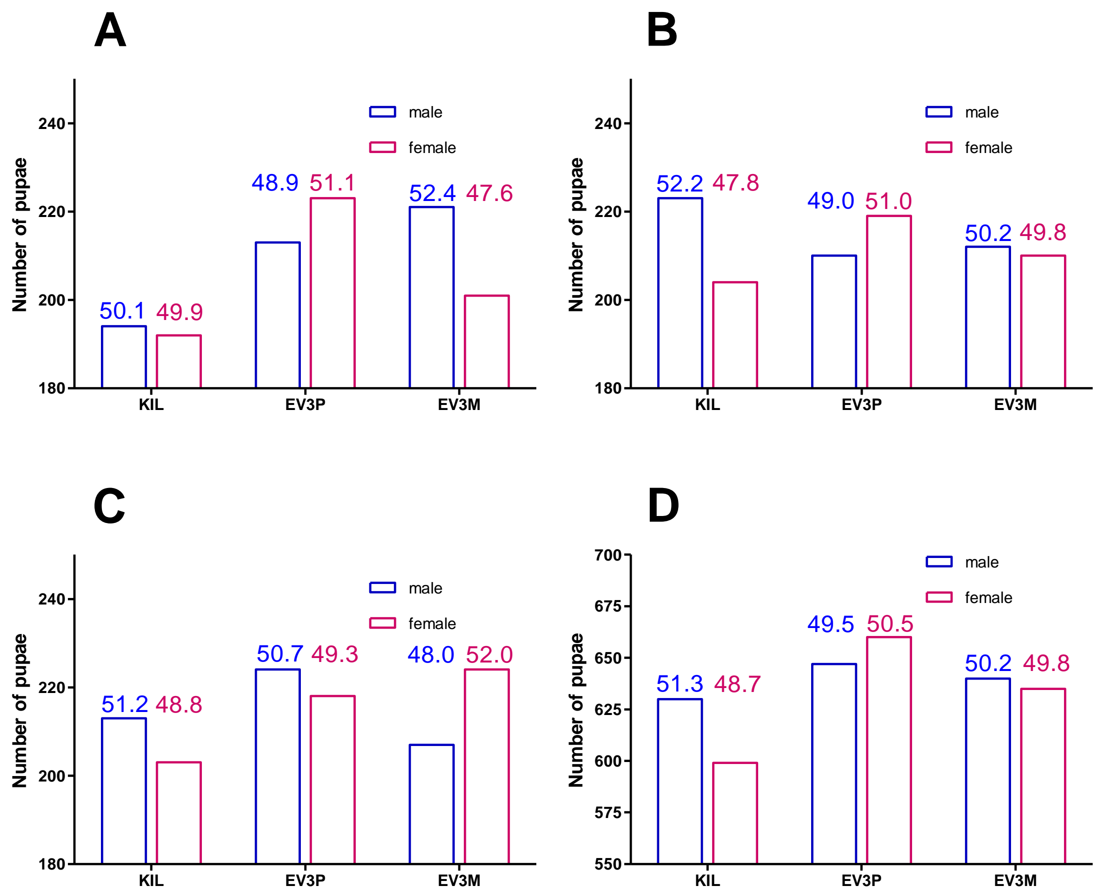


Figure 4.2. Sex ratios of pupae in the control line KIL and two hemizygous transgenic lines

A) Pupal sex ratios from experiment 1; **B**) Pupal sex ratios from experiment 2; **C**) Pupal sex ratios from experiment 3; **D**) Pooled pupal sex ratios from 3 independent experiments. Chi squared analysis shows no significant difference in sex ratios between lines for pooled data (p = 0.673). The number of males and females expressed as a percentage of the total number of pupae within lines is shown above bars.

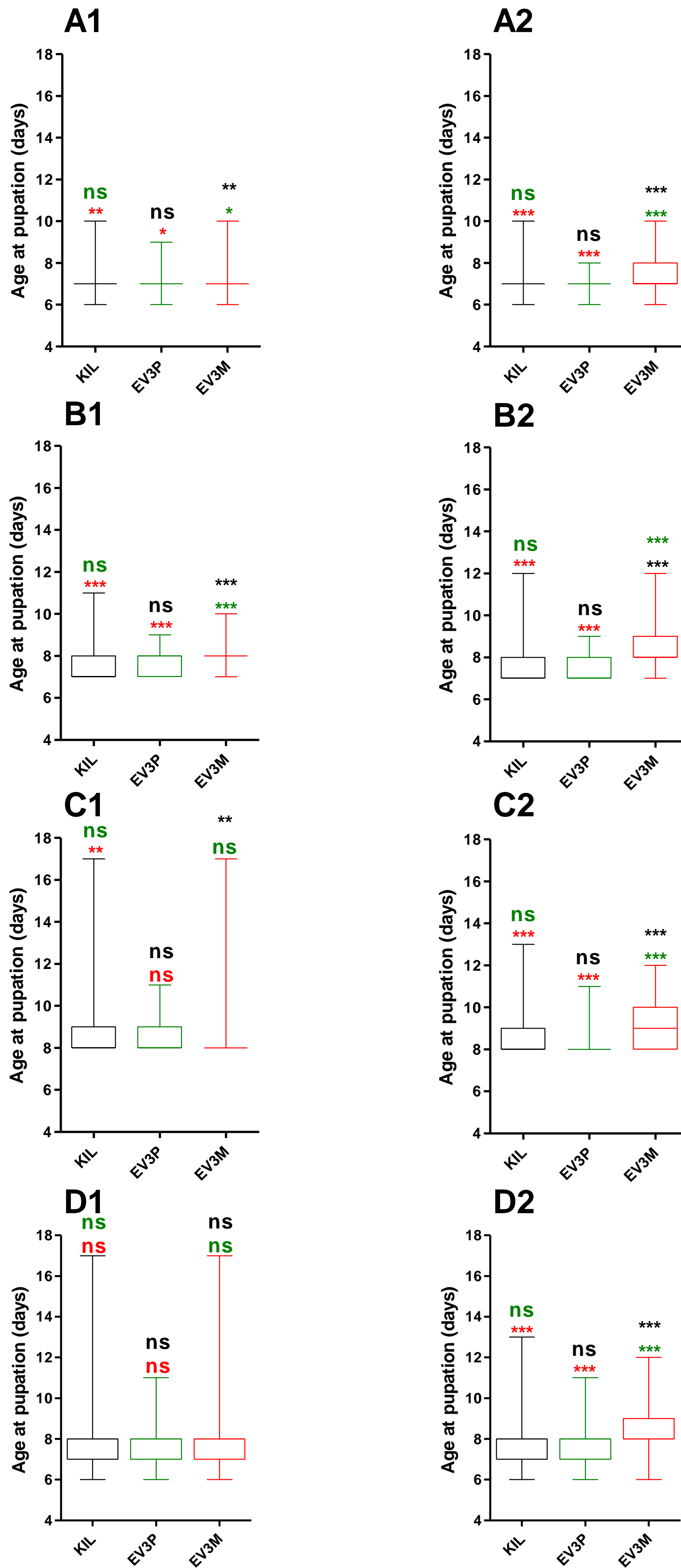
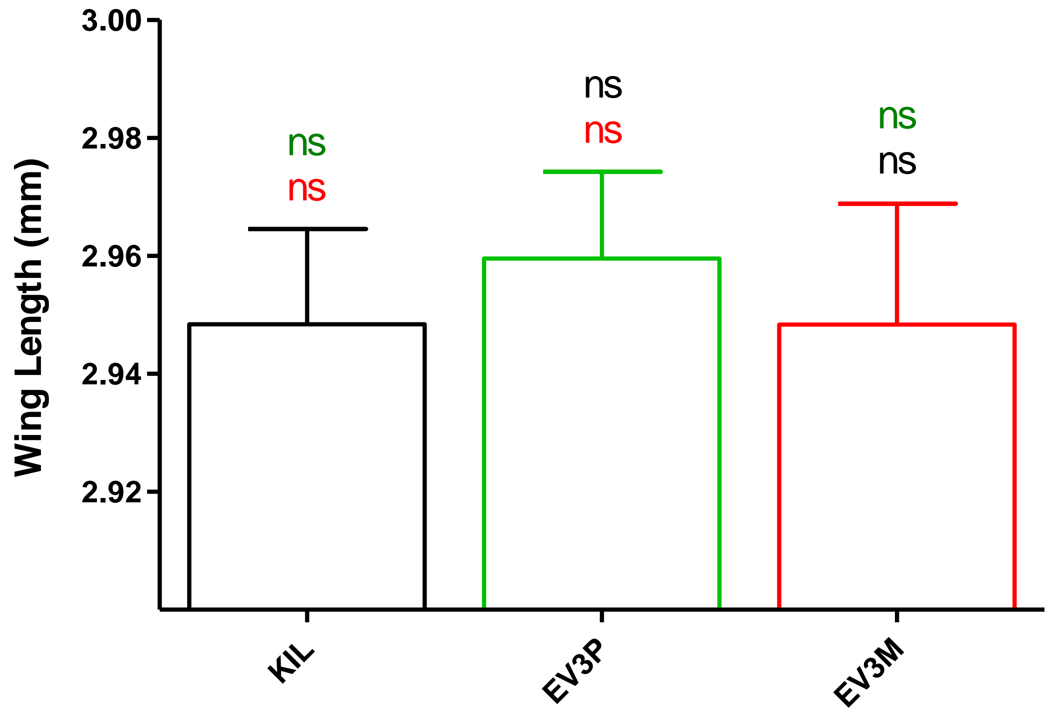

Female
Male
A1
A2
B1
B2
C1
C2
D1
D2
Age at pupation (days)
KIL
EV3P
EV3M
ns

Figure 4.3 (opposing page): Comparison of age at pupation between the KIL control line and two transgenic lines

A1) Age at pupation for female pupae from experiment 1; **A2**) Age at pupation for male pupae from experiment 1; **B1**) Age at pupation for female pupae from experiment 2; **B2**) Age at pupation for male pupae from experiment 2; **C1**) Age at pupation for female pupae from experiment 3; **C2**) Age at pupation for male pupae from experiment 3; **D1**) Age at pupation for female pupae using pooled data from experiments 1-3; **D2**) Age at pupation for male pupae using pooled data from experiments 1-3

All data was non-normally distributed with boxplots showing a line at the median. Where applicable, the interquartile range is boxed and the maximum and minimum values are indicated.

Statistical significance is shown above bars in the colour of the bar to which the significance level corresponds (***, $p < 0.0001$; **, $p < 0.001$; $p < 0.05$; ns, not significant).


Male
3.00
2.98
2.96
2.94
2.92
Wing Length (mm)
ns
ns
ns
ns
ns
ns
KIL
EV3P
EV3M

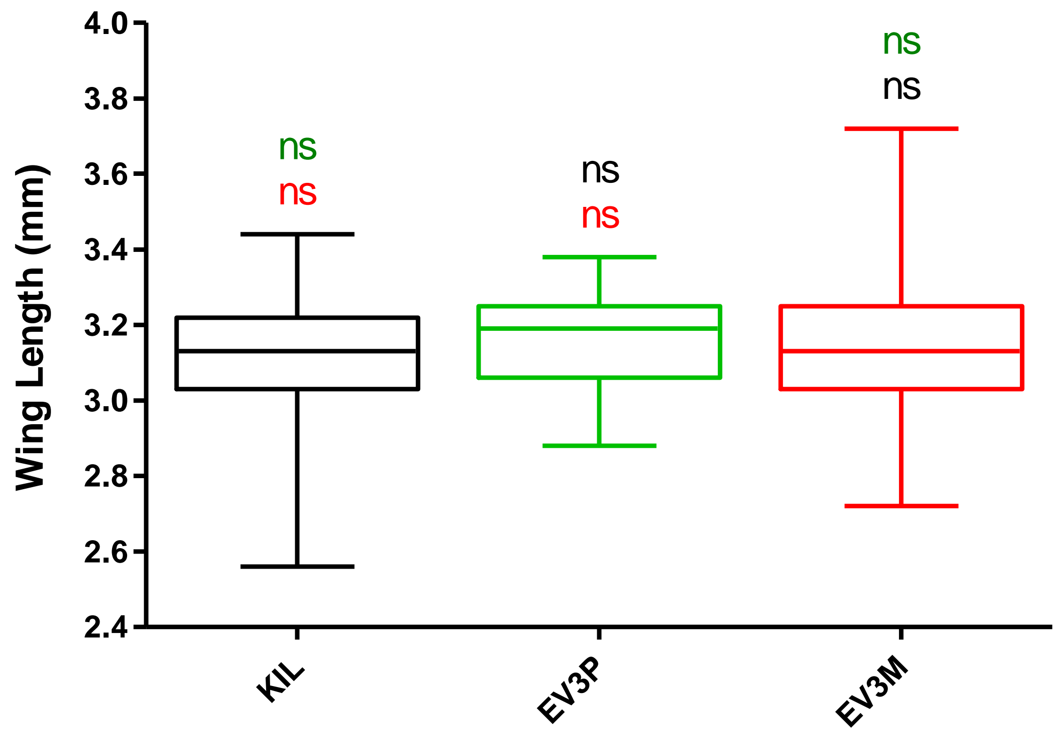
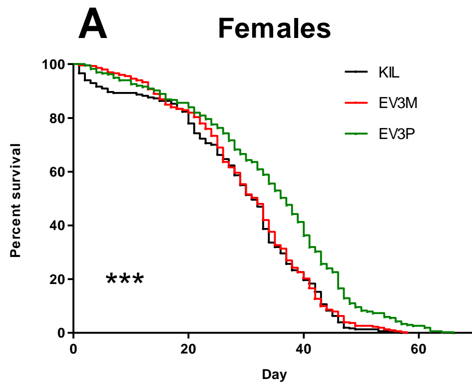
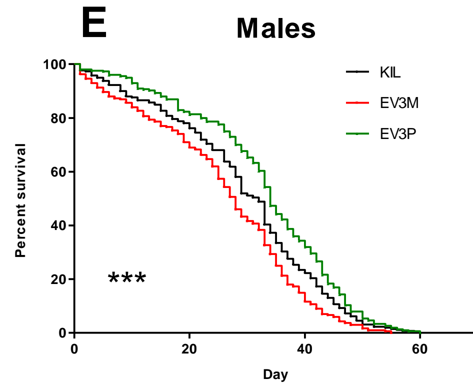

Female
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
Wing Length (mm)
ns
ns
ns
ns
ns
ns
KIL
EV3P
EV3M

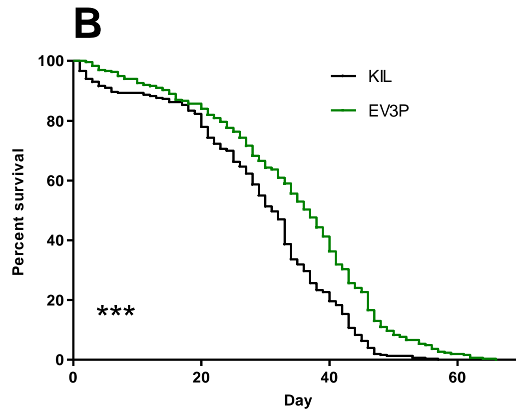
Figure 4.4 (opposing page): Comparison of wing lengths between a control line (KIL) and two hemizygous transgenic lines reared under strictly uniform conditions

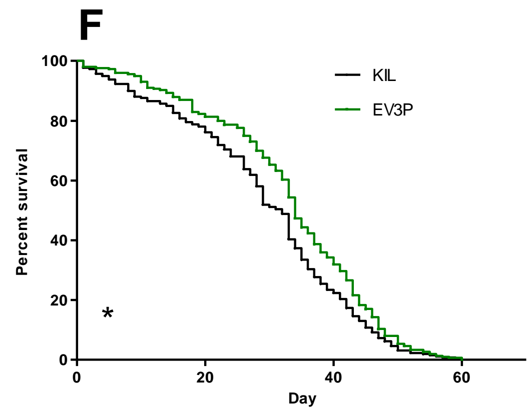
Male wing lengths followed a normal distribution therefore bars represent the mean with SEM also shown. Female data was non-normally distributed and so boxplots are representative of the data. A line indicates the median, the interquartile range is boxed and maximum and minimum values are plotted.

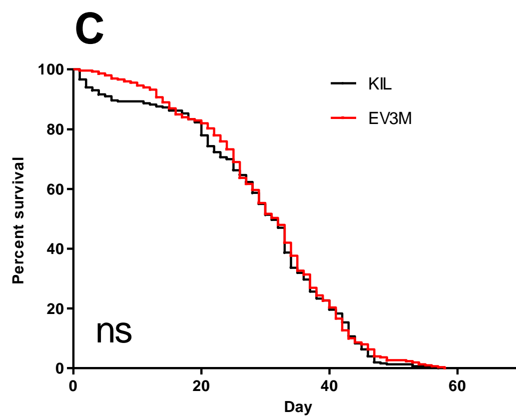
Statistical significance is shown above bars/boxplots in the colour of the bar/boxplot to which the significance level corresponds (ns, not significant).

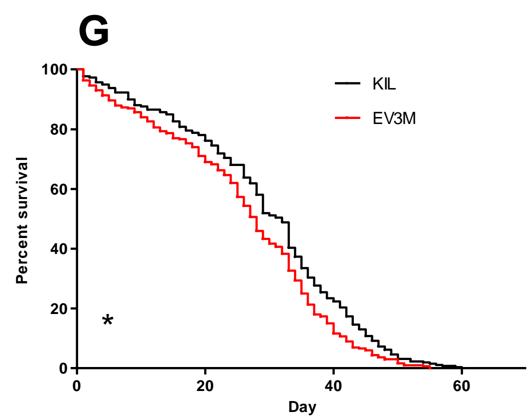

A
Females
KIL
EV3M
EV3P
Percent survival
***
Day

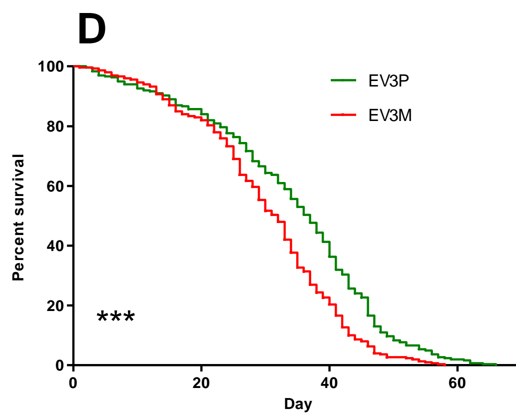

E
Males
KIL
EV3M
EV3P
Percent survival
***
Day


B
KIL
EV3P
Percent survival
***
Day


F
KIL
EV3P
Percent survival
*
Day


C
KIL
EV3M
Percent survival
ns
Day


G
KIL
EV3M
Percent survival
*
Day


D
EV3P
EV3M
Percent survival
***
Day

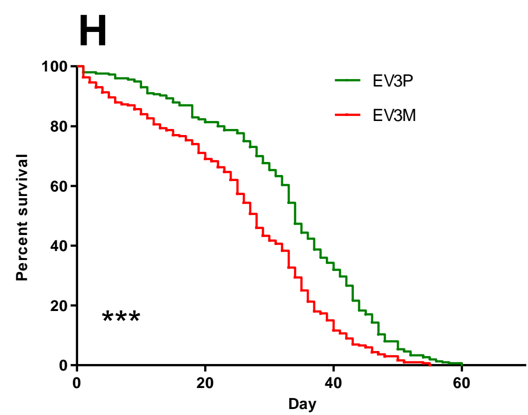
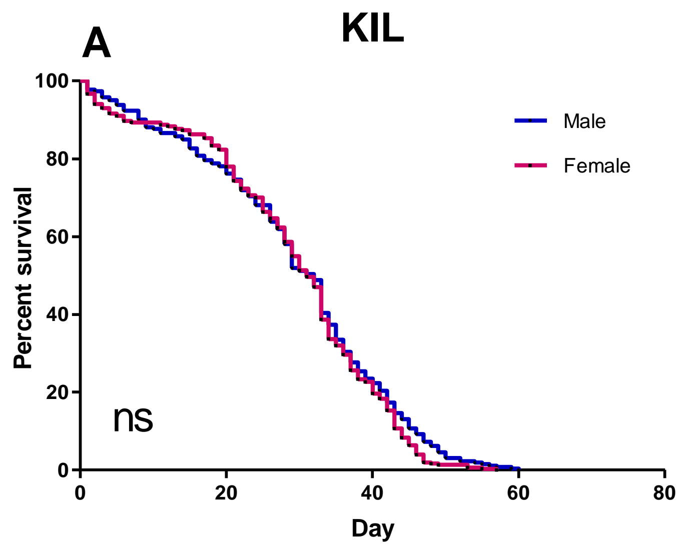
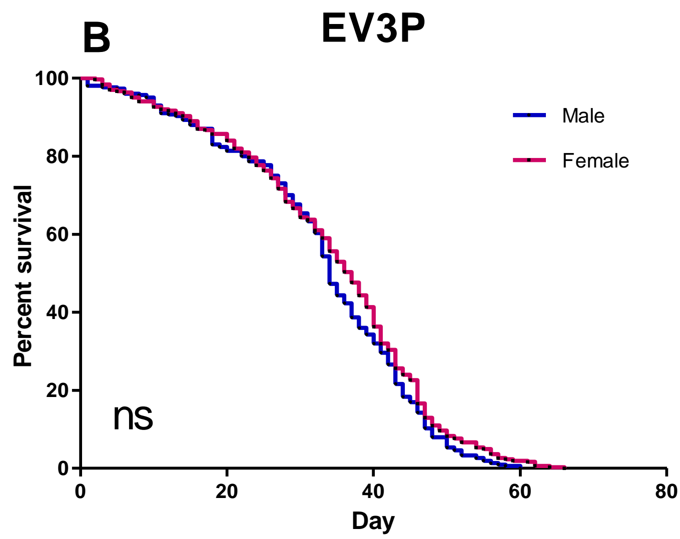

H
EV3P
EV3M
Percent survival
***
Day

Figure 4.5 (opposing page): Kaplan-Meier survival curves to compare longevity of the control (KIL) line with two hemizygous transgenic lines using pooled data from three independent experiments

Graphs A-D show female survival data and graphs E-H show male survival data. Graphs A and E show survival curves for all three lines included in the experiments. Graphs B-D and F-H compare the survival curves for each combination of pairs of lines for female and male mosquitoes respectively.

All survival data was analysed using the log-rank (Mantel-Cox) test and significance levels are displayed on each graph (***, $p < 0.0001$; *, $p < 0.05$; ns, not significant).


A
KIL
100
80
60
40
20
0
Percent survival
ns
0
20
40
60
80
Day
Male
Female


B
EV3P
100
80
60
40
20
0
Percent survival
ns
0
20
40
60
80
Day
Male
Female

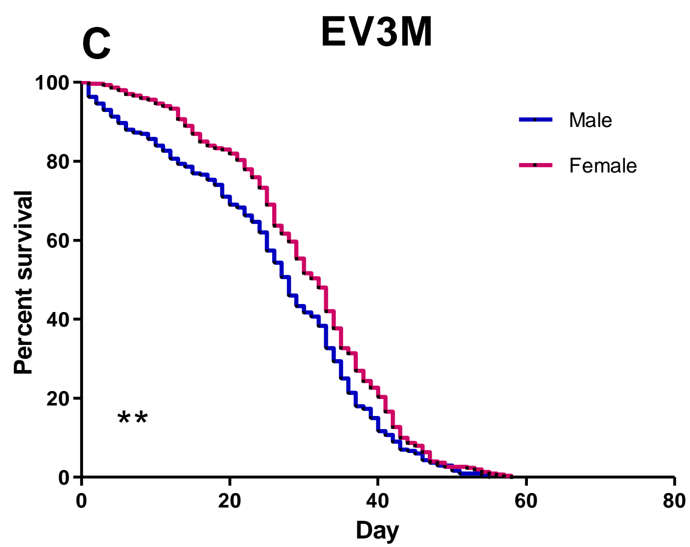

C
EV3M
100
80
60
40
20
0
Percent survival
**
0
20
40
60
80
Day
Male
Female

Figure 4.6 (opposing page): Kaplan-Meier survival curves to compare longevity of males and females for each line using pooled data from three independent experiments

A) Survival curve comparing male and female longevity in the control (KIL) line; **B**) Survival curve comparing male and female longevity in the hemizygous transgenic EV3P line; **C**) Survival curve comparing male and female longevity in the hemizygous transgenic EV3M line.

All survival data was analysed using the log-rank (Mantel-Cox) test and significance levels are displayed on each graph (**, $p < 0.001$; ns, not significant).

4.4. Discussion

4.4.1. Use of hemizygous transgenics for fitness studies

In the fitness studies undertaken here, it was decided that comparisons would be made between a control strain of *An. gambiae* and hemizygote transgenic strains rather than homozygous strains. Previous studies into transgenic fitness have used both homozygous transgenic lines (Bargielowski *et al.*, 2011; Koenraadt *et al.*, 2010; Li *et al.*, 2008) and hemizygous lines (Amenya *et al.*, 2010; Moreira *et al.*, 2004). Homozygous lines are used in fitness studies as transgenics are maintained as homozygotes in the laboratory in order that rearing is easier as homozygosity negates the need for screening at every generation. It is the homozygous state that would be released into the field should the transgenic be used as part of a control strategy because introgression of the transgene into the wild type mosquitoes in the field would be more efficient (Li et al., 2008). Hitchhiking effects of recessive, yet potentially deleterious, alleles present near the insertion site may only be noticeable in homozygous lines. Therefore if multiple transgenic lines carrying the same transgene are generated and made homozygous, the line displaying the greatest fitness can be selected for use (Li *et al.*, 2008). Thus it is unsurprising that various studies into the fitness of transgenic lines compare wild type (control) lines with transgenic lines in the homozygous state especially for those lines considered ready for release as part of a control program in the field (Bargielowski *et al.*, 2011). However, several studies highlight the effect of inbreeding on fitness (Koenraadt *et al.*, 2010; Li *et al.*, 2008) which can be reduced by out crossing to a wild type strain. Comparisons made between transgenic lines and wild type controls in terms of fitness parameters are therefore more likely to indicate that any differences observed between these lines are due to the effect of the transgene and not inbreeding. Li *et al.* (2008) also suggest that it is the introduction of competition which

causes a reduction in transgene allele frequency in mosquito populations as homozygote populations are stable over many generations. However, introduction of wild type alleles causes a dramatic loss of transgene alleles which could indicate that transgenic males are less able to compete with wild type males for females (Li *et al.*, 2008). When released into a field situation, which is the ultimate goal of transgenic mosquito creation, any transgenic mosquito in both homozygous and hemizygous form has to be able to successfully compete with their wild type counterparts in order that the control program is effective. Diaz *et al.* (2011) model the population dynamic effect of the introduction of a genetically modified mosquito into a wild type population, based on well-documented epidemiological data. The authors conclude that it is the relative fitness of the resulting hemizygotes which is crucial for the success of the release thus highlighting the need for extensive studies into the reproductive fitness of transgenic mosquitoes destined for release (Diaz *et al.*, 2011). It was therefore concluded that the fitness studies conducted here would compare the wild type strain to transgenic lines in their hemizygous state. Bargielowski *et al.* (2011) report that the differences between the transgenic line and wild type seen in their fitness studies may be due to a difference in genetic background. Therefore, for the experiments described here, the wild type control was the host strain from which the transgenics were created. From evidence shown here, it seems likely that the use of hemizygote transgenic mosquitoes removes certain confounding factors highlighting differences between wild and transgenic lines that are caused by the effect of transgenesis itself.

4.4.2. Larval mortality

Statistical analysis of mean larval mortality showed that there was no significant difference between control and transgenic lines. The striking result from investigation of this fitness parameter is that, on average, 9% of KIL larvae die prior to reaching pupation compared to

only 3.2% and 5.6% respectively for EV3P and EV3M. Although not a statistically significant result, biologically it is interesting as two out of three independent experiments follow this pattern. Although experiment 2 does not show a greater average loss of KIL, it does indicate a larger variation within this line between replicates thus suggesting that it is KIL which is more prone to larval mortality. Furthermore, all three experiments indicate similar losses of transgenic larvae in each experiment. It is unlikely that possession of the Vida3 transgene confers a fitness advantage at the larval development stage when compared to a wild type strain. Given that the difference observed is not statistically significant, it is more likely that it is caused by a small confounding factor not accounted for in the experimental design. The study involved transferring 30 larvae from oviposition vessels to each replicate. Oviposition vessels were all the same size and contained the same volume of water. Cages from which the oviposition vessels were taken were set up with the same number of female and male mosquitoes in so that similar numbers of eggs would be laid into the vessels. This was designed to ensure that each line was subject to the same density effects as L_1 larvae hatch out. However, it may be likely that either more females of the KIL line take a blood meal in the time offered, or fewer hemizygous larvae hatch out, or a combination of both, thus resulting in a greater density of L_1 larvae in the wild type cage oviposition vessel. This may result in these larvae being weaker and could account for the small yet universal increased larval mortality in the control line when compared to the transgenic lines. It could be expected that a significant decrease in survival to pupation would decrease larval competition thus prompting eclosion to occur sooner. Though significant differences between lines were not observed in this experiment, this does not mean that the slightly raised larval death levels in the KIL line would not have an effect on pupation. However, this did not appear to be the case, perhaps because larval mortality was not high enough to affect pupation, or because age at pupation

is controlled mainly by factors other than density, such as genetics and temperature especially when density is already at relatively low levels (and food supplies plentiful), when compared to the likely situation in the field. *An. gambiae* in the field are reported to be subject to larval mortality rates as high as 92-98% due to varying factors such as presence and/or absence of parasites, pathogens and predators within larval pools, inter and intra-specific competition and abiotic factors such as temperature and rainfall (Paaijmans *et al.*, 2007; Kirby and Lindsay, 2009). It is worth noting, however, that when considering only intra-specific (or intra-line) competition, higher larval mortality rates may lead to the production of larger, more fecund adult females and thus have no net fitness disadvantage (Koenraadt *et al.*, 2010). Mortality rates in this study are significantly lower than those encountered in the field as the factors affecting this parameter are not present in the laboratory. This raises the question of how well laboratory-reared and field-released mosquitoes would actually survive, especially when in competition with field mosquitoes thus making it clear that for accurate modelling to be possible for releases, it would be necessary to take fitness studies out of the laboratory and into the field.

4.4.3. Sex ratio

There was no deviation from the expected 1:1 ratio of males to females in the control (KIL) line or in either of the hemizygous lines, EV3P or EV3M. This suggests that presence of the Vida3 transgene did not influence the sex ratio of either of the hemizygous lines. The transgene is known to be located on chromosome 3R, and is 2.5kb from any known gene (Meredith *et al.*, 2011), therefore it would not be expected to distort the sex ratio.

4.4.4. Age at pupation

There is no significant difference in female age at pupation between KIL, EV3P and EV3M when considering the pooled data. This is true for comparisons between KIL and EV3P in all three independent experiments. However, when considering each of the three independent experiments separately it can be seen that there are significant differences between age at pupation of female KIL and female EV3M. In experiments 1 and 3, on average, KIL pupates sooner than EV3M but in experiment 2, it pupates later thus balancing out in the pooled data. Furthermore, in experiments 1 and 2, there is a significant difference in age of pupation of females between the two hemizygous lines which is not then reflected in the pooled data set. This is explained when looking more closely at the data which shows that the EV3P line pupates significantly later, on average, than the EV3M line in experiment 2 whereas in experiment 1, it pupates significantly sooner. The variation seen between experiments may be due to conditions in the insectary changing between repeats which could not be accounted for, such as outside temperatures affecting the environment inside the insectary. Anecdotal evidence suggests that, although insectary temperature and humidity are under control, there is certainly variation depending on ambient temperature outside. It is clear that with each successive experiment there is a general increase in age at pupation for all genotypes, though pupation across all lines within experiments began on exactly the same day. It's likely that external factors have influenced the timing of pupation, but have affected all lines to the same degree. It remains possible that this has had an effect on the data, causing anomalies in the statistics when data is pooled, though this is only seen to affect females. Given the slight inconsistency across experiments, it is most likely that pooled data reflects more accurately any differences observed between female age at pupation between lines, and so it can be

concluded that the presence of the transgene does not significantly affect female age at pupation.

When analysing pooled data, it is clear that there are significant differences in age at pupation between KIL and EV3M males and also between the two hemizygous lines, EV3P and EV3M. There is no significant difference in age at pupation between KIL and EV3P males. All three experiments exactly reflect these differences. Mating in *An. gambiae* occurs in swarms composed of males which compete for arriving females. These swarms seem to lack a female choice component thus placing more weight on male competition (Diabate *et al.*, 2011). Therefore, if males belonging to the EV3M strain take longer to reach pupation than wild type counterparts, this may have a negative effect on transfer of the transgene to subsequent generations. Li *et al*. (2008) suggest that slower development of transgenic larvae delays the male mosquito in reaching sexual maturity which would have consequences when competing in the field with their wild type males. This means that EV3M may have a significant disadvantage in terms of fitness. What is interesting is that males from the EV3P line reached eclosion within a similar timeframe to those from the KIL line which could mean that the transgene, when maternally inherited, is impacting on genes which influence pupation. The mechanism for this is unclear. Conversely, and although not observed in the hemizygous lines used within this study, transgenic lines which reach pupation sooner than wild type, a trait possibly under selection pressure in the laboratory when mass rearing, could incur a fitness disadvantage when away from laboratory conditions (Bargielowski *et al*., 2011).

4.4.5. Wing length

Wing length has been documented as a good indicator of body size for anopheline mosquitoes (Koella and Lyimo, 1996). Furthermore, body size provides a good indicator

of the female's capacity for blood and therefore can affect fecundity (Lyimo and Takken, 1993; Hogg *et al.*, 1996). Kirby and Lindsay (2009) showed that body size is related to development time in *An. gambiae* and *An. arabiensis* thus linking age at pupation to body size. Their study shows that at higher temperatures, *An. gambiae* are smaller than *An. arabiensis* so their development time is quicker, although they develop too quickly to accumulate enough food to support pupation and therefore do less well than *An. arabiensis*. Lyimo and Takken (1993) also report that a shortage of resources during development can have a detrimental effect on adult mosquitoes. Using wing length as a measure of body size, their study indicated that the smaller mosquitoes (wing length of less than 3mm) hand-caught from the field in Tanzania, are more often pre-gravid than the larger females caught, and therefore are not fecund after a blood meal. Instead they require two or three blood meals to complete their first gonotrophic cycle. Further mating studies have shown that male *An. gambiae* will select larger females even when large numbers of males are competing for a smaller number of females thus suggesting that these females are picked due to their increased egg-carrying capacity (Okanda *et al.*, 2002). Implications of body size do not only affect females but can have a large effect on the mating competitiveness of male *An. gambiae*. A study into sexual selection in mosquito swarms suggests that the most successful males were not the largest, but were of intermediate size, and these mosquitoes were six times more likely to achieve copulation with a female during one swarming event (Nghabi *et al.*, 2008). However, the study also showed that the largest males, which were on average 10% larger than intermediate-sized males and had two to four times more energy reserves, were 13% longer-lived than their intermediate-sized counterparts (Nghabi *et al.*, 2008). Interestingly, within size groups, and consistent across the three size groups, males which mated were, on average, larger than those which did not mate (Nghabi *et al.*, 2008). The authors suggest three reasons for the success of

intermediate-sized males. The first is that it is a trade-off between agility and lower energy reserves, and the second is that these males are closer to females in size, though the experiment was not designed to test for size-assortative mating. The third reason being that males of this size cohort are mostly closely following the averages of natural sexual dimorphism for this characteristic. However this would represent female choice as a mechanism behind mating behaviours within the swarm and much evidence suggests that it this is not the case with *An. gambiae* (Nghabi *et al.*, 2008).

Numerous experiments indicate that adult size plays an important role in reproductive fitness of *An. gambiae*. It is therefore clear that this parameter is an important one and one which must be carefully considered when generating transgenics with a view to future release in control programs. The study conducted here shows quite clearly that there is no significant difference between the size of wild type lines (KIL) and either hemizygous line (EV3P and EV3M) carrying the Vida3 transgene. This is true of both males and females. Use of transgenic mosquitoes as part of a control program would involve the release of a large number of homozygous male mosquitoes. It is these mosquitoes which would then have to out-compete wild type males for females. Thus it would be important that homozygote males are of a similar body size, on average, to their wild type counterparts in the field. Consequently it would be beneficial to include body size analysis of the homozygous EVida3 line. Preliminary data shows that there is no significant difference between adult size of KIL and homozygous EVida3 (data unpublished).

The correlation between body size of females and capacity for a blood meal also indicates that females of a similar size will take on board a similar infection when encountering infected hosts. Infection with the malaria parasite has an effect on reproductive fitness of *An. gambiae* by reducing fecundity (Hogg and Hurd, 1995b). In theory, a transgenic mosquito which will take a smaller blood meal, and therefore take on board a lower level

of infection (leaving aside fitness costs associated with smaller blood meals) than wild type females may be of benefit in terms of controlling disease. However, it is likely that fitness costs associated with being smaller than wild type females may outweigh any benefits gained from being exposed to fewer parasites. Furthermore it has been documented that infection levels in wild populations are maintained at very low levels with the average number of oocysts found on the midgut of field-caught infected mosquitoes being between 1 and 3 (Dawes *et al.*, 2009). It is unknown whether this reflects the fact that those with high infection levels die and are thus not available for sampling (Lyimo and Koella, 1992), or whether it is due to other factors, such as the mosquito immune response. However, these mosquitoes are likely to be better adapted at controlling natural infection with *Plasmodium spp*. than transgenic mosquitoes, and so even if they are exposed to higher parasitaemias in mammalian hosts, they are still likely to out-compete the transgenic line in terms of reproductive fitness.

4.4.6. Adult longevity

Analysis of pooled data shows that there are significant differences in longevity between KIL and hemizygous transgenic lines. This is the case for both males and females although the differences do not follow the same patterns in both sexes. When comparing longevity of females, there is no significant difference between KIL and EV3M but highly significant differences between KIL and EV3P and between EV3P and EV3M. In both of these cases, it is the EV3P line which is, on average, the longest lived, out-living even the wild type line. The difference between the transgenic lines, EV3P and EV3M is also reflected when comparing males, whereby EV3P is more long-lived than EV3M. Comparisons of KIL and EV3P males show that, as with females, it is the hemizygous strain which is, on average, the longer-lived, however the difference is less pronounced in

males. In females, there is no significant difference in longevity between KIL and EV3M but in males, KIL invividuals live significantly longer on average. Although levels of significance varied, all three experiments showed essentially consistent trends among both sexes when comparing wild type mosquitoes with transgenic hemizygous lines (Kaplan-Meier longevity curves for independent experiments 1 to 3 are found in Appendix 7.3; Figs. 7.3.1-7.3.6). In general therefore, it can be concluded that pooled data is highly representative of longevity in the three lines observed. Results from the longevity study show that not only are there significant differences between wild type and transgenic lines, which might be expected, but also that there are significant differences between the two transgenic lines. These differ only in the route of inheritance of the transgene, either through the male of female parent. This was an unexpected result but one that was also evident from the data on age at pupation.

Longevity of mosquitoes, or indeed any vector insect, is quite important in terms of its vectorial capacity. The malaria parasite takes around 2 weeks to complete its life cycle within the mosquito host and become the infective sporozoite stage within salivary glands (Barillas-Mury and Kumar, 2005). Female *An. gambiae*, to be an efficient vector of malaria, must live long enough for this life stage to be reached. It is also beneficial to the mosquito to live longer, and therefore have the increased potential to undergo more gonotrophic cycles. Indeed, a decrease in longevity has been targeted as a mechanism by which the vectorial capacity of *Ae. albopictus* can be reduced. The aim was to reduce the lifespan of females so as they could still live long enough to reproduce, but not long enough to act as a vector of disease. A species of *Wolbachia* (*w*MelPop), a natural symbiont of *Drosophila*, was transferred to the mosquito which then caused a significant shift in the age structure of the population of females towards those which are younger (Suh *et al.*, 2009). The authors do however, report that *w*MelPop reduces fecundity as well

as longevity, and causes a high embryonic lethality. As such, it is less likely to spread to field populations of *Ae. albopictus* so its use as a control strategy is limited due to its association with such high fitness costs (Suh *et al.*, 2009). This highlights the need to ensure that any mosquito released into the field which would need to compete successfully with field populations is reproductively fit. Longevity clearly forms part of this reproductive fitness; however the significance of longevity in mosquitoes in terms of fitness may differ between males and females. In theory, females need to live longer than males in order to find a mate, copulate, feed and complete at least one gonotrophic cycle, which takes around 48 hours in *An. gambiae*. Clearly more successful females will be those who can complete more than one gonotrophic cycle. Males need to live long enough to successfully mate, which can occur with several females within a short period of time once they have matured, thus their theoretical life span requirement is shorter than for females. Males may even have a competitive advantage if they mate many times earlier in life and so longevity may not be as important as in females. However, there is evidence to suggest they have a limited mating capacity due to resource costs (Dao *et al.*, 2010). This means that the resources that males emerge as adults with would not be sufficient to allow them to find a reproductively successful number of females with which to mate and would thus argue for the advantage of increased male longevity. Conversely, if males are less competitive, i.e. compromised in terms of reproductive fitness in other ways, perhaps because they take longer to pupate, and do not mate successfully early in life, clearly their reproductive fitness will also be compromised by having a reduced longevity. A study looking at the comparative physiological fitness of free-living and laboratory-reared *An. gambiae* showed that free-living males had higher lipid stores than their counterparts, possibly due to a better quality larval diet than that provided in the laboratory. Incidentally, carbohydrate stores were lower in free-living male *An. gambiae* than in those

which were laboratory-reared, probably because sugar was available *ad libitum* to these mosquitoes (Huho *et al.*, 2007). The higher lipid content was expected to lengthen life expectancy of free living males as long-term survival is associated with high lipid abundance and larger body size (Briegel, 1990). If presence of higher lipid reserves available from eclosion outweighs benefits of higher carbohydrate levels from sugars available post-eclosion, then field free-living males will have higher physiologically determined reproductive potential than their laboratory-reared counterparts (Huho *et al.*, 2007). This adds further weight to the question as to whether greater longevity is truly important for male mosquitoes in terms of fitness. It is known that carbohydrate stores in males are important to their ability to maintain flight and therefore impact on their ability to swarm. There is evidence to suggest that in field caught mosquitoes these reserves increase in older mosquitoes therefore suggesting that reproductive fitness of males does not decrease with age (Huho *et al.*, 2007). The same study also indicated that the lower lipid content present in laboratory-reared males (which is linked to a shorter life span), may impact on fitness should longevity be an important parameter (Huho *et al.*, 2007). Clearly, transgenic mosquitoes are laboratory-reared and therefore are likely to exhibit similar, if not greater, fitness deficits to those highlighted in this study comparing free-living and lab-reared male *An. gambiae*. Nghabi *et al.* (2009) showed that male *An. gambiae* of intermediate size were between six times more likely to achieve copulation than larger mosquitoes, however larger mosquitoes were consistently longer-lived than the intermediate-sized males. The authors do suggest however, that although longer lived and larger males have more opportunity to mate over their life time, this may not offset the disadvantage their size confers (Nghabi *et al.*, 2008).

The study conducted here suggests that KIL males lived significantly longer than EV3M males but had a significantly shorter life span than EV3P males. A comparison of

longevity between the two hemizygous line indicates that EV3P males are very significantly longer lived than those from the EV3M line. Therefore it is hard to conclude that the presence of the Vida3 transgene has a detrimental effect on the longevity of *An. gambiae* males. In terms of fitness of transgenic strains, a longer-lived male would be useful in terms of release, as the longevity may offset any reduction in competitiveness exhibited by the transgenic strains in mating swarms. However female longevity also has to be considered since it is they which express Vida3 and which are the vector for *Plasmodium spp*. Analysis of pooled data from longevity studies conducted here indicates that, whilst EV3P females are significantly longer lived than wild type (KIL) females, EV3M female longevity is not significantly different to that of the KIL line. Again, and as with males, it is difficult to conclude that the presence of the transgene has a detrimental effect on the longevity of females, as there is a significant difference in longevity between hemizygous lines. However it seems more likely that the presence of Vida3 in females is not detrimental in terms of longevity. As discussed previously, it would actually be beneficial to cause an age shift in the female population towards younger mosquitoes as they would be less efficient vectors, especially when this is coupled with the anti-parasitic effect of Vida3 expression in the midgut of the females. What is interesting about the longevity data is that although there appears to be a difference in longevity between hemizygous lines (which differ in the route of inheritance of the transgene), there is a difference in longevity when comparing males and females within lines only for EV3M. If there is a genetic effect on fitness which is associated to a greater degree with maternal inheritance of the transgene, one may expect females to exhibit a greater fitness cost for longevity and other fitness parameters, than is apparent in males. This is not the case in these studies, and in fact, the other fitness parameter where there is a clear difference between hemizygous lines is age at pupation. With this parameter, it is also EV3M which

suffers a detrimental effect, with a longer time to pupation, but this difference is only apparent in males and not females. The longevity study also shows that lifespan is reduced in males to a larger degree than in females when comparing EV3M line to KIL. This suggests that although the Vida3 gene is inherited from maternal gametes, it is somehow affecting male progeny to a greater degree. The mechanism by which this occurs is, at present, unclear.

The longevity studies carried out here are very artificial and, although they do represent the maximum longevity potential of both male and female mosquitoes, they do not take into account the situation in the field. Mosquitoes in these studies were not allowed to mate and females did not receive blood meals so consequently did not enter a gonotrophic cycle. As a consequence of this, females did not get the opportunity to express the Vida3 protein. Clearly these factors may indeed have a large impact on longevity of mosquitoes. In the field, female mosquitoes come into contact with many pathogens, *Plasmodium spp*. being just one. Up-regulation of the immune response to act against pathogens uses up valuable host resources and thus shortens the longevity of *An. gambiae*. Studies have shown that disruption of the gene encoding SRPN2 which is itself a negative regulator of the PPO pathway involved in the melanisation response, can lead to an uncontrolled immune reaction killing female *An. gambiae* after reproduction but before *Plasmodium* reaches infective stages (An *et al.*, 2011). The melanisation response, however, is rarely observed in co-evolved *Plasmodium*-anopheline combinations due to evasion of the immune response by the parasite (Lambrechts *et al.*, 2007). This may have consequences, upon field release, for transgenic lines such as EVida3, known to melanise malaria parasites which have successfully evaded attack by the Vida3 molecule itself (Meredith *et al.*, 2011). An up-regulated immune response via the melanisation pathway could be seen as advantageous, certainly when combined with expression of the transgene itself, in that the

knockdown effect on the parasite may be magnified. This may in turn cause a reduction in average longevity of female transgenic mosquitoes released in the field as discussed by An *et al*. (2011). This would be an ideal situation as it would allow for reproduction of the transgenic strains thus allowing the spread of the transgene through the population, whilst making them less susceptible to the malaria parasite, and generally increasing their immune response. Laboratory-reared transgenic mosquitoes, when released into the field will encounter many more pathogens/parasites than when in the laboratory. This may then initiate an up-regulation of the immune response, larger than that initiated in field mosquitoes in response to contact with parasites. As a consequence, transgenic mosquitoes would require a larger amount of resources to allocate to this up-regulation. This may cause a massive age shift in the population of female transgenic *An. gambiae* to a point where they were less likely to complete a gonotrophic cycle after an initial blood meal. Thus they would then be reproductively less fit than wild type counterparts and the spread of the transgene would be unsuccessful. The fitness studies conducted here cannot provide a measure of this as mosquitoes were not blood fed, and consequently any fitness effects linked to feeding on either non-infected or infected blood cannot be measured. Previous fitness studies have shown that as well as possessing no fitness burden when compared to wild type mosquitoes, females from transgenic lines expressing an antimalarial peptide (SM1) in the midgut were longer lived and more fecund than wild type when routinely fed on *P. berghei*-infected blood (Marrelli *et al.*, 2007). Furthermore, in cage experiments, transgenics gradually replaced non transgenics when maintained on infected blood. This had not been seen before when similar cage experiments were performed as part of studies looking at fitness effects of transgenes (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004; Moreira *et al.*, 2004). Although the activation of the innate immune cascade in mosquitoes is itself seen as a fitness-reducing occurrence (Ahmed and Hurd, 2006), Marrelli *et al.* (2007)

suggest that the observations of increased fitness from their experiments are due to the expression of a harmless immune peptide causing knockdown of the parasite prior to invasion of midgut epithelial cells and consequently prevention of the costly upregulation of an innate immune response. Dawes *et al*. (2009) showed, by analysing death rates in cages fed on blood infected with varying densities of *P. berghei* ookinetes, that female *An. stephensi* mortality is not only age related, but is also infection-intensity related. High death rates were seen in cages initially and were partly associated with blood feeding (Dawes *et al.*, 2009).

Dao et al. (2010) have shown that mating and reproduction affect longevity of *An. gambiae*. Their study showed that longevity of females fed on blood and sugar was significantly higher than those fed on sugar alone, regardless of whether or not they were mated. This suggests that in a laboratory environment where there is a reduced risk to blood feeding due to reduced exposure to pathogens and host response to a bite, blood provides more resources resulting in increased longevity. However, mated females had reduced longevity when compared to virgin females if fed on blood and sugar thus suggesting a cost to females in mating, though the cost of reproduction in terms of longevity is ambiguous (Dao *et al.*, 2010). For male *An. gambiae*, the same study showed that exposure to females for 6 days resulted in a reduced longevity when compared to virgin males. In mated males, higher mortality was associated with smaller sized mosquitoes (Dao *et al.*, 2010). This may be due to these mosquitoes having fewer initial resources accumulated as larvae. Forming mating swarms is thought to be costly to male mosquitoes, and therefore it is likely that longevity studies conducted in the laboratory, where males are exposed to females, are still not representative of the situation in the field. Clearly within a finite space such as a cage, swarming behaviour may not be as draining in terms of resources as they would be in the field as males are much more likely to come

across females without the same amount of time spent within swarms. Therefore laboratory studies are likely to over-estimate male longevity. In the fitness studies conducted here, males lived much longer than expected from general observations of stock populations within the same insectaries. Indeed, anecdotal evidence suggests that females outlive males by a significant period of time. There are two possible reasons for the observed increase in male survival between longevity-study males and stock males. The first is that longevity study males were virgin males, and consequently did not use up valuable resources by mating. The second is that stock males are routinely subjected to periods of around 18 hours where they have no access to sugar due to stock cages being starved to increase female blood-feeding. It is expected that this causes significant male death in stock cages. Longevity-study males, on the other hand, were able to access sugar and water *ad libitum*. It is clear that fitness experiments whereby longevity of mosquitoes is measured as a fitness parameter need to take factors such as blood feeding and mating into account before they can be used as a true representation of a field situation.

4.4.7. Conclusions

In conclusion, from five fitness parameters measured in these studies, there is no clear evidence to suggest that possession of the Vida3 transgene has a significant overall effect on the fitness of the hemizygous transgenic lines. Significant differences were seen between KIL and either of the hemizygous lines for some parameters such as longevity and age at pupation, but these were not consistent over both EV3P and EV3M lines, or between males and females. Indeed some significant differences observed suggest that hemizygous lines showed increased fitness compared to the wild type KIL line. Differences between EV3P and EV3M were observed and are difficult to interpret. What is apparent is that fitness studies such as the ones undertaken here will not conclusively determine how

transgenic mosquitoes will fare if released in the field as part of an integrated control strategy when they have to compete successfully with the indigenous mosquitoes. Factors such as the immune response to pathogens, including the target (in this case *Plasmodium*), mating behaviours and blood feeding/reproduction costs must be considered when conducting fitness experiments to get a better model for the field situation. Nevertheless, preliminary laboratory fitness studies are essential prior to taking such trials to the field as it will enable an initial assessment of newly generated refractory transgenic lines. Assessment of both hemizygotes and homozygotes is also essential as it will be homozygote lines which are maintained in the laboratory and released, but hemizygotes which will be the first generation to have to compete throughout their whole life cycle with wild type mosquitoes.

Chapter Five

5. General Conclusions

The work undertaken here highlights the technical challenges inherent in the transformation of disease vectors. The major malaria vector, *An. gambiae*, is a particularly demanding insect species to work with in a laboratory setting. Genetic modification of this species with complex plasmids such as those used in Chapter 2 is time consuming and is without guarantee of a successful outcome, even when following a protocol that has been optimised to maximise survival and potential for genomic integration of the transgene. Use of RIDL technology in *An. gambiae* has not yet been documented and it may be that integration of particularly large plasmids into this species is inefficient in comparison with other species, such as *Ae. aegypti* and *D. melanogaster*. A further consideration connected with the use of RIDL technology is the ability to mass rear the species concerned. Rearing mosquitoes containing RIDL constructs requires these lines to be able to survive well, as larvae, in water containing tetracycline. Investigations here provide evidence that rearing *An. gambiae* in such conditions is feasible, which was an initial concern for this species. Mass-rearing of *Ae. aegypti* has been successfully achieved with RIDL strains for the purpose of field trials (Derric Nimmo, unpublished data). However, the logistics of rearing *An. gambiae* is different in that it is reported as a less robust system, and one more liable to population crashes. It is possible to sex *Aedes* pupae using mechanical methods as there is a distinct size difference between male and females. This size difference between sexes is also apparent in anophelines but is less marked, making mechanical separation more difficult. In addition to this, anophelines are much more sensitive to the pressures of mechanical sorting, with a particularly high mortality rate amongst those which were

subject to this procedure (unpublished data). Both the population suppression (encompassing RIDL) and population replacement approaches to mosquito control will ultimately rely on the ability to rear huge numbers of the target strain. This makes it a key factor in the success of integrated control strategies releasing pre-established laboratory strains of genetically modified mosquitoes. Without the ability to mass rear a transgenic strain the technology becomes redundant in terms of its viability. Mass rearing of anophelines, as with *Aedes*, is possible. Small scale release of sterile *An. albimanus* has previously been carried out in El Salvador causing suppression of the local population, although this was short-lived due to the effect of immigration (Reviewed by Benedict and Robinson, 2003). For this to have been undertaken, *An. albimanus* had to be successfully mass-reared, producing millions of mosquitoes per week, thus proving mass-rearing feasible for anophelines (Nolan *et al.*, 2011). Since then, various laboratories have undertaken work to maximise the potential for mass-rearing of anopheline species for use in SIT programmes. These have been both broad-ranging (Singh, 2007) and focused on parameters such as oviposition behaviour (Balestrino *et al.*, 2010) and density-dependent effects (Gilles *et al.*, 2011). The Centre for Production and Infection of *Anopheles* (CEPIA) has been successful in mass-rearing both *An. gambiae* and *An. stephensi* (Thierry, 2007). This facility mainly rears anophelines for infection challenge experiments. They are able to supply several different research institutes with between 2 and 3 thousand female mosquitoes per day reared following strict regimes to ensure reproducibility between experiments (Thierry, 2007). Although not rearing transgenic mosquitoes, this facility proves that mass rearing of *An. gambiae* is possible and therefore allows for the possibility of using transgenic lines in future control strategies, with RIDL technology a viable option for anophelines.

Generation and subsequent characterisation of RIDL strains of *An. gambiae* would have resulted in the formation of further research questions associated with these lines regarding the effectiveness of the transgene and its sex-specific action, as well as the potential for its application downstream. This would have been of particular interest given the prior generation (Fu *et al.*, 2010) and large cage trials (Wise de Valdez *et al.*, 2011) of a flightless RIDL strain of *Ae. aegypti*. The inability to integrate the entire RIDL plasmid into the *An. gambiae* genome suggested that the size of the construct may affect efficiency of integration more in this species than with others. This is further backed up by the inability to integrate other large constructs successfully. However the microinjection protocol used here did allow for integration of a single *piggyBac* element, containing only a marker gene and associated 3xP3 promoter, which also suggests that the failure to integrate complete plasmids is due to their size and possible deleterious effects of the genes they encode.

Failure to produce a novel transgenic resulted in a change of emphasis for the investigations presented here. The EVida3 transgenic line generated by our laboratory had already been characterised, and the lines' ability to knockdown the murine malaria *P. yoelii nigeriensis* documented (Meredith *et al.*, 2011). However, there remained a great deal of questions regarding the production and action of the synthetic Vida3 peptide *in vivo* and its effect on the mosquito itself. This was both in terms of its interaction with the immune system and also fitness costs associated with production of Vida3. The work presented here takes steps to further our understanding of the biology of transgenic *An. gambiae* by investigation of both blood feeding habits and reproductive fitness parameters. There are many aspects of mosquito biology which need to be assessed in a laboratory before the long term goal of release can even be considered.

The multiple feed studies described here support the conclusions of Meredith *et al*. (2011) in terms of the ability of Vida3 to reduce parasite burden when expressed in the midgut of the transgenic line. However the effect is not the zero-sporozoite phenotype required for any mosquito to be deemed as truly refractory, as only one developed oocyst would produce enough sporozoites to allow the mosquito to become infective. Furthermore, these investigations into the effects of multiple blood-feeding on Vida3 action indicated that the Vida3 peptide cannot cross the midgut epithelium and thus its effects on the *Plasmodium* parasite cease once the parasite invades midgut cells. A multi-compartmental approach would therefore be necessary to provide a more thorough attack of the parasite within the mosquito host. This would be achieved by driving expression of an AMP by means of a different promoter such as anopheline antiplatelet protein (AAPP) promoter which is salivary-gland specific (Yoshida and Watanabe, 2006). Driving expression of Vida3 in both the midgut and salivary glands may further reduce the likelihood of the parasite being able to complete its life cycle within the mosquito thus blocking the transmission cycle and increasing the efficacy of the transgenic strain. Previous studies have shown that field populations of anophelines will take a further blood meal within one gonotrophic cycle (Briegel and Horler, 1993). Laboratory studies have also managed to illustrate this (Nirmala *et al.*, 2005). In terms of this study, it was thought likely that too many females may refuse the second blood meal in the laboratory. Thus to have enough dissected females for a statistically powerful test it would have required the initial number of females feeding on the mouse to exceed the amount ethically recommended. This meant that although conclusions can be drawn about the inability of the Vida3 peptide to cross the midgut epithelium, it is impossible to determine whether a second blood meal, within one gonotrophic cycle and therefore relatively shortly after the initial feed, would have a

greater knockdown effect purely due to the action of the Vida3 peptide on the parasite than when mosquitoes are fed only once within one cycle.

However, other conclusions can be drawn from these investigations into effects of multiple feeding. Studies here show a trend in line with those conducted by Lopes *et al*. (2007) in that a second (naïve) blood meal, given 3 days post infection, increases infection intensity. This could be due to the increased resources available to the parasite, but could also be attributed to the mosquito directing the resources from this blood meal away from an immune response and towards egg production. The second feed given was non-infective and as a consequence, would not instigate such a large immune response as the first feed and parasites present already may not initiate any further peaks in immune activity. This theory is corroborated by the observation of increased egg production in the second gonotrophic cycle when compared to the first in both lines E and EVida3. Interestingly the study also indicates a cost to the mosquito of production of Vida3 peptide as EVida3 is less fecund than line E when given an infected feed.

Further studies are required to examine the effect of second blood feeds (of varying immune and infection status) either with or between gonotrophic cycles. These experiments, with appropriate controls, would go some way towards ascertaining how these parameters affect infection within transgenic strains, such as EVida3, and how infection and production of novel immune peptide affects fecundity. More work is also required so that a consistent, reproducible system can be established for feeding peptides along with infected blood to mosquitoes in order that experiments such as the dose-dependent curve experiment can be carried out effectively.

Investigations conducted here into the general reproductive fitness of the transgenic line were carried out due to the observed effect of Vida3 production on fecundity highlighted by the multiple feeding experiments. Reproductive fitness is an important consideration

when assessing the feasibility of using transgenic mosquitoes generated and bred in the laboratory as part of an integrated population replacement (or indeed suppression) strategy. For this control strategy to be effective, released transgenic mosquitoes must be able to compete effectively for mates with the wild type population and be capable of producing and laying good-quality, viable egg batches. Modification of mosquito biology and/or physiology brought about due to gene disruption cause by transgenesis or associated inbreeding could compromise the ability of a transgenic line to compete effectively with wild type field mosquito populations. Clearly this would have a detrimental effect on release of transgenics as a reliable and effective control strategy. The investigations presented here concentrated on analysis of five parameters of fitness in studies similar to those conducted previously (Moreira *et al.*, 2004; Li *et al.*, 2008; Amenya *et al.*, 2010; Bargielowski *et al.*, 2011). Three out of five parameters measured (larval mortality, sex ratio and wing length) showed that there was no associated fitness cost with presence of the Vida3 transgene. However, both age at pupation and longevity highlighted interesting differences between wild type (KIL) mosquitoes and the hemizygous transgenic lines, EV3P and EV3M.

When considering age at pupation, the overall effect of the transgene is seen in males only. In females, there was no significant difference between KIL and either of the hemizygous lines. However, EV3M males pupate significantly later than both KIL and EV3P males. Increased age at pupation is generally considered a disadvantage in terms of reproductive fitness, especially with regard to competitiveness of male mosquitoes. Data generated by longevity studies shows, overall, a significant increase in longevity for both males and females of the EV3P line when compared to KIL. However, it cannot be concluded from this that the presence of Vida3 confers a fitness advantage, for two reasons. The first is that this increased fitness of a transgenic line is apparent only in one fitness parameter, and

it is possible that increased longevity may not be totally advantageous to a mosquito in terms of reproductive fitness. The second is that the EV3M line does not corroborate these results by exhibiting increased longevity when compared to KIL. Longevity data for the EV3M line are more consistent with data generated from assessment of age at pupation. In both cases (if decreased longevity is taken to confer a fitness cost to the mosquito), EV3M males exhibit a greater fitness cost in comparison to their male KIL counterparts. Genetic mechanisms behind these differences between hemizygous strains and between sexes within the EV3M strain are unclear. Since the Vida3 transgene is autosomal, but differences are seen between reciprocal crosses and between sexes in one particular hemizygous cross, then it appears possible that such differences are due to inbreeding impacting on functionality of the X chromosome. Analysis of the EVida3 line in its homozygous state should give rise to further evidence corroborating this hypothesis. Therefore, as all X chromosomes are affected in a homozygous line, the fitness costs in relation to age at pupation and longevity should be more pronounced in the homozygous line. Anecdotal evidence, from working with stock populations in the insectary suggests that, in general, homozygous lines pupate later than other stock lines within the insectaries, although this has not been stringently measured. It would also be expected, (assuming a cumulative effect), that, within the homozygous lines, females would be more greatly affected as opposed to males in the hemizygous state. This is because females possess two copies of the X chromosome, both of which would be affected, and males have only one.

Laboratory fitness studies fall into one of two categories. The first is analysis of life-table parameters, and the second is competitiveness (Scolari *et al.*, 2011). The studies undertaken here look only at a comparison of life-table parameters between a wild type laboratory strain (KIL) and the hemizygous EVida strain. Only when both categories are studied in full can the real fitness cost associated with presence of the Vida3 transgene be

gauged and more relevant fitness studies carried out in the field. Currently the competitiveness of the EVida3 strain is under assessment (Douglas Paton, personal communication). Further studies into fecundity are also necessary to complete life table analysis with regard to reproductive fitness indices. Given the reports of a transgenic line exhibiting a fitness advantage over wild type laboratory strains when fed on infected blood (Marrelli *et al.*, 2007), it would be ideal to (re)assess certain life-table parameters, such as longevity and fecundity, when maintaining EVida3 on both infected and non-infected blood. This would be an ideal next step in examining fitness parameters in the laboratory.

There are still a great many unanswered questions in terms of researching the genetics and biology of transgenic insects which must be addressed prior to taking such insects into the field. It is likely that conclusions drawn from any investigations into a particular transgenic line will not be applicable across all lines generated. However, investigations carried out here are both relevant and necessary in furthering our understanding of the biological interactions of transgenic insects.

Chapter Six

6. References

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Chapter Seven

7. Appendices

7.1. Appendix I: Detailed, adapted and modified methods

Protocol for transformations into bacterial cells

The protocol uses XL10 Gold Ultracompetent cells (Agilent Technologies, Santa Clara, USA) and is adapted from , and therefore differs slightly to, the manufacturer's protocol.

- Turn water bath to 42°C
- Pre-chill required number of 14ml BD Falcon polypropylene round-bottom tubes on ice (one tube per experimental transformation)
- Take one tube (150µl) of cells and thaw on ice
- Add 4µl of the β-ME mix provided with this kit to the cells. Swirl the tubes gently and incubate the cells on ice for 10 minutes, swirling occasionally
- Whilst the cells are incubating, add 2µl of each ligation reaction into chilled Falcons and leave on ice (only 0.5µl is required if transforming from a Miniprep)
- Divide competent cells between appropriate number of Falcon tubes (must be a minimum of 10-13µl cells per tube (when transforming from Minipreps only 5µl of competent cells are required). Ensure cells are placed on DNA droplet in Falcon tubes
- Mix cells gently and keep on ice for ≥ 30 minutes.
- Heat shock cells at 42°C for 30 seconds (timing of this is critical)
- Incubate the tubes on ice for 2 minutes

- Add 200µl of transformation medium to each tube and incubate the tubes at 37°C for 1 hour with shaking at 225-250 rpm
- Plate ≤200µl of the transformation mixture on LB agar plates containing the appropriate antibiotic and incubate the plates at 37°C overnight
    - When plating cells transformed from Minipreps, 50µl of transformation mixture is plated, and when plating cells containing the pJet vector, 100µl is plated out. All plating is undertaken in hood using aseptic techniques

Protocol for preparation of genomic DNA from 10 mosquitoes

The protocol uses Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, USA) and is adapted from, and differs slightly to, the manufacturer's protocol.

Cell Lysis

- Place a 1.5ml centrifuge tube containing 500 µl Cell Lysis Solution on ice
- Add 10 headless adults (0.5-2.0mg) to the chilled Cell Lysis Solution and homogenise thoroughly using a microfuge tube pestle. Place the sample back on ice until next step
- Incubate the lysate at 65°C for 15 minutes

RNase Treatment

- Add 2.5µl RNase A Solution (4 mg/ml) to the cell lysate
- Mix by inverting the tube 25 times and then incubate at 37°C for 15-60 minutes

Protein Precipitation

- Cool the sample to room temperature
- Add 165µl of Protein Precipitation Solution to the RNase A-treated cell lysate

- Vortex the tube vigorously for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate. Place the sample on ice for 5 minutes
- Centrifuge the tube at 13,000-16,000 x g for 3 minutes. The precipitated proteins form a tight pellet

DNA Precipitation

- Transfer the supernatant containing the DNA (leaving behind the precipitated protein pellet) to a 1.5 ml centrifuge tube containing 500µl 100% isopropanol (2-propanol). Mix by inverting gently 50 times
- Centrifuged the solution at 13,000-16,000 x g for 5 minutes
- Remove the supernatant and leave the tube to drain on clean absorbent paper. Add 500µl of 70% ethanol and invert the tube several times to wash the DNA pellet.
- Centrifuged at 13,000-16,000 x g for 1 minute. Then carefully pour the ethanol off.
- Invert the tube and drain on clean absorbent paper and allow to air dry for 10-15 minutes.

DNA Hydration

- Add 50µl of DNA Hydration Solution to the pellet.
- Re-hydrate the DNA was by incubating the sample for 1 hour at 65°C or overnight
- at room temperature.
- Store the DNA at 4°C. For long-term storage, store DNA at -20°C or -80°C.

Generation of transposase mRNA

For the experiments described, transposase mRNA was produced by reverse-transcription of the transposase coding sequence in the plasmid, OX3081 (Oxitec Ltd. UK).

Transposase mRNA was generated using mMESSAGE mMACHINE T7 Ultra and MEGAclear Kits (both from Applied Biosystems/Ambion, Texas, USA) with modifications to the manufacturers protocols.

Clean gloves, filter tips and dedicated 'RNase free' reagents were used throughout. All 1.5ml and 0.5ml microcentrifuge tubes used in this protocol are individually sealed tubes which are guaranteed RNase/DNase-free. (Safe-lock Tubes, Biopur standard, Eppendorf)

Linearisation of the plasmid (OX3081)

- Generate an endotoxin-free maxiprep of OX3081 plasmid to ensure starting material is of optimal quality
- Linearise 25µg of OX3081 plasmid by digestion with 5units of *Xba*I within a 100µl volume reaction and incubate at 37°C for one hour
- Run a (0.5µl) sample of the digest on a 1% TAE gel after one hour to check the degree of cutting by comparing linearised plasmid to uncut plasmid (allow the reaction to continue while the gel is running)
- When the plasmid is totally linearised, terminate the reaction according to mMessage protocol
    - I. Add 1/20 volume of 0.5M EDTA
    - II. Add 1/10 volume of 5M NH4OAc (as included in kit)
    - III. Add 2 volumes of ethanol (use RNase-free EtOh)
- Mixed the reaction well and chilled at -20°C overnight
- Pellet the DNA by centrifugation for 15 minutes at maximum speed
- Remove the supernatant, re-spin the tube and remove any traces of residual supernatant by means of a fine-tipped pipette
- Re-suspend the pellet in 25µl of water (nuclease-free) from the Ambion kit

- Assess the yield of the linearised DNA plasmid and store at -20°C prior to *in vitro* transcription of mRNA

Transcription Reaction

Manufacturer's instructions include a TurboDNase Step and Poly(A) tailing step. Both of these steps were not included in the protocol followed here as they are unnecessary. The coding region within the plasmid contains a polyadenylation coding sequence.

- Assemble the reaction in a 1.5ml microcentrifuge tube
- Vigorously vortex the 10xT7 reaction buffer and the T7 2xNTP/ARCA solution until both are completely in solution and spin briefly to ensure reagents are collected in bottom of the tube
- Add the components of the reaction in the order they are listed below

Amount	**Component**
to 20µl	Nuclease free water (use water supplied with Ambion kit)
10µl	T7 2xNTP/ARCA
2µl	10xT7 Reaction Buffer
1µg	Linear template DNA
2µl	T7 Enzyme mix

- Flick the tube was gently to mix then briefly spin to collect reaction mix at bottom of tube.
- Incubate the reaction for 2hours at 37°C
- Remove 2.4µl of the reaction to run on a RNase-free 1% TAE agarose gel to assess RNA quality. Remove a further 1µl of the reaction to run on a RNase-free 1% TAE-agarose gel to asses RNA quality after MEGAclear Clean-Up. Remove a fsecond 1µl sample to quantify using the NanoDrop (1 in 500µl dilution)

Recovery of the RNA

Follow Ambion MEGAclear kit protocol with following notes/steps

- Make up 1ml of fresh wash solution in a microcentrifuge tube (800µl Ethanol: 200µl Wash Conc.). Make the wash fresh each time, immediately prior to use
- Pre-heat a heating block at 65-70 °C for RNA elution step
- Bring the RNA sample up to 100µl volume with elution solution, then mix gently but thoroughly
- Add 350µl of Binding Solution Concentrate to the sample and again, mix gently by pipetting
- Add 250µl of 100% ethanol to the sample and mix gently by pipetting
- Apply the sample to the filter
- Insert a filter cartridge into 1 of the Collection/Elution tubes supplied
- Pipette the RNA mix onto the filter cartridge then centrifuge for 1min at 13,000rpm
- Discard the flow-through was discarded
- Apply 500µl of the fresh wash solution to the filter and centrifuge for approx. 50 seconds at 13,000rpm until it is eluted as before in the tube
- Repeat with a second 500µl aliquot of wash solution
- Discard the wash solution and spin the filter again for 1 minute to remove any last traces of wash solution
- Follow RNA elution Option 1 of the Ambion protocol
    - Place the filter cartridge into a new Collection/Elution tube
    - Apply 50µl of elution solution to the centre of the filter cartridge. Close the tube-cap and incubate in a heating block at 65-70°C for 10 minutes
    - Recover the eluted RNA by centrifuging for 1minute at room temperature at 13,000rpm

- Maximised RNA recovery by repeating the elution step with a second aliquot of 50µl of Elution Solution.. Collect the eluate was collected into the same tube

Precipitation of the RNA

- Pre-chill the centrifuge to 4°C
- Prepare 500µl of 70% ethanol (per reaction) and chill at -20°C
- Add 1:10 volume of 5M Ammonium Acetate (NH4Ac) to the purified RNA to the sample
- Add 2.5 volumes of 100% ethanol. Mix the sample well and incubate overnight at -20°C
- Spin the supernatant at maximum speed for 15 minutes in a pre-chilled 4°C centrifuge
- Carefully remove and discard the supernatant
- Wash the pellet with 500µl 70% fresh cold ethanol, centrifuge again and remove the 70% ethanol
- To remove the last traces of ethanol, quickly re-spin the tube and any remove any residual fluid with a fine-tipped pipette.
- Air-dry the pellet overnight at room temperature
- Re-suspend the pellet in 15µl RNase-free water (from Ambion kit).
- Remove 2µl for analysis:
    - Use 1ul for quantification by using NanoDrop.
    - Run a very clean TAE-RNA gel (Section 7.1.4) of 1µl of the sample. Run samples with Formaldehyde Load Dye in 3:1 ratio as specified in the mMessage mMachine protocol alongside any sample removed and stored from earlier in the protocol. Run gel at ~70mV for ~1 hour.

RNase-free agarose gels

When possible, all in vitro transcribed mRNA was checked on a 1% TAE agarose gel to check both quantity and quality of RNA produced. Quality of transcribed mRNA was of utmost importance given its downstream application within microinjection techniques. Therefore, 1% TAE-agarose gels were made following standard procedure but with several adaptations to ensure they remained as RNase-free as possible to avoid denaturing of RNA.

- Make up 1 x TAE using Molecular Grade water in individual RNase-free Falcon tubes
- Make up 1% agarose in RNase-free Falcon tubes using TAE made as above
- Clean the gel tank out by soaking in 1% SDS (made using Molecular Grade water). Soak combs and dams in 1% SDS as well
- After soaking for approx. 1 hour, rinse gel tank, combs and dams with 70% ethanol (made again using Molecular Grade water). Allow the tank to dry out prior to use.
- Load the gel and run as soon as possible after pouring and cooling

7.2. Appendix II: Solutions

10% Glucose solution for feeding mosquitoes

- Dissolve 100g glucose in 900 ml distilled water.
- Add streptomycin/penicillin (Sigma-Aldrich, Poole, UK) to a concentration of 0.28%.
- Adjust volume to 1L with distilled water.
- Filter sterilise before use.

Water-saturated Halocarbon Oil

- Approximately 10ml of Halocarbon oil 700 (Sigma-Aldrich Company Ltd, Dorset, UK) is contained in a sterile 30ml universal plastic tube with approximately 4ml of sterile water..
- The tube is inverted a number of times and then ready for use.

10x microinjection buffer

- 50mM KCl
- 1mM $NaPO_4$
- pH 7.2

Stock solution of Tetracycline water (3mg/ml)

- To 1L distilled water add:
  - 3g Tetracycline powder
- Mix until dissolved

- Aliquot into 50ml tubes and freeze (tetracycline degrades in sunlight so ensure powder and stock solutions are kept in the dark)

Tetracycline water for rearing mosquitoes (0.03mg/ml)

- Defrost 1 aliquot of stock tetracycline solution
- Dilute in a total of 5L of distilled water
- This will be enough for 5 standard rearing trays

Luria Bertani (LB) broth (1L)

- To 800ml of distilled water add:
    - 10g Bacto-tryptone.
    - 5g yeast extract.
    - 10g NaCl.
- Stir until the contents have dissolved entirely then adjust pH to 7.5 with NaOH.
- Adjust volume to 1L with distilled water.
- Sterilize by autoclaving.

LB Agar Recipe Luria Bertani (1L)

- To 800 ml distilled water add:
    - 10g Bacto-tryptone
    - 5g yeast extract
    - 10g NaCl
- Adjust pH to 7.5
- Add 15g Agar. Melt agar into solution in the microwave.
- Adjust volume to 1L with distilled (or milipore) H2O.

- Sterilize by autoclaving.
- Cool until warm to touch (approximately 50°C). At this point add antibiotics to the
- LB Agar.
- Add ampicillin to 100µg/ml concentration or kanamycin to 50µg/ml.

Orange loading dye, 6x

- 10mM Tris-HCl, pH7.5
- 50mM EDTA
- 10% Ficoll 400
- 0.4% OrangeG

10% SDS (1L)

- To 900ml distilled water add:
    - 100g SDS
    - Heat to 68°C
    - pH to 7.2 with HCl
- Adjust to 1L with distilled water

SOC broth (1L)

- Add the following to 900ml of distilled water.
    - 20g Bacto Tryptone.
    - 5g Bacto Yeast Extract.
    - 2ml of 5M NaCl.
    - 2.5ml of 1M KCl.
    - 10ml of 1M MgCl2.

  - 10ml of 1M MgSO4.
  - 20ml of 1M glucose.
- Adjust to 1L with distilled water. Sterilize by autoclaving

TAE 50x Buffer (1L)

- 242g Tris Base
- 57.1ml Glacial acetic acid
- 100ml 0.5M EDTA, pH8.0
- Adjust to pH7.2 and bring the final volume to 1 litre with distilled water

1x PBS buffer (1L)

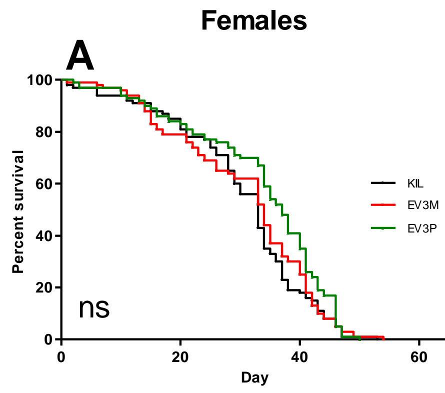
- Dissolve the following in 800ml distilled H_2O.
  - 8g of NaCl
  - 0.2g of KCl
  - 1.44g of Na_2HPO_4
  - 0.24g of KH_2PO_4
- Adjust pH to 7.4.
- Adjust volume to 1L with additional distilled H_2O.
- Sterilize by autoclaving

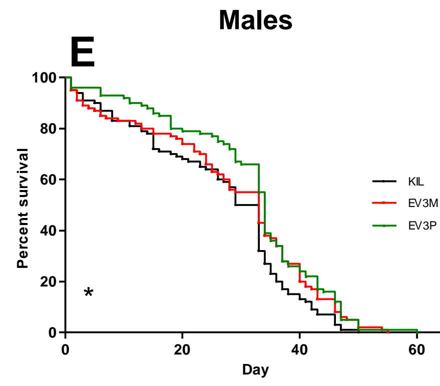
7.3. Appendix III: Kaplan Meier longevity curves illustrating longevity data for independent experiments 1-3

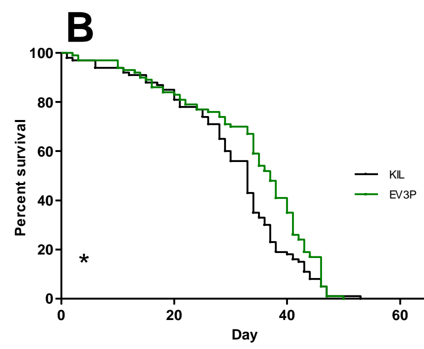
Graphs for experiments 1-3 follow on subsequent pages

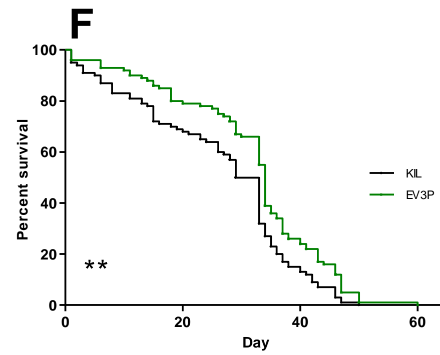
Females

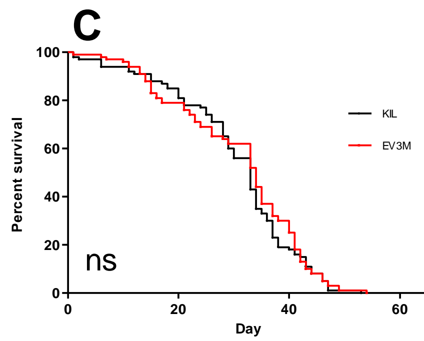
Males

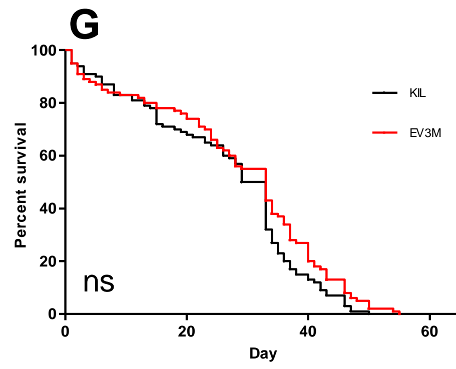


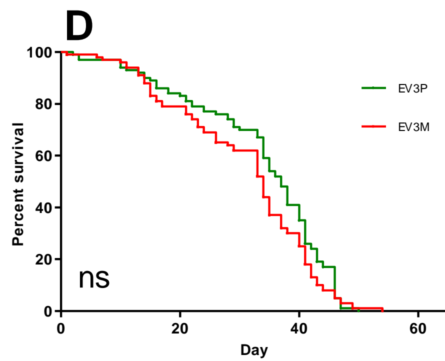












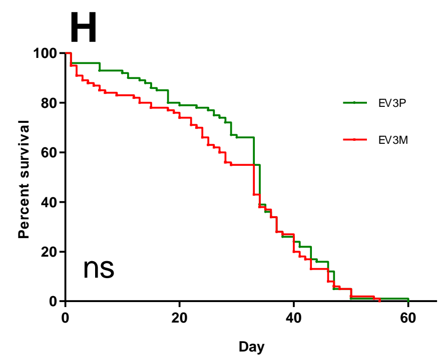
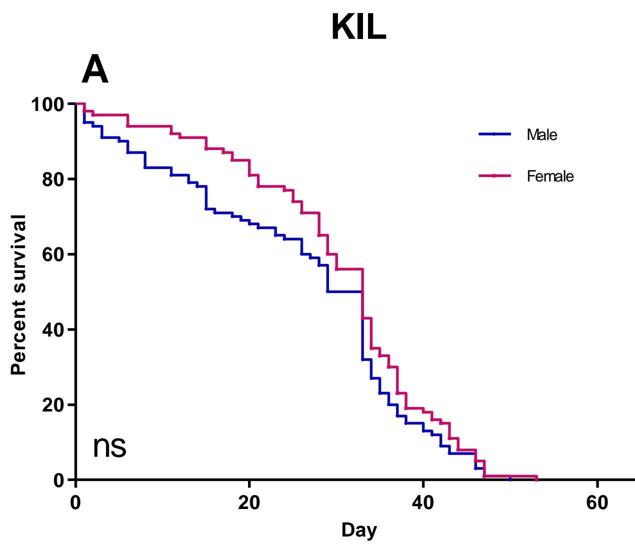


Figure 7.3.1 (previous page): Kaplan-Meier survival curves to compare longevity of the control (KIL) line with two hemizygous transgenic lines using data from Experiment 1

Graphs A-D show female survival data and graphs E-H show male survival data. Graphs A and E show survival curves for all three lines included in the experiment. Graphs B-D and F-H compare the survival curves for each combination of pairs of lines for female and male mosquitoes respectively.

All survival data was analysed using the log-rank (Mantel-Cox) test and significance levels are displayed on each graph (**, $p < 0.001$; *, $p < 0.05$; ns, not significant).


KIL
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Percent survival
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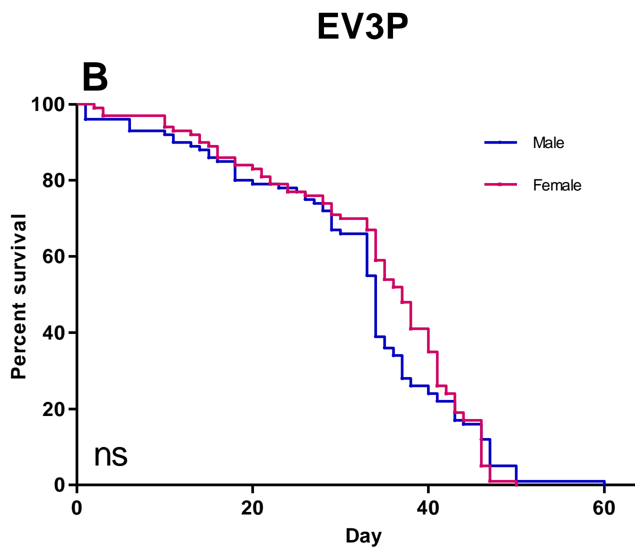
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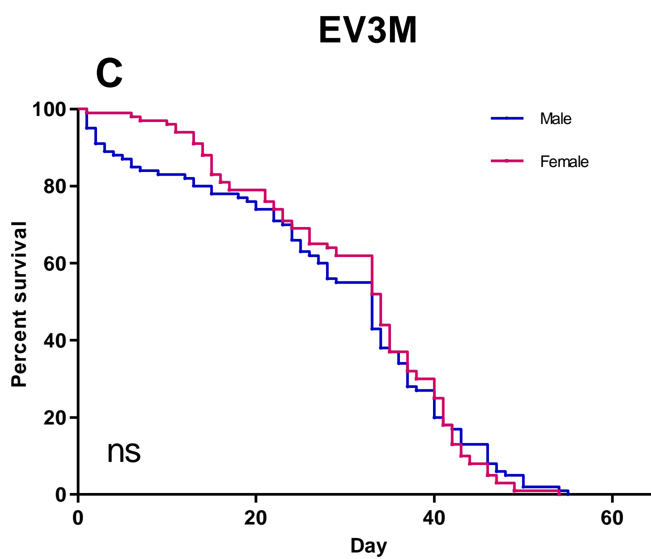
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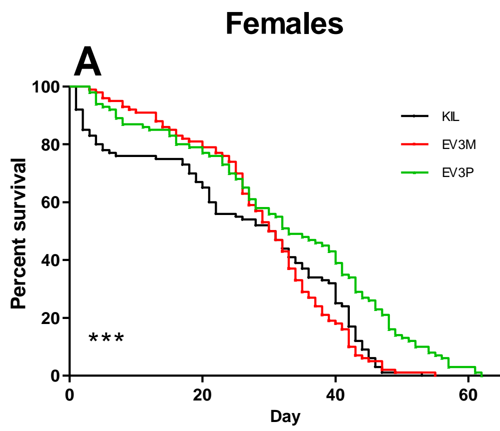
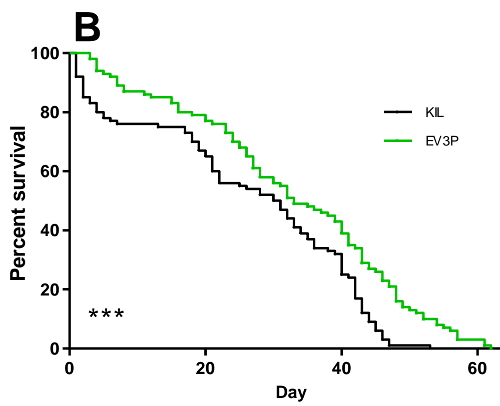
Figure 7.3.2 (previous page): Kaplan-Meier survival curves to compare longevity of males and females for each line using pooled data from Experiment 1

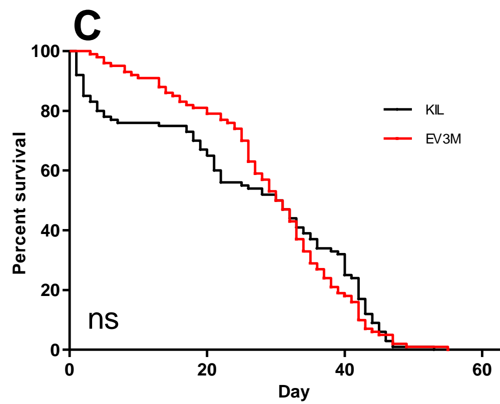
A) Survival curve comparing male and female longevity in the control (KIL) line; **B**) Survival curve comparing male and female longevity in the hemizygous transgenic EV3P line; **C**) Survival curve comparing male and female longevity in the hemizygous transgenic EV3M line.

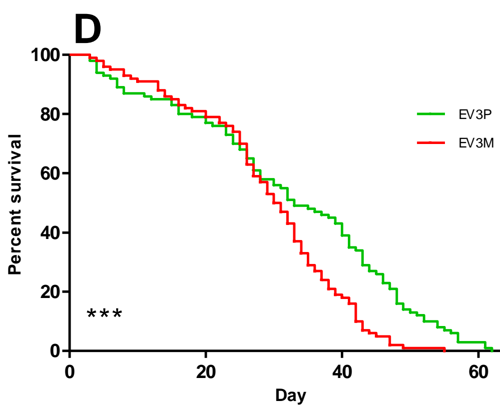
All survival data was analysed using the log-rank (Mantel-Cox) test and significance levels are displayed on each graph (ns, not significant).

Females

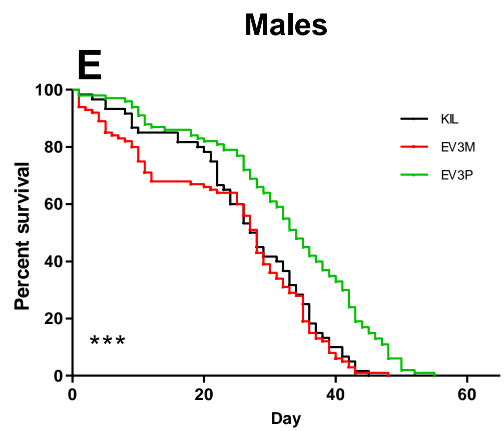


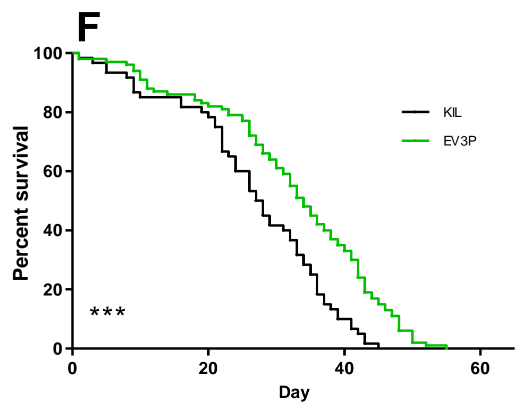


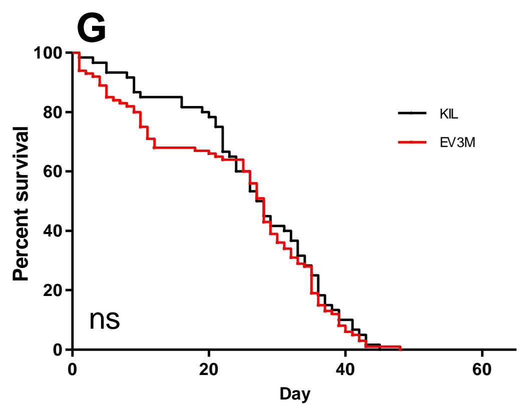




Males







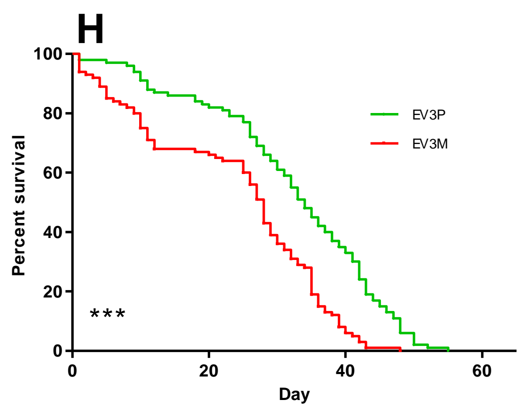
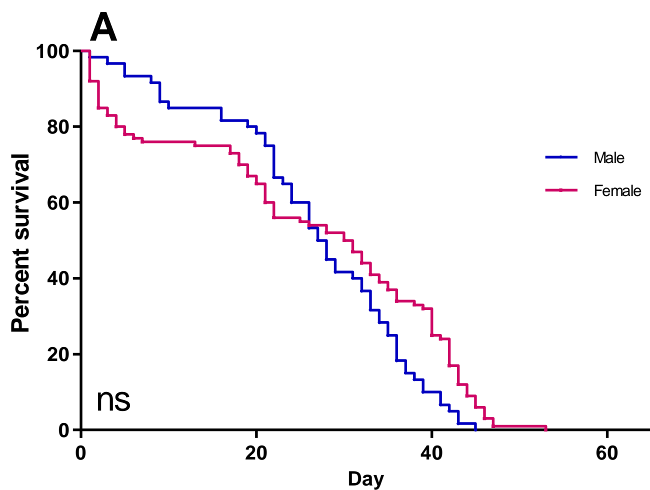


Figure 7.3.3 (previous page): Kaplan-Meier survival curves to compare longevity of the control (KIL) line with two hemizygous transgenic lines using data from Experiment 2

Graphs A-D show female survival data and graphs E-H show male survival data. Graphs A and E show survival curves for all three lines included in the experiment. Graphs B-D and F-H compare the survival curves for each combination of pairs of lines for female and male mosquitoes respectively.

All survival data was analysed using the log-rank (Mantel-Cox) test and significance levels are displayed on each graph (***, $p < 0.0001$; not significant).


KIL
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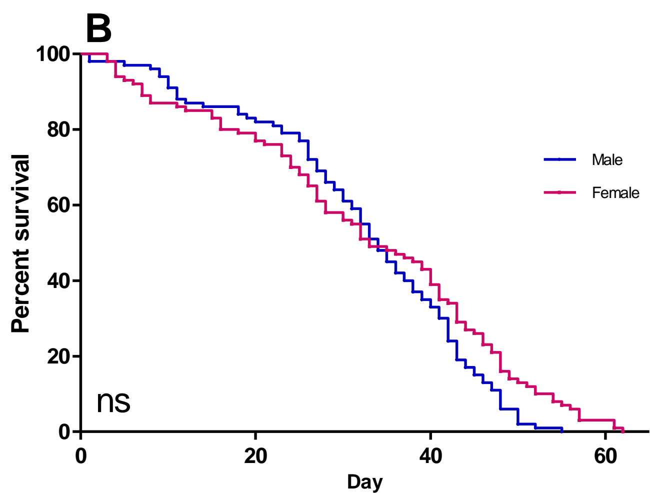
EV3P
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Male
Female

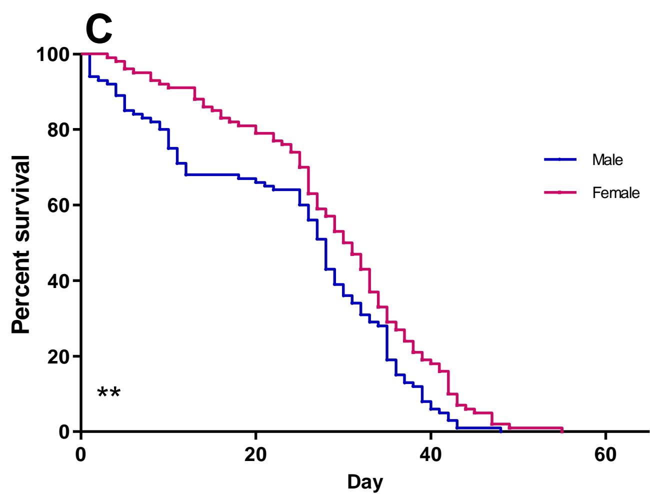
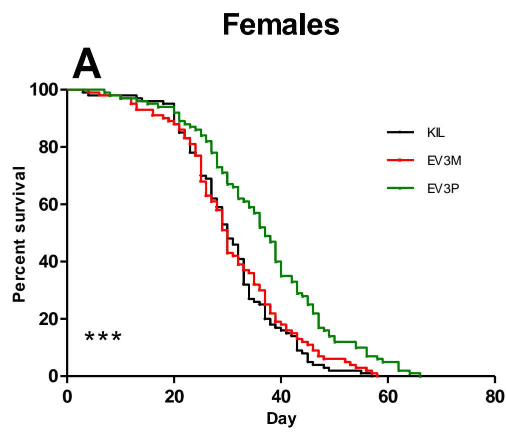

EV3M
C
Percent survival
100
80
60
40
20
0
**
0
20
40
60
Day
Male
Female

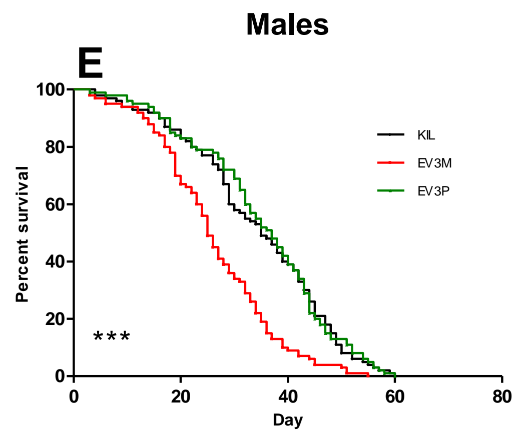
Figure 7.3.4 (previous page): Kaplan-Meier survival curves to compare longevity of males and females for each line using pooled data from Experiment 2

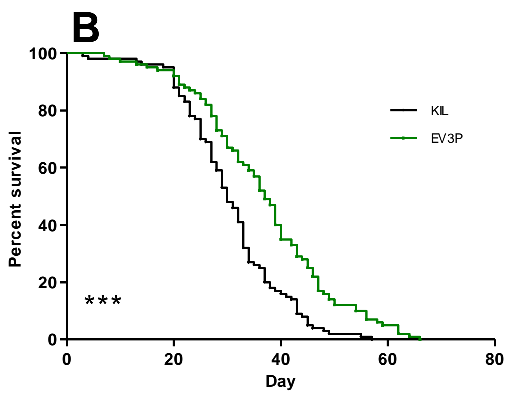
A) Survival curve comparing male and female longevity in the control (KIL) line; **B**) Survival curve comparing male and female longevity in the hemizygous transgenic EV3P line; **C**) Survival curve comparing male and female longevity in the hemizygous transgenic EV3M line.

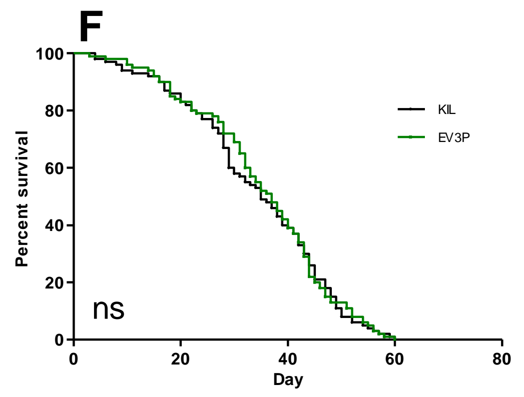
All survival data was analysed using the log-rank (Mantel-Cox) test and significance levels are displayed on each graph (**, $p < 0.001$; ns, not significant).

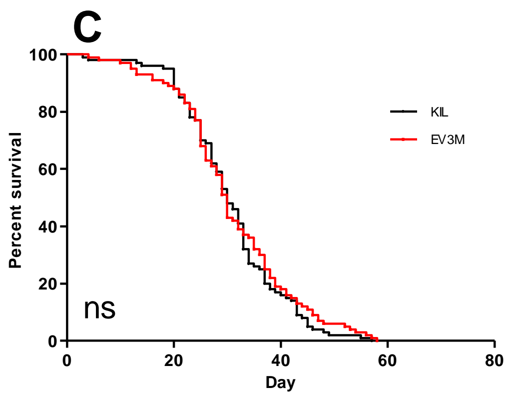
Females | Males

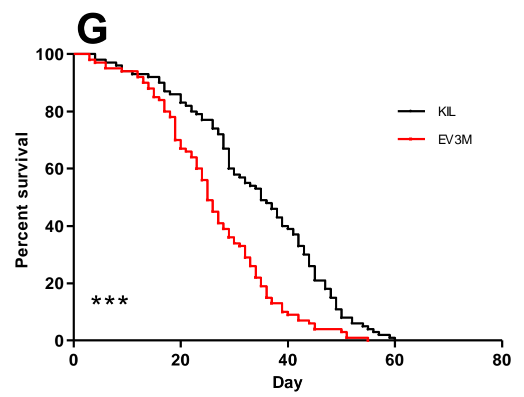


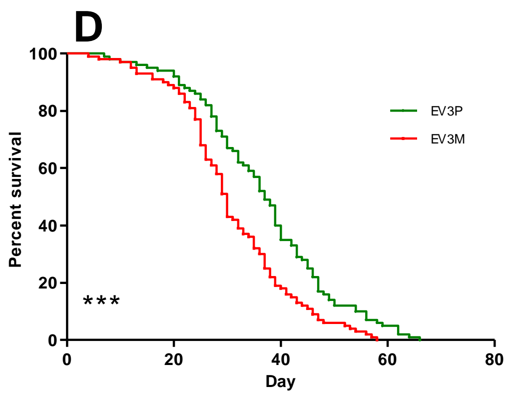












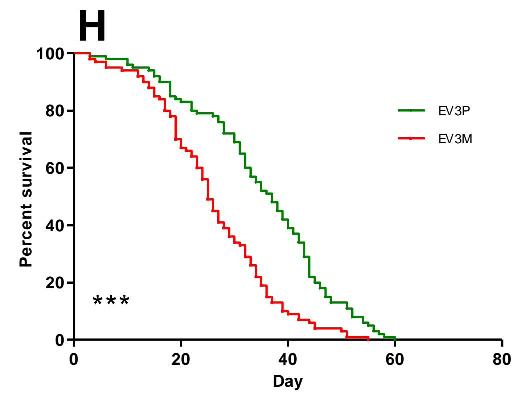
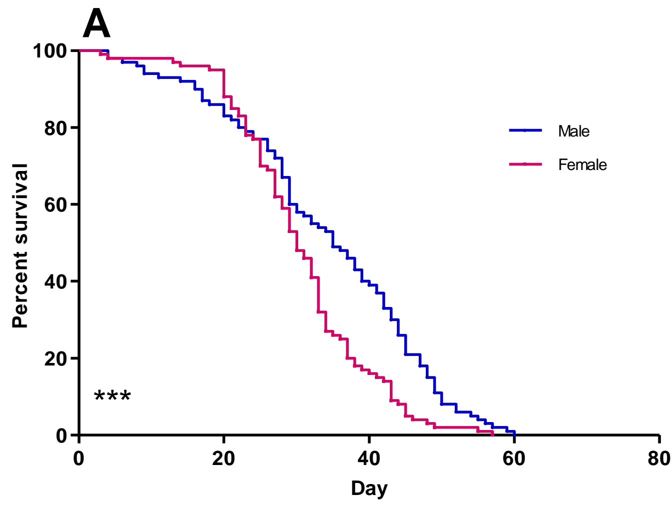
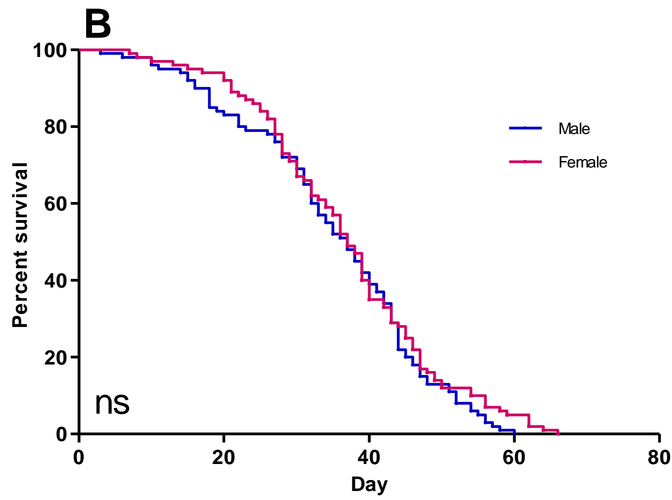


Figure 7.3.5 (previous page): Kaplan-Meier survival curves to compare longevity of the control (KIL) line with two hemizygous transgenic lines using data from Experiment 3

Graphs A-D show female survival data and graphs E-H show male survival data. Graphs A and E show survival curves for all three lines included in the experiment. Graphs B-D and F-H compare the survival curves for each combination of pairs of lines for female and male mosquitoes respectively.

All survival data was analysed using the log-rank (Mantel-Cox) test and significance levels are displayed on each graph (***, $p < 0.0001$; not significant).


KIL
A
100
80
60
40
20
0
Percent survival
***
0
20
40
60
80
Day
Male
Female


EV3P
B
100
80
60
40
20
0
Percent survival
ns
0
20
40
60
80
Day
Male
Female

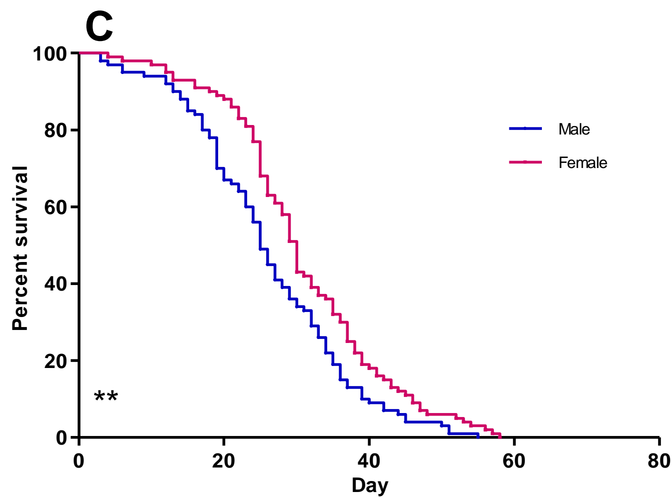

EV3M
C
100
80
60
40
20
0
Percent survival
**
0
20
40
60
80
Day
Male
Female

Figure 7.3.6 (previous page): Kaplan-Meier survival curves to compare longevity of males and females for each line using pooled data from Experiment 3

A) Survival curve comparing male and female longevity in the control (KIL) line; **B**) Survival curve comparing male and female longevity in the hemizygous transgenic EV3P line; **C**) Survival curve comparing male and female longevity in the hemizygous transgenic EV3M line.

All survival data was analysed using the log-rank (Mantel-Cox) test and significance levels are displayed on each graph (***, $p < 0.0001$; **, $p < 0.001$; ns, not significant).