Molecular genetic analysis of *Anopheles* mosquitoes when challenged by *Plasmodium* parasites

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Declaration

I declare that this PhD thesis is the product of my own work and that it has not been submitted before for any degree or examination at any other university. Wherever contributions of others were involved, every effort was made to indicate this clearly, with due references to the literature, and acknowledgements of collaborative research and discussion.

Signed: 

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Dedications

To my parents. Thanks for all you've done.

To my friends. Thanks for being there.

Ethical clearance

STRICTLY CONFIDENTIAL

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APPLICANT: PROF M. COETZEE

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PROJECT TITLE: MALARIAL INFECTIVITY EXPERIMENTS IN LABORATORY COLONIES OF *ANOPHELINE* VECTOR MOSQUITOES.

SPECIES	NUMBER	TYPE OF APPLICATION
MOUSE	420	EXPERIMENTAL

i) Approval is hereby given for the experiment/routine procedure described in the above application.

The use of these animals is subject to the National Code 1990 Guidelines as used by the NHLS AEC. If an application for a routine procedure then the recommended guide lines or SOP must be followed. It is limited to the procedure specified in the application form and to:

SIGNED---------- DATE: 15.03.2007
(Chairperson: Animal Ethics Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1) (C) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

SIGNED---------- DATE: 15.03.2007
(Registered Veterinarian)

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Abbreviations and acronyms

AMP	Antimicrobial peptides
CTL	C-type lectin
DDT	Dichloro-diphenyl-trichloroethane
GNBP	Gram-negative binding protein
IRS	Indoor residual spraying
IP	Inter-peritoneal/ly
JAK	Janus kinase
LRIM1	*Leucine-rich immunity protein 1*
MR4	Malaria Research and Reference Reagent Resource Centre
NF-κB	Nuclear Factor kappa B
NICD	National Institute for Communicable Diseases
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PGRP	Peptidoglycans recognition protein
PGRPLC	Peptidoglycans recognition protein long chain
PRR	Pathogen recognition receptor
PPO	Preprophenoloxidase
RBC	Red blood cell

SRPN2	Serine protease inhibitor 2
STAT	Signal-transducer and activator of transcription protein
TEP	Thioester-binding protein
VCRL	Vector control reference laboratory
WHO	World Health Organization

Related conference presentations and journal articles

International Congress of Parasitology (ICOPA) XII, Melbourne, Australia, 15-20 August 2010. Infection of *Anopheles* species with the rodent malaria parasite *Plasmodium berghei*. Oral presentation.

Wits Faculty of Health Sciences research day, 22 September 2010. Infection of *Anopheles* species with the rodent malaria parasite, *Plasmodium berghei*. Poster presentation.

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Abstract

Malaria is the most serious tropical infectious disease in humans, caused by parasites of the *Plasmodium* genus and transmitted by anopheline mosquitoes. The interaction between the parasites and vectors has become a focus for malaria research as it may present an alternative disease control method by enhancing anti-plasmodial factors within the mosquito to impede parasite development and transmission. *Anopheles gambiae* is the best studied African malaria vector and is often used with the murine malaria parasite, *Plasmodium berghei*, for investigating parasite-vector interactions in the laboratory setting. *Anopheles funestus* has not been studied and its interactions with *Plasmodium* were unknown, until now. Although the Vector Control Reference Laboratory routinely maintains *An. funestus* and a number of *An. gambiae* colonies, none have been infected with *Plasmodium* since their establishment. This study aimed to use *P. berghei* to determine the vectorial capacity of these colonies and to examine the involvement of the 2La paracentric chromosomal inversion and antimicrobial peptides during *Plasmodium* infection in *An. gambiae* and *An. funestus*, respectively.

Most of the *An. gambiae* complex colonies were susceptible to *P. berghei*, but the range of feeding and infection rates varied considerably. The infection rates for some of the older colonies were lower than previously documented. *Anopheles funestus* colonies were all viable

vectors and there was an inverse correlation between the insecticide resistance profile and parasite susceptibility. Increased detoxification enzyme activities may have been contributing to a greater degree of parasite elimination.

In *An. gambiae*, molecular karyotyping of the 2La inversion using PCR was validated against traditional cytogenetic techniques. The PCR was shown to be a reliable substitute for identifying the inversion. Using molecular karyotyping on 2La polymorphic colonies infected with *P. berghei*, it was found that infected females were more likely to carry the 2La inversion, indicating possible correlation between the inversion and susceptibility to parasites.

In *An. funestus*, the expression of antimicrobial pepetide genes during *P. berghei* infection was examined using real-time PCR. Although all three genes showed increased activity at certain points of the infection, none displayed significant anti-plasmodial properties. However, in the less parasite susceptible strain, expression of two genes was higher towards the end of the infection, which was not observed in the other strains. It is possible that the co-expression of both peptides has led to a decrease in parasite load in late infection, but given the multi-factorial nature of the parasite-vector interaction, further investigation is required.

Chapter 1: General introduction

1.1 Introduction

Malaria, caused by parasitic protozoans of the *Plasmodium* genus and transmitted by mosquitoes belonging to the *Anopheles* genus, is the most important vector-borne infectious disease in humans. The disease affects up to 40% of the world's population, and kills up to 700,000 people a year, most of which are children below the age of five (WHO, 2011). Interventions via anti-malaria drugs and vector control are feasible means to reduce, or possibly even eliminate malaria, particularly if multiple deterrents are utilized.

The term malaria is derived from the Italian "mal'aria", as the disease was originally associated with the "bad air", "evil air" or "corrupted air" of the marshy areas. The final form of the word currently used to describe the disease was only first seen in 1838 (Neghina *et al*., 2010). It is a disease that has plagued humans since antiquity under various names, with records from Ancient Egypt intimating its presence (Garnham, 1966; Neghina *et al*., 2010). The disease was prevalent not only in the tropics and sub-tropical regions worldwide, but was also found in temperate areas, including parts of North America, Europe and Asia. There are records of malaria cases as far north as Netherlands, Siberia and Manchuria (Faust *et al*., 1970). Little was known about the disease or how to treat it effectively for centuries. The first viable anti-malarial in the form of cinchona bark was found in South America and brought to Europe in the 1600s; the active compound acting against malaria in the bark, quinine, was isolated in 1820 by French chemists (Garnham, 1966).

The real breakthrough with the characterization of the disease came in the late 19th century. First, the malaria parasite was discovered by Laveran in Algeria in 1880, although this wasn't generally accepted until 1885 (Belding, 1965). During the next five years, Italian researchers identified a number of parasite species. The mosquito was eventually confirmed as the vector for malaria parasites by Ross in India in 1897. Using avian malaria, he proved that transmission of the parasite occurred through the bite of infected mosquitoes. Around the same time, and independently of Ross, Grassi and Bignami in Italy were able to successfully infect a human volunteer with malaria parasites by allowing mosquitoes to feed on the person, demonstrating the mosquitoes' involvement in malaria transmission (Garnham, 1966). In 1907, Ross further confirmed that only anopheline mosquitoes are human malarial vectors, and that the parasites are injected into the host as the mosquito is feeding.

Understanding the mosquitoes' participation in the spread of malaria led to increased public health and sanitation awareness, which reduced malaria transmission in many areas, but did not eliminate the disease. The first ever documented use of insecticides as a control agent against malaria was in South Africa, by Park-Ross (1936) and de Meillon (1936). Pyrethrum, a botanical insecticide extracted from chrysanthemum flowers, first tested by Giemsa (Harrison, 1978) was used to great effect in the field. Park-Ross was primarily concerned with monitoring the decline of malaria cases, while de Meillon demonstrated the viability of indoor spraying to eliminate endophilic (preferring to rest indoors) malarial vectors. Around the same time, the first effective artificial anti-malaria drug, chloroquine, was synthesized in Germany, but its true effectiveness was only realized towards the end of World War II (Harrison, 1978). There was a dramatic upswing in development of viable insecticides during and after World War II (Asa & Clarke, 1961). The most important of these was dichloro-diphenyl-trichloroethane, more popularly known as DDT, which is a chlorinated hydrocarbon. Two

other classes of insecticides also arose during this period, the first was organophosphates (OPs), discovered in the 1930s, and methylcarbamates (MCs) in the 1940s (Casida & Quistad, 1998).

With DDT as a highly effective insecticide, comprehensive indoor house-spraying programs were put in place during World War II by various countries in the Mediterranean region and on islands in the Pacific to try and reduce, if not eliminate malaria. In 1955, the World Health Organization (WHO) launched the Global Malaria Eradication Programme, which aimed to interrupt malaria transmission by using DDT as the sole indoor residual spray (IRS) component (International Development Advisory Board, 1956). The initiative was immensely successful in Latin America, the Caribbean, the Balkans, and parts of the Pacific Rim region (Trigg & Kondrachine, 1998; WHO, 2009). By this point, the search for effective anti-malarial compounds to replace quinine had finally led to the testing and proof of chloroquine (initially called Resochin by its creator) as being an effective drug, and it was approved as the anti-malarial for general public consumption (Coatney, 1963). The combination of the new anti-malarial drug, insecticides and better understanding of the mosquitoes, more or less brought about the current malarial distribution: The United States of America has been free of malaria since the late 1950s; Europe eliminated the last vestige of the disease in the 1960s; parts of Asia, typically islands or archipelagos (Singapore and Taiwan in South-east/East Asia, for example) were also successful in eliminating it (TAMRI & WHO malaria team in Taiwan, 1958; WHO, 2009). While isolated landmasses can be an advantage for the elimination of malaria, it is not an automatic guarantee that this will happen, as islands like Sri Lanka and Madagascar have returned to being stable transmission areas after initial successes. Malaria prevalences on Zanzibar and Pemba islands were greatly reduced for at least 10 years from the late 1950s, with no malaria related deaths reported (WHO, 1976), but when vector control

stopped, the disease reappeared and has persisted to this day. The islands of Mauritius and Reunion, on the other hand, managed to eliminate most of the vector species and this has in effect eliminated malaria from the islands (WHO, 1976). The last class of effective insecticides, the pyrethroids, were introduced in the 1980s. This is the synthetic analogue for pyrethrin, the active component in pyrethrum, thus providing similar benefits and disadvantages as the natural compound, but can now be mass produced with an effective residual life of up to six months (WHO, 2009).

The bulk of malaria transmission was, and still remains, in the Afrotropical Region, where it is considered to be endemic, and in many parts holo- or hyper-endemic (WHO, 2009, 2012). The endemicity of a region is defined by the combined results of 1) the resistance of the human population to malaria (taking into account spleen rate, parasite rate), 2) the capacity of the vector/s to infect (including bloodmeal preference, sporozoite rate, longevity, adult population density), and 3) the capacity of the terrain and climate to provide a vector population above critical density (WHO, 1963). The continent was actually excluded from the Global Malaria Eradication program in 1955 because it was thought that very little success would be achieved in interrupting malaria transmission - Africa was “too difficult” (WHO, 1976). The only successes in control via insecticide spraying were at the edges of tropical Africa where malaria was unstable (South Africa, Swaziland, Zimbabwe and the islands of Madagascar, Mauritius, Reunion; WHO, 1976).

South Africa is one of the few African countries where malaria is not stably transmitted but still has transmission hotspots, particularly on the north-eastern borders (South African Department of Health, unpublished data). Malaria transmission also remains stable in forested areas of Latin America, South and South-east Asia (WHO, 2009). Areas where malaria has

been eliminated, but are still climatically hospitable to the vector mosquitoes, may be exposed to sporadic outbreaks primarily due to infected individuals or infective mosquitoes coming in from endemic regions (Signorelli *et al*., 1990; Marty *et al*., 1992; Layton *et al*., 1993; Muentener *et al*., 1999).

Currently, the elimination of malaria from the Afrotropical Region is not possible for several reasons. Implementation of control programs in Africa is often difficult due politico-social instabilities, a lack of will to sustain the policies correctly and for a sufficiently long period, or simply the wrong control strategy being used (WHO, 2007). The situation is exacerbated if control programs are initiated and then abandoned, as the exposure of the parasites or mosquitoes to drugs and insecticides without continuance can easily lead to resistance, which will leave future control programs worse off (Bruce-Chwatt, 1978). The resistance problem is further hampered by the lack of new, effective insecticide and anti-malarial drugs. Since the 1960s, field collections in various parts of Africa have shown that insecticide resistance in anopheline mosquitoes is prevalent throughout the continent, and has increased in more recent times (WHO technical report 655, 1980; Ranson *et al*., 2009) making it a challenging task to control the vectors using insecticides alone (Hargreaves *et al*., 2000; Okoye *et al*., 2008a; Morgan *et al*., 2010 and Djouka *et al*., 2011 are just a few examples detailing insecticide resistance in Africa).

1.2 The malaria parasite

Plasmodium parasites require two hosts in order to complete their lifecycle – a vertebrate host and a mosquito vector. In the first instance, there is a wide range of vertebrates that different *Plasmodium* species have adapted to, ranging from reptiles and birds, to mammals (Garnham, 1966). Despite the diverse evolutionary paths that the parasites have taken in their choices of

vertebrate hosts, the infection process remains remarkably similar throughout the genus. Of the more than 100 known *Plasmodium* species, four are commonly found to infect humans under natural conditions: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (Garnham, 1966). *Plasmodium vivax*, *P. ovale*, and *P. malariae*, are in the subgenus *Plasmodium*, while *P. falciparum* is in the subgenus *Laverania* together with a few malaria species for non-human primates (Kreier & Baker 1987). A number of primate and monkey malaria parasites have also been shown to be able to infect humans experimentally (Garnham, 1966), but in most instances, no naturally occurring cases have been found. The one exception is *P. knowlesi*, a primate parasite in South-East Asia, which seems to be evolving through zoonosis and has become the fifth human malaria parasite (Singh *et al*., 2004; White, 2008).

Of the four human *Plasmodium* species present in Africa, *P. falciparum* is the most dangerous. Infected erythrocytes can block capillaries and if this occurs in the brain, it leads to cerebral malaria which is often fatal, particularly in infants and pregnant women (WHO, 2012). Severe dysfunction of other vital organs including lungs, kidneys and liver is also possible (Carter & Mendi, 2005). In addition, *P. falciparum* infection can often lead to severe anaemia which can also be lethal. The species accounts for the vast majority (86%) of malaria-related deaths in children below the age of five in Africa (WHO, 2012). *Plasmodium vivax*, the most globally widespread of the four species, is found in the tropics as well as temperate regions of the world (Carter & Mendis, 2005). It can cause severe anaemia, but mild anaemia is the more common symptom, so fatalities are rare compared with *P*. *falciparum*. *Plasmodium ovale* is the rarest of the four, mainly found in Central and West Africa as well as some islands of the Western Pacific (Lysenko & Beljaev, 1969; Collins & Jeffrey, 2005), as opposed to *Plasmodium malariae*, which also has a world-wide distribution in the tropics and sub-tropics, albeit at lower frequencies than *P. vivax*. Deaths from *P. ovale* and *P. malariae* infections are

also rare (Collins & Paskewitz, 1995). Although fatalities are typically associated with *P. falciparum*, the other three species have the ability to cause long bouts of infection, or recurring relapses in the long term. *Plasmodium malariae* has been known to have long-lasting blood stage infections, which can persist asymptomatically for decades (Carter & Mendis, 2005; Tuteja, 2007), while *P. vivax* and *P. ovale* have dormant liver stages that can last for weeks, up to years, before re-emerging and causing a relapse of infection (Chin & Coatney, 1971; Collins & Paskewitz, 1995). In such instances, if the appropriate vector is in the vicinity and feeds on the relapsing individual, it can lead to unexpected minor outbreaks of malaria in areas otherwise no longer associated with the disease (Garnham, 1966; Carter & Mendis, 2005).

1.3 The *Plasmodium* lifecycle

It is essential for the *Plasmodium* species' survival to have access to both the vector and host, not only because the vector is needed to transmit the parasite to new hosts, but important life stages occur in both vector and host, which cannot be bypassed. In the vertebrates, the parasite has an emphasis on asexual reproduction, with precursor cells for sexual reproduction; all the life stages here are haploid (Burkot & Graves, 2000). In the mosquito, sexual reproduction takes place, which then switches back to asexual reproduction to increase the number of parasites. Interestingly, the mosquito is actually considered the definitive host for the parasite, as sexual reproduction occurs in the insect (Garnham, 1966). Alternatively, the lifecycle can be divided into three stages of cell division and development: merogony/schizogony (asexual), gametogony (sexual), and sporogony (asexual) (Burkot & Graves, 2000).

An infected adult female mosquito releases sporozoites from her salivary glands into the

bloodstream of the vertebrate host upon taking a bloodmeal. The sporozoites use the host's circulatory system to get to the liver, which can happen within a space of a few minutes (Shin *et al*., 1982). On reaching the liver, the sporozoites may migrate through several hepatocytes before finding the optimal host cell (Mota & Rodriguez, 2001), where they undergo asexual replication known as exo-erythrocytic schizogony, or in certain species, enter a dormant stage. The replication takes 9 - 16 days, whereupon merozoites are produced and released into the bloodstream. The merozoites invade the erythrocytes, and undergo asexual reproduction once more, going through distinct stages of development in the red blood cells (RBCs) that can be observed using light microscopy. At the end of intra-erythrocytic replication, mature schizonts containing around 20 merozoites are produced, and they are released into the bloodstream with the lysis of the original RBCs, to infect more erythrocytes. This mass, synchronized release of the merozoites is what leads to the chills and fever associated with malaria, and can also lead to severe anaemia. Depending on the species, the erythrocytic infection cycle repeats every 48 hours (*P. falciparum*, *P. ovale* and *P. vivax*) or 72 hours (*P. malariae*), which gave rise to the terms tertian and quartan malaria, respectively (Garnham, 1966). At some point in this invasive stage, typically when the reproduction has slowed, gametocytes, the precursor to the sexual gametes, are produced (Brey, 2003). The male and female gametocytes are morphologically different; they are inert in the bloodstream and remain so until taken up by an anopheline mosquito female during feeding.

When the gametocytes are taken up by the mosquito during feeding, the sexual stage of the parasite development occurs very quickly in the mosquito midgut. This is triggered by changes in temperature, pH (Billker *et al*., 1997) and presence of xanthurenic acid (Billker *et al*., 1998). The male gametocytes exflagellate, releasing 8 flagellated male microgametes that will seek out the sessile female macrogametes to undergo sexual reproduction. The production

of the microgamete occurs within a space of less than 10 minutes, where three endomitotic cycles are completed along with the assembly of the axoneme (Billker *et al*., 2004). Successful fertilization of the macrogametes by the exflagellated microgametes results in another transformation of the parasite, producing motile ookinetes.

The ookinete has to cross the physical barriers posed by the chitinous peritrophic membrane and the midgut epithelium in the mosquito. Once the ookinete is able to lodge itself in the basal lamina of the midgut, it develops into a sessile oocyst. In the case of human malaria, oocysts can be detected three to four days after the ingestion of the bloodmeal (the Malaria Research and Reference Reagent Resource Centre (MR4) methods in *Anopheles* research laboratory manual, 2007). The oocyst is now safe from elimination and begins to undergo multiple asexual replication processes to produce sporozoites. The number of oocysts at this stage can vary significantly even in natural vector-parasite pairing, ranging from single digits to over 60 oocysts in one individual female (Shute *et al*., 1965). Each oocyst is capable of releasing thousands of sporozoites upon maturation. The number of sporozoites released per oocyst was initially estimated at around 1,000 by Ross (1910) but higher numbers have been reported, typically around 10,000 per oocyst (Pringle, 1965). Therefore, even if only a single oocyst survives development through the midgut wall, the mosquito can become infective, as the amplification factor in oocyst-sporozoite transition is 2,000 - 8,000 fold (Christophides *et al*., 2004). The length of time taken to complete sporozoite development is dependent on the species of mosquito as well as the climate. Sporozoites may be released into the haemolymph as early as one week, or any time up to two weeks, post-infected bloodmeal (Collins & Paskewitz, 1995)

Upon release from the oocysts, the sporozoites have to make their way into the salivary

glands of the mosquito. The mosquito salivary glands are paired organs located in the anterior end of the thorax. In anophelines, each gland is formed by two similarly constructed lateral lobes, with a shorter, wider medial lobe. The medial lobe's cul-de-sac is made up of large secretory cells, and a narrow channel of non-secretory cells connects it to the other lobes (Wright, 1969). The lateral lobes have a proximal and distal region, where the distal region is made up of secretory cells similar to those of the medial lobe, and it is in here where the sporozoites congregate. It still needs to be fully elucidated whether the sporozoites actively move toward the salivary glands by chemotaxis (Akaki & Dvorak, 2005) or are passively carried by the haemolymph circulation (Rodriguez & Hernández-Hernández, 2004), but there is evidence supporting both mechanisms. Upon arrival at the salivary glands, the sporozoites gain a parasitophorous vacuole by hijacking part of the host cell plasmalemma. The sporozoites then penetrate the gland cells. Once inside, they lose the vacuole, and create another one to break out of the cells and into the secretory cavity. Inside the cavity, the second vacuole disintegrates, and the sporozoites accumulate as tightly packed bundles (Rodriguez & Hernández-Hernández, 2004). The salivary glands typically carry anywhere between 10^2 - 10^6 sporozoites, depending on the species, although sporozoites numbering in the hundreds of thousands have been found (Shute, 1945; Beier *et al*., 1991a) Although in most feeding events less than 25 sporozoites pass into the host, the mosquito can release up to 300 sporozoites (Rosenberg *et al*., 1990; Beier *et al*., 1991; Li *et al*., 1992). The number of sporozoites released has been suggested to be an indication of the severity of the impending infection (Mcgregor, 1965), but there is evidence contrary to this (Sinden *et al*., 2007). In addition, infected mosquitoes have been shown to be infective more than 70 days post-infected bloodmeal (James, 1926; Shute, 1945), which is already considerably beyond the average lifespan of the adult female. Therefore the mosquito can be considered to be permanently infective should the parasite make its way into the salivary glands.

While the various life stages of the parasites were fairly well established soon after Ross' discovery – guides for malarial study noting the lifecycle have been in print since the early 20th century (Stephens & Christophers, 1908) – actual molecular mechanisms remain largely unclear. For example, details of the invasion mechanisms in both vector (the midgut and salivary gland invasion) and the host (the hepatocytes and erythrocytes) are not fully elucidated. Better understanding of interactions at these stages may provide possible points of intervention for novel strategies to disrupt malaria transmission.

1.4 The murine malaria parasite, *P. berghei* and others

The rodent infection system is important in malaria research for a number of reasons. The progression of the murine *Plasmodium* is comparable to the human malaria and uses the same genus of mosquitoes as vectors, which makes them favourable comparative models. Although one could argue that *in vivo* primate malaria models may provide more suitable comparisons, the practicalities of cost, time, space and expertise needed to maintain primates, as well as the ethical considerations, makes them far less attractive. In contrast, rodent malaria maintainance is relatively easy and techniques for synchronising and enhancing infection are well described (Mons & Sinden, 1990). When compared to using human malaria species for experimentation, rodent *Plasmodium* is safer as it cannot be transmitted to any other mammals (Brey, 1999), thus alleviating significant biosafety concerns. It also provides a complete infection system, where it is possible to take the parasite through both host and vector *in vivo*. This is obviously not possible using human malaria parasites. Such apparent obstacles have led to innovative infection systems such as using *in vitro* hepatocyte models (Sacci *et al*., 1992), or using SCID or recombinant mice to develop humanized mouse models (Morosan *et al*., 2006). These systems would be useful to test the efficacy of vaccines and anti-malarials, but still cannot

compare to a complete *in vivo* system in other respects.

The first rodent malaria parasite, *P. berghei*, was surprisingly only described some decades after the discovery of the human and avian parasites (Vincke & Lips, 1948). Three other murine parasites were found subsequently: *P. vinckei* (Rodhain, 1952), *P. chabaudi* (Landau, 1965) and *P. yoelii* (Landau & Chaubaud, 1965). A number of strains and sub-species have also been isolated for these species (for review of this, see Garnham 1966; Landau & Boulard, 1978). Prior to the discovery of murine parasites, avian *Plasmodium* parasites were used extensively to study the parasite biology and for anti-malarial treatments. However, the avian *Plasmodium* species infection process is somewhat dissimilar to those of mammalian malarias, as they have prominent exo-erythocytic development stages in macrophages and endothelial cells of organs, whereas in mammal *Plasmodium* species, exo-erythrocytic forms are less obvious (Garnham, 1966). This is particularly true in *P. falciparum*, and is the main reason that it is in a separate subgenus, *Laverania*, and not the subgenus *Plasmodium*. The vectors of avian malaria are also different (*Aedes* and *Culex* mosquitoes), while it is the same genus for all mammalian malarias (*Anopheles* species) (Garnham, 1966).

Plasmodium berghei was discovered in the Congo, although Vincke may have already observed the parasite in its natural vector a few years earlier (van den Berghe, 1954; Bruce-Chwatt, 1978). *Plasmodium berghei*'s natural habitat is highly localized, restricted to the gallery forests in Katanga between 1,000 - 1,700 m above sea level. Different strains have been isolated from nearby localities with similar biotopes, but the parasite has never been found to occur naturally outside of Central Africa (Landau & Boulard, 1978). The parasite's natural host is a number of sylvatic rodents that live in the forest, primarily tree rats of the *Thamnomys* genus. It is transmitted solely by *Anopheles dureni* in nature, even though there

are a number of other anopheline species in sympatry (Garnham, 1966; Killick-Kendrick, 1978). There has been no success in colonizing *An. dureni* but fortunately, the rodent parasite has been found to be compatible with other mosquito species (Yoeli, 1965).

Early attempts to infect other mosquito species aside from *An. dureni* consistently ended in failure. While oocysts were often detected, the sporozoites were not. When sporozoites were seen, these were often few in number and highly sporadic in the infected cohort (Box *et al*., 1953; Bray, 1954). The inability to breed both the natural vector (which is still the case) and vertebrate host in captivity led to an impasse in establishing rodent malaria as a viable laboratory model for over a decade despite its promise. This was eventually resolved when Yoeli discovered it is essential to maintain the infected mosquitoes at 19 - 21°C, which is the ambient temperature in the gallery forests (Yoeli *et al*., 1964; Vanderberg & Yoeli, 1965, 1966). The revelation led to successful infection of *An. quadrimaculatus* and other anophelines, thus bypassing the need for *An. dureni* (Yoeli, 1965). The parasite was also tested on a large variety of rodent species as possible hosts, and attained successful cyclical transmission in a number of species (Bruce-Chwatt, 1978). To date, almost all common laboratory mouse strains are susceptible to *P. berghei* and the infection is typically fatal. Hamsters are viable options as well, particularly for gametocytes (Sinden *et al*., 2002). Some rat strains are susceptible and usually survive the infection, while guinea pigs will fight the infection off and rarely show symptoms of infection. Voles, adult rabbits and North American gerbils are also resistant to the parasites (Belding, 1965). There is a proviso for successful infection of the rats and mice – the rodents need to be young adolescents whose immune system hasn't fully developed (Sinden, 2002). While the adult mice will eventually succumb to the infection if the initial parasitaemia is high enough, the initial time to first detection of parasite can vary, which would make it difficult to plan for experiments.

Although all four murine parasites have been used in research, *P. berghei* is the most popular choice by far and research using *P. chabaudi*, *P. vinckei* and *P. yoelii* are greatly in the minority (Sinden, 2002). The infection in rodents proceeds very similarly to that observed in human malaria, just a little faster as observed by Yoeli (1965). The mice are infective to mosquitoes three to five days after initial exposure to the parasite, and depending on the strains used, as well as the initial level of parasitaemia used for infection, the mice will succumb to the parasites any time between a week to 15 days after infection.

Compared to the four human malaria parasites, *P. berghei* pathogenesis is most similar to that of *P. vivax*. However, mice typically do not survive the infection, while humans rarely succumb to *vivax* infections, so such comparisons are not precise. With regard to the genetic makeup, the *Plasmodium* genus has fairly conserved genomes, with approximately 3,900 orthologous genes (accounting for around 26% of the *Plasmodium* genome) (Aguilar *et al*., 2005). Therefore, alternative *Plasmodium* species infections are still viable and can produce results that, to some degree, reflect the processes in *P. falciparum* infection. There are also differences in the physiological response of *An. gambiae* to *P. berghei* and *P. falciparum.* Development of *P. berghei* ookinetes can involve a large number (~50), whereas rarely more than a handful of *P. falciparum* ookinetes survive in the *An. gambiae* midgut (Aguilar *et al*., 2005). The midgut invasion pathways also seem to differ, with *P. falciparum* using an intercellular route, as opposed to *P. berghei*'s intracellular approach (Tahar *et al*., 2002). Expression of the mosquito genes during parasite infection also varies, with *P. berghei* triggering three times as many *An. gambiae* midgut genes compared to *P. falciparum* (Dong *et al*., 2006).

Taking these points into consideration, it may be easy to disregard the *P. berghei*-anopheline model, but the similarities and differences in responses provide an excellent gateway for preliminary studies. The use of unnatural vector-parasite pairing is useful for observing response mechanisms otherwise not seen in normal interactions, such as melanotic encapsulation (Collins *et al*., 1986), and parasite lysis (Vernick *et al*., 1995). By comparing these results to field studies and data from natural pairings, it would provide a better picture of the infection and may provide alternative options for eliminating the parasite-vector interaction (Brey, 1999).

1.5 The vectors - anopheline mosquitoes

Mosquitoes are invertebrate arthropods classified under the Order Diptera, Family Culicidae. They are efficient vectors of etiological agents for a number of diseases including malaria, filariasis, yellow fever, dengue and other arboviruses (Gillies & de Meillon, 1968; Kettle, 1984). Female mosquitoes are haematophagous and most species are anautogenous, requiring nutrients from vertebrate blood for successful egg development.

Human malaria can only be spread by mosquitoes belonging to the Genus *Anopheles*. There are over 420 *Anopheles* species known worldwide, of which about 60 are natural malaria vectors but only half of these are considered to be medically important (Service, 1993). In the Afrotropical Region, malaria vectors are dominated by four species: *An. gambiae sensu stricto* (*s.s.*), *An. coluzzii, An. arabiensis* and *An. funestus*. *Anopheles gambiae s.s.* (hereafter referred to as *An. gambiae*), *An. coluzzii* and *An. arabiensis* belong to the *An. gambiae* complex, a group of eight recognized sibling species that are morphologically identical to each other (Gillies & Coetzee, 1987; Coetzee *et al.*, 2013). *Anopheles funestus* belongs to, and is the nominal member of another sibling group, which is not quite as morphologically

obscure as the *An. gambiae* complex (Gillies & de Meillon, 1968).

1.5.1 The *An. gambiae* complex

The eight currently recognized species of the *An. gambiae* complex are widespread in Africa and the adjacent islands as well as nearby regions of the Arabian Peninsula. These species were initially considered to be a single species, *Anopheles gambiae*, when it was first described in 1902, with ecological salt-water variants (Gillies & de Meillon, 1968). Subsequent studies of the species showed highly varied biology and behaviour in larval and adult stages. The larvae can be found in different water bodies ranging from shallow, open sun-lit pools to edges of swamps and brackisk water (de Meillon, 1937; Vincke & Parent, 1944; Muirhead-Thompson, 1951). The extent of the adults' endophily and anthropophily was also highly varied depending on regions where collections were performed (for review, see Gillies & de Meillon, 1968).

The saltwater variant in West Africa was the first to be separated from *An. gambiae*, as it is distinctly different at all life stages. This species was named *Anopheles melas*. *Anopheles gambiae* remained as a single species for over six decades until 1962, when three individual publications detailed the separation of the East African saltwater-breeding form from the freshwater form (Paterson, 1962; Kuhlow, 1962), and that the freshwater form itself consisted of two mating groups (forms A and B) (Davidson & Jackson, 1962). Cross-mating of these forms produced sterile males, demonstrating reproductive isolation between them. The East African saltwater form was named *An. merus*, form A remained *An. gambiae*, while form B became *An. arabiensis* (Mattingly, 1977). A third mating form (form C) was found in southern Africa (Paterson, 1964), which is a non-vector for human malaria (White, 1974). This species was named *Anopheles quadriannulatus* (Mattingly, 1977). An Ethiopian variant of *An.*

quadriannulatus was found to be distinctly different cytogenetically from those in southern Africa (Hunt *et al*., 1998) and was recently named *Anopheles amharicus* (Coetzee *et al*., 2013). A seventh species (form D) was confirmed in Uganda, through cross-mating experiments and cytogenetic characterization (Davidson & White, 1972; Hunt, 1972). It is a highly localized species, and was named *Anopheles bwambae*, after the region where it was found.

The evolution of *An. gambiae* is still in progress, however, with genetical data showing that the species is currently undergoing incipient speciation (Coluzzi, 1984; della Torre *et al.*, 2001; Gentile *et al.*, 2001). Although hybrid male sterility from cross-mating was an early key feature for separating the cryptic species of the *An. gambiae* complex, identification by analysis of polytene chromosome banding patterns was more practical. Consequently, a large volume of data has accumulated with respect to the chromosomal variation in many *An. gambiae* populations across Africa. The changes in banding patterns are the results of fixed paracentric inversions, and *An. gambiae* has the highest complexity of the seven species (Coluzzi, 1984). A number of the paracentric inversion polymorphisms on the second chromosome have been associated with ecotypic differences in populations. Furthermore, the observed frequency of these inversions often deviate significantly from Hardy-Weinberg equilibrium and has been linked to incipient speciation within *An. gambiae* (Coluzzi *et al*., 1984). *Anopheles gambiae* has been divided into five chromosomal forms, based on non-Linnean nomenclature: Forest, Savanna, Mopti, Bamako and Bissau (Coluzzi *et al.*, 1985). Non-random distribution of inversions was observed in these chromosomal forms, particularly those on chromosome 2R, and often with significant deviation from Hardy-Weinberg equilibrium (della Torre *et al*., 2001). Although there are no morphological differences nor reproductive barriers (under laboratory conditions) between the chromosomal forms,

cytogenetic analysis showed little or no intergradation between some of the forms in nature (Bryan *et al*., 1982; Coluzzi *et al.*, 1985; Touré *et al*., 1983, 1994, 1998). This genetic discontinuity is strongest in Mali and Burkina Faso, where Savanna, Mopti and Bamako occur in sympatry and no, or very few, heterokaryotypes exist. Analyses of intergenic spacer (IGS) and internal transcription spacer (ITS) regions of the ribosomal DNA (rDNA) showed no difference between Savanna and Bamako forms, but Mopti is distinctively different from the other two (Favia *et al*., 1997; Gentile *et al.*, 2001). More recent evidence has shown the presence of two distinct molecular forms, M and S, that only loosely correlate with the chromosomal forms (della Torre *et al.*, 2005). Recently, the M form has been named *Anopheles coluzzii*, making it the eighth named species in the complex, while S form remains *An. gambiae* (Coetzee *et al*., 2013).

Of the eight species in the complex, *An. gambiae*, *An. coluzzii* and *An. arabiensis* are the major malaria vectors, while *An. merus*, *An. melas*, and *An. bwambae* are only considered important in certain regions due to their restricted distribution. *Anopheles quadriannulatus* and *An. amharicus* are non-vector, cattle-feeding species restricted to southern Africa and Ethiopia respectively (Gillies & Coetzee, 1987; Sinka *et al.*, 2010). *Anopheles gambiae* and *An. arabiensis* are the most widespread across the continent while *An. coluzzii* is found in West and Central Africa (Sinka *et al.*, 2010; Coetzee *et al.*, 2013). All three species utilize freshwater breeding habitats with *An. gambiae* and *An. arabiensis* found in almost any temporary water source, ranging from natural depressions where flood or rain water has collected, to hoof prints on the edge of ponds, water-filled road ruts and car-tracks. On the other hand, *An. coluzzii* prefers more permanent breeding sites such as flooded rice paddies (Gillies & de Meillon, 1968; Sinka *et al.*, 2010; Coetzee *et al*., 2013). All three species are endophilic and anthropophilic (somewhat less so for *An. arabiensis*), and their evolution

seems to have coincided with the settling of humans from hunter-gatherers to permanent residency, often exploiting breeding sites made available from human agriculture activities (Alvarado, 1963; Powell *et al*., 1999). The close association of *An. gambiae* and *An. coluzzii* with human habitations makes them vulnerable to vector control by indoor house spraying while *An. arabiensis* can be found both indoors and outdoors and this difference in resting behaviour makes it more difficult to control.

1.5.2 The *An. funestus* group

Anopheles funestus is one of nine named (and two as yet unnamed) species in the *An. funestus* Group and is the only significant malaria vector species (Gillies & de Meillon, 1968). It is highly endophilic and anthropophilic and breeds in permanent, vegetated swamps or slow-moving streams. It can become the dominant vector in a region, especially in the dry season, when the temporary water sources favoured by the *An. gambiae* complex species become limited (Gillies & de Meillon, 1968). The other members of the group, *An. vaneedeni*, *An. leesoni*, *An. rivulorum*, *An. parensis*, *An. fuscivenosus*, *An. aruni*, *An. brucei* and *An. confusus* are all mostly zoophilic (Gillies & de Meillon, 1968). However, *An. rivulorum* has been implicated as a minor vector in Tanzania (Wilkes *et al*., 1996; Coetzee & Koekemoer, 2013) and *An. vaneedeni* has demonstrated vectorial capacity in the laboratory but not in nature (de Meillon *et al*., 1977).

Anopheles funestus also possesses paracentric chromosomal inversions like *An. gambiae*, and can also be divided into chromosomal forms based on karyotypes. Populations sampled from Burkino Faso showed two chromosomal forms: Kiribina with the standard chromosomal arrangement, and Folonzo, which is highly polymorphic for several alternative arrangements (Costantini *et al*., 1999). Sympatric populations of the chromosomal forms deviate

significantly from Hardy-Weinberg equilibrium with a marked lack of heterozygotes, suggesting that the forms are undergoing incipient speciation similar to the *An. gambiae* situation (Costantini *et al*., 1999; Guelbeogo *et al*., 2005). Further investigation will be necessary to determine whether these chromosomal forms can be considered good species.

Anopheles funestus is widespread in tropical regions, especially savanna areas, and can thrive in closed canopy forests. It is also present on some islands off the African continent (Sinka *et al.*, 2010). Due to the high degree of anthropophily and endophily, the species is very susceptible to IRS. In most regions, *An. funestus* has a lower infection rate (2 - 5%) compared with *An. gambiae*, but this is not always the case and infection rates may be over 10% (de Meillon, 1933). Its preference for breeding in permanent water bodies also leads to more stable *An. funestus* populations, allowing for year-round endemic transmission of malaria.

Anopheles funestus has a longer generation time compared to members of the *An. gambiae* complex, taking on average two to three weeks from egg to adult and up to a month or longer in cooler conditions. The adult is also longer-lived, able to survive up to 40 days. Due to the long development time, *An. funestus* populations usually only start to increase in the middle of the wet season and reach a peak at the end of it and into the early part of the dry season (Fontenille *et al*., 1997). This is in contrast to *An. gambiae*, which undergoes a population explosion soon after the first rain and the numbers peak in the middle of the wet season (Gillies & De Meillon, 1968). This separation in seasonal abundance is unfortunate, as it results in large numbers of vectors throughout large parts of the year in many localities across Africa.

1.6 Vector-parasite interactions

The complete reliance of *Plasmodium* species on mosquitoes for a large part of their lifecycle

provides opportunities in terms of novel disruption strategies. Even though Huff (1927) proposed that mosquito immune responses may be an important determinant for parasite infectivity, for the most part it was thought that the mosquito is a passive carrier of the parasite. However, new and improved molecular techniques have provided data in recent years showing that this is not the case at all.

As mentioned previously, the *Plasmodium* lifecycle in the mosquito involves both sexual and asexual reproduction, and undergoes multiple transformations in order to complete the cycle (for review of this, see Ghosh *et al*., 2000; Sinden, 2002). The parasite must not only first survive in the midgut, but also has to traverse two epithelial barriers, and finally successfully navigate the haemolymph to arrive at the salivary glands (Abraham & Jacob-Lorena, 2004; Siden-Kiamos & Louis, 2004; Vlachou *et al*., 2004; Michel & Kafatos, 2005). These are obvious points where the mosquito's immune system can attack the parasite.

Mosquitoes, like all other insects, rely on a well-conserved innate immune system to fend off pathogens and foreign microbes. The innate immune system exists in all eukaryotes and is the only line of defense for invertebrates against pathogens (Christophides *et al*., 2002, 2004). Two categories of reactions are generated against infections in innate immunity: tissue/cellular and humoral responses (Christophides *et al*., 2004). The first category involves what can be considered macroscopic responses, such as phagocytosis, cellular encapsulation and induction of apoptosis. It can also include physical barriers like the peritrophic matrix and midgut epithelium that the invading organisms have to traverse. The humoral, or molecular responses are much more sophisticated, involving multiple steps and elicit a variety of responses to deal with pathogens. Potential pathogens are identified through conserved pathogen-associated molecular patterns (PAMPs), which are capable of non-self recognition.

These then interact with host pattern recognition receptors (PRRs) which may be cell-bound or circulating in the haemolymph. These receptors are able to activate a diverse range of responses including antimicrobial peptides (AMPs) and proteolytic cascades (of which melanization is a part). Signal transduction pathways are also engaged in immune responses, two of which – Toll and Imd (Immunodeficient) pathways – are nuclear factor kappa B (NF-κB) related, and the third, the JAK/STAT pathway acts through kinase and proteins of its namesake (for reviews of these pathways, see Christophides *et al*., 2004; Lehan *et al*., 2004; Cirimotich *et al*., 2010).

In anophelines, the PRRs that have been shown to be involved in *Plasmodium* infection include peptidoglycan recognition proteins (PGRPs), thioester containing proteins (TEPs), C-type lectins (CTLs), and Gram-negative binding proteins (GNBPs). PGRPs are the most well known insect PRRs; they participate in AMP synthesis, melanization cascade, and phagocytosis of Gram-negative bacteria. In *Anopheles*, knockdown of PGRPLC (a member of PGRP) not only increases oocyst numbers, but also infection prevalence and percentage of melanized oocysts (Meister *et al*., 2009; Michel & Kafatos, 2005). Fifteen TEPs have been identified in the *An. gambiae* genome thus far and are typically active during bacterial infection. Several of them have also been shown to be upregulated after *Plasmodium* infection (Blandin *et al*., 2004; Christophides *et al*., 2002). Among these, TEP1 has been shown to interact with *P. berghei* ookinetes, leading to parasite killing by lysis in the basal labyrinth of the midgut epithelium (Blandin *et al*., 2004). CTLs are involved in bacterial responses by promoting phagocytosis, haemocyte nodule formation, and activation of prophenoloxidase (PPO), which leads to melanization. RNA silencing of two CTL genes, *CTL4* and *CTLMA2*, showed significant increase in melanization of *P. berghei* ookinetes in *An. gambiae* (97% and 48% respectively) (Osta *et al*., 2004). Although GNBPs are induced in the midgut and

salivary glands at the different parasite stages (Dimopoulos *et al*., 1998), there has been little evidence to show that they have significant impact on parasite development.

The Toll and Imd pathways are highly conserved in insects (Hoffmann, 2003). Intracellular components of both pathways have been identified in *An. gambiae* (Christophides *et al*., 2002), and are primarily involved in immune responses (Toll is also responsible for embryogenesis in *Drosophila*). The Toll pathway is induced by Gram-positive bacteria and fungal infections, whereas Imd is involved in Gram-negative bacteria infections. Activation of either pathway triggers movement of the REL (a NF-κB-like protein)/NF-κB transcription factor families into the nucleus, which recruit other proteins into the nucleus and degrade inhibitor proteins of the pathway to allow the signal cascade to continue. This leads to the production of antimicrobial peptides that are primarily effective against bacteria, but recent research has shown that certain AMPs are able to interact with *Plasmodium* parasites as well. The JAK/STAT pathway acts through a separate set of proteins and receptors – the janus kinase (JAK) and signal-transducer and activator of transcription protein (STAT) – to respond to bacterial infection (Agaisse & Perrimon, 2004). This pathway's participation in insect immunity was first demonstrated in *An. gambiae* (Barillas-Mury *et al*., 1999).

Antimicrobial peptides are small, positively charged peptides, produced in fat bodies, haemocytes and epithelia. They are released in high concentration into the haemolymph upon bacterial or fungal infection (Hoffmann *et al*., 1996). The AMPs bind to the cell walls of micro-organisms and are thought to kill by causing lethal damage to the membrane. Four classes of AMPs have been discovered in *An. gambiae* so far: defensins, cecropins, gambicins and attacins. *In vitro* experiments where infected mosquitoes were directly injected with synthesized or non-anopheline AMPs showed these molecules can affect oocyst development

(Gwadz *et al*., 1989; Shahabuddin *et al*., 1998). Subsequent experiments have shown that this is usually the case *in vivo*. Transgenic *An. gambiae* expressing cecropin A (or cecropin 1) 24 hours after a bloodmeal reduces oocyst number by 60% (Kim *et al*., 2003). Gambicin, a novel AMP discovered in *An. gambiae* is able to act on both Gram-positive and Gram-negative bacteria and has marginal lethality against *P. berghei* (Vizioli *et al*., 2001). The one peptide that has not shown any inherent anti-plasmodial property is defensin 1 (defA) (Blandin *et al*., 2001), even though exogenous infusion of the peptide indicated otherwise (Shahabuddin *et al*., 1998).

Proteolytic cascades, which involve serial activation of serine proteases, are also able to initiate the Toll pathway and can cleave PPO zymogen to trigger melanotic encapsulation, which immobilizes and kills the pathogen with free radicals and toxic intermediates that are generated during the encapsulation process (Osta *et al*., 2004; Michel *et al*., 2005). Inhibitors for these serine proteases thus have significant impact on the mosquito's immunity. There are at least 27 known families of serine protease inhibitor (Patston, 2000); in anophelines, the most important is the serpin family. Serpins 1 - 3 are orthologous to *Drosophila melanogaster*'s Spn27A, and silencing Serpin 2, the functional ortholog, reduces *P. berghei* oocysts (Michel *et al*., 2005). Encapsulation can also occur through aggregation of haemocytes around the pathogen, but this has not been reported with *Plasmodium* oocysts. Lastly, phagocytosis may play a role in eliminating the parasite, but the only evidence for this thus far is in an unnatural pairing of *Ae. aegypti* and *P. gallinaceum*, where phagocytosis of the sporozoites by the granulocyte subpopulation of the mosquito was the primary immune mechanism (Hillyer *et al*., 2003).

The immune responses are a critical factor in determining the viability of vector-parasite

pairing in both nature and laboratory setting. Such a response is obvious in the L3-5 mosquito strain, bred from a normally parasite-friendly *An. gambiae* strain, which melanizes *P. berghei* and allopatric strains of *P. falciparum* ookinetes (Collins *et al*., 1986), or the lysis of *P. gallinaceum* ookinetes in the *An. gambiae* SUAF2 strain (Vernick *et al*., 1995).

Aside from the more obvious interactions between the mosquito immune mechanism and parasite invasion, there are also genetic components of the mosquito's resistance to the *Plasmodium* parasite, and natural resistance alleles seem to occur at high frequency in some areas (Niare *et al*., 2002). As mentioned earlier, the *An. gambiae* genome is littered with a large number of paracentric chromosomal inversions. One such inversion, 2La, not only has a role in ecotypic adaptation for the species (Powell *et al*., 1999), is associated with insecticide resistance (Brooke *et al*., 2000), but also contains genes and loci that influence immune responses to *Plasmodium* infection. Four *Plasmodium*-resistance loci influencing melanization or infection intensity have been found within or near the inversion, and this region is considered a major *Plasmodium*-resistance island (PRI) of the *An. gambiae* genome (Riehle *et al*., 2006). Furthermore, the PRI was also found to include two novel leucine-rich repeat (LRR) proteins, named *Anopheles-Plasmodium*-responsive leucine-rich repeat 1 (APL1) and 2 (APL2). The APL1 protein was shown to have anti-oocyst activity, further implicating the PRI's role in the immune response against *Plasmodium* infection (Riehle *et al*., 2006).

1.7 Rationale

There is no doubt that malaria remains one of the deadliest infectious diseases in the world, but current efforts in reducing the burden are severely limited by resistance from the parasite and the vector to drugs and insecticides, respectively. Better understanding of the biological interactions between the vectors and parasites is necessary to discover new avenues for

disrupting transmission. Of the two primary African malaria vector species, *An. gambiae* and *An. funestus*, the former has been studied in great detail, even though much remains unknown, particularly with respect to molecular mechanisms in presence of the *Plasmodium*. The lack of data is even greater with *An. funestus*, primarily due to the lack of success in colonizing this species.

Numerous mosquito species have been tested to determine whether they are viable vectors for *P. berghei*. There are a number of human malaria vectors that can carry *P. berghei* successfully (*An. gambiae*, *An. quadrimaculatus* and *An. stephensi*), but *An. funestus* was classified as a non-vector in an experimental situation (van den Berghe, 1954; Vincke, 1954). However, there have been no other recorded attempts after these early studies to show that the species is indeed a vector/non-vector (but see below). This is particularly important if one considers the advances that have been made in the re-classification of the Afrotropical mosquito species in the last five decades. With the separation of both *An. gambiae* and *An. funestus* into distinct species complexes or groups, revision of what is considered vectors and non-vectors would be essential to further research. It is likely that *An. funestus* is actually a viable vector for *P. berghei*, as other human malaria vectors are often compatible with non-human malaria parasites, albeit not at an efficient level (Alavi *et al*., 2003) and indeed, this has been demonstrated in *An. funestus* twice this year (Xu *et al.*, 2013; Lo & Coetzee, 2013), the latter paper being a product of research for this thesis.

It has been mentioned previously that there are discrepancies between data from *P. falciparum* and *P. berghei* infections in the same vectors. Furthermore, given the view that each vector-parasite pairing would generate a unique pattern of interaction between the two organisms (Sinden *et al*., 2004), it would be illogical to expect that *P. berghei* would generate the same

responses in *An. gambiae* and *An. funestus*. It is therefore of utmost importance to establish a viable and replicable infection system using *An. funestus* and *P. berghei* to enable investigations of the interactions between them and to compare these with similar interactions in *An. gambiae.*

The establishment of two *An. funestus* colonies by the Department of Medical Entomology, South African Institute for Medical Research (now Vector Control Reference Laboratory (VCRL) at the National Institute for Communicable Diseases), Johannesburg, South Africa, in 2000 (Hunt *et al.,* 2005) has opened up new research options, one of which is the creation of an infection model in this important malaria vector that previously was unattainable. In addition, numerous strains of members of the *An. gambiae* complex are currently being maintained at the VCRL which made the research listed below a viable option.

1.8 Aims and Objectives

1. To set up an infection system using strains of *An. gambiae* and *P. berghei*.
2. To optimize and establish an infection system using *An. funestus* and *P. berghei*, and characterize the infection cycle.
3. Screen the infectivity of the colonies available in the VCRL Botha de Meillon insectary using the infection model.
4. Optimize the 2La inversion molecular karyotyping technique for *An. gambiae* colonies and determine if the inversion has any association to *Plasmodium* infection in local strains.
5. Determine expression profiles of selected immunity genes during *P. berghei* infection in the various *An. funestus* colonies.

Chapter 2: The *Anopheles-P. berghei* infection system

2.1 Introduction

The first rodent malaria parasite was found in the Congo in the region of Katanga, and described and named *P. berghei* by Vincke and Lips (1948). Its natural vector, *Anopheles dureni* was described a decade earlier, and later found to be infected with sporozoites, but the association between the mosquito and the parasite was only made in the early 1950s (Garnham, 1966; Bruce-Chwatt, 1978). In spite of knowing both the vector and the parasite, *P. berghei* only became a viable research model after another decade of intensive research, after obstacles to cyclical transmission were resolved (Vanderberg, 1964; Yoeli, 1965). Since then, the use of *P. berghei* in malaria research has been one of paramount importance, providing significant contributions to knowledge of mosquito and parasite immunology and chemotherapy (Bruce-Chwatt, 1978).

The susceptibility of a large number of *Anopheles* mosquitoes to this *Plasmodium* species (Sinden *et al*., 2002) has allowed establishment of numerous *in vivo* infection systems to investigate vector-parasite interactions on both a physiological and molecular level. However, although *An. funestus* is a major African malaria vector, data on vector-parasite interactions are severely lacking, primarily due to the absence of viable laboratory colonies. Hunt *et al*. (2005) were successful in establishing colonies of *An. funestus* from Angola (colony designated FANG) and Mozambique (colony designated FUMOZ) that are currently maintained in the NICD insectaries in Johannesburg. The FANG strain is fully susceptible to all insecticides while FUMOZ was established from a wild population that is resistant to pyrethroids and carbamates. Through selection over successive generations, a strain

(FUMOZ-R) has been established that is almost 100% resistant to permethrin (a pyrethroid used for treating bed nets) (Hunt *et al.,* 2005).

In addition, the NICD houses over a dozen *Anopheles gambiae* complex colonies (see Appendix 1). These include *An. gambiae* (ranging from G3 established by the London School of Hygiene & Tropical Medicine in 1975 from Gambian material (Malaria research and reference reagent centre, http://www.mr4.org) to TONGS from Cote d'Ivoire that was established just over a year ago), *An. arabiensis* (KGB established over 35 years ago to AMAL established last year), *An. merus* and *An. quadriannulatus*. A number of these strains (especially the older *An. gambiae* strains such as G3 and PALA) have been used as experimental vectors successfully in laboratory settings elsewhere (Al-Mashhadani *et al*., 1980; Collins *et al*., 1985). However, successful infection of a strain reared in one laboratory is no guarantee that infection will occur in other laboratories as it is known that genetic composition of a strain will change over time (Chevillon *et al*., 1995; Scarpassa *et al*., 2008; o'Donnell & Armbruster, 2010). Therefore the infectivity status of the VCRL colonies needed to be established in order to identify appropriate strains to be used as controls for the various experiments carried out in this study.

Substantial information exists for setting up an infection system using *P. berghei* with *An. gambiae* complex colonies. The one recorded attempt at infecting *An. funestus* with *P. berghei* (van den Berghe, 1954) used wild caught populations and was carried out at a time when the critical development temperature for the parasites (19 - 20°C) was unknown. The present study, therefore, aimed to establish a functional *P. berghei* infection system and test the infectivity of the *An. gambiae* complex strains available at the NICD. The process would then be adapted to infecting *An. funestus* with *P. berghei*, which would provide proper clarity on

whether *An. funestus* is a viable vector for the rodent malaria parasite, and if so, how effective the species is in hosting parasite development.

2.2 Materials and methods

2.2.1 Establishment of *An. funestus* susceptible colony

In order to assess any possible confounding influence of insecticide resistance on mosquito infectivity, the *An. funestus* base colony FUMOZ, which is partially resistant to pyrethroids but not under any selection pressure, was back-selected to establish a susceptible strain. Forty individual females were randomly selected from the general FUMOZ colony and placed into separate egg-laying vials. The vials had wet filter paper on the bottom to collect the eggs, and the females were provided with a bloodmeal every two to three days until their deaths. Eggs collected from each female were reared separately through to adulthood, at which point half the population from each egg batch were exposed to 0.75% permethrin for one hour, per WHO exposure assay protocols (WHO, 1998). The knockdown was recorded immediately after exposure and deaths were recorded after 24 hours. The offspring from each egg batch that were considered susceptible (greater than 85% mortality) were pooled together in a cage for mating. The new colony was designated FUMOZ-BS (Back Selection) and was maintained at standard insectary conditions (25 ±2°C, 80 ±10% relative humidity, and 12 hours light/dark cycle with intermediate 30 minutes dusk/dawn cycle). The colony was given access to guinea pigs for bloodfeeding two to three times a week, and the females were allowed to lay eggs twice a week to continue the colony. A separate back selection process was performed when FUMOZ-BS reached the 10^{th} generation (F_{10}) to supplement and increase the colony numbers.

2.2.2 Mice infection

The mice infection followed established protocols (Sinden *et al*., 2002) with some modifications. *Plasmodium berghei* ANKA strain was obtained from Imperial College, London (courtesy of Prof. R. Sinden) and also from MR4 (MRA-311) and stored in liquid nitrogen until needed. Infected blood with parasitaemia of at least 10% or higher was used to initiate infection in the mice. The laboratory mouse strain C57BL/6 (C57 Black strain 6) was used as the vertebrate host for the parasite infection. Three to four days prior to being used for mosquito feeding, 0.2 - 0.3 ml of the infected blood was injected intra-peritoneally (IP) into six to eight weeks old female mice. On the day of feeding the mosquitoes, the mice were anaesthetized (Rompun [2% Xylazine HCL 20 mg/ml] + Anaket [Ketamine hydrochloride 100 mg/ml], 1:1 mixture; 0.2 - 0.3 ml per mouse), and placed on top of the cages for a minimum of 15 minutes for bloodfeeding. The infected mice were maintained at standard conditions for rodents (20 ±1°C, ~50% relative humidity (RH)) with constant access to food and water.

To monitor the parasitaemia in the infected mice, thin blood smear slides were prepared at least every alternate day after feeding the mosquitoes. The blood smear slides were air-dried, fixed with absolute methanol for 10 - 20 seconds, and allowed to air dry again. The slides were stained with Giemsa (10% Giemsa in Giemsa buffer) for 20 - 30 minutes then washed with slow running water and left to air dry. The parasites were observed under 600 - 1,000X magnification using light microscopy. To determine the parasitaemia level, infected cells were counted from at least 1,000 red blood cells (RBCs) to give a percentage infection.

When the parasitaemia was over 10% (typically 9 - 13 days after infection), the mice were anaesthetized using the same mixture as previously described, and sacrificed by cardiac

puncture. Blood was drawn directly out of the heart using a 22G needle and 1 ml syringe. A small volume of freezing solution (30% glycogen-PBS solution) was used to prevent coagulation during the cardiac puncture. The blood was mixed at a 2:3 volume ratio with freezing solution, and stored in liquid nitrogen in cryogenic vials (Corning, New York, USA). The blood was placed in liquid nitrogen storage within 20 minutes of removal from the mouse to minimize death and damage to the parasites. The infected blood stocks were stored indefinitely in liquid nitrogen until needed. If the blood was not needed, the mouse was terminated using carbon dioxide.

Direct blood passage was the easiest method for infecting the mice, but cyclical transmission was also necessary, as it was not recommended to use direct blood passage stock for infection more than eight times in succession (Sinden *et al.*, 2002). Infected mosquitoes were allowed to feed on healthy mice 18 - 21 days after the infected bloodmeal. The mice infected in this way were also monitored using blood smears, and were sacrificed five to seven days after the feeding. Storage of the P_0 blood was in the same manner as described above.

Ethical clearance for the use of mice for *P. berghei* infection was obtained from the NICD animal ethics committee (ethical clearance number: 110/07, 2007) and a copy provided on page iv.

2.2.3 *An. gambiae* infection

Anopheles gambiae infection was performed using the established protocols of Sinden *et al.* (2002). The *An. gambiae* G3 strain, known to carry *P. berghei* infection in other studies (Collins *et al.*, 1986; Crews-Oyens *et al.*, 1993; Brey *et al.*, 1995; Abraham *et al.*, 2005) was used initially to establish the infection system. Seventy to ninety newly eclosed female

mosquitoes were collected from the colony, maintained at standard insectary conditions, given sugar water (10% w:v) as sustenance, and left for three to four days before feeding. Sugar water was removed 18 - 24 hours prior to feeding, to starve the females and maximize the number of females that would feed on the mice. At the same time, the mosquitoes were moved to a cooler insectary room to facilitate the parasite development (20 ±1°C, 85 ±10% RH). The mosquitoes were allowed to feed on the mice for a minimum of 15 minutes to maximize the feeding numbers and feeding to repletion. Females that had not fed were removed from the cages. Four to six days after the initial feed, the mosquitoes were again starved overnight, and allowed to feed for a second bloodmeal. Feeding rates were calculated based on the number of females that did feed against the total number of females offered a bloodmeal.

The bloodfed females were kept at 20 ±1°C, 85 ±10% RH for at least 16 days, during which time they were provided with sugar water for sustenance. After 16 days, the salivary glands were dissected to screen for sporozoite presence using light microscopy (see section 2.2.5). Mosquitoes were considered infective if sporozoites were detected in the salivary glands. When necessary, infective populations were allowed to feed on a healthy mouse to initiate cyclical transmission as described above. The infection rates were calculated based on the number of females that had infected salivary glands against the number of females that took a bloodmeal.

The three *An. gambiae* complex species that are considered malaria vectors were tested (*An. gambiae s.s, An. arabiensis* and *An. merus*) as well as the non-vector member *An. quadriannulatus*. The following colonies were tested: BOA, CIG, G3, GAH, IANP20, NAG, PALA, SUA (*An. gambiae s.s.*); SANGWE (*An. quadriannulatus*); ARER, KGB, MA, MALPAN, MBN-DDT (*An. arabiensis*); MAF and ZAM (*An. merus*) (see Appendix 1).

Infection for all colonies was performed at least three times to determine whether they were viable hosts. Colonies that were able to carry the parasites were infected at least three more times to determine their competence as vectors.

2.2.4 *An. funestus* infection

Anopheles funestus was tested for infection in a similar manner to *An. gambiae* infection. Newly eclosed *An. funestus* adult females were maintained in the same way as *An. gambiae*, but were kept for 10 - 14 days at standard insectary conditions, before being moved to the cooler conditions and deprived of sugar water prior to feeding on infected mice. The females were also given a second bloodmeal four to six days after the infected bloodmeal. Dissection of midguts for oocysts started 10 days after the infected bloodmeal, and salivary gland dissections for sporozoites from 18 days after infected bloodmeal (see section 2.2.5).

Both *An. funestus* base colonies (FUMOZ and FANG) were initially tested. The more susceptible of the two, FUMOZ, was used for further infection experiments along with the colonies selected from FUMOZ (FUMOZ-R permethrin-resistant and FUMOZ-BS permethrin-susceptible). All the *An. funestus* colonies were infected at least three times to determine their vectorial capacity. Feeding and infection rates for *An. funestus* colonies were calculated in the same manner as for *An. gambiae* colonies. A cohort of F_1 females reared from eggs produced by wild caught *An. funestus* from Ghana were also subjected to feeding on infected mice. The feeding experiment for this cohort was only performed once as only a small number of adults were available. Two-sample T-tests and one-way ANOVA using the Statistix 7 analysis software (Analytical Software, USA) were performed to compare the feeding and infection rates between the strains, as well as to compare the oocyst and sporozoite numbers.

2.2.5 Mosquito dissection

Dissections were carried out according to the protocols set out in *A practical guide for malaria entomologists in the African region of WHO* (WHO, 1961) and *MR4 methods in* Anopheles *Research Laboratory Manual* (version 5.2, 2009). Bloodfed *An. funestus* females were dissected at two time points to determine the appearance of the different life stages of the parasite. The midguts were dissected from 10 days post-infected bloodmeal for oocysts, while the salivary glands were dissected from 16 days post-infected bloodmeal for sporozoites. *Anopheles gambiae* were only dissected for sporozoites to confirm infection.

To dissect the midgut, the mosquito was knocked out using ether and placed on a drop of PBS buffer on a glass slide. The legs and wings were removed for easier access (and also to prevent the mosquito from escaping if it woke up prematurely). Under 25 - 50X magnification, the thorax and abdomen were first separated and an incision was made at the second last segment of the abdomen. The posterior end of the midgut was pulled out by slowly separating the last segments from the main sections of the abdomen. The midgut was cleaned by removing the attached Malphigian tubules, and then moved to a new slide and resuspended in a drop of PBS. A coverslip was gently placed onto the slide and the midgut observed under 100 - 400X magnification for oocysts.

In some instances, the midgut was stained in 1% mercurochrome for 20 minutes to emphasize the oocysts. Excess mercurochrome was then washed off with PBS, the midgut resuspended in PBS on a new slide, and observed under the same magnification as above.

For salivary glands dissection, the mosquitoes were knocked out and treated on the slide in

the same manner as for the oocyst dissection. The head and the thorax were separated using dissecting needles under 25 - 50X magnification and the salivary glands, a pair of almost transparent trilobular structures, were gently teased out from the tissues. As much of the surrounding tissue was removed as possible, while taking care not to break the glands. The salivary glands were moved to a clean slide and resuspended in a drop of PBS. A coverslip was gently placed onto the slide and the salivary glands observed under 400 - 600X magnification for sporozoites. It was sometimes necessary to gently press or tap on the coverslip to rupture the salivary glands to detect the sporozoites.

2.3 Results

2.3.1 *An. gambiae* complex infections

A number of *An. gambiae* complex colonies of different species and from different localities were tested to see whether they could carry the infection. In general, they required little incentive to feed on the mice and often fed to repletion with 15 minutes. Although previous infection data with other *Anopheles* mosquitoes (*An. quadrimaculatus* and *An. stephensi*) suggest the adults reared and maintained at 21°C, or moved to 21°C after eclosure feed better (Yoeli, 1965), the *An. gambiae* complex colonies at the VCRL actually fed better when they were first maintained at standard insectary temperature, and only moved to the cooler temperature at the time when the sugar water was removed. Immediate exposure to the cooler temperature after emerging from pupae led to a 10 - 20% decrease in feeding rates compared to those held at the higher temperatures. At the lower temperature, the lifespan of *An. gambiae* s.l. was extended, with individuals surviving past 40 days after eclosure. Dissections of the salivary glands of some these individuals showed that sporozoites were still present.

Comparing species, the *An. gambiae* s.s. colonies in general fed the best with 40 - 50% feeding rates, and also had the highest infection rates (above 10%) (Table 2.1). However, there were also two *An. gambiae* colonies that were resistant to *P. berghei* infection, both from Nigeria (Table 2.1). *Anopheles arabiensis* colonies were less successful at feeding and also had lower rates of infection, typically below 5%. The two *An. merus* colonies fed at rates comparable to *An. gambiae*, but infection was below 5%. Lastly, *An. quadriannulatus* had one of the lowest feeding rates and was completely resistant to infection (Table 2.1).

The strain most frequently used for *P. berghei* infection (for cyclical transmission and as a positive control) was G3, as it had both the highest feeding and infection rates. More recently established colonies (SUA, GAH, COG) were also easy to infect, and showed consistently higher levels of infection (10 - 20%) compared to the other strains (usually below 5%), and the latter group often required multiple attempts to achieve successful infections. These three colonies were also used for cyclical transmission and as positive controls if G3 was unavailable for any reason.

Plasmodium berghei infection in *An. gambiae* complex colonies was consistent with published data. Midguts were not examined for oocysts as the lifecycle in *An. gambiae* is well known, therefore data regarding the oocysts were not collected. The earliest time-point when the sporozoites were seen was 14 days post bloodmeal, in G3, but sporozoites were usually detected in the salivary glands around 16 days after feeding.

Table 2.1: *An. gambiae* complex colonies feeding and infection rates.

Species	Colony (sample size, n =)	Average percentage fed (±std dev)	Average percentage infected (±std dev)
An. gambiae	BOA (225)	35.6 (±2.9)	2.5 (±2.1)
"	CIG (247)	48.6 (±8.5)	4.3 (±1.8)
"	COG (270)	45.9 (±6.4)	10.7 (±4.4)
"	G3 (265)	51.7 (±7.4)	35.1 (±12.1)
"	GAH (244)	46.5(±8.4)	21.9 (±8.9)
"	IANP20 (246)	45.9 (±5.1)	6.3 (±1.8)
"	NAG (237)	36.6 (±5.7)	0.0
"	PALA (233)	36.9 (±4.0)	0.0
"	SUA (267)	45.4 (±10.8)	20.7 (±5.9)
An. arabiensis	ARER (229)	24.0 (±4.7)	2.2 (±3.8)
"	KGB (247)	38.4 (±35.8)	3.8 (±2.8)
"	MA (238)	25.8 (±9.1)	5.4 (±2.2)
"	MALPAN (223)	24.2 (±6.4)	4.3 (±3.8)
"	MBN-DDT (215)	27.4 (±2.7)	1.8 (±1.6)
An. merus	MAF (219)	37.8 (±2.3)	2.5 (±2.4)
"	ZAM (213)	31.4 (±8.3)	3.8 (±3.1)
An. quadriannulatus	SANGWE (235)	27.7 (±2.1)	0.0

2.3.2 *An. funestus* infections

Based on data obtained from infection of the *An. gambiae* complex colonies, *P. berghei* infection was tested on the two base *An. funestus* colonies, FANG and FUMOZ. The initial results showed that FUMOZ was the better colony for infection as it not only fed better on mice, but was also more prone to *Plasmodium* infection (Table 2.2). The feeding and infection rates between FANG and FUMOZ were significantly different ($p<0.01$). The F_1 progeny

obtained from wild caught *An. funestus* fed at an extremely low rate even with starving. There was no infection found in the bloodfed females in this cohort. Although *An. funestus* females could be persuaded to feed on mice three to four days after eclosure, those that did were in the vast minority (typically less than 5% of the cohort). To achieve maximum feeding rates, the females were left for at least 10 - 14 days before being allowed to feed on infected mice. The females were maintained at standard insectary conditions during this time as the feeding rate also decreased, like *An. gambiae* colonies, if they were maintained at 21°C immediately after eclosure.

Table 2.2: *An. funestus* base colonies and F_1 progeny (Ghana) feeding and infection rates.

Colony (n =)	**Average percentage fed (±std dev)**	**Average percentage infected (±std dev)**
FANG (567)	30.3 (±3.7)	4.6 (±1.2)
FUMOZ (622)	42.2 (±4.5)	10.4 (±3.9)
Ghana F_1 (195)	3.0 (±1.5)	0.0

Just prior to feeding, the females were disturbed by gentle tapping on the cage and by exhaling into the cage. These steps were often necessary to coax *An. funestus* into feeding on the mouse even after being deprived of sugar water. *Anopheles funestus* took longer to feed to repletion compared with *An. gambiae*, usually requiring 15 - 20 minutes to do so. The lower temperature for parasite development also lengthened *An. funestus* lifespan, and adults living for more than 40 days after eclosure were common as long sugar water was provided. Females that lived longer than 60 days have been observed, but they were in the minority.

Based on these data, subsequent infection experiments thus only utilized FUMOZ and its derivative colonies. The feeding rates in the FUMOZ strains were better than in the *An. gambiae* colonies, but the infection rates varied significantly, with the base colony having an

infection rate comparable to the best of the *An. gambiae* infections, and the FUMOZ-R colony with the lowest infection rate (Table 2.3).

Table 2.3: *An. funestus* FUMOZ and variant colonies' feeding and infection rate

Colony (n =)	**Average percentage fed (±std dev)**	**Average percentage infected (±std dev)**
FUMOZ (1968)	52.1 (±4.1)	20.4 (±7.8)
FUMOZ-R (2139)	46.6 (±3.3)	6.1 (±5.6)
FUMOZ-BS (2023)	50.7 (±3.8)	15.4 (±6.3)

To visualize the oocysts, mercurochrome staining was typically the recommended method. However, the staining was found not to be very useful here as the whole midgut is stained red and the oocysts were only stained a little more strongly than their surroundings (Figure 2.1).

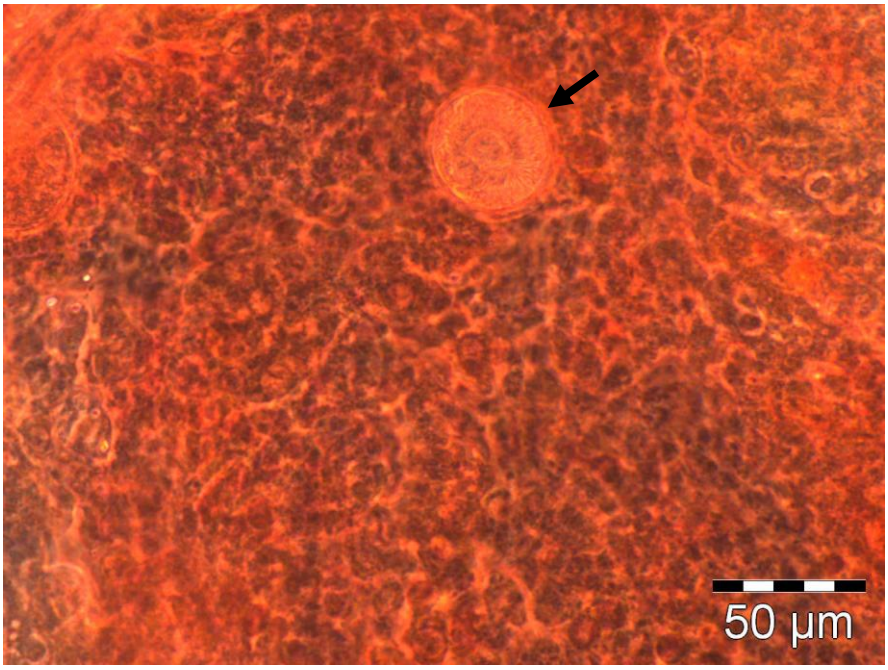


Figure 2.1: Infected midgut stained with 1% mercurochrome. The oocyst is visible as a doughnut shaped structure (phase contrast, 400X magnification).

Normal visualization was often sufficient for identifying oocysts, and it was sometimes easier to visualize all the oocysts by manipulating the phase contrast filters (Figures 2.2 and 2.3). Differential interference contrast microscopy was also used to examine the oocysts, and was useful in producing more definitive imaging of sporozoites within oocysts (Figure 2.4). Oocysts were seen on the midgut from around day 10 and persisted past day 20. *Plasmodium berghei* oocysts were 30 - 40 μm in diameter, and were not concentrated in a specific region of the midgut. Depending on how far the development was, immature sporozoites could be seen as faint lines within the oocysts (Figures 2.1, 2.3 - 2.5). By rupturing the oocysts, sporozoites under development could be seen attached to the blastophores (Figure 2.6), ranging from first stages of development to the almost mature vermicule form (Figure 2.7). Loose immature sporozoites that had yet to achieve the typical crescent shape were also released in the rupturing.

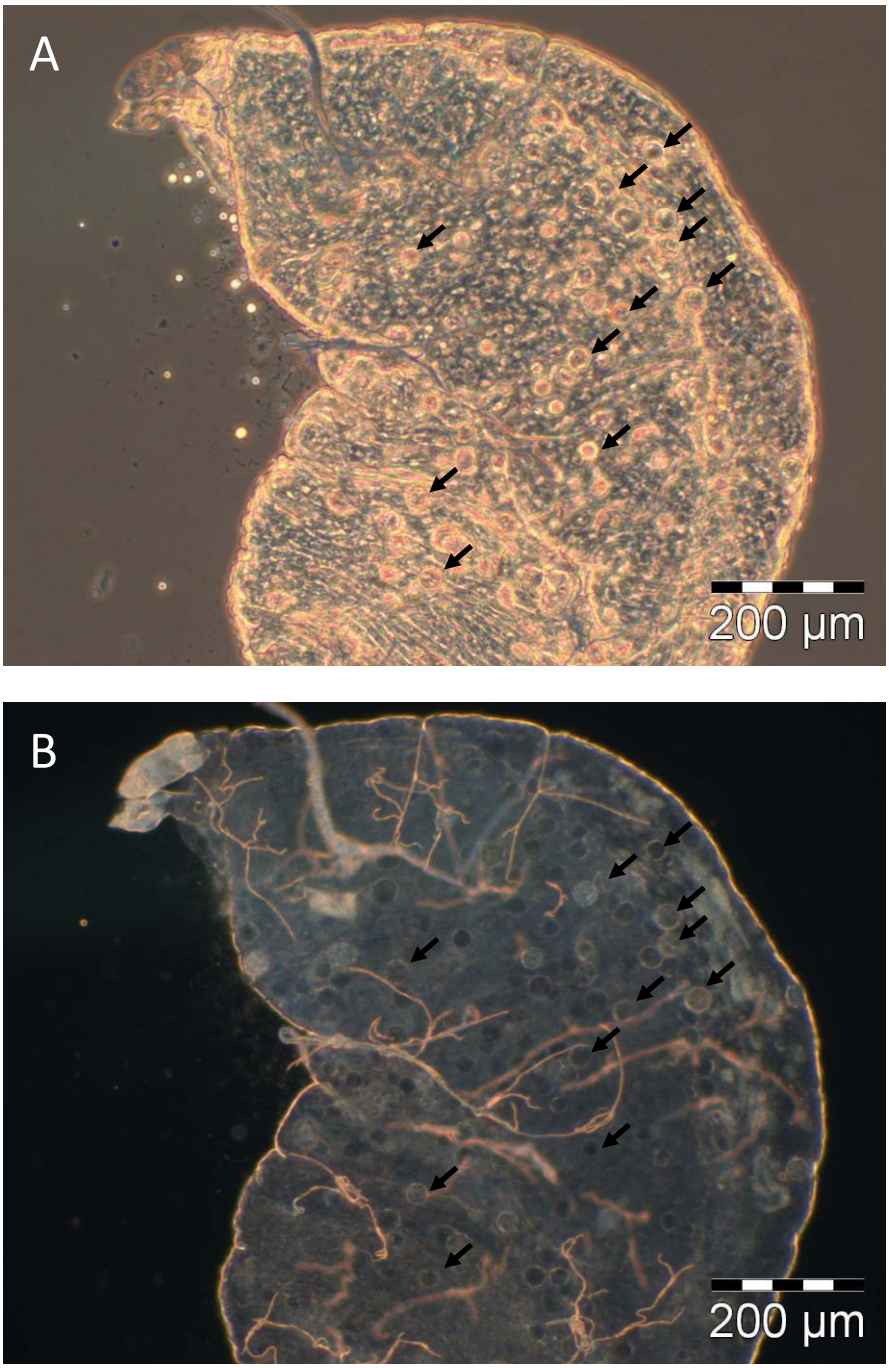


Figure 2.2A: Midgut dissection of *An. funestus* at 20 days post-infected bloodmeal (phase contrast, 100X magnification). 2.2B: The same midgut as the previous figure, but captured with the phase contrast filters out of alignment, which emphasized the oocysts as darker circles (ventral side) or lighter circles (dorsal side). Arrows are pointing out some of the oocysts.

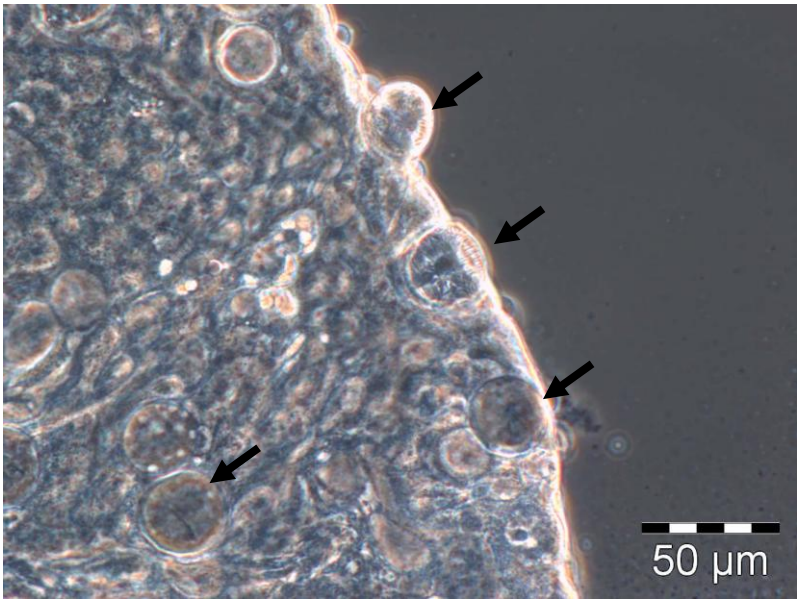


Figure 2.3: Oocysts on *An. funestus* midgut under phase contrast microscopy. The oocysts are at different stages of maturation, as some can be seen containing immature sporozoites while others have indistinct dark contents (400X magnification).

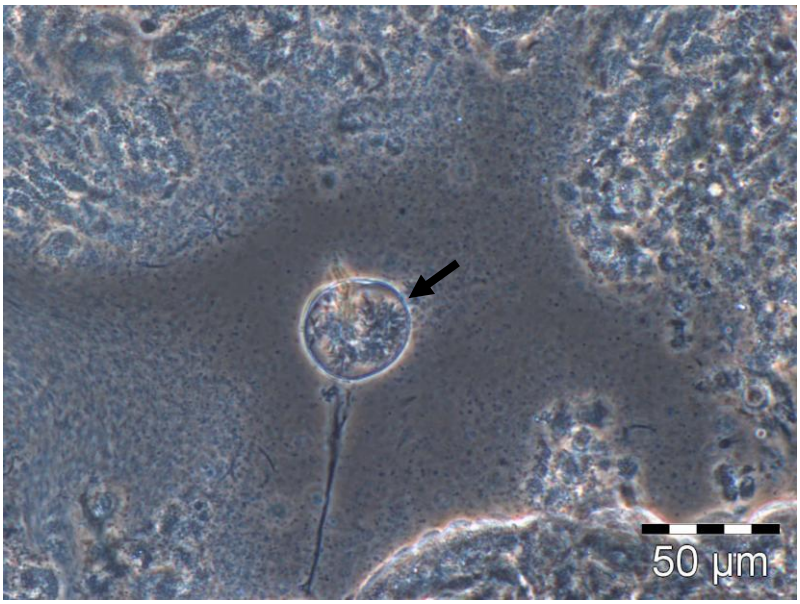


Figure 2.4: An unbroken oocyst detached from the midgut. Developing sporozoites can be seen within as short, black lines (phase contrast, 400X magnification).

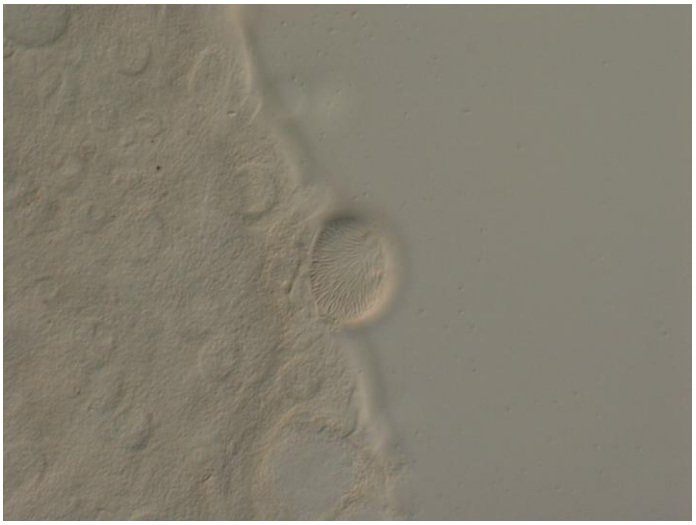


Figure 2.5: A mature oocyst on *An. funestus* midgut under differential interference contrast microscopy. The sporozoites can be seen as short vermicules completely filling the interior (400X magnification).

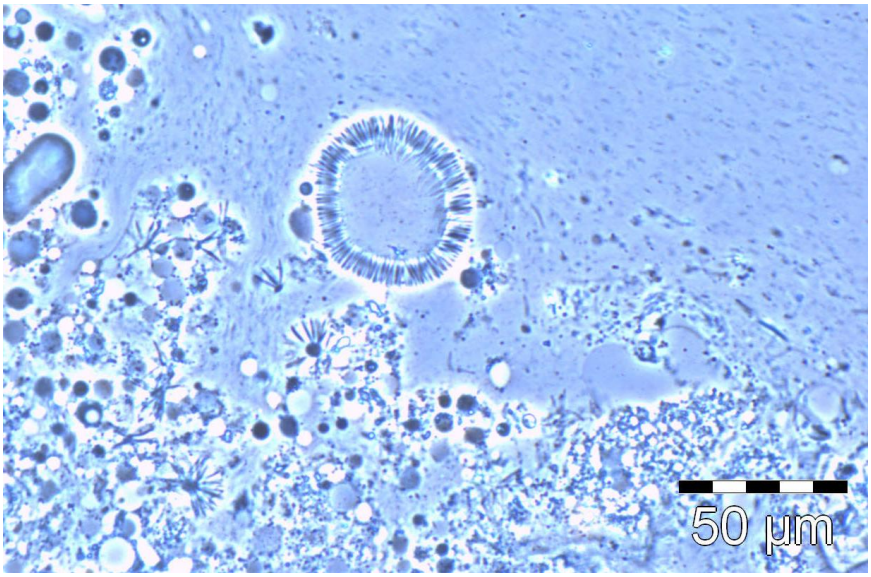


Figure 2.6: Immature sporozoites attached to a large blastophore in the midgut. The blastophore may have become distended when the midgut was ruptured. Smaller blastophores are also present, along with loose, immature sporozoites that have not achieved the crescent form (phase contrast, 400X magnification).

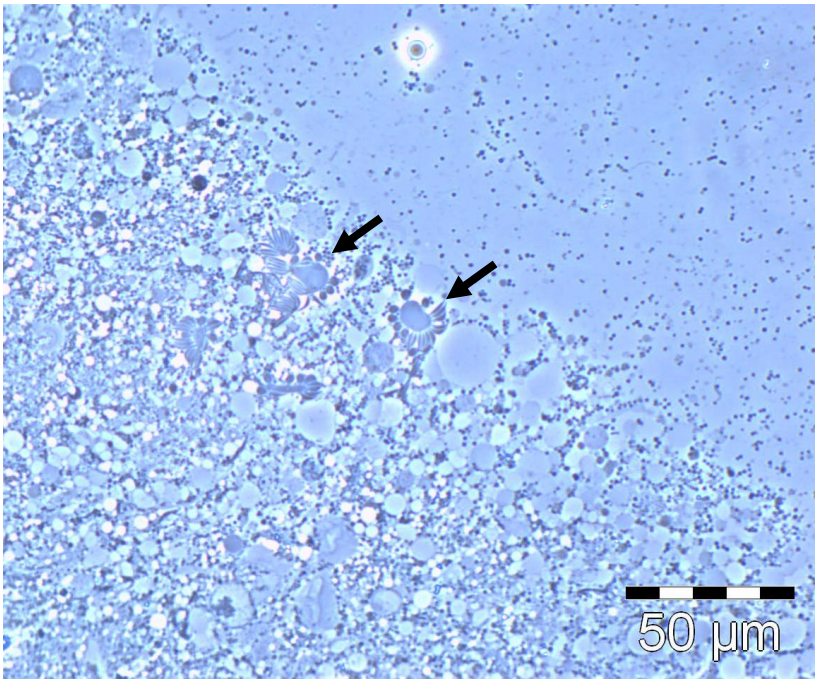


Figure 2.7: Sporozoite assembly on the blastophores from burst oocysts in a ruptured midgut. Sporozoites of at different stages of development can be attached to blastophores, ranging from nascent circular forms to short immature vermicules (phase contrast, 400X magnification).

Interestingly, dissections of the midguts from infected females showed there were incongruencies in the infection rate and oocyst numbers in FUMOZ and FUMOZ-BS. The higher infection rate in FUMOZ was not a direct correlation with oocyst numbers, as FUMOZ-BS had significantly higher average number of oocysts (Table 2.4). When the data were broken down further, it was noted that FUMOZ typically had low numbers of oocysts, but had a wide range, with some individuals carrying more than 50 oocysts. On the other hand, FUMOZ-BS was capable of carrying a high number of oocysts with the majority supporting 50 or more oocysts on the midgut.

Table 2.4: Comparison of oocyst load in FUMOZ strains.

Colony (n =)	Average oocyst numbers (±std dev)	Oocyst numbers			
		1 - 10	11 - 30	31 - 50	51 - 100
FUMOZ (59)	25 (±35)	58%	10%	0	32%
FUMOZ-R (64)	8 (±7)	81.3%	18.7%	0	0
FUMOZ-BS (65)	55 (±28)	0	0	38.5%	61.5%

Compared to *An. gambiae, An. funestus* salivary glands were approximately 40 - 60% smaller (Figure 2.8), but were still able to carry *P. berghei*. While infected salivary glands were fairly resistant to mechanical motions during the dissection process, the application of a coverslip once the glands were on the slide tended to rupture them (over 90% of the time), no matter how gently the coverslip was placed. Whenever possible, both salivary glands were extracted for observation. In these instances, if one salivary gland was observed to be infected, the other would be as well. *Plasmodium berghei* sporozoites were usually 12 - 14 μm in length, and concentrated in the two longer lateral lobes of the salivary glands, particularly in the distal cul-de-sac (Figure 2.9). Sporozoites were only detected higher up the lateral lobes in FUMOZ and FUMOZ-BS if the sporozoite count was high. No sporozoites were observed in the medial lobe at any point. The earliest time when sporozoites were observed was 16 days post infected bloodmeal, in FUMOZ. Sporozoites were typically detected in the salivary glands after day 18, and sometimes as late as day 22. The sporozoites in the salivary glands had the classical crescent shape (Figure 2.10), and were often packed densely on top of each other (Figure 2.11).

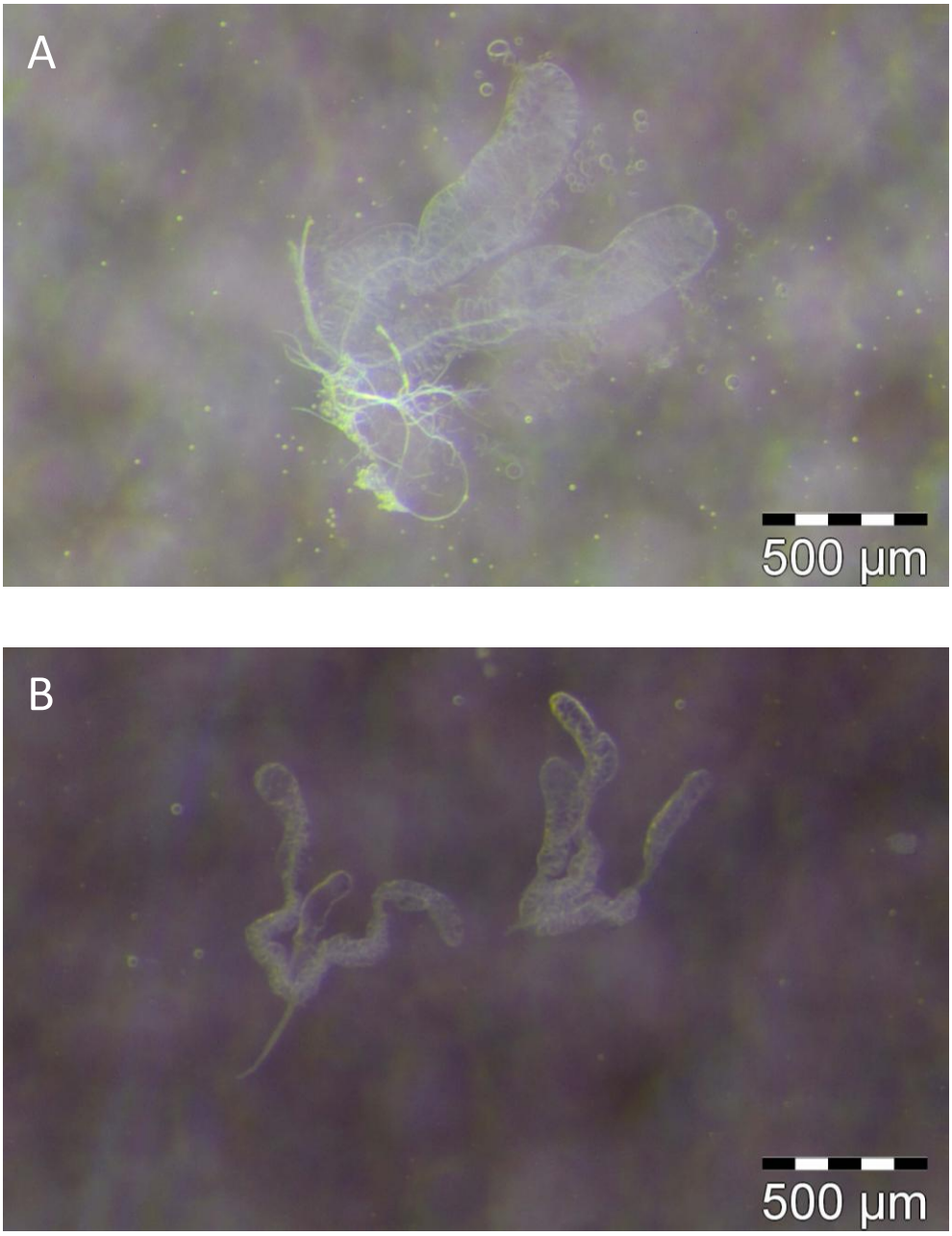


Figure 2.8: Uninfected *An. gambiae* (A) and *An. funestus* (B) salivary glands. *Anopheles funestus* salivary glands are at least 40 - 60% smaller those of than *An. gambiae* (dissection/light microscope, 100X magnification).

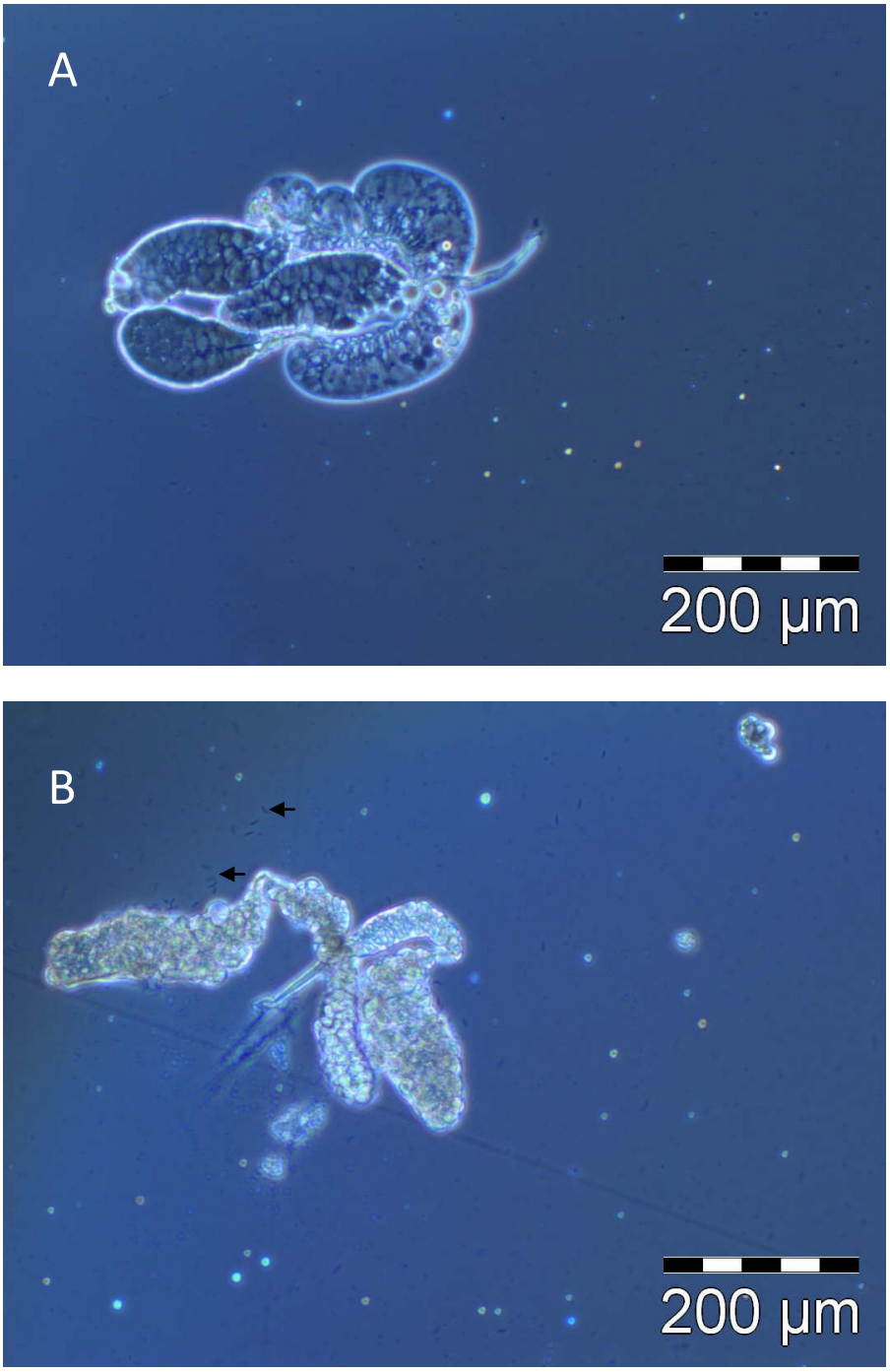


Figure 2.9: Uninfected (A) versus infected (B) *An. funestus* salivary gland. The sporozoites are clustered in the distal end of the lateral lobes, causing opaqueness compared to the transparent lobes in uninfected gland. Some loose sporozoites can be seen near the lateral lobes, indicated by the arrows (phase contrast, 100X magnification).

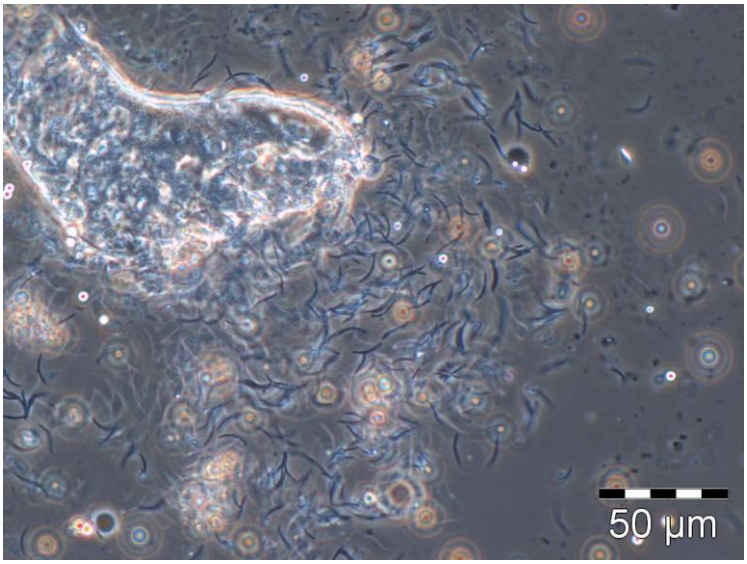


Figure 2.10: A partially ruptured *An. funestus* salivary gland. The *P. berghei* sporozoites have the mature crescent form. A significant percentage of the sporozoites are at different focal planes, leading to the faint, unfocused crescent shapes scattered throughout the image (phase contrast, 400X magnification).

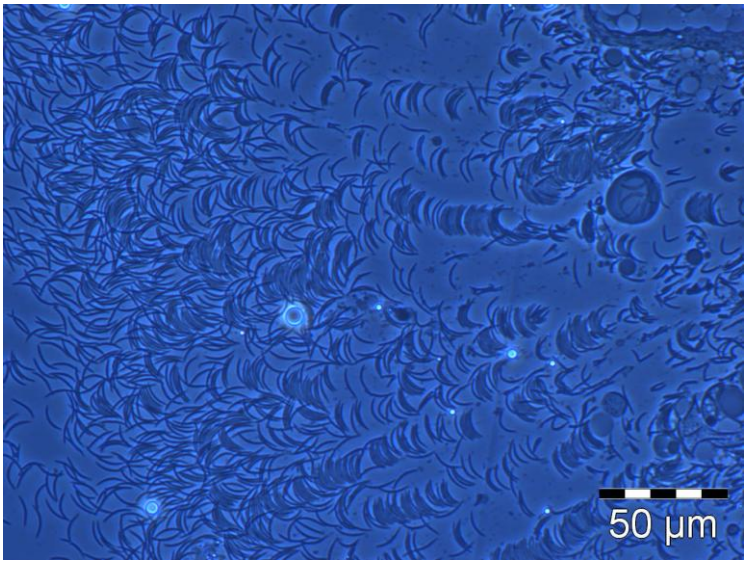


Figure 2.11: Sporozoites released from a fully ruptured *An. funestus* salivary gland. A percentage of the sporozoites are seen still packed tightly on top of each other (phase contrast, 400X magnification).

In terms of sporozoite counts in the salivary glands, the numbers corresponded with the oocyst count, with FUMOZ-BS carrying a very high number in the salivary glands, while FUMOZ and FUMOZ-R had much lower sporozoite counts (Table 2.5). A more detailed breakdown showed that FUMOZ-BS salivary glands often contained more than 10,000 sporozoites, while majority of FUMOZ-R hosted 2,000 or less sporozoites. The base colony had the widest range in the number of sporozoites found in its salivary glands, but never carried more than 10,000 sporozoites (Table 2.5). The feeding rates between FUMOZ and its derivative strains were not significantly different ($p>0.01$) but the infection rates were ($p<0.01$).

Table 2.5: Comparison of sporozoite load in FUMOZ and related sub-strains.

Colony (n =)	**Average sporozoite numbers (±std dev)**	**Sporozoite numbers**				
		1 - 1 000	**1 001 - 2 000**	**2 001 - 5 000**	**5 001 - 10 000**	**10 000 - 15 000**
FUMOZ(59)	2248 (±1173)	20%	30%	30%	20%	0
FUMOZ-R(64)	1586 (±1044)	27%	53%	25%	0	0
FUMOZ-BS(65)	7648 (±4986)	7.7%	15.4%	7.7%	23.1%	46.1%

2.4 Discussion

2.4.1 General observations

Results of the mice infections using C57/Black strain were similar to data reported elsewhere (Killick-Kendrick, 1978; MacKey *et al*., 1980). The infection is always fatal in laboratory mice; the rodents typically succumb 10 - 15 days after infection, depending on the initial injected parasitaemia, and the number of blood passages the parasite has been through (Sinden *et al*., 2002). Infections using infected bloodstock that has undergone one or less

blood passage (P_0 - P_1) were more virulent and usually led to death within a week. With two to four blood passages, the mice survived up to two weeks before becoming terminally ill. Five or more passages increased the mice survival time to over 20 days.

Although the popular laboratory mice strain for *P. berghei* infection is Balb/c, it was not used for this study as we found that the local strain was highly susceptible to the parasites when injected with the same dosage of *P. berghei* as C57BL/6. The majority of Balb/c mice had to be sacrificed within a week because of the severity of the infection. Due to the speedy progression of the infection, Balb/c often displayed symptoms of illness three to four days after IP infection and were unlikely to survive the anaesthetics for bloodfeeding. Ethically, this was also an issue as it meant prolonging the suffering of the mice.

A longer survival time in C57BL/6 mice compared to Balb/c during *P. berghei* infection is consistent with published data (Contraras *et al*., 1980; Mackey *et al*., 1980). However, the infected mice in the current study often do not live past two weeks, which was a shorter period than expected. It is possible that the consistent cyclical transmission (no more than five direct blood passages were performed before restarting a new transmission cycle) allowed the parasite to remain more virulent than continuous direct blood passage transmission. A higher starting parasitaemia level in the IP injections may also have contributed to the shorter survival time. It is interesting that in wild *P. berghei* infections, the pathogenesis is typically mild and rarely leads to the tree rats' deaths, whereas in a laboratory setting, the infected rodent (if susceptible to the parasite), whether tree rats, hamsters or mice, invariably die (Yoeli, 1965). This pattern holds true for the mice infected in this study, and it has been hypothesized that in nature, young tree rats gain protective substances and antibodies from their mothers during suckling (Yoeli, 1965). The difference in this disease progression has not

been investigated in detail during the four decades the infection model has been in existence.

Mice infection was also possible through cyclical transmission, whereby infected mosquitoes were allowed to feed on healthy mice 18 - 21 days after the infected bloodmeal. The infection progresses much faster in this instance, typically leading to fatality within five to seven days. The parasitaemia level in cyclical transmission rarely reaches 10% in the short infection time. Cyclical transmission is essential in maintaining infectious rodent malaria parasites in the laboratory setting (Sinden *et al*., 2002). It not only demonstrates the biological viability of the infection model but also helps to reduce the genetic damage or resulting from changes from direct passage. Direct blood passage is recommended for no more than eight direct passages, as the parasites seem to suffer irreparable genetic damage without passage through the invertebrate host (Sinden *et al*., 2002).

There were a number of observations that applied to the mosquito infection regardless of the species. For each infection, 70 - 90 females were needed for feeding. This was considered the minimum viable number for feeding as most strains had feeding rates between 20 - 50%. Initial attempts at using 50 females often led to too few feeding, and not all bloodfed females will survive the parasite development time even under optimal conditions. Using 100 or more females did not significantly increase the feeding prevalence, as it merely meant there were more females trying to feed over the same amount of surface area, and often bloodfed females will not move away from the mouse until disturbed externally. It also brought up concerns that the mouse may be losing a significant amount of blood, especially with *An. gambiae* strains that tend to cause blood splatter when feeding to repletion.

The second bloodmeal given within a week after the initial infected bloodmeal has been

highly recommended as it not only increases survival of the females, but also bolsters the parasite infection (Ponnudurai *et al*., 1989; Sinden *et al*., 2002). This had a positive effect on both *An. gambiae* and *An. funestus* survival, and death during the parasite development was reduced to 20% or less of the population. Infection rates were also boosted by 2 - 5% depending on the colony, with *An. funestus* colonies benefiting from the second bloodmeal more than *An. gambiae*.

Maintainance of the bloodfed population at 21°C significantly lengthened the lifespan of the adult mosquitoes in all species. Most females will live at least a week longer than the average lifespan at standard insectary conditions. This is likely due to the lower temperature also lowering the base metabolism level in the adults, which allowed them survive for longer. Furthermore, the adults were also seen to be rarely moving except during feeding, so activity was minimized, further reducing the energy expenditure. The infectious status of the mosquitoes remained the same throughout the adult lifecycle, and successful infection of the mice was possible using infected *An. gambiae* more than 35 days old and *An. funestus* more than 50 days old.

Environmental parameters also had an impact on infection. It has been shown that temperature is a significant factor in parasite development, where at higher temperatures (over 24°C) complete parasite development is non-existent, and at lower temperatures (under 18°C), the oocyst-to-sporozoite development is reduced (Yoeli, 1965). Although the effect of temperature on infection was not part of the study, it was noted when the temperature deviated from the optimal range, infection were often adversely affected. This was consistent with results obtained elsewhere, where higher temperature was detrimental on the infection, typically terminating the parasite development (Vanderberg & Yoeli, 1965). At lower

temperatures, the parasite development was slower (sporozoites were usually observed two to three days later than usual), and infection rate was also lower. Due to this, infections in summer were occasionally less successful as the temperature control sometimes could not cope with the higher outside temperatures, and the insectary exceeded 23°C for some hours during the day. Low humidity was also observed to have a negative effect on the development: sporozoites were not observed below 65% RH. There was no obvious effect on parasite development when the humidity was higher than normal (≥85%).

2.4.2 *P. berghei* infections in *An. gambiae* complex colonies

Most of the *An. gambiae* complex colonies tested were able to carry the infection, some more successfully than others. Infection of old colonies (having been in existence for over a decade) produced highly varied results. The G3 colony, which was established in 1975, has been used previously as a viable *P. berghei* vector (Collins *et al.*, 1986) and was the most susceptible *An. gambiae* s.s. colony. However, PALA, established in 1967, was completely resistant to *P. berghei* in this study despite being susceptible elsewhere (Al-Mashhadeni *et al.*, 1980). Considering the significant time span that these two strains have been in existence, it is not surprising that the genetic makeup of the two is different. Collins *et al*. (1986) showed that *Plasmodium* refractoriness is in part linked to simple Mendelian genetics, and selection can create lines resistant to the parasite within three generations, creating long-lasting patterns of susceptibility/resistance. There is also evidence that "wild type" mosquitoes are refractory to the parasite (Riehle *et al.*, 2006). It is possible that in the case of the PALA strain, the initial starting population for the VCRL sub-colony was biased towards *Plasmodium* resistance and selection favouring the resistance phenotype was unknowingly facilitated due to long-term isolation, or other unforeseen events, such as unexpected bottlenecking or adaptation to slightly different insectary conditions and resources. Alternatively, it may have reverted back

to the ancestral genotype, thus becoming refractory to *Plasmodium*. Even in the G3 strain, the infection rate obtained here compared to the data of Collins *et al.* (1986) are significantly different (below 50% versus over 90%), and the same factors mentioned above may have impacted on the infection rate. These differences in results reinforce the idea that long-standing colonies may be quite different, not only to the wild populations from which they originated, but offshoot colonies reared in different laboratories can also be different, especially if significant time has passed since their creation/isolation (Black *et al*., 1988a, b; Chevillon *et al*., 1995; Scarpassa *et al*., 2008; o'Donnell & Ambruster, 2010). For all intents and purposes, it may be useful to consider them as distinct populations that have adapted to different locales, and therefore results from one strain should not be taken as applicable to the same colony based elsewhere. More data on the impact of *Plasmodium* susceptibility in *An. gambiae* with long term inbreeding may be of use in understanding the genetic differentiation of the species through time.

In the remainder of the old colonies, all but one (NAG) were able to carry the parasite, albeit not as efficiently as G3, with average sporozoite indices around 5%. The average feeding rates in these colonies were lower even when more time was given for feeding. The newer colonies (COG, GAH, SUA) were more efficient vectors compared with the old colonies, but none was as effective as G3 in either feeding rate or infectivity. Although *An. gambiae* is assumed to be a malaria vector no matter where it comes from (there are no data to indicate otherwise), there will be differences in infection rates from different localities due to genetic differences, seasonal fluctuations and other environmental factors. Therefore, the initial starting population for any colony may have diminished vectorial capacity to begin with, and the likelihood of this increases if the starting numbers were small. Evolution and genetic drifts towards total *Plasmodium* resistance is clearly possible, but appears to be less likely than a

low level of susceptibility, as only two out of nine strains tested here were fully refractory to the parasite.

Anopheles arabiensis, as the other major malaria vector, is often found in sympatry with *An. gambiae*, while *An. merus* is a minor vector in the coastal region of East Africa, Madagascar and parts of southern Africa (also in sympatry with the major vectors). To the best of my knowledge, the results presented here are the first infection data for both species using *P. berghei*. Colonies of both species were able to carry the parasite, albeit at a low frequency, and often less than the *An. gambiae* colonies. In *An. arabiensis*, most of the colonies had a lower average feeding rate to start with, even after starving and disturbing the cages prior to feeding. The lower feeding rate limited the number of possible infections, so it was unsurprising that the resulting infection rate was low. The infection rate with *P. berghei* was comparable to infections of human malaria sporozoites in wild caught *An. arabiensis* around human settlements that have rarely exceeded 5% (Taye *et al*., 2006; Abdalla *et al*., 2008). The *An. arabiensis* colonies have therefore maintained a baseline susceptibility to *Plasmodium* infection, and the baseline is consistent regardless of the regions that the colonies came from (Eritrea, Mozambique, South Africa, and Zimbabwe).

In the two *An. merus* colonies, the average feeding rates were better than for *An. arabiensis*, but the sporozoite index was of a similar level to the *An. arabiensis* colonies. *Anopheles merus* is only of minor importance in malaria transmission, as it is usually found in sympatry with the major vectors, typically accounting for less than 10% of mosquitoes, if found at all (Shiff *et al*., 1995; Temu *et al*., 1998; Pock Tsy *et al*., 2003; Cuamba & Mendis, 2009). Infection rates of wild *An. merus* are usually low, at three to four percent (Mosha & Petrarca, 1983; Cuamba & Mendis, 2009), but higher rates have been detected (Temu *et al*., 1995). The

P. berghei infection rate observed here was slightly lower than what is recorded in the wild populations with human malaria. This may be due to either long-term isolation like the *An. gambiae* colonies, or that the colonies were established from *An. merus* populations in South Africa, where it has never been incriminated in malaria transmission in nature.

A single *An. quadriannulatus* colony, SANGWE, was also tested. *Anopheles quadriannulatus* and its sister species *An. amharicus* are the only medically unimportant members of the *An. gambiae* complex, as human *Plasmodium* parasites have never been detected in the wild populations, and the mosquitoes were thought to be zoophilic (Pates *et al*., 2001). The colony had a low feeding rate, but since this is also the case during routine maintenance, it is likely a carryover effect that influenced the infection experiments. The colony was completely resistant to *P. berghei* infection, which is in line with published data (Habteworld *et al*., 2008). However, it has also been shown elsewhere that *An. quadriannulatus* can be infected with *P. falciparum*, albeit at a much lower rate than *An. gambiae* and *An. stephensi* (Takken *et al*., 1999). It is possible the discrepancy between the results here and previously published data arose from the different strains of *An. quadriannulatus* used. Both published papers utilized the SKUQUA strain from South Africa, while the SANGWE strain, used here, was from Mozambique.

2.4.3 *P. berghei* infections in *An. funestus*

When *An. funestus* was successfully colonized, two baseline colonies were established (FANG and FUMOZ), along with a line selected for high permethrin resistance from FUMOZ (FUMOZ-R) (Hunt *et al*., 2005). As FUMOZ was partially resistant to permethrin, it was thought that a third, permethrin-susceptible colony would be useful for a proper comparative study between insecticide susceptible versus resistant strains. Although FANG is fully

susceptible to permethrin, it is from the other side of the continent to FUMOZ (Angola and Mozambique, respectively), which means comparison between the two colonies may not be valid due to geographic variance. Furthermore, initial infection data showed that FANG was not only less prone to feed on mice, but the infection rate was also significantly lower than for FUMOZ. A stable susceptible colony created from FUMOZ, along with FUMOZ-R, would provide a more useful comparison not only for the current infection model, but also for future work where insecticide resistance (or lack thereof) may have an impact. The establishment of FUMOZ-BS showed it was possible by simple selection methods to re-establish some degree of insecticide susceptibility in the strain.

It should be mentioned that although FUMOZ-BS was considered the susceptible variant of the three strains, it was not strictly in accordance with the WHO definition of insecticide resistance/susceptibility. The WHO defines mosquito susceptibility to an insecticide as mortality of 98 - 100%, and 80 - 97% mortality as "possibility of resistance that needs to be confirmed" (WHO CDS CPC MAL 98.12 document, 1998). As the selection process for FUMOZ-BS used families that had at least 85% mortality, the strain would fall into the latter category. However, it was impractical to try and select only families that had 98 - 100% mortality as this would have greatly reduced the number of families that could be retained for breeding. Consequently, the degree of insecticide susceptibility was set at 85% to facilitate the establishment of the colony. It may be possible to further select a proper permethrin-susceptible colony in the future, but as this was not the main aim of the study, the use of a semi-susceptible colony was thought to be sufficient for the required purposes.

The establishment of an infection system using *An. funestus* required much optimizing. The main obstacle was getting the usually highly anthropophilic *An. funestus* to feed on mice.

Even though the colonies are routinely maintained using guinea pigs as the source of the bloodmeal, the females were still reluctant to feed on the smaller mouse. This was particularly obvious when compared to most *An. gambiae* colonies, which required little incentive to feed on the mice. Starving *An. funestus* females prior to providing a bloodmeal became even more important to induce feeding, as it increased the feeding percentage quite significantly. The second issue was when to provide the *An. funestus* with the bloodmeal. *Anopheles gambiae* were usually fed three to four days after eclosure for infection or mating purposes. Although *An. funestus* of the same age will feed, it is only a small number that would feed on mice, even with starving. It was thought that because the *An. funestus* live longer than *An. gambiae*, their maturation process would take longer. The bloodmeal was therefore shifted back to at least a week after eclosure, and it was eventually found that bloodmeals given 10 - 14 days after emerging led to the greatest number of females feeding on the rodents. A small cohort of F_1 progeny from wild *An. funestus* caught in Ghana was also used in the infection process and although subjected to the same conditions as the colonies, the females fed very poorly. The feeding preference of the species thus still remains an obstacle.

Average oocyst number in infected FUMOZ-R females was in the single digits compared with the other two *An. funestus* colonies. The oocyst number is also the lowest ever observed compared to any *P. berghei* vectors to date (Yoeli, 1965). On the other hand, the average number of oocyst in FUMOZ-BS was comparable with *An. dureni* (60 - 80), but not as high as *An. quadrimaculatus*, which is able to carry 100 - 500 oocysts (Yoeli, 1965) – the number of oocysts never exceeded 100 in FUMOZ-BS. FUMOZ was the median species, covering both ranges, with a bias towards the lower oocyst numbers. This was logical considering that the base colony gave rise to the other two colonies, and as parasite refractoriness seems to be heavily influenced by genetic traits, the original colony should have individuals with

genotypes ranging from somewhat resistant to the parasites to being very susceptible.

The average sporozoite numbers in the three colonies correlated with the oocyst numbers, with FUMOZ-R being the lowest, followed by FUMOZ and FUMOZ-BS. Sporozoite number in FUMOZ-R was significantly higher than observed in *An. quadrimaculatus* (100 - 300), despite carrying less oocysts. Infected FUMOZ-BS females' sporozoite numbers were similar to *An. stephensi* (6,000 - 8,000), but the range in the former is much greater, from about 500 to just over 13,000. The majority of the infections exceeded 5,000 sporozoites per female, so the strain can be considered highly susceptible to the parasite. FUMOZ as the median colony displayed a low average sporozoite number. Its range was quite evenly spread, but the sporozoites never passed 9,000, and were not lower than 200. The persistence of sporozoites for the remainder of the infected females' lives is also consistent with data from other mosquitoes (James, 1926), and shows that the *An. funestus* salivary glands is a non-threatening environment for the parasite.

The combined feeding and infection data showed the base colony had the highest rate in both respects, followed by FUMOZ-BS and FUMOZ-R, whereas FUMOZ-BS had the highest oocysts and sporozoites loads. Thus there was no association between the most frequently infected strain (FUMOZ) and the strain most amenable to parasite development (FUMOZ-BS).

This is the first known instance showing a possible link between *Plasmodium* parasite susceptibility and insecticide susceptibility in *An. funestus*. Existing data for other anophelines are somewhat contradictory, as most studies seem to support insecticide resistance being associated with increased parasite survival (Vontas *et al*., 2001, 2004; Christophides *et al*., 2004), but the correlation is not consistent (Vontas *et al*., 2004). This difference in results has

been attributed to different vector and parasite species, thus more work is necessary to fully elucidate the association. Currently there is no known related interaction or pathway that acts in both insecticide and *Plasmodium* resistance mechanisms in malaria vectors. The most appropriate data regarding such interaction is found in *Culex quinquefasciatus* and the lymphatic filariasis parasite, *Wuchereria bancrofti*. Insecticide resistant *Cx. quinquefasciatus* based on increased esterase activity affects the development of *W. bancrofti* larvae in the gut cells (McCarroll *et al*., 2000). The correlation was easier to quantify in *Cx. quinquefasciatus* as the esterase amplicon duplication is the dominant resistant mechanism, found in over 80% of insecticide resistant *Cx. quinquefasciatus* (Hemingway & Karunaratne, 1998). It may be more difficult to correlate the two phenotypes in anophelines as the malaria vector has a number of insecticide resistant mechanisms which are involved depending on the type of insecticide the mosquitoes are exposed to.

Susceptibility to insecticides is likely an ancestral phenotype for both *An. gambiae* complex and *An. funestus* group species as mosquitoes had never been exposed to high amounts of toxic chemicals until human intervention - both in public health and agriculture - began on a mass scale in the mid-20th century (WHO, 1979). The current understanding of insecticide resistance is that it is primarily based on biochemical pathways, typically relying on up-regulation of detoxification enzymes to minimize the impact of neurotoxic compounds (Hemingway & Ranson, 2000). Anopheline refractoriness to *Plasmodium* is also likely the ancestral phenotype (Riehel *et al*., 2006; Habtewold *et al.*, 2008), but the varying degree of infection here showed that it is rare for strains to be fully refractory to the parasite even when the environment is optimal for the insect. The mosquito's susceptibility or refractoriness to *Plasmodium* could be described as a gradient (Beier, 1998), and it is very rare under wild or laboratory conditions to find cohorts that are either totally (i.e. 100%) susceptible or resistant

to the parasite. If parasite refractoriness was indeed the ancestral phenotype, it suggests that such a phenotype was skewed towards being highly refractory, which may be part of the reason why the vast majority of wild mosquitoes are not infected.

To determine the development of *P. berghei* in *An. funestus*, dissections for midgut and salivary glands were essential. It was unknown if the parasite developed at a different rate in *An. funestus* compared with other *P. berghei* vectors, particularly considering the longer adult lifespan of *An. funestus*. Midgut dissections revealed oocysts consistently only 10 days after the infected bloodmeal, and maturation of oocysts takes at least 15 days after the bloodmeal, with maturing oocysts still observed at 20 days post feeding. This development time is much longer than in the natural vector *An. dureni*, which takes 8 - 11 days to oocyst maturation. It is also longer than the development in the experimental vectors *An. quadrimaculatus* and *An. stephensi*, which takes 13 - 14 days to oocyst maturation (Yoeli, 1965). Salivary glands dissection was initiated from 14 days post bloodmeal, and the earliest time when sporozoites were seen in the glands was 16 days after the infected bloodmeal, in line with the slow oocyst development. The average time for sporozoite invasion was around day 18 - 21, and sometimes as late as day 24. Compared to the recently publication by Xu *et al*. (2013) using the same vector-parasite pairing, the appearance of oocysts in this study was slower by three days, but the invasion of salivary glands was very similar, around day 18 - 20. The slight differences in development time may be due to using *An. funestus* from different localities, and it may be of interest to compare the two strains for similar genetic markers (e. g. anti-*Plasmodium* gene expression) in the future.

The other major difference in infecting *An. funestus* versus *An. gambiae* complex species, was that instead of using three-to-four days old adults, *An. funestus* females needed to be 10 - 14

days old before being given the infected bloodmeal. This is probably due to the longer adult lifespan of *An. funestus* compared to *An. gambiae*. Another theory is that as *An. funestus* is highly anthropophilic, they are less inclined to feed on other animals. However, as oogenesis in anopheline mosquitos is anautogenous and requires nutrients from vertebrate blood, delaying the bloodmeal may eventually force the females to feed on whatever source of blood is given. This could be correlated to the observation that although it is possible to get *An. funestus* females to feed on rodents three to four days after eclosure, the feeding rate is very low (typically less than 10%) and successful sporogony is even lower out of the bloodfed population (less than 5% of fed females).

Although early data showed that *An. funestus* was not a vector for *P. berghei* (van den Berghe, 1954), this has been proven not to be the case (Xu *et al.*, 2013; Lo & Coetzee, 2013). Both wild and laboratory colonized *An. funestus* can in fact be viable vectors for rodent malaria, possibly as good as *An. stephensi*, the traditional experimental vector used for supporting *P. berghei*. The main reason for this dramatic change in results is the increased understanding of the conditions required for *P. berghei* infections. The first recorded attempt of infecting *An. funestus* was in the early 1950s, soon after its discovery when much about the parasite was unknown (particularly the lower temperature requirements) and infection attempts using unnatural vectors were often unsuccessful (Bruce-Chwatt, 1978). Successful cyclical transmission in the laboratory was only achieved by Yoeli in 1963, where established *Anopheles* colonies were tested. This naturally excluded *An. funestus* since no colony existed at that point. Secondly, *An. funestus* underwent some reclassification after the 1960s (Gillies & de Meilon 1968; Gillies & Coetzee; 1987). It is likely that the original infectivity test performed by van der Berghe (1954) was done on *An. funestus* s.s., as the species has a wide distribution in Africa, but this cannot be proven conclusively. It would have also been difficult

to persuade the wild *An. funestus* cohort to feed on infected rodents due to the species' highly anthropophilic nature, which may have also contributed to the failed infection.

The data presented here have shown conclusively that *An. funestus* is a viable vector for *P. berghei*, albeit with slower development time compared to other known vectors of the parasite. In the case of FUMOZ-BS, the species was highly competent, as the highest *P. berghei* sporozoite rates ever documented were seen in individuals of this colony. Furthermore, some association was observed between insecticide resistance and parasite refractoriness, where strains with lower insecticide resistance carried more intense infections, and vice versa. The successful infection result will hopefully provide impetus to use *An. funestus* in future vector-parasite research, and the association between insecticide resistance and parasite refractoriness should be further examined in *An. funestus* and other malaria vectors.

Chapter 3: Distribution of 2La in *An. gambiae* colonies and correlation with dieldrin and *P. berghei* exposure

3.1 Introduction

Detailed information on how to maintain *Anopheles gambiae* colonies in a laboratory setting has existed for six decades (Moores, 1953; Shute 1956). This has led to the establishment of numerous strains and colonies in a controlled environment. Wild populations, insecticide resistant variants, and genetically altered cohorts are all available for research purposes (see MR4 catalogues). However, their genetic profiles are often unknown, which may influence resistance to insecticide, malaria vectorial capacity, and the species' ability for ecological adaptation/differentiation. Furthermore, colonized populations are likely to show reduced genetic variation compared to the wild, ancestral cohort from which they were established. Local random genetic drift is known to influence genetic differentiation in a number of mosquito vectors, including *Culex pipiens* (Chevillon *et al*., 1995), *Aedes aegypti* (Scarpassa *et al*., 2008) and *Aedes albopictus* (O'Donnell & Armbruster, 2010). Physiological and genetic characteristics may also be significantly influenced as a consequence of genetic drift during local population establishment due to a small starting number of individuals, or collection from a reduced population (Black *et al*., 1988a, b). Certain colonies have also been in captivity for decades under standard insectary conditions (e. g. the G3 and KGB strains have been maintained for more than 3 decades), and are likely to have diverged from the original populations due to long-term genetic isolation, inbreeding, or bottlenecks. It is therefore important to assess at least some of the genetic variation within a colony,

particularly those important genotypes that can influence insecticide resistance and adaptations. At the VCRL Botha de Meillon insectary, a large number of *An. gambiae* colonies are maintained for experimental purposes as well as controls (see Appendix 1).

In *An. gambiae* complex mosquitoes, there are over 30 paracentric chromosomal inversions, and ten of these can be used to differentiate between morphologically identical sibling species (Coluzzi *et al*., 2002). Some of the paracentric inversions are also non-randomly distributed, suggesting that these rearrangements are present due to selection pressure on alleles trapped within the inversions, which may be able to confer adaptive advantages in different ecological niches (Coluzzi *et al*., 2002). Indeed, a number of these inversions have been linked to environmental, physiological and biological adaptations (Coluzzi *et al*., 1979; Petrarca & Beier, 1992; Brooke *et al*., 2000; Riehle *et al*., 2006; Gray *et al*., 2009). Although chromosomal inversions do not alter gene function (unless the gene sequence spans the breakpoint), the recombination of alleles situated in the inverted segment is suppressed in the heterokaryotypes (Kirkpatrick & Barton, 2006). This cross-over suppression allows for the maintenance of association between single or multiple alleles and particular inversion arrangements that can also inadvertently provide adaptive advantages in contrasting conditions, which in turn enable the organism to adapt to highly divergent habitats and widen their range (Kirkpatrick & Barton, 2006).

A particularly good example of this is the 2La inversion. This is a large region on the left arm of chromosome 2 of the *An. gambiae* complex, which can be inverted during chromosomal recombination; it is the only inversion on 2L and is highly polymorphic (White *et al*., 2007). Inversion 2La's close correlation to the humidity cline was described in *An. gambiae* over 30 years ago (Coluzzi *et al*., 1979). is almost fixed in dry regions and decreases in frequency

with increasing humidity. Individuals carrying the inverted arrangement are also more likely to be found indoors due to the nocturnal saturation deficit (Sharakhov *et al*., 2006). More recently, inversion 2La has been associated with mosquito-parasite interactions in *An. gambiae*. Two immune genes, APL1 and LRIM1, act against a number of *Plasmodium* species and are found within the 2La inversion (Osta *et al*., 2004; Riehle *et al*., 2006), along with four *Plasmodium*-resistant loci that influence the degree of parasite encapsulation and infectivity (Riehle *et al*., 2006). The 2La inversion has also been correlated with enhanced thermal tolerance in larvae (Rocca *et al*., 2009), and increased resistance to dessication in early adulthood of female mosquitoes (Gray *et al*., 2009). Lastly, the assortment of this inversion closely associates with dieldrin resistance/susceptibility in certain *An. gambiae* laboratory colonies (Brooke *et al*., 2000) because the rdl locus associated with dieldrin resistance occurs within inversion 2La (Holt *et al*., 2002). It is therefore important to determine the 2La inversion's frequency in a given *An. gambiae* s.s. population, be it wild or laboratory, as it can affect the species' adaptation and fitness in various ways.

The traditional method of karyotyping inversions is by cytogenetics (Coluzzi, 1968; Green, 1972; Hunt, 1972), but the technique has several restrictions which have been limiting factors in advancing our understanding of 2La. There are two possible stages of mosquito development for obtaining viable polytene chromosomes for inversion karyotyping: 1) from the salivary glands of 4th instar larvae (Frizzi, 1947), or 2) from the large nurse cells in the ovaries of half-gravid females (Coluzzi & Sabatini, 1968). The latter is the easier to obtain but it is still restricted by practical issues. The length of the gonotrophic cycle is dependent on ambient environmental conditions where warmer conditions accelerate the process (Gillies, 1953), so it can be difficult to ascertain the best time to collect the nurse cells. This automatically precludes the possibility of performing cytogenetic analysis on archived

samples that have not been prepared properly, nor can it be done on males. In addition, cytogenetics is time consuming in terms of preparation and analysis of the samples, requiring considerable expertise to analyze the banding patterns correctly.

Certain advances in the past decade have helped to alleviate dependence on cytogenetics for inversion 2La analyses. Sequencing of the entire *An. gambiae* genome (Holt *et al*., 2002) provided the means for detailed analyses of the 2La breakpoint structures (Sharakhov *et al*., 2006) and allowed for the design of a Polymerase Chain Reaction (PCR-based) method for karyotype analysis. The primer pairs that generate the PCR products span the breakpoints, thus forming the basis for discrimination between inversion arrangements (White *et al*., 2007). The molecular karyotyping is not only faster and easier to analyze, but also can be used on males and any archived specimens, which provides significant advantages over cytogenetics.

The aim of this chapter was to: a) determine if the PCR method of White *et al*. (2007) is an appropriate substitution for inversion 2La karyotyping by cytogenetic analysis using GAH, an *An. gambiae* laboratory colony known to be heterozygous for the inversion (B. Brooke, personal communication); b) determine the frequencies of inversion 2La in other polymorphic *An. gambiae* laboratory colonies that have not been monitored for over a decade (Brooke *et al*., 2000) or have never been examined not at all; c) compare the frequencies of 2La karyotypes between a base *An. gambiae* colony and a dieldrin-resistant selected strain in order to determine if there is a long-term association between the 2La inversion and dieldrin resistance; and d) infect samples of 2La polymorphic colonies with *P. berghei* in order to determine if the assortment of the 2La inversion associates with parasite infectivity.

3.2 Materials and methods

3.2.1 *An. gambiae* colonies

The following *An. gambiae* laboratory colonies, known to be polymorphic for the 2La inversion, were used: CIG (Cote d'Ivoire), GAH (Ghana), IANP20 (Nigeria), COG and COG-EXP (Republic of Congo). COG-EXP is an insecticide resistant selected line derived from COG. The selection procedure was based on adult exposures to 0.05% deltamethrin, 4% dieldrin and 4% DDT every generation as per WHO (1998) exposure protocols. Survivors from each set of exposures constituted the breeding cohort for the next generation, thus maintaining the colony's resistance profile. All colonies were maintained under standard insectary conditions.

3.2.2 Cytogenetics for 2La karyotyping

Cytogenetics was performed largely according to previously established protocols (Green 1972; Hunt, 1973). Adult female *An. gambiae* mosquitoes less than a week old were collected from the GAH colony, provided with a bloodmeal, and dissected 26 - 30 hours later once they had reached the half-gravid stage. The females were anaesthetized with diethyl ether and their ovaries were dissected from the posterior region of the midgut. The ovarioles were then suspended in Carnoy's fixative solution (1:3 ratio of glacial acetic acid:absolute ethanol) for at least 24 hours. The ovaries were stored in Carnoy's solution at 4°C while the remainder of the carcasses were stored individually on silica gel for later use in correlated PCR assays of White *et al*. (2007).

The polytene chromosomes were prepared for microscopy as follows. A small volume of 50% propionic acid was mixed with 2% lacto-aceto orcein dye on a slide. Ovaries with associated

nurse cells were removed from the Carnoy's solution and transferred onto the slide. It was important to prevent the ovaries from drying out during the transfer. The tissues were allowed to absorb the propionic acid and dye by osmosis for approximately three minutes whereafter as much of the stain as possible was removed by dabbing with filter paper. The ovarioles were resuspended in 50% propionic acid and a coverslip was gently lowered onto the prepared tissue. Pressure was firmly applied to the coverslip by tapping with a modified dissecting needle in order to rupture the nurse cells and release the polytene chromosomes. These chromosome preparations were viewed and photographed under phase-contrast microscopy. The banding sequences of the polytene chromosomes in question were compared to those of a reference cytogenetic map (Green, 1972; Coluzzi *et al*., 1977) in order to karyotype inversion 2La. At least fifteen females for each genotype (2La homozygote, 2La/+^{a} heterozygote, and 2L+^{a} homozygote) were identified and their respective carcasses were then processed for molecular karyotyping.

3.2.3 PCR for detection of 2La inversion

DNA was extracted from preserved carcasses using Collin's method (1987). All plastics (including pipette tips, eppendorff tubes, etc.) were autoclaved for 20 minutes at 121°C before the extraction. The grinding buffer was composed of 1.6 ml NaCl (1M), 1.095 g sucrose, 2.4 ml EDTA (0.5M), 1 ml SDS (10%), and 2 ml Tris-Cl (pH 8.6, 1M). The final volume was made up to 20 ml using distilled water. Each specimen was placed into a 1.5 ml microcentrifuge tube and was ground as finely as possible using a pestle. The pestle was left inside while 200 µl grinding buffer was added. The specimen was ground again and the pestle was removed while ensuring as little as possible of the material and buffer remained on the pestle. The mixture was incubated at 70°C for 30 - 60 minutes, and 28 µl KAc (8M) was added. This was mixed by vortexing and then incubated on ice for 30 minutes. The mixture

was then centrifuged for 20 minutes at 13,000 rpm, and the supernatant was carefully transferred to a clean 1.5 ml microcentrifuge tube without disturbing the pellet which was then discarded. Ice cold 100% ethanol (400 µl) was added to the supernatant, mixed (by inverting the tube briefly) and left overnight at -20°C. The mixture was then centrifuged for 30 minutes at 13,000 rpm. The supernatant was removed without disturbing the pellet. Ice cold 70% ethanol (200 µl) was added to the pellet and gently mixed. The mixture was centrifuged again for 30 minutes at 13,000 rpm. The supernatant was again removed, and the pellet was allowed to air dry. The DNA pellet was resuspended in 150 - 200 µl TE buffer (1X).

The PCR assay was adapted from the protocol of White *et al*. (2007), with some modifications. The three PCR primer sequences were those designed by White *et al*. (2007), but were renamed as 2La (27A2), 2L+ (DPCross5), and universal (23A2) for easier identification. The PCR conditions were as follows: 94°C for two minutes; 35 cycles of 94°C for 45 seconds, 55°C for 60 seconds, 72°C for 90 seconds; a final extension at 72°C for 10 minutes. For each 12.5 µl PCR reaction, the following reagents were added: 1X PCR reaction buffer, 0.8 mM of each primer, 2.5 mM $MgCl_2$, 0.4 mM dNTPS, 0.5 U of Dreamtaq (Fermentas, USA, cat. #EP0701), 100 - 200 ng DNA, and the remaining volume made up with double distilled water. The PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. The gel was run at 100 - 120 V for an hour to separate the products. The gel was then placed under UV light to visualize the PCR products.

PCR was performed on the karyotyped samples of the GAH strain and then on CIG and IANP20 using 50 - 55 randomly selected individuals from each colony. Initial baseline sampling of COGS was performed using samples drawn from the F_9 to F_{13} generations. Random sampling of COGS and COGS-EXP (35 - 40 adults) was then performed every

alternate generation from F_{15} - F_{25} to determine whether the inversion distribution was being influenced by exposure to insecticide. At F_{25}, both strains were subjected to exposure to dieldrin as per the WHO (1998) insecticide exposure protocol. DNA was extracted from both the surviving and dead cohort, and subjected to PCR to determine the 2La genotypes of each phenotypically characterised specimen.

The PCR results were used to determine if the cohorts were in Hardy-Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$) for 2La, where 'p' is the frequency of one arrangement, and 'q' the frequency of the alternate arrangement. Chi-square (X^2) Tests were also performed to determine if the inversion frequencies varied significantly between generations, or between COGS and COGS-EXP of the same generation, or between dieldrin resistant and susceptible specimens.

3.2.4 Molecular karyotyping of 2La in *P. berghei* infected mosquitoes

Adult females from COGS, IANP20 and GAH were infected with *P. berghei* as described in chapter two (section 2.2.3). Eighteen days post bloodmeal, the salivary glands were dissected to determine if sporozoites were present. The female carcasses were separated according to their infectivity status; DNA extraction and PCR followed the same methods as described in 3.2.2. At least 30 infected females from each colony were processed in this manner.

The infected cohorts were also examined for Hardy-Weinberg equilibrium with respect to inversion 2La. Chi-square Tests were performed to determine if the inversion frequencies varied significantly from between the infected cohort and the uninfected population.

3.3 Results

3.3.1 Comparative study of 2La karyotyping between cytogenetic analysis and PCR

The 2La inversion was karyotyped according to banding patterns on the standard chromosome map (Figures 3.1 - 3.3). 2L+^{a}/a heterozygotes were the easiest to identify due to the typical looping that occurs as a consequence of the two arrangements (Figure 3.1). Using PCR, 2L+^{a} homozygotes gave rise to a 207 bp fragment, 2La homozygotes a 492 bp fragment, and 2L+^{a}/a heterozygotes produced both bands (Figure 3.4), as described by White *et al*. (2007). The comparative study using cytogenetics and PCR karyotyping produced a good correlation between the two sets of results. Out of 54 samples of mixed genotype karyotyped by cytogenetics, three samples (5.6%) could not be correlated due to poor PCR amplicon quality. All three samples were heterogygotes according to cytogenetic analysis.

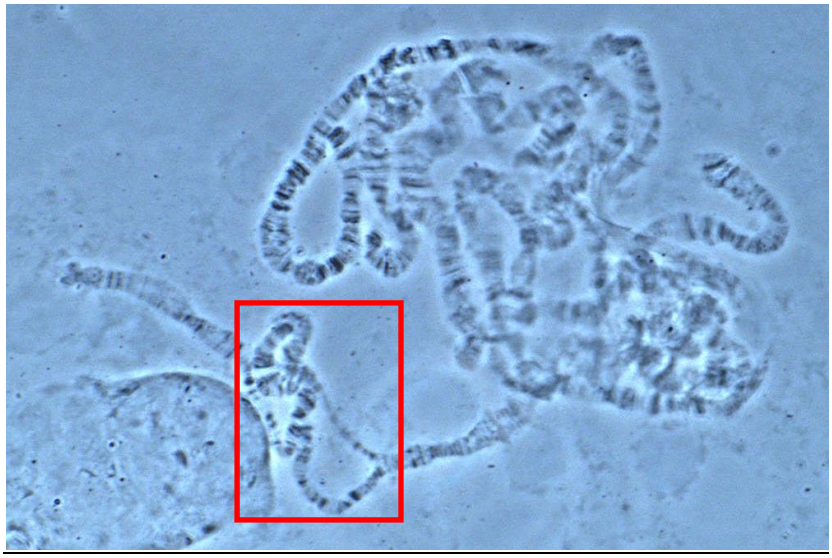


Figure 3.1: 2La/+^{a} heterozygote in GAH female showing the typical chromosome looping that occurs in heterozygotes (phase contrast, 60X magnification).

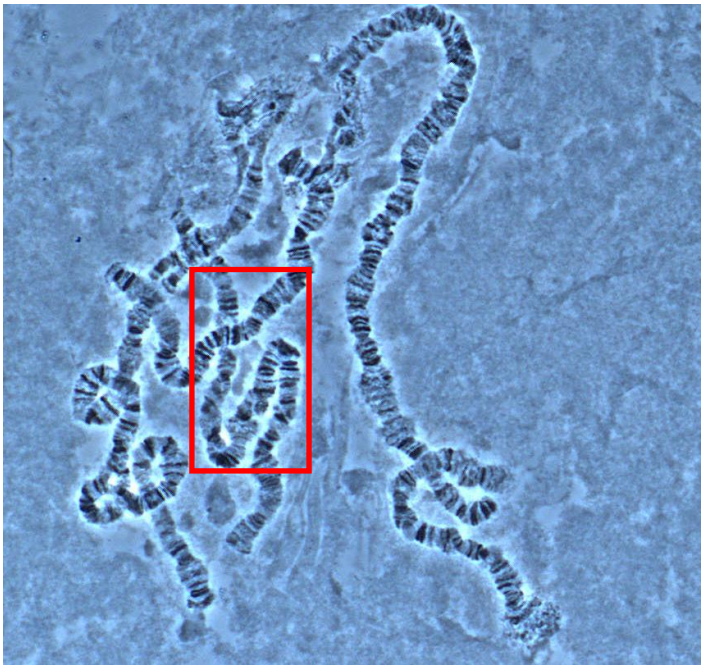


Figure 3.2: 2L+^{a} homozygote in GAH female (phase contrast, 60X magnification).

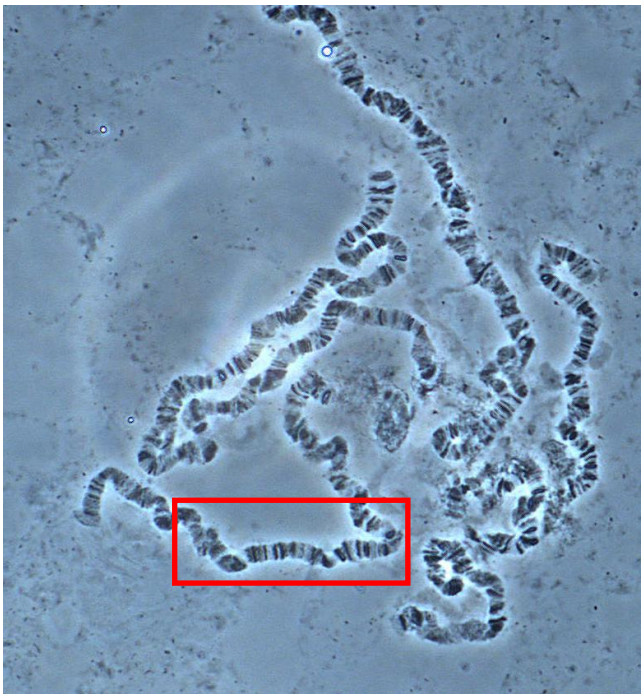


Figure 3.3: 2La homozygote in GAH female (phase contrast, 60X magnification).

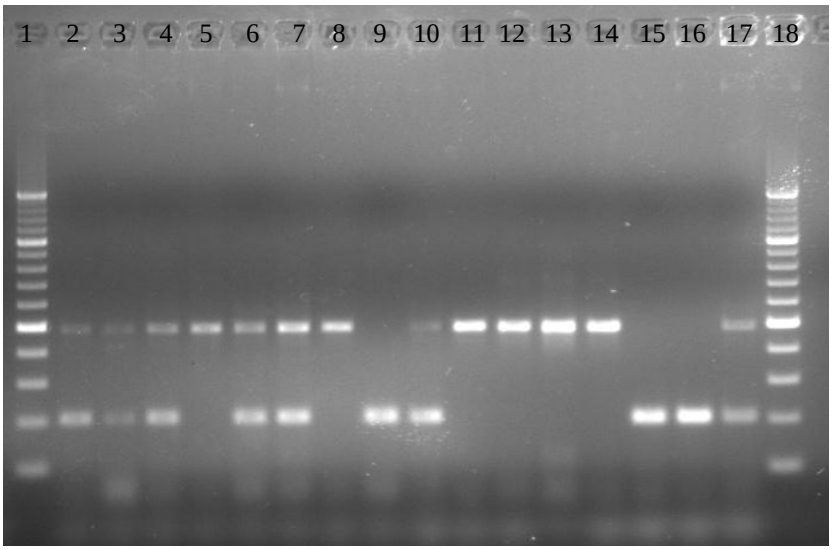


Figure 3.4: Inversion 2La karyotype PCR results on 1.5% agarose gel. The samples were run with 100 bp molecular markers in lanes 1 and 18. $2L+^{a}$ homokaryotypes gave rise to a single 482 bp fragment (lanes 5, 8, 11 - 14), while 2La/a produced the smaller 207 bp band (lanes 9, 15 - 16). Heterokaryotic samples produced both bands (2 - 4, 6, 7, 10, 17).

3.3.2 Frequency of 2La in *An. gambiae* laboratory colonies

PCR was used to determine the 2La inversion frequencies in GAH, CIG, IANP20 and COGS, by random sampling of the colonies (Figure 3.5). Each of the colonies displayed quite different profiles for the inversion. Allelic frequencies for all colonies were in Hardy-Weinberg equilbrium (Table 3.1), but Chi-square analysis showed the genotype frequencies were rarely in equilibrium (Table 3.2). In GAH, the population (n = 55) is dominated by the heterozygotes (72.7%), with a minority (27%) of 2La homozygotes. On the other hand, 2La homozygotes were dominant (92%) in CIG, with heterozygotes (8%) making up the remainder of the population (n = 52). In COGS (n = 37) and IANP20 (n = 53), 2La homozygotes were also dominant (67.6% and 86.8%, respectively) although $2L+^{a}$

homozygotes and heterozygotes were both present (in COGS 16.2% for both; 5.7% and 7.5% in IANP20).

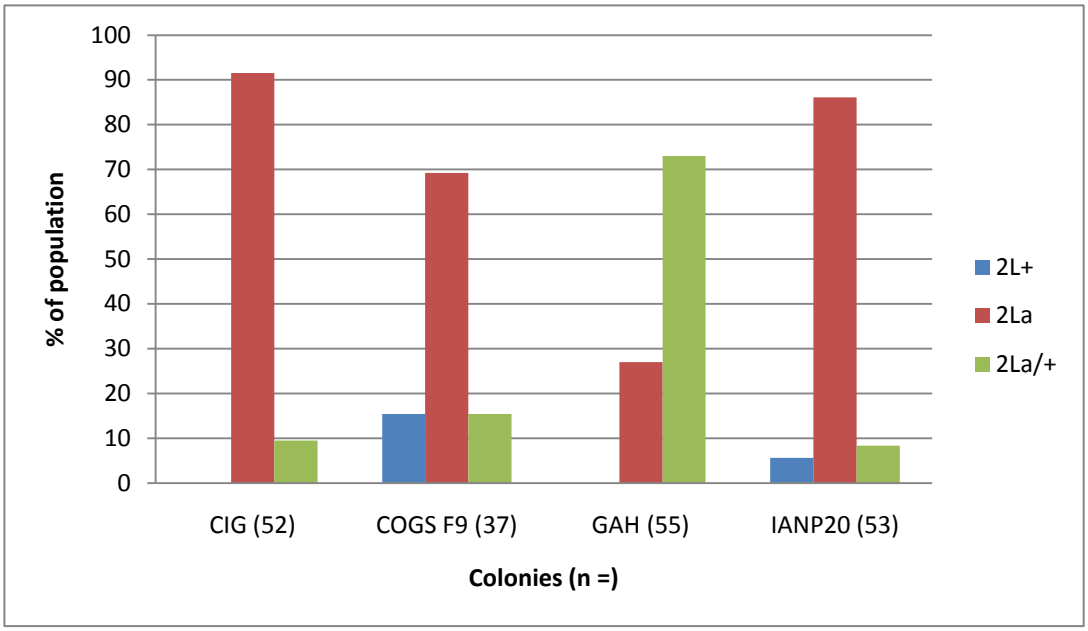


Figure 3.5: Frequencies of the 2La inversion arrangements in four *An. gambiae* polymorphic laboratory colonies.

Table 3.1: Frequency of the 2La alleles in the PCR karyotyped cohorts and subsequent Hardy-Weinberg equilibrium calculations.

Colony/generation	Alleles (2n =)	2La (p)	2L+ (q)	p^2	2pq	q^2
CIG	104	100	4	0.925	0.074	0.001
COGS F_9	76	58	18	0.582	0.362	0.056
GAH	110	70	40	0.405	0.463	0.132
IANP20	106	96	10	0.820	0.171	0.009
COGS F_9 infected	28	21	7	0.5625	0.375	0.0625
GAH infected	34	31	3	0.831	0.161	0.008
IANP20 infected	10	10	0	1.000	0.000	0.000
COGS F_{11}	70	46	24	0.432	0.451	0.117
COGS F_{13}	72	43	29	0.357	0.481	0.162
COGS F_{15}	80	42	38	0.276	0.499	0.225
COGS-EXP F_{15}	76	16	60	0.044	0.332	0.624
COGS F_{17}	78	34	44	0.190	0.492	0.318
COGS-EXP F_{17}	74	49	25	0.438	0.447	0.115
COGS F_{19}	72	44	28	0.374	0.475	0.151
COGS-EXP F_{19}	78	51	27	0.428	0.453	0.119
COGS F_{21}	78	36	42	0.213	0.497	0.290
COGS-EXP F_{21}	80	32	48	0.16	0.48	0.36
COGS F_{23}	70	26	44	0.138	0.467	0.395
COGS-EXP F_{23}	76	18	58	0.056	0.362	0.582
COGS F_{25}	74	25	49	0.114	0.447	0.439
COGS-EXP F_{25}	76	32	44	0.177	0.488	0.335
COGS F_{25} dieldrin exposure	96	42	54	0.191	0.492	0.317
COGS-EXP F_{25} dieldrin exposure	100	62	38	0.384	0.472	0.144

Table 3.2: Chi-square Tests for generational 2La karyotype comparisons in *An. gambiae* colonies.

Colonies	**X^2 value**	**p ≤**
CIG	598.6	0.005
COGS F_9	31.28	0.005
GAH	32.1	0.005
IANP20	69.62	0.005
COGS F_9 infected	52.33	0.005
GAH infected	379.5	0.005
IANP20 infected	0	1
COGS F_{11}	61.27	0.005
COGS F_{13}	39.33	0.005
COGS F_{15}	10.31	0.01
COGS-EXP F_{15}	29.29	0.005
COGS F_{17}	7.075	0.1
COGS-EXP F_{17}	214.5	0.005
COGS F_{19}	173.4	0.005
COGS-EXP F_{19}	320.7	0.005
COGS F_{21}	17.54	0.005
COGS-EXP F_{21}	86.11	0.005
COGS F_{23}	8.967	0.25
COGS-EXP F_{23}	29.52	0.005
COGS F_{25}	0.704	0.9
COGS-EXP F_{25}	5.769	0.5
COGS F_{25} dieldrin exposure survivor	11.11	0.005
COGS F_{25}F25 dieldrin exposure knockdown	49.27	0.005
COGS-EXP F_{25} dieldrin exposure survivor	440.6	0.005
COGS-EXP F_{25} dieldrin exposure knockdown	67.32	0.005

The COGS colony and its insecticide-exposed sub-colony (COGS-EXP), were monitored for 2La assortment over 10 generations from F_{15} to F_{25} (Figure 3.6). In the base colony, all three karyotypes were present, although it was initially dominated by 2La. However, as time progressed, 2La began to decline in frequency, and either $2L+^{a}$ homozygotes or heterozygotes formed the majority in the population, depending on the generation examined. The COGS-EXP line was significantly skewed towards karyotypes containing $2L+^{a}$, as almost no 2La were detected for most of the generations. Only in F_{25} did 2La appear in fair numbers again. Linear regression analysis of the 2La arrangement through the generations showed that in COGS there was a slight trend of $2L+^{a}$ slowly increasing ($R^2 = 0.7115$), but it was not highly significant ($p \leq 0.1$). In COGS-EXP there were no significant trend to speak of ($R^{2} = 0.1994$; $p \leq 0.5$)

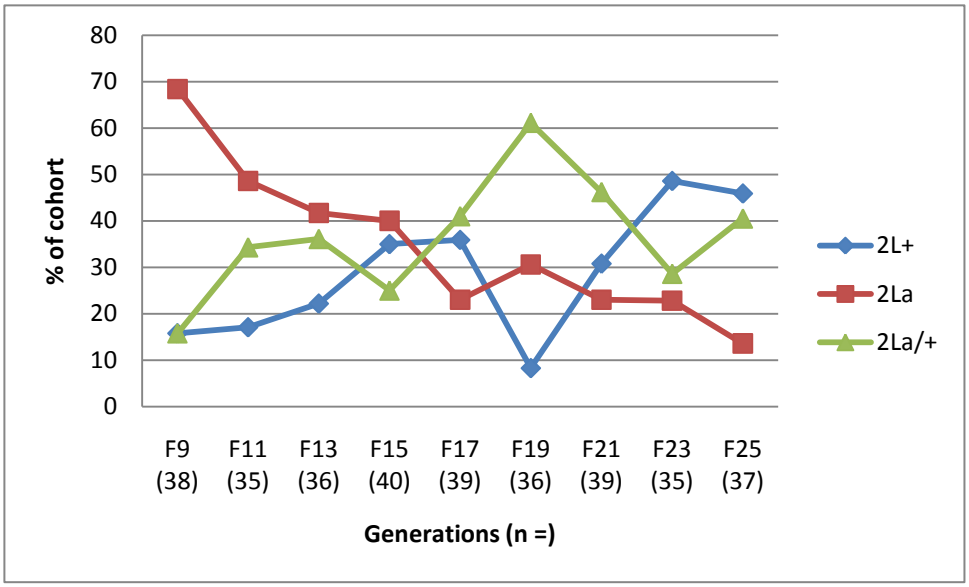


Figure 3.6: 2La frequency in COGS strain over 16 generations.

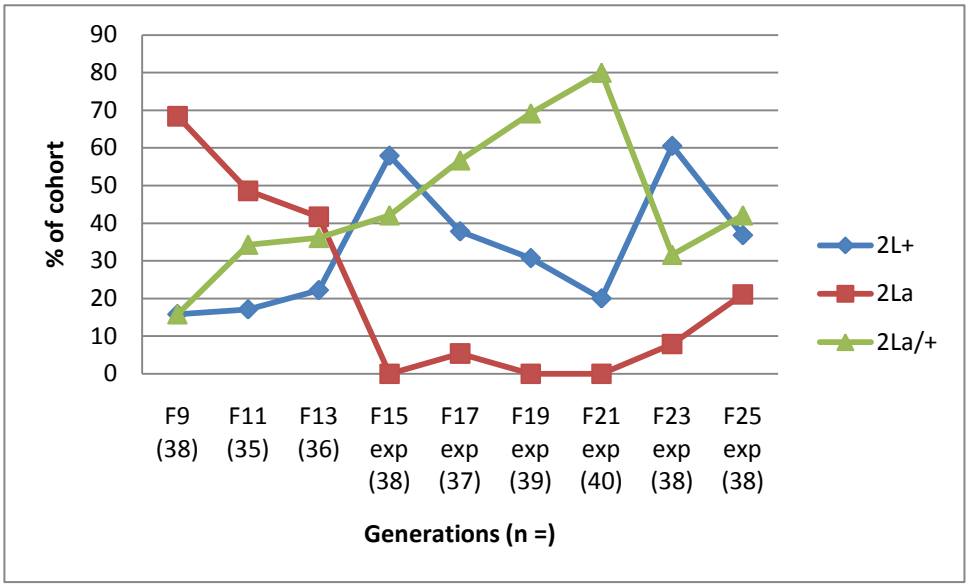


Figure 3.7: 2La frequency in COGS-EXP strain over 10 generations.

Dieldrin exposure at F_{25} in COGS (n = 48) and COGS-EXP (n = 50) lines showed that all survivors carried either the $2L+^{a}$ or $2La/+^{a}$ arrangements (Figure 3.7). In COGS, the dieldrin susceptible specimens covered all three karyotypes, with close to half of the knockdowns being heterozygotes (45.7%) and the remainder fairly evenly split between the two homozygotes (32.6% for $2L+^{a}/+^{a}$ and 22.9% for 2La/a). In contrast, the majority of dead individuals in COGS-EXP were 2La homokaryotypes (70.0%). The remainder of the knockdowns were slightly biased towards the heterokaryotypes than $2L+^{a}$ homokaryotype (18.0% versus 12.0%). None of the cohorts exposed were in Hard-Weinberg equilibrium with regard to the genotype distribution (Table 3.2). Comparison of COGS and COGS-EXP of the same generation showed significant difference in 2La genotype distribution (Table 3.4). Although there was already a significant difference between F_{25} genotype distributions of COGS and COGS-EXP, the difference was even greater when comparing mosquitoes of the two strains following dieldrin exposure (Table 3.2).

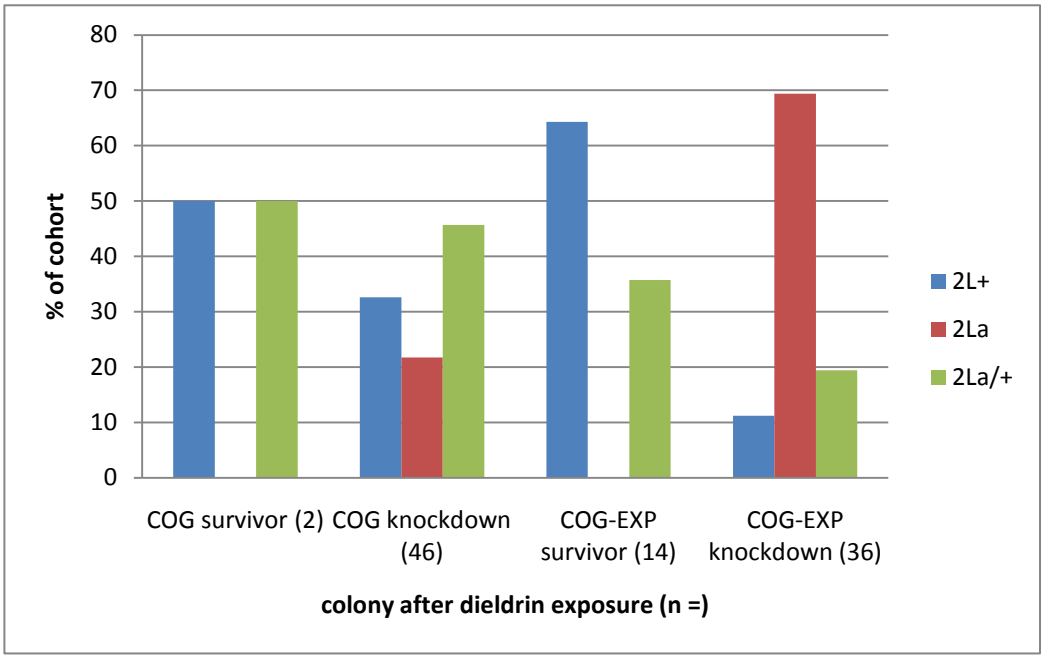


Figure 3.8: 2La distribution in COGS and COGS-EXP line at F_{25} after dieldrin-exposure assays.

3.3.3 Frequency of 2La in *P. berghei*-infected *An. gambiae* colonies

The frequency of sporozoite infection in the three 2La polymorphic *An. gambiae* colonies ranged from 4.3% (IANP20; n = 113) to 15.0% (GAH; n = 114). COGS (n = 124) was the median, with 11.3% infection rate. The values were within expected range as reported in the previous chapter. PCR showed that in all three colonies, 2La was present in the vast majority of the infected females, with all infected IANP20 homozygous for the 2La arrangement (Figure 3.9). Only in COGS was $2L+^{a}$ homozygotes infected, which accounted for a minority (17.6%) of the infection. Heterokaryotype infections accounted for a low percent of the infection in both COGS and GAH (21.4% and 14.3%, respectively). Both cohorts were not in Hardy-Weinberg equilibrium (Table 3.2), and the 2La genotype frequency of the infected cohort was significantly different to the infected cohort (Table 3.5).

Table 3.3: Chi-square Tests for generational 2La karyotype comparisons in *Anopheles gambiae* COGS and COGS-EXP laboratory colonies.

Colonies	X^2 value	p ≤
F_9 vs. F_{11}	21.66	0.005
F_{11} vs. F_{13}	2.46	0.1
F_{13} vs. F_{15}	10.86	0.005
F_{13} vs. F_{15} EXP	100.09	0.005
F_{15} vs. F_{17}	17.488	0.005
F_{15} EXP vs. F_{17} EXP	12.043	0.005
F_{17} vs. F_{19}	33.58	0.001
F_{17} EXP vs. F_{19} EXP	9.49	0.01
F_{19} vs. F_{21}	66.5	0.001
F_{19} EXP vs. F_{21} EXP	5.42	0.05
F_{21} vs. F_{23}	16.99	0.005
F_{21} EXP vs. F_{23} EXP	111.29	0.001
F_{23} vs. F_{25}	12.35	0.005
F_{23} EXP vs. F_{25} EXP	34.83	0.001

Table 3.4: Chi-square tests for 2La karyotype comparisons between COGS and COGS-EXP strains of the same generations.

Colonies	X^2 value	p ≤
F_{15} vs. F_{15} EXP	66.68	0.001
F_{17} vs. F_{17} EXP	19.581	0.005
F_{19} vs. F_{19} EXP	91.674	0.001
F_{21} vs. F_{21} EXP	51.52	0.001
F_{23} vs. F_{23} EXP	12.94	0.005
F_{25} vs. F_{25} EXP	4.514	0.1
F_{25} vs. F_{25} EXP (dieldrin exposure assay)	53.6	0.001

Table 3.5: Chi-square tests for 2La karyotype comparisons between uninfected and *P. berghei* infected *An. gambiae* laboratory colony.

Colonies	X^2 value	p ≤
COGS F_9 vs. infected	2.633	0.1
GAH vs. infected	155.71	0.001
IANP20 vs. infected	16.14	0.005

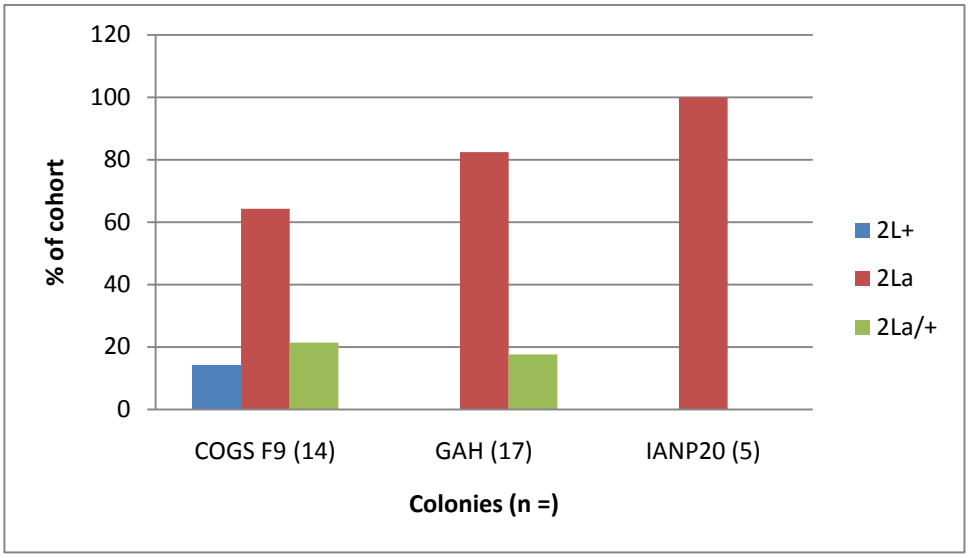


Figure 3.9: Distribution of inversion 2La karyotypes in *P. berghei*-infected females of three *An. gambiae* colonies.

3.4 Discussion

3.4.1 Comparison of cytogenetic and molecular karyotyping

Molecular karyotyping of the 2La inversion was shown to be suitably reliable when compared to the cytogenetic method (White *et al*., 2007). However, unexpected non-specific amplicons in addition to the expected fragments were produced when used elsewhere. These additional fragments have previously been detected and do not appear to be PCR artefacts or derived from another unlinked locus (Obbard *et al*., 2007; Ng'habi *et al*., 2008). Due to these unexpected PCR products, it was necessary to validate the PCR with traditional cytogenetics. Furthermore, even though PCR is widely accepted as a robust technique, adapting protocols from other laboratories sometimes require re-optimization, caused by variations in starting materials, reagents and PCR machines. These nuances further necessitated a comparative study of results between molecular and cytogenetic karyotyping.

All three possible karyotypes were observed in the GAH colony by cytogenetic analysis and were then subjected to the PCR assay. Along with the two expected fragments, additional fragments were also amplified (around 400 bp and 600 bp), but these were subsequently eliminated after optimization of the PCR. The PCR at the original conditions was tested on COG and IANP20, and a 400 bp non-specific band also appeared in the former. In some of the IANP20 samples, a non-specific band of approximately 780 bp was present, which is similar in size to one of the derivative fragments detected by Obbard *et al*., (2007). However, the re-optimized PCR eliminated all of these fragments, indicating that these were all non-specific amplifications, and not fragments derived due to polymorphisms in the amplicon region. These results caution against adopting the PCR assays blindly as a substitute for cytogenetic

analysis in determining 2La status.

Almost all 2La-related publications since the establishment of the molecular karyotyping method have utilized the PCR technique to quantify the inversion's distribution in wild populations (Obbard *et al*., 2007; Stump *et al*., 2007; Ng'habi *et al*., 2008; Gray *et al*., 2009; Rocca *et al*., 2009; Fouet *et al*., 2012). This indicates the general acceptance that PCR is a suitable substitute for cytogenetic karyotyping. However, due to the ambiguities brought on by derivative fragments, cytogenetics ought to be used in conjunction with PCR to validate the results (Ng'habi *et al*., 2008). In summary, although polymorphisms in the amplified region of 2La may lead to distortion of results, the lower level of expertise required for molecular karyotyping makes profiling of the inversion in any given population much easier. It is also less time-consuming and can be performed on individuals of either sex. It is thus a good technique for quick karyotyping of the inversion, and useful for population genetics where large sample numbers may need to be processed. However, cytogenetic karyotyping cannot be totally disregarded, as it can provide details within the inversion where the PCR is unable to do so.

3.4.2 Distribution of 2La in *An. gambiae* colonies

A number of the *An. gambiae* colonies maintained in the VCRL insectary are known to be polymorphic for 2La but the frequency of the inversion has not been quantified in recent times (and in the case of newer colonies, not at all). The CIG and IANP20 colonies were previously karyotyped for 2la with evidence of positive heterosis in both (Brooke *et al*., 2000). The frequencies have changed after a decade with both colonies now heavily dominated by 2La homozygotes (91.5% for CIG, and 86.1% for IANP20). There was no selective pressure applied by insecticides, where dieldrin resistance has been being linked to the inversion

(Brooke *et al*., 2000). It is possible that due to the lack of selective pressure over time, in addition to long-term genetic isolation, bottlenecking and genetic drift, both populations have shifted back to 2La, now considered the ancestral arrangement (Sharakhov *et al*., 2006). More investigations would be required to understand the pattern of 2La selection in long-term insectary colonies.

With respect to the new colonies, GAH and COG (both less than 5 years old), their 2La frequency has never been monitored. The GAH strain was dominated by heterozygotes with some 2La homozygotes. It was the only colony tested that is dominated by the heterokaryotype (73%). No $2L+^{a}$ homokaryotypes were found during the process, although they should exist considering the presence of the heterokaryotype. The absence of $2L+^{a}$ homokaryotypes from the IANP20 cohort may have been due to the slightly small sample size (n = 55). $2L+^{a}$ homozygous mosquitoes have been observed previously as being less fit than other genotypes, as they were usually smaller, less prone to take bloodmeals, and showed slow ovarian development (R. Hunt, MSc dissertation, University of the Witwatersrand, 1984), which may have also contributed to its absence in GAH, and the low frequency in other colonies. This may be due to the presence of lethal genes within the inversion, but the hypothesis has yet to be tested.

The COGS colony, along with its derivative colony, COGS-EXP, underwent more extensive profiling. The initial baseline data over seven generations (from F_9 - F_{15}) showed that although 2La began as the dominant arrangement, homozygous individuals steadily declined in numbers (Figure 3.6). Although it never fully disappeared, 2La/a became the minority, and the strain was dominated by $2L+^{a}/+^{a}$ or heterozygous individuals. Both colonies were typically not in Hardy-Weinberg equilibrium, but the 2La genotype frequencies at any given

generation were significantly different when compared to generations two previous or after it, except for between F_{11} and F_{13}, where the X^2 value was fairly small compared to the others. Given the fact that these laboratory colonies are not infinitely large, it is not surprising that 2La and related genotype is not in equilibrium as time passes. What sort of effect (if any) the disequilibrium will have on the colonies is unknown, and perhaps should be explored further.

Linear regression of the inversion showed no significant pattern of selection in either COGS or COG-EXP, but the former was somewhat tending towards higher frequencies for $2L+^a$. This was surprising as $2L+^a$ is supposed to offer some resistance to dieldrin, yet it was not selected for in the dieldrin exposed colony. However, the data have been somewhat misleading as for most of the generations monitored, the 2La frequency was decreasing, and only in F_{23} and F_{25} was there an increase of the arrangement. It is unknown what could have led to this increase, and without further data, it is difficult to hypothesize. The COGS colony maintained all three karyotypes throughout the profiling, with the pattern of 2La/a being in the minority continuing through the generations. In contrast, the COGS-EXP population only showed low frequencies of the homokaryotype in some generations. The selection pressure for $2L+^a$ by the insecticide was fairly evident, despite the results of the linear regression analysis. To show that the 2La inversion was linked to dieldrin resistance, individuals from F_{25} of both colonies were exposed to dieldrin. The results clearly showed 2La homokaryotype individuals are fully susceptible to dieldrin, and the $2L+^a$ arrangement, whether present as a single or double copy, confers some resistance to the insecticide. The correlation was particularly obvious in the COGS-EXP strain, as the vast majority of knockdowns were 2La homokaryotypes. Correlation between dieldrin resistance and $2L+^a$ was not as conclusive in the COGS colony. Although the survivors carried $2L+^a$, the presence of $2L+^a$ was not a guarantee that the mosquito would survive dieldrin exposure, as all three karyotypes were

found in the knocked down individuals, and close to half of the deaths were heterokaryotypes. The results reinforce the idea that insecticide resistance is multifactorial and that there are likely to be metabolic detoxification mechanisms acting in parallel to the resistance locus (Hemingway *et al*., 2004). In general, the results were consistent with previous data obtained from CIG and IANP20 (Brooke *et al*., 2000). The main difference was that in the earlier study, there were survivors carrying 2La/a, and no $2L+^{a}$ homokaryotes were knocked down, whereas in COGS and COGS-EXP there were no 2La/a survivors, and some $2L+^{a}$ homokaryotes did not survive. As the colonies were not from the same locations and have been maintained as insectary colonies for different lengths of time, inherent genetic and resistance profiles were likely different as well, leading to variations in results.

3.4.3 Distribution of 2La in *P. berghei* infected females

Of the four *An. gambiae* colonies initially tested for 2La frequency, only three were used for *P. berghei* infection. The CIG colony was omitted as infection rates for the strain were very low compared with the other three colonies (see Chapter Two). In the three *An. gambiae* populations tested, all infected females were skewed towards the 2La arrangement, particularly 2La homokaryotic individuals. Infection rates of 2La homokaryotes were at least three times higher compared to the other arrangements. For COGS, the 2La frequency of infected females was of a similar percentage to what was detected in the randomized sampling of the colony at the same generation, where 2La homozygotes were dominant and the other two karyotypes in more or less equal distribution. In IANP20, it was only the 2La homokaryotes that showed infections, which was perhaps not surprising as the base population was already heavily dominated by 2La/a. The cohort that were successfully infected may have been too small (n = 5) to detect infected heterokaryotype or $2L+^{a}/+^{a}$ individuals, as GAH has showed that females carrying $2L+^{a}$ can be infected. The most

unexpected distribution of the 2La inversion was in infected GAH females. The base population was dominated by heterokaryotes with 2La homokaryotes in the minority, yet in the infected cohort the proportion was inverted, with the homozygotes dominant, and present at over four times the number compare with 2La/+^{a} females. All cohorts barring IANP20 were not in Hardy-Weinberg equilibrium, but this is not surprising given the very small number of infected females in IANP20. The 2La genotype frequencies of the GAH and IANP20 were significantly different compared to their respective uninfected cohorts when subjected to Chi-square Test. COGS F_9 was not as significantly different compared to the other two strains. The smaller sample size of the infected cohorts may be a slight concern, but the fact that in all three strains 2La homozygotes were more likely to be infected, indicates that there is a trend there.

The results presented here are in contrast to data collected on wild populations from the Kisumu region, where it was found that the 2L+^{a} homozygotes were more likely to be infected with *Plasmodium* than the 2La homozygotes (Petrarca & Beier, 1992). However, a cautious approach to interpreting data should always be taken when making comparison between wild-caught populations infected with *P. falciparum* and insectary-based cohorts infected with *P. berghei* as it is known that *P. falciparum* and *P. berghei* can trigger different immune responses in mosquitoes (Tahar *et al*., 2002; Riehle *et al*., 2006). More detailed research on the 2La region also revealed such differences, where paralogs of the APL1 family exhibit mutually exclusive protection against different *Plasmodium* species, and the protection is mediated by different signalling pathways (Mitri *et al*., 2009).

Variations in mosquito physiology between wild and laboratory-reared populations, as well as those brought on by spatial and temporal separation, temperature and other environmental

variables, are well acknowledged (Carey, 2001; Huho *et al*., 2007). Consequently, it would be more useful to take both sets of results into consideration given the different circumstances, rather than eliminate one data set altogether, especially considering that each vector-parasite pairing, whether natural or unnatural, tends to have its own unique pattern of interaction (Sinden *et al*., 2002). It is also important to not assume the data obtained from one vector-parasite pairing is not necessarily applicable to another, particularly as laboratory models often have not been systematically validated against natural host-pathogen interactions (Mitri & Vernick, 2012).

The prevalence of 2La in *P. berghei* infected females in the present study seems to indicate a greater degree of susceptibility to *Plasmodium* linked to the inversion. A number of immune genes that affect *Plasmodium* infection and transmission are found within or near the 2La region (Osta *et al*., 2004; Cohuet *et al*., 2006; Riehle *et al.*, 2006). While none of these immune genes occur at the inversion break points with their functions and expression directly affected by the rearrangement, it is unknown what effect the inversion has on these genes. There may be cis- or trans-acting mechanisms targeting these genes that become less effective (or even non-operational) once the locations of the genes have shifted, but there are no molecular data available for this hypothesis with regard to the 2La inversion. Conversely, genes outside of the inversion may be influenced due to displacement of loci that previously interacted with them. More research is thus necessary to elucidate the interactions and effects the 2La inversion may have on *Plasmodium* infection.

Chapter 4: Expression of immune genes in *An. funestus* during *P. berghei* infection

4.1 Introduction

The malaria parasite is completely reliant on anopheline mosquitoes as both the obligatory host, and as a vector to transmit it to the vertebrate host. The traditional view of the mosquito-parasite relationship maintains that the vector is a passive carrier of the parasite, and does not act against the parasite's presence, nor does it suffer from adverse effects. However, studies in recent decades have shown this to be untrue. Ookinetes passing through the midgut leads to tissue damage (Ramasamy *et al*., 1997); amino acids level in haemocoelomic fluid are altered (Mack *et al*., 1979) and physiological modifications such as egg production and feeding behaviour are also likely (Hogg & Hurd, 1995; Koella *et al*., 1998; Rossignol, *et al*., 1984). Selection based on basic Mendelian genetics has also shown it is possible to create an *An. gambiae* strain that is refractory to the parasites (Collins *et al*., 1986). The *Plasmodium* resistance mechanisms have been further elucidated through the *An. gambiae* genome (Holt *et al*., 2002), and advances in molecular techniques, such as gene silencing by RNA interference (RNAi) in the same species (Blandin *et al*., 2002). This has led to discovery of a number of genes and associated regulatory pathways mediating mosquito susceptibility to *Plasmodium*, which are all involved in the mosquito's innate immunity (Abraham *et al*., 2005; Michel *et al*., 2005; Meister *et al*., 2005; Vlachou *et al*., 2005; Dong *et al*., 2006).

Much of anopheline innate immunity has been resolved by comparative studies with the *Drosophila melanogaster* immune system, coupled with some descriptions from orthologous

pathways in mammals (Cirimotich *et al*., 2010). Innate immunity involves both humoral and cellular factors, typically responding to microbial challenges within the haemolymph. Circulating haemocytes can phagocytose or encapsulate microbes; serine protease cascades can lead to melanization or release of free radicals to kill the pathogens (Meister *et al*., 2004; Osta *et al*., 2004). PRRs binding to PAMPs of pathogens will lead to production of antimicrobial effector molecules (Meister *et al*., 2004; Osta *et al*., 2004). On a cellular level, intracellular immune responses detect pathogens using PGRPs, which activate one of three immune signaling pathways (Toll, IMD or JAK/STAT). Activation of these pathways usually results in up-regulation of immune-related genes, including AMPs (Choe *et al*., 2002; Yassine & Osta, 2010).

Of the multitude of immune responses the mosquito can mount, the most diverse is perhaps the AMPs. These peptides have been well studied in other insects (Hoffmann, 1995; Hoffmann *et al*., 1996), and were amongst the first group of immune-related molecules to be studied in mosquitoes (Richman *et al*., 1996; Dimopoulos *et al*., 1997, 1998; Vizioli *et al*., 2000, 2001) Insects mount potent anti-bacterial/anti-fungal responses through production of AMPs in the fat body (Hoffmann *et al*., 1996). Molecular studies have also revealed that some of the AMPs also have anti-parasitic properties when mosquitoes are exposed to malaria parasites (Richman *et al*., 1996; Dimopoulos *et al*., 1997, 1998; Vizioli *et al*., 2000, 2001). Insect AMP can be divided into four major classes according to sequence similarity: cecropins, cysteine-rich peptides, glycine-rich polypeptides and proline-rich peptides (Meister *et al*., 1997). Defensins are cysteine-rich peptides that were first discovered in *Drosophila*, expressed by fat bodies and some haemocytes during bacterial infection and mechanical injuries (Hoffmann & Hetru, 1992). Defensin 1/defensin A (*AgDfn1*) was subsequently isolated in *An. gambiae* (Richman *et al*., 1996), and three more defensins were found in the

annotated genome (Christophides *et al*., 2002). Defensins primarily act against Gram^{+} bacteria and some filamentous fungi (Vizioli *et al*., 2001) but the anti-*Plasmodium* effect of defensin is still ambiguous at this point. Exogenous defensin or up-regulation of defensin through bacterial challenge affected *P. gallinaceum* oocysts and sporozoites in *Ae. aegypti* (Shahabuddin *et al*., 1998; Lowenberger *et al*., 1999), as does co-overexpression of endogenous defensin A with cecropin A (Kokoza *et al*., 2010). However, knockout of defensin in *An. gambiae* during *P. berghei* infection showed no significant changes in the parasite's midgut developmental stages (Blandin *et al*., 2002).

Cecropins are found in both insects and mammals (Boman, 1994). In *An. gambiae*, three cecropins were initially isolated (Vizioli *et al*., 2000; Zheng & Zhang, 2002) followed by a fourth in the genome sequence (Christophides *et al*., 2002). Cecropins have a broad spectrum of antimicrobial activity in mosquitoes. The peptides usually act against Gram^{-} bacteria but have been found to act against a number of species of Gram^{+} bacteria, yeasts and fungi (Vizioli *et al*., 2000). Cecropin 1/cecropin A (cec1/cecA) expression is induced during *Plasmodium* infection (Vizioli *et al*., 2000), and expression beginning 24 hours post-infected bloodmeal causes significant reduction in the number of *P. berghei* oocysts in *An. gambiae* (Kim *et al*., 2004).

Gambicin is a novel AMP that was first isolated in *An. gambiae* (Vizioli *et al*., 2001) and later found in other dipteran vectors such as *Cx. pipiens pipiens* (Bartholomay *et al*., 2003), *Cx. quinquefasciatus* (Ribeiro *et al*., 2004) and *Ae. aegypti* (Hillyer *et al*., 2005). It is a unique AMP with no sequence homology to other known insect AMPs. The peptide works against both Gram^{+} and Gram^{-} bacteria, with some lethality to *P. berghei* ookinetes (Vizoli *et al*., 2001). Attacin was the only novel class of AMP discovered in the *An. gambiae* genome

(Christophides *et al*., 2002), with the other peptides having being isolated earlier. Although it is known to have antimicrobial activities in other insects (Hultmark *et al*., 1983; Sugiyama *et al*., 1995), only recently have there been data showing some activity during *Plasmodium* infection in *An. albimanus* (Herrera-Ortiz *et al*., 2011).

It is likely that *An. funestus* uses similar mechanisms in response to *Plasmodium* invasion, but without a functional infection model, it was not feasible to validate the immune responses in *An. funestus*. However, having established the *An. funestus-P. berghei* infection model, this chapter aimed to examine the immune response in the mosquito during *Plasmodium* invasion and development. Three AMP genes – cecropin 1, defensin 1 and gambicin – were amongst the first AMPS to be studied in *An. gambiae,* and were chosen for that reason. Assessment of their expression during *P. berghei* infection at various time points, in three *An. funestus* strains with varying insecticide susceptibility, will provide new information for *An. funestus* infection, and also allow a comparison with results obtained previously from *An. gambiae*.

4.2 Materials and methods

4.2.1 *An. funestus* infection

Adult female *An. funestus* were infected with *P. berghei* as described in chapter two (section 2.2.3). The three FUMOZ colonies were all infected and maintained as for normal *P. berghei* development until the time points required for RNA extraction. The infection status of the females was determined retroactively, that is, females were collected from the fed cohort at various time points, and only used for gene expression analyses if the females of the same cohort had infected salivary glands when dissections were performed. The mosquitoes were knocked down with ether, their abdomen removed and kept on ice. RNA was extracted

immediately following the dissection. Alternatively, the dissected abdomens were stored at -70ºC and RNA extraction took place within 48 hours of the dissection.

4.2.2 RNA extraction

Total RNA was extracted from all three colonies at the following time points: 24 hours post infection (pi), 7 days pi, 14 days pi and 21 days pi. Three biological replicates for each strain were obtained for each time point. Each replicate contained 10 - 12 mosquito abdomens. RNA was also extracted from adult females that were given uninfected bloodmeals at the same time as 24 hours pi cohorts. These uninfected females were used as baseline material in real-time PCR.

RNA was extracted using a modified TRIzol method. The dissected mosquito abdomens were placed in 100 µl TRI reagent (Sigma-aldrich) and homogenized in a microcentrifuge tube, the suspension incubated at room temperature for five minutes and then centrifuged at 12,000 g for 10 minutes at 4ºC. The liquid was transferred into a new microcentrifuge tube and homogenized for 15 seconds with 20 µl chloroform. It was again incubated at room temperature for 15 minutes, and then centrifuged again at 12,000 g for 15 minutes at 4ºC. The top aqueous phase of the three layers was transferred into a fresh tube; 50 µl isopropanol was added and gently mixed to precipitate the RNA. This was incubated at room temperature for 10 minutes and then centrifuged at 12,000 g for 10 minutes at 4ºC. The supernatant was removed and the precipitated RNA was washed in 100 µl 70% ethanol, vortexed briefly, then centrifuged at 7,500 g for 5 minutes at 4ºC. The ethanol was removed and the RNA pellet allowed to air dry before being suspended in 35 µl DEPC water.

To eliminate DNA that may have carried over during the extraction, the resuspended RNA

was treated with DNase I. For each sample, 1 µl DNase I and 4 µl 5X RT buffer was added, and incubated at 37ºC for 15 minutes. The DNase was then heat inactivated at 70ºC for 10 minutes, and then immediately placed on ice, or into the freezer for storage.

4.2.3 RNA quality assessment

To determine the quality of the total RNA extracted, two methods were used: the traditional formaldehyde gel and Bioanalyzer (Agilent, USA).

4.2.3.1 RNA formaldehyde gel

To verify the RNA quality, RNA samples were electrophoresed on a 1.2% formaldehyde gel. RNase-free water (44 ml) was mixed with 0.6 g agarose in an RNase free container, brought to the boil and swirled until the agarose had dissolved. The mixture was moved to the fume hood, allowed to cool to 55 - 60ºC, before 5 ml 10X 3-(*N*-morpholino)-propanesulfonic acid (MOPS) formaldehyde gel running buffer was added under the fume hood, followed by 1.5 ml 37% formaldehyde solution. Air bubbles were avoided (or removed after) while adding and mixing the two formaldehyde-based solutions. Ethidium bromide (2 µl; 10mg/ml) was also added and mixed. The liquid was then poured into a gel template and allowed to set for at least 30 minutes before use.

To prepare the RNA samples for electrophoresis, the following reagents were mixed together: 2.5 µl 10X MOPS running buffer, 4.4 µl 37% formaldehyde solution and 12.5 µl formamide. The RNA sample was then added (2 - 10 µg) and DEPC water was added to make up a final volume of 25 µl. The mixture was incubated at 55ºC for 15 minutes, and 5 µl orange loading buffer added to each sample.

The formaldehyde gel had to sit in 1X MOPS gel running buffer for at least 30 minutes to equilibrate before electrophoresis. The RNA samples were loaded onto the gel and electrophoresed with RNA molecular weight markers (Fermentas, EU; cat # SM1821) at 5 V/cm for at least 45 minutes. The RNA products were visualized under ultraviolet (UV) illumination.

4.2.3.2 Bioanalyzer

The Agilent (California, USA) 2100 Bioanalyzer is an alternative to RNA formaldehyde gels, requiring less input material and can provide more information about the RNA sample than the traditional gel. The RNA and reagents were prepared as the manual described from the Agilent RNA 6000 Nano Kit (Agilent Technologies, USA). In brief, gel matrix was spin-filtered at 1,500 g ±20% for 10 minutes, and mixed with an aliquot of the dye concentrate. The mix was spun at 13,000 g for 10 minutes before loading onto the RNA Nano chip using a special syringe and chip priming station. This was followed by 5 µl marker into each of the samples wells and the ladder well. RNA samples were heat denatured at 70ºC for 2 minutes, and 1 µl of each sample along with 1 µl of the RNA ladder were loaded onto the chip. The chip was then vortexed and placed into the Bioanalyzer for analysis. The software generates a gel and electropherogram for each samples, as well as analyzes the amount of intact and degraded RNA. By comparing the data to the RNA ladder, the RNA Integrity Numbers (RIN) for the samples were calculated, where on a scale of 1 to 10, samples with intact RNA would score more highly than degraded samples.

4.2.4 cDNA synthesis

cDNA synthesis was performed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). The process was performed exactly as described in the kit manual. Up to 5 µg of total RNA was first primed with Oligo $(dT)_{20}$, incubated at 65ºC and placed on ice. The cDNA synthesis mix was then added and incubated at 25ºC for 50 minutes. The synthesis was terminated by incubation at 85ºC for 5 minutes and then chilled on ice. The residual RNA was removed by addition of RNase H and incubation at 37ºC for 20 minutes. The Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific, Massachusetts, USA) was used to assess the quantity of cDNA produced. The cDNA was stored at -20ºC or used immediately for other downstream applications.

4.2.5 Primer design

PCR primers for the three *An. funestus* immune genes were obtained and tested using several approaches: 1) primers designed for amplifying the immune genes in *An. gambiae* were tested on *An. funestus*; 2) degenerate primers based on the degeneracy of the amino acid sequence in the region where the *An. gambiae*-specific primers were located were designed and tested (only defensin); and 3) primers based on known amino acid and nucleotide sequences of homologous genes from other mosquito species, including *Ae. aegypti*, *An. stephensi*, and members of the *An. gambiae* complex were tested. Primers for cecropin were designed using *An. stephensi*, *An. gambiae* and *An. arabiensis*. Defensin primers were designed using the same species plus *Ae. aegypti*. Gambicin primers were designed using consensus sequences from all members of the *An. gambiae* complex (see Appendix 2 for all three versions of primer sequences).

A number of housekeeping/reference genes were also designed by locating conserved sequences across multiple mosquito species. These were needed for real-time PCR to establish baseline gene expression. The reference genes designed included the following: GAPDH, ND5, rpS7, rpL8, rpL19, 18S, rpS26, CO1 (see Appendix 2).

All designed primers were subjected to NetPrimer to validate their functionality and stability (e.g. no hairpin, palindrome, or significant primer dimer formation). Primer sequences with potential to adversely affect amplification were redesigned. The primers used for PCR (both conventional and real-time) are listed in Appendix 2.

4.2.6 PCR

Conventional PCR was performed on the Bio-Rad C1000™ series thermal cycler (Bio-Rad Laboratories, California, USA). Optimal amplification of the genes was achieved using DreamTaq DNA polymerase (Fermentas, EU), using primers designed for targeting conserved regions across mosquito species. The mastermix used and conditions for each of the PCRs were as follows.

Cecropin: 0.5mM final concentration for both primers, 1X PCR buffer, 2.5mM $MgCl_2$, 0.5mM of each dNTP, 200 - 300 ng DNA, 0.5U of DreamTaq DNA polymerase and made up to a final volume of 20 µl with double distilled water. The reactions were amplified using the following parameters: initial denaturation at 95ºC for 3 minutes; 40 cycles of 94ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 45 seconds; final extension at 72ºC for 10 minutes, and hold at 4ºC indefinitely.

Defensin: 1mM final concentration for both primers, 1X PCR buffer, 2.5mM $MgCl_2$, 0.5mM

of each dNTP, 0.5U DNA polymerase, 200 - 300 ng DNA, and made up to a final volume of 20 µl. The reactions were amplified using the following parameters: initial denaturation at 95ºC for 3 minutes; 40 cycles of 95ºC for 30 seconds, 55ºC for 45 seconds, and 72ºC for 45 seconds; final extension at 72ºC for 10 minutes, and hold at 4ºC indefinitely.

Gambicin: 0.5mM for both primers, 1X PCR buffer, 2.5mM $MgCl_2$, 0.5mM of each dNTP, 200 - 300 ng DNA, 0.5U of DNA polymerase and made up to a final volume of 20 µl with double distilled water. The reactions were amplified using the following parameters: initial denaturation at 95ºC for 3 minutes; 40 cycles of 94ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 45 seconds; final extension at 72ºC for 10 minutes, and hold at 4ºC indefinitely.

To determine the size of the fragments, the products were electrophoresed on 1% agarose gels stained with ethidium bromide (0.5 µl in 80 ml). The samples were mixed with a marker dye, loaded on the gel with a DNA molecular weight marker (Fermentas, EU, cat. #SM0321), and electrophoresed at 100 - 120 V for 60 minutes. The products were visualized under UV illumination.

4.2.7 Cloning

PCR products for defensin and gambicin were cloned. Prior to cloning, the PCR product was electrophoresed as described previously. The DNA fragments of interest were then excised from the gel and extracted from the agarose using the QIAquick Gel Extraction Kit (Qiagen Inc, California, USA, cat. #28704), as stated in the kit manual. The piece of gel containing the DNA fragment was weighed, and 3 volumes of Buffer QG were added to 1 volume of the gel (every 100 mg ≈ 100 µl) excised. The mixture was incubated at 50ºC for 10 minutes with intermittent vortexing. To maximize the DNA yield, 1 gel volume of isopropanol was then

added and mixed. The solute was added to the QIAquick spin column/collection tube and centrifuged for 1 minute at 17,900 x g (13,000 rpm). The flow-through was discarded, and 0.5 ml buffer GQ was added to the column and centrifuged for 1 minute at 17,900 x g. The DNA was washed by adding 0.75 ml buffer PE to the spin column and centrifuged for one minute at the same speed. The column was spun for another minute to remove excess buffer and then placed into a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 µl of buffer EB was added to the centre of the column, allowed to stand for a minute, and then centrifuged for a minute. The purified DNA's concentration was determined using the Nanodrop® ND-1000 spectrophotometer (Thermo Scientific, Massachusetts, USA), and stored at -20ºC for storage or processed for sequencing.

The Promega pGEM® -T Easy Vector Systems (Wisconsin, USA, cat. #A1360) was used for the cloning. For optimal cloning, the insert:vector ratio was calculated to be 8:1 for all three genes, and the total volume of DNA per reaction was adjusted accordingly. The rest of the reaction consisted of 1X rapid ligation buffer T4 DNA ligase, 50 ng pGEM®-T Easy Vector, 3 Weiss Unit of T4 DNA ligase, and made up to a final volume of 10 µl with nuclease free water. The reaction was gently mixed and incubated at room temperature for 1 hour, or at 4ºC overnight to maximize the number of transformants.

Transformation of the ligation reaction and selection of successfully transformed colonies was performed using the *E. cloni*® 10G Chemically Competent Cells (Lucigen Corp, New York, USA, cat. #60106-1), a derivative of *Escherichia coli* (*E. coli*) that has high transformation efficiency by heat shock. The *E. cloni*® cells were taken from the -70ºC freezer and thawed completely on ice. Sterile culture tubes were chilled on ice (one tube for each transformation reaction) and 40 µl of the thawed cells were added per tube. The ligation reaction was heat-

inactivated (70ºC for five minute) and 1 - 4 µl was added and gently stirred while on ice. The cells were incubated on ice for 30 minutes, followed by heat shocking at 42ºC for 45 seconds. The mixture was then chilled on ice for another two minutes and 960 µl recovery medium (warmed to room temperature) was added. The cells were placed in a shaking incubator at 250 rpm for one hour at 37ºC.

The nutrient agar plates were made up of LB medium (10 g Baco-tryptone , 5 g Bacto-yeast, 5 g NaCl mixed in 1 L distilled water and adjusted to pH 7.0 with NaOH). Agar (15 g) was then added and the medium autoclaved. Once the medium had cooled to approximately 50ºC, ampicillin was added (final concentration of 100 µg/ml). It was further supplemented with IPTG (0.5mM), and X-Gal (final concentration of 80 µg/ml). The agar was poured into culture plates to set. The plates were used immediately, or stored at 4ºC for up to a month. Up to 100 µl of the transformed cell was plated per nutrient agar plate and incubated overnight at 37ºC. Successfully transformed cells would have the *LacZ* gene function disrupted and would appear white as they were unable to process X-Gal (untransformed colonies were blue). Individual colonies were picked off the plate using a sterile pipette tip and resuspended in 20 µl double sterile water. The selected colonies were amplified using the Bio-Rad C1000™ series thermal cycler using the standard SP6 and T7 primers. Each reaction contained 0.2mM for both primers, 1X PCR buffer, 1.0mM $MgCl_2$, 1.0mM of each dNTP, 0.5 µl resuspended cells, 0.5U of DNA polymerase and made up to a final volume of 20 µl with double distilled water. The amplification conditions were as follows: initial denaturation at 95ºC for 3 minutes; 35 cycles of 95ºC for 30 seconds, 50ºC for 30 seconds, 72ºC for 45 seconds; final extension at 72ºC for 10 minutes and hold indefinitely at 4ºC. The PCR products were electrophoresed on a 1.0% agarose gel stained with ethidium bromide, and visualized under UV illumination.

4.2.8 DNA sequencing

To verify the cloning (for defensin and gambicin) and PCR (cecropin) amplicons, the products were sequenced using an automated sequencer. Prior to sequencing, the DNA was purified using either the QIAquick Gel Extraction kit, as described earlier, or the QIAquick PCR purification Kit (Qiagen Inc., cat. #28104). For purification of PCR products, each sample that was to be sequenced was mixed with 5 volumes of buffer PB. This was added to the QIAquick spin column/collection tube assembly and centrifuged at 17,900 x g for 1 minute. The bound DNA was washed with 0.75 ml buffer PE and centrifuged again at the same speed for a minute. The spin column was centrifuged one more time to get rid of any excess liquid. The QIAquick column was then placed in a clean microcentrifuge tube and 30 µl buffer EB was added to elute the DNA. The column was allowed to stand for a minute and centrifuged for a minute at the same speed. The concentration of the purified DNA was determined using the Nanodrop ND-1000 spectrophotometer.

Sequencing was initially performed in-house using the Applied Biosystems (California, USA) 3130 automated DNA sequencer. For each sequencing sample, two sequencing reactions (one for forward primer, the other for reverse primer) were prepared using the BigDye Terminator Cycle Sequencing kit (Applied Biosystem, cat. #4337455). Each reaction contained 1 µl terminator ready reaction mix, 1.5 µl 5X sequencing buffer, 50 - 100 ng of cleaned PCR product, 5µM of either forward or reverse primer for the target gene, and made up to 10 µl with Sabax water. The reactions were amplified using the following conditions: 94ºC for 1 minute, followed by 25 cycles of 95ºC for 30 seconds, 50ºC for 20 seconds, and 60ºC for 4 minutes. The sequencing products were then cleaned using the DyeEx 2.0 Spin kit (Qiagen, cat. #63204) as per kit manual. The dried sample was resuspended in 10 µl hi-di formamide (Applied Biosystem, cat. #4311320), vortexed and spun briefly. It was heated at 95ºC for 2

minutes and then placed on ice for 1 minute. The sample was vortexed and spun down again and pipetted onto the 96-well sequencing plate. The plate was sealed with a septum cover and placed into the sequencer. The resulting sequences were viewed/analyzed using the Lasergene DNASTAR software, or were viewed using the FinchTV software (Geospiza Inc., Washington, USA).

Later, sequences were sent to Inqaba Biotech (Pretoria, South Africa), utilizing a similar automated sequencer setup.

4.2.9 Semi-quantitative real-time PCR

Real-time PCR was performed on the Bio-Rad CFX96™ Real-Time PCR detection system. All three genes (cecropin, defensin and gambicin) were amplified along with two reference genes (S26 and CO1) in accordance to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline to calculate relative expression of the immune genes. The eight reference genes initially designed were narrowed down to two by examining the Cq or raw data values between uninfected and infected cohort. The BestKeeper© (Pfaffl *et al*., 2004) and NormFinder software (Andersen *et al.,* 2004) were then used to determine the best reference genes.

Cecropin and gambicin amplification were performed using the Bio-Rad iQ™ Master SYBR® Green Supermix (cat. #170-8880). Each reaction contained the following: 0.5 mM of both primers, 1X SYBR master mix, 50 - 60 ng DNA, and made up to a final volume of 20 µl with double distilled water. Amplification was as follows: initial denaturation at 95ºC for 5 minutes; 40 cycles of 95ºC for 10 seconds, 55ºC for 15 seconds, and 72ºC for 15 seconds; final extension at 72ºC for 5 minutes, and melt curve capture from 60 - 95ºC with increments of

0.5ºC for 5 seconds and fluorescence capture for SYBR Green (510 - 530 nm) at every increment.

Defensin was amplified using the Applied Biosystem SYBR kit (cat. #4304886). Each reaction contained the following: 0.4mM of both primers, 1X SYBR master mix, 0.5mM of each dNTP, 2.5mM $MgCl_2$, 50 - 60 ng DNA, and made up to a final volume of 20 µl with double distilled water. Amplification was as follows: initial denaturation at 95ºC for 5 minutes; 40 cycles of 95ºC for 10 seconds, 56ºC for 15 seconds, and 72ºC for 15 seconds; final extension at 72ºC for 5 minutes, and melt curve capture from 60 - 95ºC with increment of 0.5ºC for 5 seconds and fluorescence capture for SYBR Green (510 - 530 nm) at every increment.

Relative expression levels of the immune genes were all performed in triplicate runs, and calculated using Cq values obtained for the target gene and accompanying housekeeping genes (Pfaffl *et al*., 2001). The formula used is as provided below.

$$\text{Relative expression ratio} = \frac{(\text{Efficiency of target})^{\Delta \text{Cq Target (treated - untreated)}}}{(\text{Efficiency of HKG})^{\Delta \text{Cq Target (treated - untreated)}}}$$

4.3 Results

4.3.1 *An. funestus* infection

Infection rates for the cohorts used for RNA extraction and subsequent AMP expression analysis varied between 5 - 20% (Table 4.1), which were within the expected range for the strains.

Table 4.1: Infection rate for the cohorts used for RNA extraction.

Colony (total no. for three replicates)	Infection rate (±std dev) (%)
FUMOZ (n = 32)	15.5 (± 6.0)
FUMOZ-R (n = 33)	16.3 (± 2.9)
FUMOZ-BS (n = 35)	5.2 (± 0.3)

4.3.2 RNA quality assessment

All samples were initially subjected to formaldehyde gel electrophoresis or Agilent RNA Nano chip analysis to determine the integrity of the RNA. Gel electrophoresis typically showed strong 18S bands and weak 28S, 5.8S and 5S bands (Figure 4.1). The bioanalyzer calculated the integrity of the RNA based on the amount of 28S and 18S RNA as well as other smaller fractions (Figure 4.2). RNA samples used had at least a value of 5.5 (out of 10) before proceeding to the next steps.

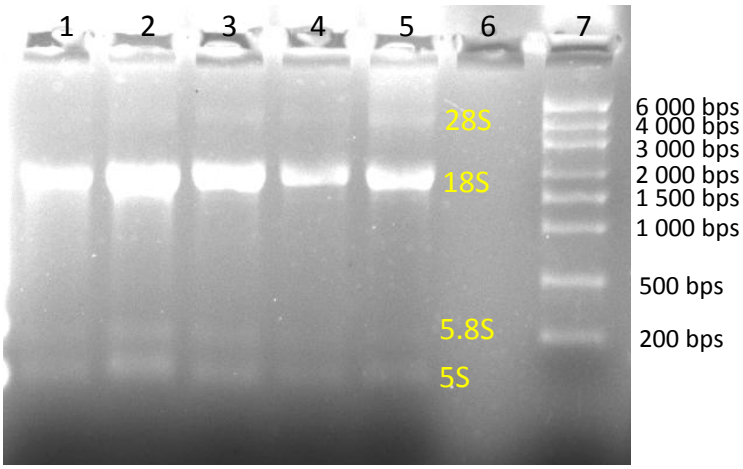


Figure 4.1: Extracted RNA on a formaldehyde gel. Lanes 1 - 2: FUMOZ; 3 - 4: FUMOZ-BS; 5: FUMOZ-R; 6: negative control; 7: molecular weight marker (Fermentas High Range RNA ladder; cat. #SM1821).

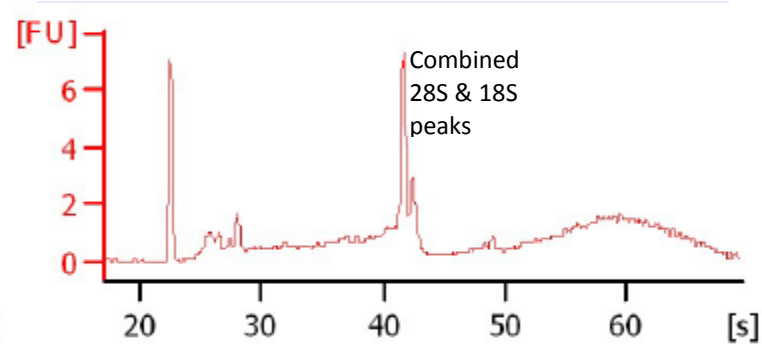


Figure 4.1: A bioanalyzer electropherogram of a RNA sample.

4.3.3 Immune genes PCR

PCR for the three immune genes using the *An. gambiae* primers produced zero amplification or highly non-specific results (Figure 4.3). The primers were designed to produce fragments of 400 - 600 bps depending on the gene targeted, but a number of varying size products were seen, along with streaks showing non-specific amplifications. Degenerate primers for defensin were designed to amplify shorter fragments of 300 - 400 bps, but again highly non-specific results were obtained producing streaks as opposed to short segments of DNA (Figure 4.4). Furthermore, in both sets of primers, the target fragments (where present) were either extremely weak compared to the non-specific products or non-existent. Optimizations on both sets of PCRs included varying temperature, primer concentration, starting material concentration, $MgCl_2$ concentration, and using different types of Taq polymerase, but none of these optimization produced satisfactory results. It was difficult to isolate and clone the expected products due to the constant streaks often seen in the PCRs.

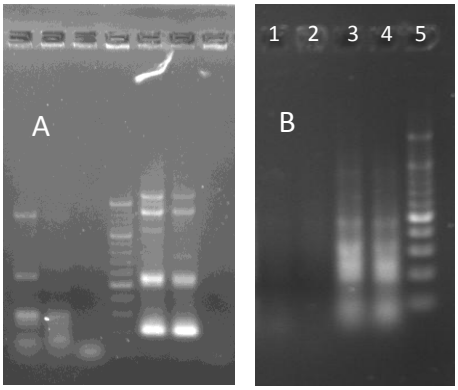


Figure 4.2: A and B: Defensin, gambicin and cecropin PCR on *An. funestus* samples using An. gambiae specific primers. A: lanes 1 – 3, defensin; lane 4, molecular marker (100 bps); lanes 5 – 7, gambicin. B: lanes 1 – 4, cecropin; lane 5, molecular marker (100 bps).

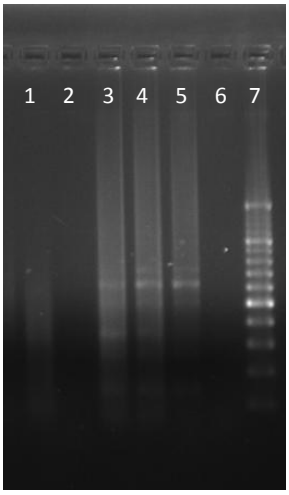


Figure 4.3: Defensin PCR using *An. funestus* with degenerate primers. Lanes 1 - 6: defensin amplification; lane 7: molecular marker (100 bps).

PCR using primers targeting conserved regions across multiple mosquito species produced the best results of the three approaches. The primers were designed to produce amplicons between 100 - 250 bps in size. Cecropin PCR produced only one amplicon with almost no optimization required. Gambicin produced some non-specific products along with the theoretical target. All fragments produced were less than 300 bps, which was a significant improvement, as this was closer to the expected product size. The defensin PCR had more

non-specific products, but was also an improvement as all the amplicons were below 300 bps and there were less non-specific fragments compared to the *An. gambiae* and degenerate primer sets (Figure 4.5).

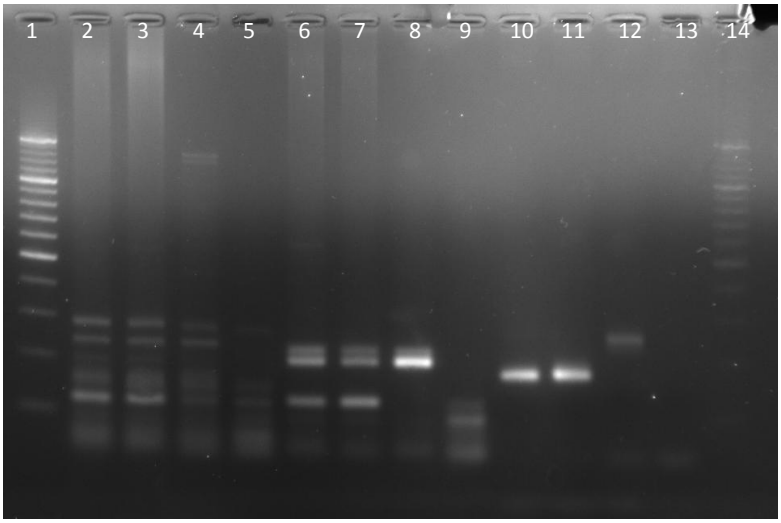


Figure 4.5: Defensin, gambicin and cecropin PCR using cross-species conserved region primers on An. funestus. Lanes 1 and 14: molecular marker (100 bp); lanes 2 - 5: defensin; lanes 6 - 9: gambicin; lanes 10 - 13: cecropin.

Verification of the PCR was subsequently based on using the primers designed using multiple mosquito species. As defensin and gambicin PCRs produced multiple amplicons which were close to the expected product, all the amplicons were cloned prior to sequencing. For each product, 20 - 30 clones were selected and further amplified using the SP6T7 PCR (Figure 4.6). Three to five clones were then selected and processed for sequencing. As cecropin PCR only produced a single product, it was directly processed for sequencing.

The contigs produced by the clones were subjected to the Basic Local Alignment Search Tool (BLAST) which matched them to sequences in the National Centre of Biological information

(NCBI) Genbank nucleotide database. For all three genes, the amplicon of the expected size was able to align with homologous genes in other mosquito species. The amplicons were all within 20 bps of the originally designed size, and were 95 - 99% similar to the genes in other mosquito species (see Appendix 3). The products did not align with any other known genes in the mosquito, indicating the primers' specificity. Once products from the gambicin and defensin PCR were confirmed to be correct, the PCR were optimized to produce only the single amplicon (Figure 4.7)

Reference genes were designed in a similar manner, and underwent the same process to confirm that the correct sequences were amplified in *An. funestus*.

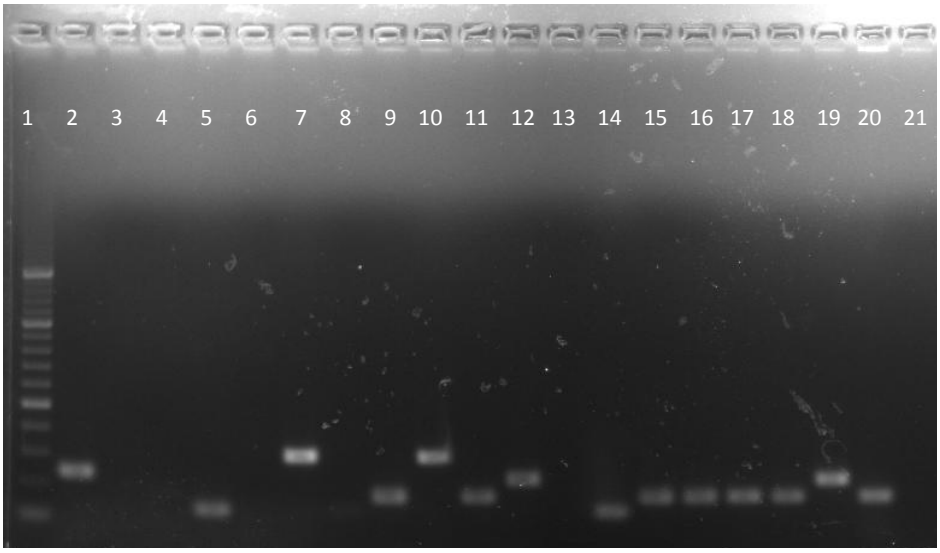


Figure 4.6: SP6T7 PCR from gambicin cloned inserts. Lane 1: molecular marker (100 bps); lanes 2 - 20: PCR products from selected transformed colonies; lane 21: negative control.

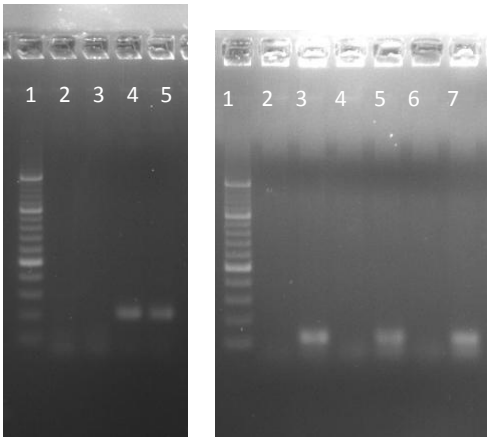


Figure 4.7: gambicin (A) and defensin (B) PCR products using redesigned primers. A: lane 1, molecular marker (100bp); lanes 2 – 3, negative control; 4 – 5, gambicin. B: lane 1, molecular marker (100bp); lanes 2, 4, 6, negative controls; 3, 5, 7, defensin.

4.3.4 Real-time PCR

Relative quantification of expression for all three genes was carried out using real-time PCR. Two reference genes were selected with the target genes to obtain meaningful data. Using the Bestkeeper and Normfinder software, CO1 and S26 were considered two of the better reference genes to be used in the study (see Appendix 4). The Normfinder software considered ND5 and rpL8 to be the best pair of reference genes to be used in combination, but when the real-time PCR runs were performed, there were non-specific amplicons for these genes at the amplification parameters designed for the immune genes. As a result, the two genes with the best stability values were considered - CO1 and S26. With regard to Bestkeeper, both CO1 and S26 were considered viable, thus there was agreement between the two software packages for the reference genes used in this study.

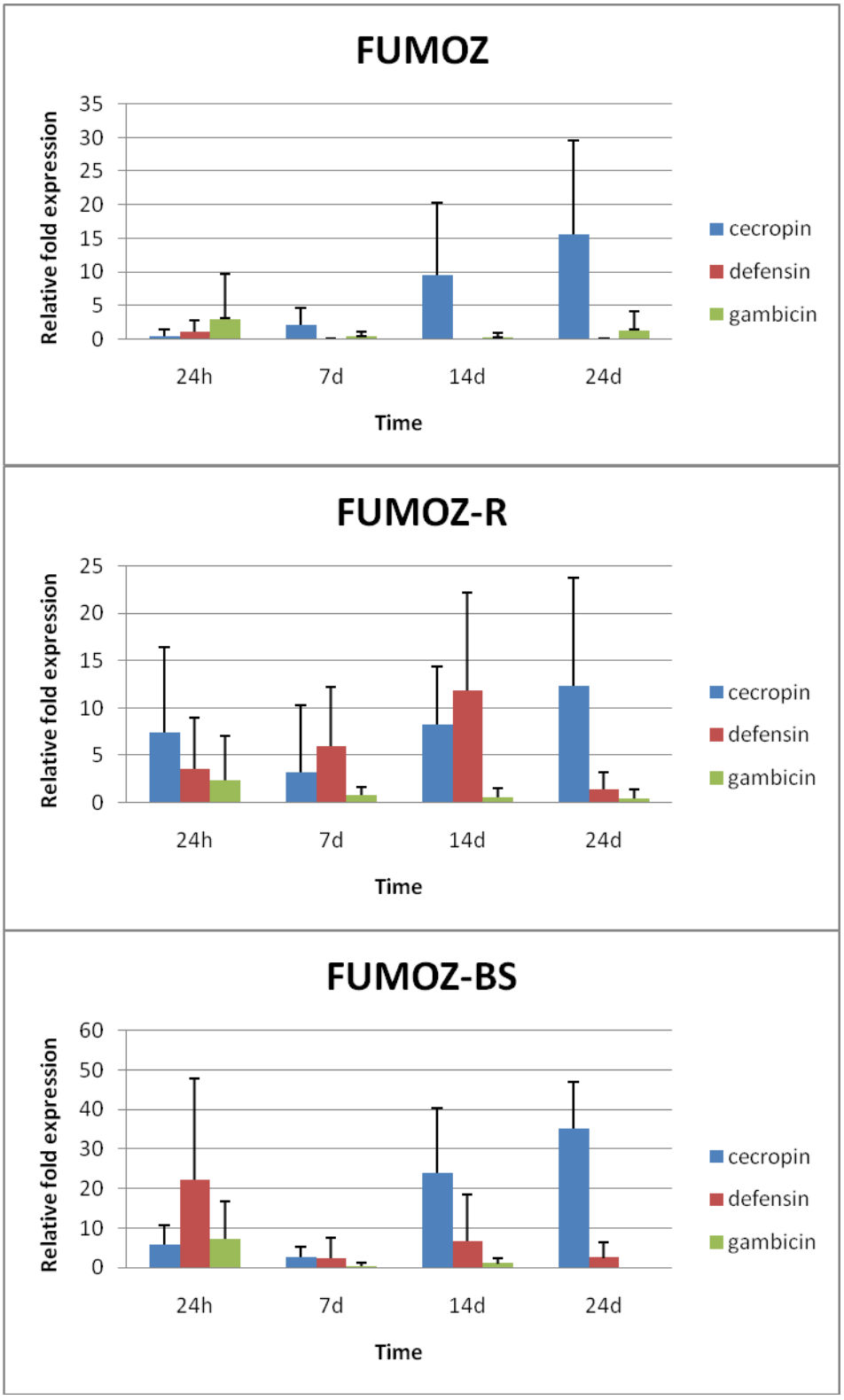


Figure 4.8: Expression of AMP genes in individual *An. funestus* strains, plotted against both reference genes.

When the immune genes were analyzed against both reference genes, gambicin was consistently the lowest expressed across all three *An. funestus* strains. Gambicin expression was typically highest 24 hours pi and decreased thereafter, although there was a slight increase at 14 days pi in FUMOZ-BS. Cecropin expression in all three strains showed increased activity as the infection progressed, although the expression level at 24 hours pi differed with FUMOZ-R initiating a much stronger expression than FUMOZ and FUMOZ-BS. However, during the other time points, cecropin expression was always highest in FUMOZ-BS, followed by FUMOZ and FUMOZ-R (Figure 4.8).

Defensin was the most varied of the three, maintaining very low levels in FUMOZ throughout the infection. Expression of the gene was at its peak at 14 days pi in both FUMOZ-R and FUMOZ-BS, but in FUMOZ-R defensin expression was low while FUMOZ-BS began with high expression. Co-expression of the AMPs was the strongest in FUMOZ-R with defensin and cecropin both having similar levels of increased expression through most of the time points (Figure 4.8). Combined results from triplicate experiments showed large variations between each discrete run.

4.4 Discussion

In order to isolate and analyze expression of the AMP genes in *An. funestus*, some assumptions had to be made in light of the relative absence of genomic data (particularly when compared to other well studied mosquito vectors). It has been established that the dipteran innate immunity system is a highly conserved one, with the same pathways activated by the same pathogens and producing highly similar, if not identical effector molecules. This is further supported by the presence of AMPs across the mosquito genera (*Culex*, *Anopheles*, and *Aedes*), with largely homologous sequences and activation pathways for these peptides

despite divergent evolution (Holt *et al*., 2002; Shin *et al*., 2002; Bartholomay *et al*., 2003). Given these data, it was assumed that *An. funestus* AMPs were highly likely to have similar sequences to *An. gambiae*, especially when the genes have been conserved across different genera of mosquitoes.

The first attempt at isolating the AMP genes in *An. funestus* was using the primers designed for *An. gambiae*. This was based on the assumption that *An. gambiae* and *An. funestus* immune genes should be extremely similar to each other so that the *An. gambiae* primers would be able to bind to the same region in *An. funestus*. This was not the case for any of the genes as the targeted fragments were often weakly amplified and a large number of non-specific fragments were also produced. Degenerate primers based on amino acid sequences were then designed to target the same region as the *An. gambiae* primers. This design process was only performed on defensin as a trial attempt. When it was found that the degenerate primers were more problematic than using the *An. gambiae* primers (only non-specific products were produced), this approach was abandoned.

In light of the inefficient degenerate primers, the third round of primer design relied on both amino acid and nucleotide sequences, targeting conserved regions elsewhere on the AMP genes. In addition, the primers were designed to amplify smaller regions (typically less than 200 bps), which was more in line with the optimal amplicon size when using real-time PCR. For cecropin, this worked exceptionally well, as only one amplicon was produced, and it was confirmed to be the desired product using DNA sequencing. Defensin and gambicin were subjected to cloning due to a number of non-specific products under normal PCR conditions. Once the target amplicon was identified by sequencing, the PCR was optimized to produce only the single band. Real-time PCR protocols were then created from the optimized

conventional PCR to quantify the gene expression. Sequences from the amplified regions in all three genes showed greater than 90% similarity to other known anopheline AMP genes. As the primers were targeting conserved regions of the genes, the high degree of sequence homology was expected. Whether this homology extends to the complete sequence remains to be determined.

The three AMP genes' expression levels were evaluated against the reference genes rpS26 and CO1 by examining parasite infected samples at various times during the infection, and a comparison made across all three *An. funestus* strains. Some associations have been made between AMPs and anti-parasitic responses in *An. gambiae* and other vectors, but the data are mostly limited to the early stages of infection and little is known about the interactions between the AMPs and sporozoites. It was initially hoped that the gene expression study could be performed on the abdomen and the salivary glands, particularly in the late stage of the infection, but isolation of sufficient material to perform cDNA synthesis proved to be difficult. The effects of *P. berghei* infection on AMPs expression were thus limited to the abdominal carcass here.

AMP expression profiles in *An. funestus* suggested that of the three peptides, gambicin was least affected by *P. berghei* infection. In *An. gambiae*, gambicin is expressed throughout the whole organism barring the head and extremities. It was found to be significantly up-regulated in the midgut and abdomen 24 hours post infected bloodmeal, and was also up-regulated from 11 - 21 days when sporozoites were released into the haemolymph for salivary gland invasion (Vizioli *et al*., 2001). The level of expression detected in *An. funestus* was initially similar to that of *An. gambiae*, but then decreased to two-fold or less in the older infected *An. funestus*. The peptide is therefore still up-regulated in late infection, but not as

significantly so in *An. funestus*.

Defensin had the most varied expression profile in *An. funestus*, as there were no consistent patterns across the strains. In FUMOZ, the peptide was constitutively expressed, except at 24 hours pi where there was a slight increase in expression. In FUMOZ-R and FUMOZ-BS, however, it reached high levels at 24 hours and 14 days pi. Furthermore, defensin expression in FUMOZ-R peaked at 14 days, while in FUMOZ-BS it was at 24 hours. The one commonality was that at 24 days, the expression level was very low in both strains. This suggested that defensin responded to the ookinete transition at 24 hours and possibly the early stages of sporozoite release into the haemolymph. This is similar to the expression profile in *An. gambiae*, where defensin was detected in *An. gambiae* midgut and salivary glands, and was highly expressed in the midgut/abdomen during early stages of infection, with lower activity around 10 - 15 days, but was then up-regulated at the salivary glands during sporozoite invasion (Dimopoulos *et al*., 1998).

Cecropin expression at 24 hours pi varied between the species. FUMOZ-R and FUMOZ-BS strongly expressed the peptide while the expression level was low in FUMOZ. However, cecropin expression in all strains reached high levels as the infection progressed. This is significantly different to the other two peptides that were at low levels by 24 days pi. Furthermore, the level of expression after 24 hours pi seemed to correlate with the parasite infection intensity of the strains, as FUMOZ-BS, with the highest parasite load, had the highest cecropin expression level, and FUMOZ-R had both low parasite load and low cecropin expression. It is possible that unlike the other two AMPs, cecropin is induced by the increase in the number of sporozoites circulating in the mosquito as the infection reaches the end stage. However, if this is the case, it also demonstrated that cecropin alone is not very

effective at reducing the number of parasites, as all three *An. funestus* strains remained infective despite increasing expression of the AMP gene. Currently there is no other data examining the effect of cecropin against sporozoites *in vivo* or *in vitro*, so further investigation may be necessary to understand the interaction between cecropin and parasite infection in the mosquito.

When the AMPs expression levels were examined individually, only cecropin displayed a pattern correlating to parasite development and infection intensity. If the AMPs expression were examined together, however, it appeared that in the more parasite-refractory strain, FUMOZ-R, there was more consistent co-expression of cecropin and defensin through the infection. Although FUMOZ-BS also had some increased co-expression at 14 days pi, it was not as high as in FUMOZ-R, and the defensin expression was lower than cecropin. Therefore, while the stand-alone response from cecropin is clearly inadequate in dealing with the parasites, the co-expression of defensin and cecropin may be a viable combination to reduce sporozoite load as demonstrated by Kokoza *et al.* (2010) and may warrant further investigation.

The large standard deviations that were typically seen throughout the gene expression results were likely due to individual samples, sample size and the varying infection rates. As the infection rate in *An. funestus* was low (20% at most), in any of the biological replicates collected, the majority of the individuals would have been uninfected. With the current state of technology, it was impossible to determine the infection status of each and every female used in the cohorts for the gene expression, particularly in the early stages of the infection, as the parasite would be present in the mosquito but that is no guarantee that the infection would have been successfully completed. Later in the infection, it is possible to determine the

infection status by dissections, but to do so would lead to the loss of significant amount of material (and contamination) that could be processed for RNA. This led to the compromise where the infection rate had to be determined retrospectively. There is also no way of determining the infection intensity without losing material, and thus the parasite load can vary between infected individuals, leading to further variations in the result. It may be of use to devise a method where it is possible to determine the early infection stages, as well as a more efficient molecular method to detect the presence of oocysts, as opposed to relying on microscopy.

Although progress has been made in identifying components of the mosquito immune system, much of the details regarding the mechanisms and interactions remain unclear, particularly in the late stage of the infection. The identification of AMPs and other immune molecules like TEP and LRIM acting in the early stages of *Plasmodium* infection showed that the mosquito immune system has a wide array of responses at its disposal (Dimopoulos *et al*., 1997, 1998; Christophides *et al.*, 2004). However, the typical immune responses are clearly not sufficient to eliminate the parasite. This is likely an effect of co-evolution between the vector and parasite, a balancing act for the mosquito between fitness cost and succumbing to *Plasmodium* infection, but even the true effect remains unresolved due to inconsistent data (Ferguson & Read, 2002). Nonetheless, research into artificially bolstering the immune reactions against *Plasmodium* in the vectors has shown some success and AMPs are possible candidates in such approaches (Cohuet *et al*., 2006; Kokoza *et al*., 2010).

Chapter 5: General discussion, future research and conclusion

5.1 General discussion

The large body of work dealing with *An. funestus* up to now has been concerned with physiological characterization and behavioural patterns (e.g. Gillies & de Meillon, 1968; Charlwood *et al.*, 2003; Spillings *et al.*, 2010; Wondji *et al*., 2011; Coetzee & Koekemoer, 2013), with some focus on cytogenetic data (Sharakhov *et al*., 2002; Wondji *et al*., 2005, 2007). There are also some comparative analyses with other malaria vectors (Coetzee *et al*., 2006; Charlwood, 2011). More recently, with the advent of molecular biology techniques, inroads have been made with regard to the species' insecticide resistance profile and related mechanisms (Amenya *et al*., 2008; Okoye *et al*., 2008a, b; Spillings *et al*., 2008; Coetzee & Koekemoer, 2013). These studies are important for understanding how best to control *An. funestus* using the available arsenal of insecticides. Efforts are now also being directed towards obtaining comprehensive sets of genomic/protein data, such as the sialome (Calvo *et al*., 2007), salivary gland proteome (Fontaine *et al*., 2012), as well as the complete genome sequence for the organism (*Anopheles* Genomes Cluster Committee, 2008). However, one critical issue that has not been addressed thus far is the vectorial capacity of *An. funestus* in the laboratory setting.

5.1.1 *An. funestus-P. berghei* infection

Anopheles funestus is a highly competent vector of human malaria due to its extremely anthropophilic and endophilic nature. This, however, does not necessarily make it a useful vector model in the laboratory. The failure to infect the species over six decades ago using *P. berghei* (van den Berghe, 1954) may have been more due to a lack of understanding of both

vector and parasite biology than a true indication of the vector's permissibility towards the parasite. In the intervening decades, malaria vectors were often found to be susceptible to infection with other *Plasmodium* species that they would never come into contact with naturally (Garnham, 1966; Bruce-Chwatt, 1978). These unnatural pairings have advanced our understanding of vector-parasite interactions, and the question arose as to whether *An. funestus* can be a host of *P. berghei* under the correct conditions. The hypothesis has been proven correct (Xu *et al.,* 2013; Lo *et al.,* 2013) and in the present study all *An. funestus* colonies were shown to be viable vectors, albeit at different rates.

Trends and patterns seen in other *Plasmodium* infection models are generally consistent with those observed thus far in the *An. funestus-P. berghei* infection system. The malaria parasite, for the duration of its lifecycle in the invertebrate host, seems to be constantly living on a knife's edge as only a small minority of the mosquitoes is ever infected, and numerous bottlenecks during development in the insect severely limit parasite numbers. However, this is possibly a false interpretation of how vulnerable the parasite is in the mosquito, as the parasite is consistently able to survive under these adverse conditions. This demonstrates that extensive co-evolution and co-adaptation has occurred between the two organisms, where both suffer to some extent but are still able to thrive. There is obviously no such co-evolution between unnatural vector-parasite pairings, but the fact that *P. berghei* still follows a similar pattern of development in *An. funestus* indicates that there are significant similarities between *Plasmodium* species to make *An. funestus-P. berghei* infection system a useful alternative in malaria research. The *An. funestus-P. berghei* infection system thus provides new research opportunities for studying the immune system in *An. funestus* and for comparative studies with other vector-parasite systems.

Perhaps the most significant result from the successful infection of *An. funestus* was that the insecticide resistant phenotype was present alongside low parasite infection intensity, and vice versa. There are very little data concerning the overlap between vector-parasite interactions and vector-insecticide interactions for anopheline mosquitoes, and some of these results are in conflict with one another (Christophides *et al*., 2004; Vontas *et al*., 2004). This is the first indication that there is a possible association between the two phenotypes in *An. funestus*, which could have significance for vector control and malaria transmission.

5.1.2 2La and *P. berghei* infection in *An. gambiae*

The association of phenotypes (e.g. insecticide resistance) with genotypes (e.g. paracentric inversions) has long been an important area of research for *An. gambiae*. A particularly important genotype is the 2La paracentric inversion, which has been linked to numerous environmental adaptations, and evolutionary divergence of the species (Coluzzi *et al*., 1979; Petrarca & Beier, 1992; Brooke *et al*., 2000; Riehle *et al*., 2006; Gray *et al*., 2009). Quantitative trait loci (QTL), co-localized with mutations in the sodium channel gene and the γ-aminobutyric acid (GABA) receptor associated with insecticide resistance, have also been shown to be in the inversion or in the vicinity thereof (Holt *et al*., 1996; Ranson *et al*., 2004; Zheng *et al*., 1996). What prevented the use of 2La as an efficient marker in routine vector surveillance was that the cytogenetic technique was the only means of identifying inversions for almost four decades. This issue was recently overcome when the 2La breakpoints were sequenced and a protocol designed to identify the rearrangements by PCR (Sharakhov *et al*., 2006; White *et al*., 2007). The molecular karyotyping worked well in all the *An. gambiae* colonies tested in this study and corresponded perfectly to cytogenetic observations on the same individuals.

Further molecular karyotyping of *P. berghei* infected females from 2La heterozygous colonies showed a bias towards the 2La inversion even when the population from which the infected cohort were drawn were not 2La dominated. There is thus yet another possible genotype-phenotype association for 2La with regard to *P. berghei* infection. This is not the first time the 2La inversion has been associated with parasite interaction, as the PRI is located within the inversion along with a number of immune genes (Niare *et al*., 2002; Osta *et al*., 2004; Riehle *et al*., 2006).

There appears to be a commonality between insecticide resistance and parasite refractoriness, and in this study, the presence of 2La in the *An. gambiae* colonies seems to be a marker for both dieldrin susceptibility as well as parasite susceptibility. It is currently unknown whether constant parasite infection or insecticide pressure would drive the $2La^+$ arrangement to dominance. Perhaps neither would apply any selection pressure on the genotype since previously tested colonies retained $2La^+$ at high levels despite an absence of selection pressure (Brooke *et al*., 2000). Furthermore, as the 2La inversion has an effect on the ability of *An. gambiae* to adapt to drier climates, it is possible that the xeric *An. gambiae* populations may be better hosts to *Plasmodium* than their mesic counterparts. The relation between the 2La inversion and parasite refractoriness would thus need to be investigated in more detail, since it also has the potential to impact on the species' insecticide resistance and climate adaptation profiles. If 2La does influence vectorial capacity, the chromosomal arrangement's involvement in malaria transmission may be even more complex than previously thought.

In *An. funestus,* some data are available for insecticide resistance mechanisms and the correlating QTLs, where similar genes (GSTs, mixed function oxidase, cytochrome P450s) and mutations (GABA receptor) are also involved in *An. gambiae* (Brooke *et al*., 2011; Okoye

et al., 2008; Wondji *et al*., 2007). This shows that there are significant similarities between *An. gambiae* and *An. funestus* in terms of insecticide resistance mechanisms. Given that *An. funestus* has numerous paracentric inversions (albeit not as many as *An. gambiae*), it is possible that some of these inversions may have similar influences on the *An. funestus* phenotypes. Cytogenetic techniques thus may have an important place in *An. funestus* research, as data on how paracentric inversions influence *An. funestus* phenotypes remain largely incomplete.

5.1.3 Expression of AMPs of *An. funestus* during *P. berghei* infection

The successful infection of *An. funestus* strains presents not only the first set of laboratory infection data for the species, but also one of the few sets of results where both insecticide resistance and parasite refractory phenotypes are shown in the experimental cohorts. Infection in the insecticide resistant strain of *An. funestus* displayed the lowest oocyst and sporozoite counts of the three FUMOZ colonies. Conversely, the insecticide susceptible strain was the most heavily infected, with parasite numbers comparable to those detected in other anopheline vectors. *Anopheles funestus* thus demonstrates a positive correlation between insecticide resistance and parasite refractoriness. The results here are consistent with most published data (Vontas *et al*., 2001; Christophides *et al*., 2004), and it is generally thought that the higher levels of insecticide detoxification enzymes conferring resistance usually facilitate better anti-parasite responses (Vontas *et al*., 2001, 2004). Nonetheless, insecticide resistance in anophelines is brought about by a number of different detoxification mechanisms depending on the class of insecticide the insect is exposed to. Further investigations are necessary, therefore, to determine whether resistance to other insecticides aside from permethrin would lead to the same results.

As this is the first time that laboratory colonized *An. funestus* has been successfully infected with any *Plasmodium* species, no data exist for parasite resistance mechanisms, or any related information (such as resistance QTL) in this species. In other insects, the AMP proteins were among the first molecules to be examined for their properties and functions, so the three best characterized AMPs in *An. gambiae* - cecropin1, defensin1, and gambicin - were targeted in this study of *An. funestus*, and their expression levels monitored over the course of the *P. berghei* infection. All three genes had increased expression 24 hours pi, and were at least constitutively expressed throughout the remainder of the infection, showing that they were active as immune responses. Although there were different patterns of AMPs expression between the FUMOZ strains, it was clear that the AMPs had no strong anti-malarial properties as all three *An. funestus* strains were able to sustain the infection despite the increased expression.

Nonetheless, AMP participation in anti-*Plasmodium* responses should not be disregarded entirely. Mosquito immune responses are clearly multifactorial, with complex interactions between all the components. Of all the *Plasmodium*-killing mechanisms identified thus far, none has been shown to lead to total elimination of the parasite, and the molecular basis for these mechanisms is usually unresolved (Christophides *et al*., 2004). The other point that should be raised is that the AMPs were all previously examined in isolation to one another. This is obviously necessary to determine the standalone effect of the genes/proteins, but it cannot reliably inform us what the AMP's true effect is when acting in concert with other immune factors, and certainly not within a fully functional *in vivo* setting. A good example of combined factors producing more profound anti-*Plasmodium* responses than the standalone factors was demonstrated by Kokoza *et al.* (2010), where over-expression of defensin and cecropin together in *Ae. aegypti* was able to reduce *P. gallinaceum* oocyst numbers, and the

sporozoites were unable to make it to the salivary glands. This was not seen when the two AMPs were examined separately in *An. gambiae*. Different vector-parasite combinations may have led to such a difference, but the fact that higher cecropin and defensin co-expression was observed here in the more parasite refractory *An. funestus* strain, lends some credence to the idea that co-expression of AMPs may be an option in reducing parasite load in the mosquito.

Most AMP research have been focused on the effects on ookinetes and oocysts invasion (Richman *et al*., 1997; Dimopoulos *et al*., 1997; Dimopoulos *et al*., 1998; Vizioli *et al*., 2000; Vizioli *et al*., 2001, 2001a; Tahar *et al*., 2002). The reason for the focus is that if the early stages can be eliminated, the parasite cannot develop further and spread. The immune reaction against sporozoites in the haemolymph and salivary glands is also considered to be less dramatic than those launched against oocysts and ookinetes (Christophides *et al*., 2004). With the amplification factor from oocysts-to-sporozoites being an 2, 000 - 8, 000 fold increase, any immune responses mounted is considered to have no substantial effect on parasite transmission by that stage (Christophides *et al*., 2004). However, there have been little data indicating that complete elimination of the parasite in the mosquito is possible by only targeting the pre-sporozoite stages without selecting for enhanced melanization/encapsulation phenotype. Furthermore, recent data suggest that even if low number of oocysts are present, it is possible to prevent sporozoite invasion of the salivary glands using AMPs (Kokoza *et al*., 2010). Expression of AMPs in *An. funestus* indicates that they are induced by the presence of the parasite, and even if the peptides alone are not able to reduce the parasite load, activation of other immune reactions in conjunction with AMPs may provide better responses to the infection. It would therefore be prudent to further investigate what interactions exist between the AMPs (and other immune factors) when sporozoites are released into the haemolymph and during salivary glands invasion.

The question of how insecticide resistance in mosquitoes affects parasite refractoriness (and vice versa) has largely not been addressed, not just in anophelines, but also other mosquito vectors such as *Culex* and *Aedes* species. One of the few investigation examining this interaction showed that in insecticide resistant *Cx. quinquefasciatus* (which has increased esterase activity) the development of *W. bancrofti* larvae in the gut cells were affected (McCarroll *et al*., 2000). Other attempts at correlating the two phenotypes has usually led to conflicting results that require further investigation (Yan *et al*., 1997; McCarroll *et al.,* 2000; McCarroll & Hemingway, 2002; Vontas *et al*., 2004). While the aim of this study was not to show if there are any relationships between insecticide resistance and parasite refractoriness, the results indicate that there appears to be some correlation between the two. In this case, permethrin resistance in *An. funestus* is related to increased refractoriness towards the parasite. This would have significant repercussions for vector control and malaria management. If mosquitoes with pyrethroid resistance carry less sporozoites, the increased insecticide resistance may not be a total loss, as it may be possible to induce the mosquito immune system to be a little more effective at eliminating the parasite, as opposed to an extensive modification of the immune response. It would also be interesting to determine whether insecticide resistance will drive the selection of parasite resistance (or vice versa), as currently there is no relevant data for this.

The data presented here shows in part the highly complex nature of the vector-mosquito interaction, whether it is purely between the two organisms, or with regard to other factors such as insecticide resistance. Although one of the key tenets of a good scientific experiment is to observe the effect of only one variable while controlling all others, this tends to dismiss the complexity that is inherent in an organism, or interactions between organisms. It is rare for

biological components to have a single interaction with one other component *in vivo*, thus a good experiment may not necessarily produce accurate results truly reflective of the real conditions. As mentioned above, the role of AMPs in *Plasmodium* infection is a good example of this. Characterization of the AMPs in *An. gambiae* showed largely weak anti-*Plasmodium* effects when examined separately, but when combined, they may generate more effective responses than previously thought. Similarly, parasite refractoriness has rarely been examined in conjunction with insecticide resistance, but the few results that are available seem to indicate that there are interactions between the two. The single variable experiments are also likely to have contributed to the unresolved question of fitness versus survival cost incurred by mosquitoes during *Plasmodium* infection, given the complexity of such an issue. The resolution of these multi-factorial questions may require a combination of experiments simultaneously targeting various aspects of the vector and parasite biology to truly understand the interplay between the organisms, and the environments they inhabit.

5.2 Future research

The possible uses for the *An. funestus*-*P. berghei* infection system in the future, whether as a standalone infections model or to provide a comparison with existing infection systems, are numerous. A few possible directions that link directly to results presented in this thesis are given here.

The infection of *An. gambiae* complex colonies using *P. berghei* at VCRL is the first set of infection results for these colonies since their establishment. The infection rates obtained for a number of the older colonies that have been used in laboratories elsewhere (e.g. G3 strain), are lower than previously observed. The causes of this difference are unknown and have been speculated upon in earlier parts of the thesis, but investigation into why there is a change in

infection rate may be useful if these colonies are to be used as reference or baseline controls in the future. Similarly, the change in 2La frequency in the heterozygous *An. gambiae* colonies should be investigated, especially considering that the inversion can influence a number of phenotypic expressions in the mosquito.

Paracentric chromosomal inversions like 2La in *An. gambiae* and their effects on the species' evolution and adaptations are well studied. *Anopheles funestus* does not possess as many paracentric inversions as *An. gambiae*, and it is largely unknown if there is any association between *An. funestus* inversions and physiological adaptations. Given the similarity between *An. gambiae* and *An. funestus* (e.g. overlapping habitats, vectorial capacity, insecticide resistance mechanisms), and that *An. gambiae* is highly influenced by its paracentric inversions, the inversions in *An. funestus* should be examined in greater detail. While the *An. funestus* whole genome sequence will provide a wealth of data, especially if homologous and orthologous sequences to the *An. gambiae* genome can be found, it should be remembered that cytogenetics on *An. gambiae* decades earlier, revealed significant physiological and evolutionary correlations prior to determination of the *An. gambiae* genome (a few examples are Coluzzi *et al*., 1977, 1979, 1985; Davidson & Hunt, 1973; Green, 1972; Green & Hunt, 1980; Hunt, 1972; Petrarca & Beier, 1992; Brooke *et al*., 2000). Although cytogenetic techniques require specific expertise, these skills are necessary to correlate physical traits with chromosomal variations that the molecular techniques are not as well suited for.

The quantitative study of the parasite infection indicated there were some associations between insecticide resistance and parasite refractoriness in *An. funestus*. The data on the association of the two phenotypes in mosquitoes are severely limited at this point, despite the impact these two traits have on vectors and disease control. More attention needs to be given

to understanding if and how the two phenotypes affect each other and how this may lead to better methods for controlling malaria and other vector-borne diseases.

The analysis of three AMPs in *An. funestus* during *P. berghei* infection is the first step to understanding the immune response in *An. funestus*. There remain a large number of immune-related genes/proteins that can be examined (e.g. GNBP, LRIM, TEP), provided homologues are found in *An. funestus*. Data mining the *An. funestus* genome will facilitate this process. It would also be important to examine these immune-related genes/proteins in conjunction with one another, and not as standalones, as their combined effect may be more significant than when they are examined separately.

Lastly, the infection of *An. funestus* using *P. falciparum* remains the ideal infection system to strive for. The caveat for using murine (or any other non-human) malaria models to extrapolate data regarding anopheline-*P. falciparum* infections remains. Multiple papers have addressed this issue, demonstrating discrepancies between unnatural vector-parasite pairings and the normal pairing found in nature (Dimopoulos, 2003; Aguilar *et al*., 2005; Cohuet *et al*., 2006; Dong *et al.*, 2006; Michel *et al*., 2006). Research using *P. berghei* thus may not necessarily provide results relevant to *P. falciparum*, but can be useful in conjunction with other studies and more research (Cohuet *et al.*, 2006). Until such time, the use of *P. berghei* makes a good starting point for investigating *An. funestus-Plasmodium* interactions, and infection of the species using *P. berghei* ookinete cultures may be the next step towards establishing infection with *P. falciparum.*

5.3 Conclusion

Malaria research has advanced significantly in the past few decades, in part due to better understanding of the vector biology, physiology, and vector-parasite interactions. The use of unnatural vector-parasite pairing has been the mainstay to deciphering the complex relation between the two organisms, and results here have provided new data and options for this area of research. Most of the *Anopheles gambiae* complex species tested here were shown to be competent vectors and can now be used with confidence as the targets or as references in future infection-related experiments. The successful development of *P. berghei* in *An. funestus* provides a novel infection system that represents a significant step forward for *An. funestus* research.

Infection of both *An. gambiae* and *An. funestus* with *P. berghei* produced results where the infection can be correlated to other phenotypes, genotypes and molecular expression. Understanding how the mosquitoes' genetic makeup influences its phenotypic expression is critical to vector control and disease management, especially if phenotypes of increased resistance/refractoriness are involved. Molecular mechanisms of resistant/refractory phenotypes have typically been studied in isolation, but there are indications that it is much more complex *in vivo*, where pathways and interactions can overlap, influencing more than just one trait. Furthermore, these traits often exist as a gradient, and not absolute values, thus further complicating the roles and effects of the factors and genes involved.

The increased understanding of the genetic basis of insecticide resistance and parasite refractoriness in anopheline mosquitoes makes the use of genetically modified mosquito part of the final solution to malaria eradication ever more promising. Such understanding ironically also reveals more complexities that exist within the organism and of its interactions

with *Plasmodium*. *Anopheles funestus* is no exception, as infection with *P. berghei* showed a possible correlation to insecticide resistance. The species also demonstrated immune responses similar to those seen in other mosquitoes with respect to anti-microbial peptides; furthermore, co-expression of AMPs may be a viable alternative in reducing parasite loads even though individual AMPs have little anti-plasmodial effect. More research is therefore required to understand the interaction of *An. funestus* with malaria parasites. The results presented herein are the first step in investigating parasite interaction in *An. funestus*, and hopefully will provide impetus for more research in this direction.

Appendices

Appendix 1: *Anopheles* colonies in the Botha de Meillon insectary used in the study

Colony Name	Species	Country of origin	Date colonized	Resistance status
BOA	*An. gambiae*	Cote d'Ivoire	1997	Dieldrin
CIG	*An. gambiae*	Cote d'Ivoire	1999	Dieldrin
COGS	*An. gambiae*	Congo	2010	Susceptible
COGS-EXP	*An. gambiae*	Congo	2010	Multiple
G3	*An. gambiae*	The Gambia	1970s	Dieldrin
GAH	*An. gambiae*	Ghana	2004	Multiple
IANP20	*An. gambiae*	Nigeria	1970s	Dieldrin
NAG	*An. gambiae*	Migeria	2001	Dieldrin
PALA	*An. gambiae*	Burkino Faso	1970s	Dieldrin
SUA	*An. gambiae*	Liberia	?	Malathion
ARER	*An. arabiensis*	Eritria	2000	Susceptible
KGB	*An. arabiensis*	Zimbabwe	1975	Susceptible
MA	*An. arabiensis*	Mozambique	1990	Susceptible
MALPAN	*An. arabiensis*	South Africa	?	Susceptible
MBN-DDT	*An. arabiensis*	South Africa	2003	Multiple
MAF	*An. merus*	South Africa	1988	Susceptible
ZAM	*An. merus*	South Africa	1980	Susceptible
SANGWE	*An. quadriannulatus*	Zimbabwe	1998	Susceptible
FANG	*An. funestus*	Angola	2002	Susceptible
FUMOZ	*An. funestus*	Mozambique	2002	Partial
FUMOZ-R	*An. funestus*	Mozambique	2002	Permethrin
FUMOZ-BS	*An. funestus*	Mozambique	2008	Susceptible

Appendix 2: PCR primer sequences

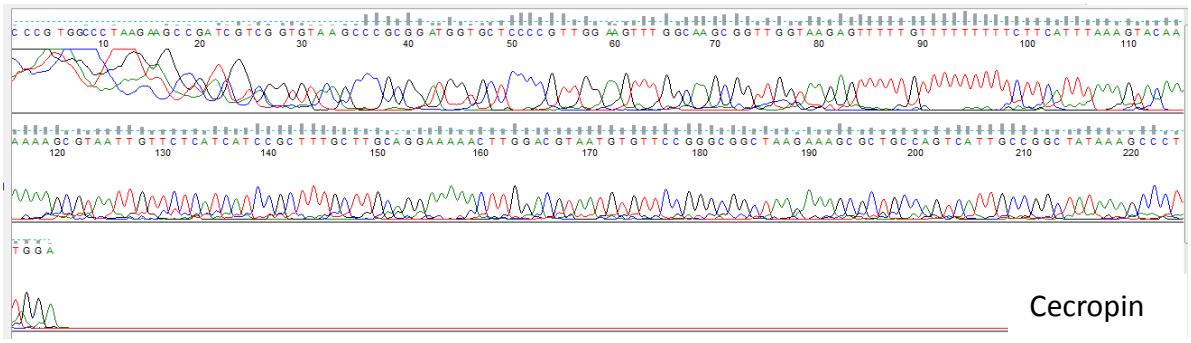
Table A2.1: Primers for antimicrobial peptide genes.

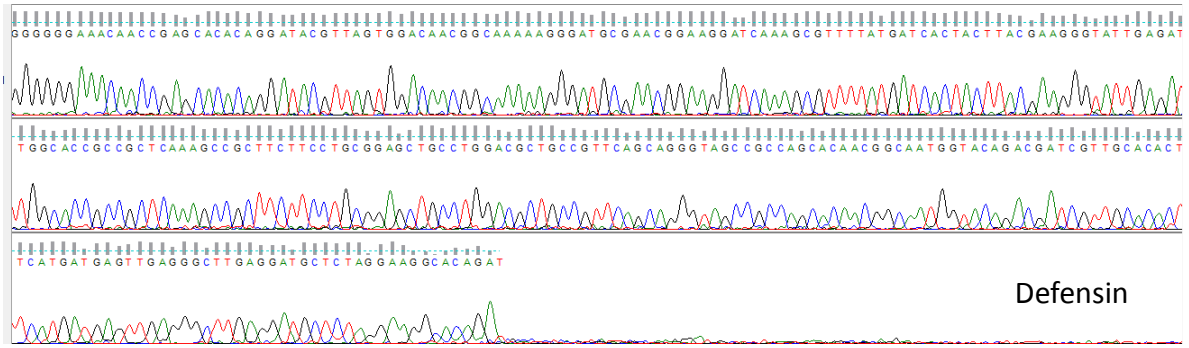
Primer	Sequence	Amplicon size
Cec F	5' - CAT CTT ATC AAC CCA GA - 3'	287 bps
Cec R	5' - GCC ATA TCA TGT TAG CA - 3'	
CecRD F	5' - AAC TTC ACA AAA CTG TTC ATT - 3'	175 bps
CecRD R	5' - CCA AGG GCT TTA TAG CCG GCA - 3'	
Dfn F	5' - CTG TGC CTT CCT AGA GCA T - 3'	403 bps
Dfn R	5' - CAC AAC CTC TTC CCA GGA T - 3'	
Dfn degen F	5' - ATR ACR CGN AAR RAN CTY GT - 3'	~400 bps
Dfn degen R	5' - CGN TCN CTY CTY CCN CAN GG - 3'	
DfnRD F	5' - GAC GAA CTG CCC GAG GAA ACG - 3'	253 bps
DfnRD R	5' - GTT GCG GCA AAC ACA CAC - 3'	
DfnRD2 F	5' - GAG AAC TAT CGG GCC AAA CGG - 3'	127 bps
DfnRD2 R	5' - CCT TAC TGT TGC AGT AAC CAC CGC - 3'	
Gmb F	5' - AAC CGG AAG GGC GTT TCG TG - 3'	265 bps
Gmb R	5' - CGT CTG GCA CTG ATT AAA CC - 3'	
GmbRD F	5' - GAA GCA AGT GTG CAT TCT TCT - 3'	150 bps
GmbRD R	5' - TAG CCA CAC TAG CGA GCA CCG - 3'	

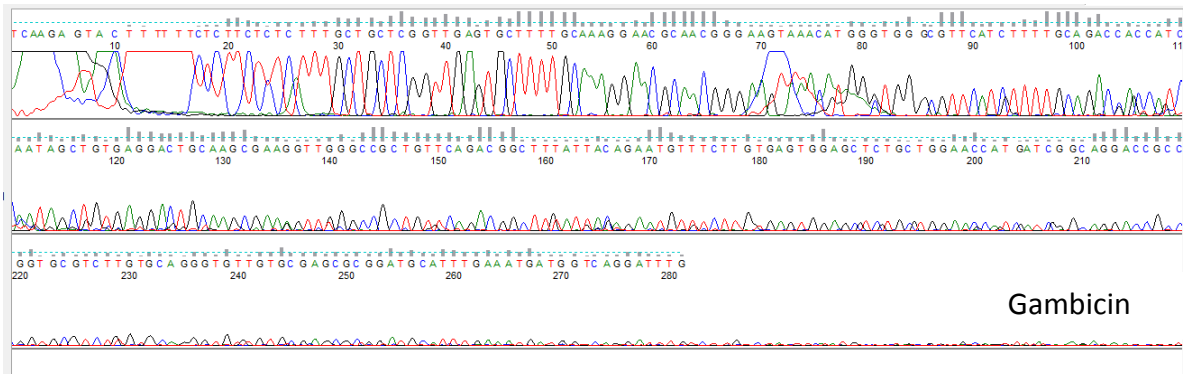
Table A2.2: Primers for candidate reference genes.

Primer	Sequence	Amplicon size
CO1 F	5' - TAG GAG CCC CTG ATA TAG CTT TC - 3'	123 bps
CO1 R	5' - ACT GTT CAT CCT GTT CCT GCT C - 3'	
GAPDH F	5' - GAC TGC CAC TCG TCC ATC - 3'	139 bps
GAPDH R	5' - CCT TGG TCT GCA TGT ACT TG - 3'	
ND5 F	5' - TAG AAT TTT ATT AGG GTG GGA TGG - 3'	122 bps
ND5 R	5' - ATC TCC AAT TCG ATT TGA TAA TGC - 3'	
rpL8 F	5' - CAT CAG CAC ATT GGT AAG - 3'	305 bps
rpL8 R	5' - GTT TTC GCT TCC CGT TTT TC - 3'	
rpL19 F	5' - GAA ACA CCA ACT CCC GAG A - 3'	223 bps
rpL19 R	5' - TCA ACA GGC GAC GCA ACA C - 3'	
rpS7 F	5' - GTG CCG GTG CCG AAA CAG AA - 3'	134 bps
rpS7 R	5' - AGC ACA AAC ACT CCA ATA ATC AAG - 3'	
rpS26 F	5' - GAT AAG GCA ATC AAG AAG TTC G - 3'	160 bps
rpS26 R	5' - TAC GGA CAA CCT TCG AGT GG - 3'	
18S F	5' - TAC CTG GGC GTT CTA CTC - 3'	116 bps
18S R	5' - CTT TGA GCA CTC TAA TTT GTT C - 3'	

Appendix 3: Gene sequences for *An. funestus* antimicrobial peptide genes







Cecropin

Anopheles stephensi salivary cecropin mRNA, partial cds
Sequence ID: gb|EU071705.1|
97% similarity

```
Query  119  GGAAAAACTTGGACGTAATGTGTTCCGGGCGGCTAAGAAAGCGCTGCCAGTCATTGCCGG  178
            ||||||||||||||||||||||||||||| |||||||||||||||| ||| |||||||
Sbjct  46   GGAAAAACTTGGACGTAATGTGTTCCGGGCTGCTAAGAAAGCGCTGCCGGTCGTTGCCGG  105

Query  179  CTATAAAGCCCTTGGA  194
             || ||||| ||||||
Sbjct  106  ATACAAAGCACTTGGA  121

Query  7    GATGGTGCTCCCCGTTGGAAGTTTGGCAAGCGGTTGG  43
            |||||||| ||||||||||||||||||||||||||||
Sbjct  11   GATGGTGCGCCCCGTTGGAAGTTTGGCAAGCGGTTGG  47
```

Defensin

Anopheles gambiae defensin (Def) gene, complete cds
Sequence ID: gb|AF063402.1|AF063402
98% similarity

```
Query  1     CCCACACCGAACCCGCTGGCCAAATCGCAGGTCGCCCGCTTGGCCCGATAGTTCTCCAGC  60
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  2439  CCCACACCGAACCCGCTGGCCAGATCGCAGGTCGCCCGCTTGGCCCGATAGTTCTCCAGC  2380

Query  61    GCGGCATGGTGCGTTTCCTCGGGCAGTTCGTCCACTAATGGGAGGGGGGAAA-CAACCGA  119
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  2379  GCGGCATGGTGCGTTTCCTCGGGCAGTTCGTCCACTAATGGGAGGGGGGAAAACAACCGA  2320

Query  120   GCACACAGGATACGTTAGTGGACAACGGCAAAAAGGGATGCGAACGGAAGGATCAAAGCG  179
             ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  2319  GCACACAGGATACGTTAGTGGACAACGGGAAAAAGGGATGCGAACGGAAGGATCAAAGCG  2260
```

Gambicin

Anopheles gambiae M isolate GamM957_A gambicin (AGAP008645) gene, complete cds.
Sequence ID: gb|GU990117.1|
98% similarity

```
Query  1    GAGTGCTTTTGCAAAGGAACGCAACGGGAAGTAAACATGGGTGGGCGTTCATCTTTTGCA  60
            ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  434  GAGTGCTTTTGCAAAGGAACGCAACGGGAAGTAAACATGGCTGGGCGTTCATCTTTTGCA  493

Query  61   GACCACCATCAATAGCTGTGAGGACTGCAAGCGAAGGTTGGGCCGCTGTTCAGACGGCTT  120
            ||| |||||||||||||||||||||||||||| ||| ||||||||||||||||||||||
Sbjct  494  GACGACCATCAATAGCTGTGAGGACTGCAAGCGAAAGTTTGGCCGCTGTTCAGACGGCTT  553

Query  121  TATTACAGAATGTTTCTTGTGAGTGGAGCTCTGCTGGAACCATGATCGGCAGGACCGCCG  180
            |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  554  TATTACAGAATGTTTCTTGTGAGTGGAGCTCTGCTGGAACCATCATCGGCAGGACCGCCG  613

Query  181  GTGCGTCTTGTGCAGGGTGT  200
            ||||||||||||||||||||
Sbjct  614  GTGCGTCTTGTGCAGGGTGT  633
```

Appendix 4: Real-time PCR

Reference gene calculation results

Data from the Normfinder software indicating the best reference gene and best possible pairing of reference genes. Genes with the lowest stability values would be the optimal choice.

Gene name	Stability value
rpS7	0.223
rpS26	0.178
rpL19	0.312
ND5	0.355
rpL18	0.403
GAPDH	0.256
CO1	0.148
18S	0.801

Best gene	CO1
Stability value	0.148
Best combination of two genes	ND5 and rpL18
Stability value for best combination of two genes	0.062

Bestkeeper software also calculates the best reference genes as the ones with the lowest score.

	rpS7	rpS26	rpL19	ND5	rpL18	GAPDH	CO1	18S
p-value	0.243	0.001	0.005	0.016	0.001	0.001	0.001	0.001

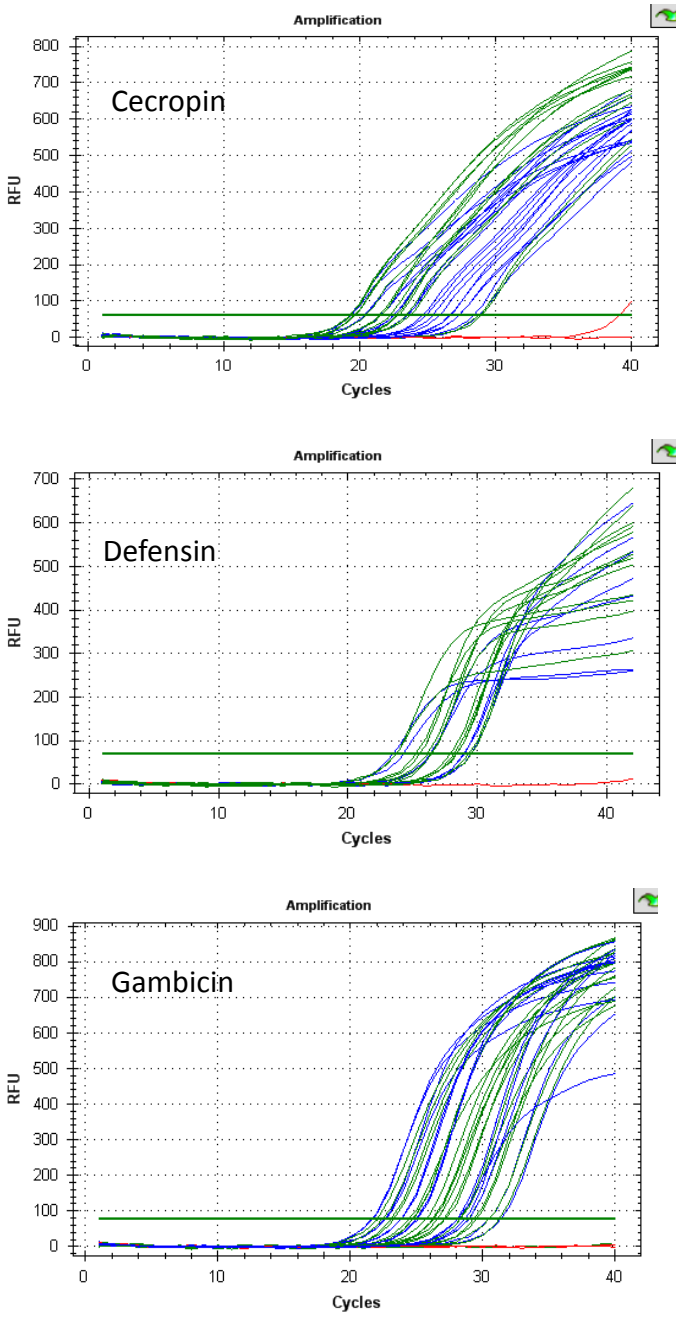


Figure: Sample of real-time PCR runs of the immune genes with standard curves (in blue).

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