Selection and characterization of a new, non-melanising line of *Anopheles gambiae s.s.* refractory to *Plasmodium falciparum*

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To

Zbyněk Zubřík Rosendorf

1974 – 2014

…I am a thousand winds that blow
I am the diamond glints on snow
I am the sunlight on ripened grain
I am the gentle autumn rain
When you awaken in the morning's hush
I am the swift uplifting rush
Of quiet birds in circled flight
I am the soft stars that shine at night…

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Abstract

This thesis describes the selection of a new, non-melanising, refractory line of *Anopheles gambiae s.s.*, denoted GU-REF, its characterisation with respect to fitness, and preliminary data on possible genes under selection. GU-REF was selected for refractoriness to *Plasmodium falciparum* clone 3D7 over 11 generations, four of which are described in this work. A control line (GU-CON) was selected at random at the same time as a control for inbreeding effects.

At the beginning of the project the GU-REF and GU-CON lines were at generation 7 of selection. However it was discovered that the colonies had become infected with the fungal pathogen microsporidia. It was therefore necessary to recover microsporidia-free colonies first, which was done by selective breeding from non-infected female mosquitoes. A further four generations of selection for refractoriness were then performed and are described here.

The final generation of mosquitoes (GU-REF11) exhibited a significantly lower infection prevalence compared to the GU-CON11 line. Moreover, the GU-REF11 was also more refractory than GU-CON11 to a different *P. falciparum* clone, HB3, which was not used in selection. This suggests that the mechanism of refractoriness is general to *P. falciparum,* and not specific to the parasite clone used for the selection process.

To establish the costs, if any, of refractoriness in the newly selected GU-REF mosquitoes relative to the GU-CON line, five fitness markers were assessed: body size, adult survival, proportion of mosquitoes laying eggs after first blood meal, length of first gonotrophic cycle, and number of eggs per egg lay. To further investigate differences between GU-REF and GU-CON that could contribute to refractoriness of GU-REF, the speed of blood meal digestion was also analyzed. The overall fitness of GU-REF line compared to the GU-CON line was not significantly negatively affected by the selection process for refractoriness.

In the final part of the project, a candidate gene, *APL1A,* was selected from the existing published data. Allele *APL1A*^{2} was found to be significantly enriched in GU-REF11 mosquitoes, compared to the GU-CON line, suggesting that it is associated with the refractory phenotype.

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Acknowledgements

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Declaration

I hereby solemnly declare that the research described within these pages is my own work except where stated, and truthfully acknowledge the contributions of all those collectively remembered, forgotten, and unrecognised, who went before.

Signed:

Lenka Richterová

February 2018

Abbreviations

3D7	*Plasmodium falciparum* clone of Dutch isolate NF54
A2M	alpha-2-macroglobulin
Ab	Antibody
AgDscam	*Anopheles gambiae* Down syndrome cell adhesion molecule
AMPs	Antimicrobial peptides
APL1A^2	*Anopheles Plasmodium*-responsive leucine-rich protein A haplotype 2
BCA	Bicinchoninic acid protein assay
Cad	Caudal
CASPL1	Caspase *Anopheles gambiae* homologue of Dredd in *Drosophila*
CC	coiled-coil
CD8+ T cells	Cluster of Differentiation 8 thymus cells
CEC	Cecropin
CLIP	corticotropin-like intermediate peptide
CRD	carbohydrate recognition domain
CTLGA	C-type lectin galactose binding
CTLMA	C-type lectin mannose binding
CTLs	C-type lectin
CTLSE	C-type lectin selectin
DCE	dopachrome conversion enzyme
DDC	dopa decarboxylase

DEF	Defensin
Dome	Domeless
Fadd	Fas-associated death domain
FBNs	Fibrinogen-like domain immunolectins = FREPs
FREPs	Fibrinogen-related proteins = FBNs
G3	*Anopheles gambiae* strain from The Gambia
G3-5 line	*Anopheles gambiae* melanising line derived from G3
GALES	Galactoside binding lectins
GAM	Gambicin
GMO	Genetically Modified Organism
GNBPs	Gram-negative binding proteins
GPI-anchor	glycosylphosphatidylinositol anchor
GU-CON	Glasgow University control strain of *Anopheles gambiae* KEELE
GU-REF	Glasgow University refractory strain of *Anopheles gambiae* KEELE
HB3	*Plasmodium falciparum* clone from Honduras isolate H1
HLH	helix-loop-helix
Hop	Hopscotch
HSPGs	Heparan sulfate proteoglycans
HWE	Hardy-Weinberg equilibrium
IBF	Infected blood-feed
IFAKARA	*Anopheles gambiae* strain from Ifakara, Tanzania

Imd	Immune deficiency
IRID	Infection responsive immunoglobulin domain
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
JAK	Janus kinase
KEELE	*Anopheles gambiae* strain established in Keele University
KIL	*Anopheles gambiae* strain from Tanzania
L3-5 line	*Anopheles gambiae* melanising line
LDLs	low density lipoproteins
LPS	Lipopolysaccharide
LRIM	Leucine-rich repeat immune protein
LRR	Leucine-rich repeat
M	Mopti
MAMPs	Microbe-associated molecular patterns
NF54	*Plasmodium falciparum* isolate from The Netherlands
NFkB	nuclear factor kappa B
NK cells	Natural killer cells
NO	nitric oxide
NOS	nitric oxide synthase
PABA	4-amino benzoic acid
PAH	phenylalanine hydroxylase
PAMPs	Pathogen-associated molecular patterns

PANGGL	Pro-Ala-Asn-Gly-Gly-Leu
PBS	Phosphate-buffered saline
Pen	*Plasmodium* encapsulation locus
PGRPs	Peptidoglycan recognition proteins
PIAS	protein inhibitor of activated STAT
PO	Phenoloxidase
PPAEs	prophenoloxidase activating enzymes
PPO	Prophenoloxidase
PRI	*Plasmodium* resistance island
PRRs	Pattern recognition receptors
QTL	quantitative trait locus
R	software for statistical computing and graphics
RBC	Red blood cell
Rel2	Relish
RH	relative humidity
RNAi	RNA interference
RPMI	medium developed at **R**oswell **P**ark **M**emorial **I**nstitute
RTS, S	Malaria vaccine: coexpressed - **R**epeat region *P.f.* CSP, **T**- cell epitopes CSP, HB**s**Ag with free HB**s**Ag
S	Savannah
SCRs	Scavenger receptors
SERPINS	serine protease inhibitors
SOCS	suppressor of cytokine signalling

STAT	signal transducers and activators of transcription
TBV	Transmission blocking vaccines
TEPs	Thioester-containing proteins
Upd	Unpaired
WHO	World Health Organisation
ZANU	*Anopheles gambiae* strain from Zanzibar
βGRP	β-1,3 glucan recognition protein
γδT cells	γδ thymus cells

1 Chapter: General introduction

1.1 Malaria burden and overview of disease transmission

Malaria is one of the most prevalent parasitic diseases in the tropics around the world. Although malaria is preventable and treatable, it was estimated that in 2015, 3.4 billion people in 91 countries lived at risk of the disease and that 212 milion people (uncertainty range 148–304 million) were infected in 2015 out of which 490,000 died (uncertainty range 235,000–639,000) (WHO, 2016). 90% of cases and 92% of deaths occurred in Africa, and most of the deaths (70%) were in infants under the age of five. The total international funding for malaria control in 2015 was estimated to be in the region of US$ 2.9 billion. Malaria also has a huge detrimental impact on the economies of the countries that are affected by the disease.

There are five species of malaria parasite that infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. These parasites are transmitted by approximately 60 Anopheline mosquito species around the world, of which 41 are considered dominant (Manguin, 2008; Sinka et al, 2010a; Sinka et al, 2011; Sinka et al, 2010b). *P. falciparum* is responsible for approximately 90% of malaria-associated deaths and most of the cases of severe malaria (www.cdc.gov/malaria). Adults living in malaria endemic regions reaching their teenage years tend to have developed immunological response decreasing the impact of the disease with local malaria strains due to continuous exposure (Langhorne et al, 2008) . Common symptoms of the disease include periodic fevers with chills, caused by the lysis of infected red blood cells (RBC), headache, nausea and fatigue. Untreated, malaria infection can lead to kidney damage, cerebral malaria, swelling of the spleen, respiratory problems, and ultimately can lead to death.

The tools that are employed in malaria control are targeted against either its vector, the mosquito, or against the parasite in the human host. The main vector control strategies consist of controlling breeding sites, the use of larvicides, insecticides and insecticide-treated nets (ITNs), and indoor residual spraying

(IRS). In 2015, there was 53% coverage with ITNs in sub-Saharan Africa, whereas IRS accounted for only 5.8% coverage of the population at risk in Africa. In the human host, malaria can be prevented using prophylactic drugs, or infected individuals can be treated with antimalarials. There is currently no vaccine used against the disease, although several candidate vaccines are in development (Birkett et al, 2013), and one (RTS, S, now renamed Mosquirix) is currently being rolled out for pilot studies in 2018 (Matuschewski, 2017) (http://www.who.int/ mediacentre/ news/ releases/ 2016/ funding-malaria-vaccine/en/).

Not all mosquito species are equally effective vectors for malaria (the phenomenon known as vector competence). Even within one mosquito population, vectorial capacity will vary and some of the mosquitoes will be completely refractory to infection (Cohuet et al, 2010; Lefevre et al, 2013). These phenomena were identified and mosquito-parasite interactions have been the subject of study for over 50 years. A better understanding of the mosquito-based mechanisms for killing the parasite would be extremely useful in designing GMO (Genetically Modified Organism) mosquitoes or transmission blocking vaccines (TBV).

In the past 85 years, six mosquito species have been selected for their refractoriness to five *Plasmodium* species (Collins et al, 1986; Feldmann, 1989; Frizzi et al, 1975; Hurd et al, 2005; van der Kaay & Boorsma, 1977; Vernick et al, 1995; Ward, 1963). The combination of parasite and vector has usually been artificial, i.e. not occurring in nature, due to the lack of cultivation techniques for *P. falciparum* gametocytes. The only *Anopheles gambiae* line refractory to *P. falciparum* is the G3-5 line, where the mechanism of refractoriness is melanisation (Collins et al, 1986). Melanisation is also the most studied mechanism of refractoriness, and research has mostly involved the use of a rodent malaria system. According to records from nature, melanisation occurs in under 1% of cases (Schwartz, 2002), where refractoriness is between 5-25% (Niare et al, 2002). These differences in representation suggest that melanisation is not a common refractory mechanism.

In this thesis, a line of *An. gambiae* (the main vector in sub-Saharan Africa) which is refractory to *P. falciparum* (the malaria species causing the most severe disease) was studied. This line was selected in the laboratory for a refractory

mechanism other than melanisation. We suggest that this mechanism is more relevant to natural conditions.

1.2 **Malaria parasite lifecycle**

Malaria is a disease caused by the *Plasmodium* parasite from the phylum apicomplexa. The lifecycle will be summarised briefly here and more detail is provided in later sections. *Plasmodium* is transmited from an infected human to another by the bite of an infected female Anopheline mosquito. The bitten person is infected with a dose of sporozoites from the mosquito's saliva injected during blood feeding. Sporozoites then travel to the liver and form liver stages within hepatocytes. The parasite undergoes multiple rounds of mitotic replication within the hepatocyte, such that the cell becomes filled with merozoites and these are then released into the blood-stream. In the blood-stream, the merozoites infect red blood cells (RBC) and form ring stages. The ring stage develops to a trophozoite which continues its development to a schizont, containing merozoites (asexual blood cycle). Some merozoites develop through an alternative pathway to form gametocytes. Gametocytes are then ingested by another mosquito taking a blood-meal. In the mosquito midgut, the gametocytes develop in to gametes, which fuse to form a zygote. The zygote elongates to form a motile ookinete, which travels through the mosquito midgut epithelia and forms an oocyst under its outer layer. In the oocyst, a sporogonic cycle leads to the development of hundreds of motile sporozoites. The sporozoites then travel to the salivary gland, where they wait for the mosquito to take its next blood-meal, in turn infecting another person **(**Figure 1.1**)**.

1.2.1 **Vertebrate host stages**

1.2.1.1 **Sporozoite invasion**

Humans are infected when a female mosquito carrying sporozoites in her salivary glands takes a blood meal. Sporozoites in the mosquito's saliva are injected through the host's skin at the beginning of a blood-meal, when the mosquito is probing the skin to find a blood vessel and injecting saliva. On average ~100 sporozoites are injected through the skin in one blood meal (Medica &

Sinnis, 2005). A proportion of these will penetrate the blood vessel and consequently travel from the site of the bite through the blood-stream. Around 20% of sporozoites end up in a draining lymph node, where they initiate an immune response. Any remaining sporozoites are destroyed or remain in the skin (Ejigiri & Sinnis, 2009).

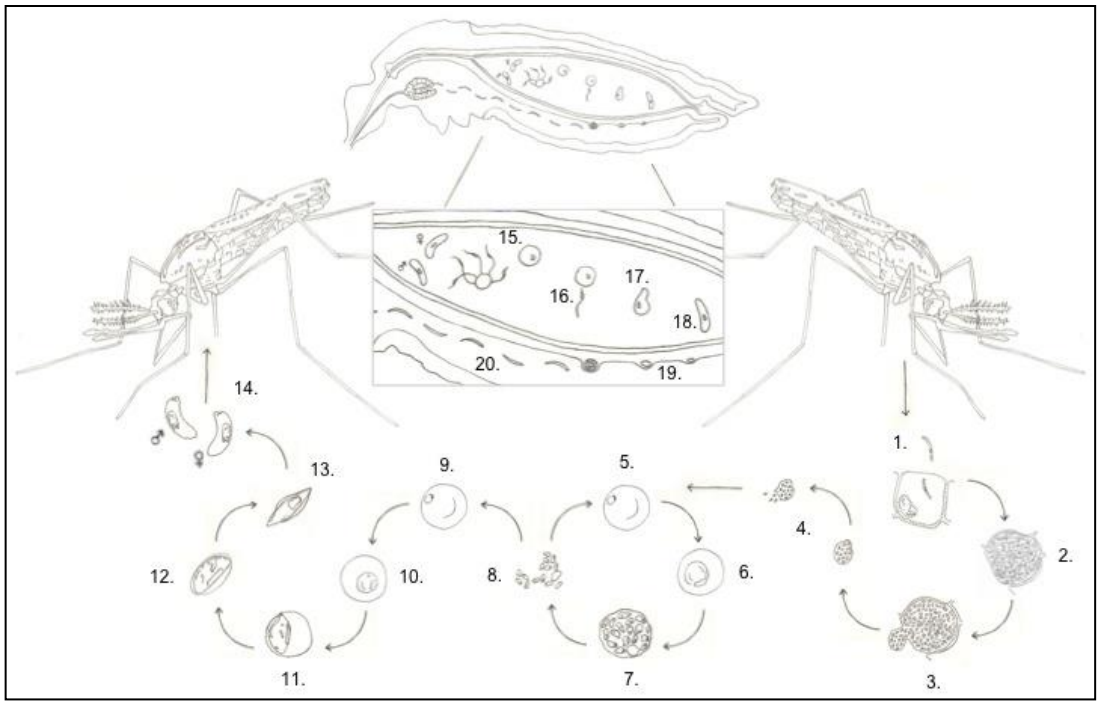


Figure 1.1. Lifecycle of *Plasmodium falciparum*.

1.-14. Human part of the lifecycle. Development in the liver takes ~5-7days. The asexual cycle in red blood cells takes ~48h. Development of gametocytes takes ~10 days. (1.) sporozoite invading a liver cell; (2.) liver schizont; (3.) merosome formation; (4.) merosome rupture and release of merozoites into the bloodstream; (5.) merozoite invades red blood cell and forms ring stage; (6.) trophozoite inside a red blood cell; (7.) red blood cell merozoite formation within a schizont; (8) free merozoites after cell rupture; (9.) ring stage precommitted to gametocyte production; (10.) stage I gametocyte; (11.) stage II gametocyte; (12.) stage III gametocyte; (13.) stage IV gametocyte; (14.) stage V circulating male and female gametocytes.15.-20. Mosquito part of the lifecycle. From gametocyte ingestion to the ookinete passing through the midgut takes ~18h. Oocyst development up to rupture and sporozoite release takes 10 days in optimal conditions. (15.) female rounded macrogamete and male microgametocytes emerging during exflagellation; (16.) fertilization of gametes; (17.) retort form; (18.) ookinete; (19.) oocyst formation; (20.) sporozoites traveling through the hemolymph to penetrate the salivary glands.

Recently, sporozoites in the skin have been suggested to be a new stage in the malaria life cycle (Guilbride et al, 2012; Sinnis, 2008). Sporozoites have been detected in skin up to 2-3h post inoculation. It has been suggested that naive CD8+ T cells are primed in skin lymph nodes by dendritic cells, and that these migrate to the spleen and liver, where they are essential for protection against sporozoites in the liver (Yamauchi et al, 2007). This finding is potentially very

important for anti-malarial vaccine development (Guilbride et al, 2012; Sinnis, 2008).

1.2.1.2 **Liver stages**

Sporozoites travel in the blood stream until they reach the liver capillary beds, which have specific surface molecules with which they interact (Coppi et al, 2007). One of the debated possible signals for liver invasion is highly sulfonated heparan sulfate proteoglycans (HSPGs) on the surface of hepatocytes (Coppi et al, 2007). The sporozoites then traverse the sinusoidal barrier and enter hepatocytes. In the liver, sporozoites appear to migrate through several hepatocytes before finally invading one and beginning to develop, although the mechanism for choosing their final resting host cell is unclear (Tardieux & Menard, 2008). Within the hepatocyte, the sporozoite begins to divide mitotically, producing a liver schizont containing thousands of merozoites. After mitosis and merozoite formation are complete, merosomes containing merozoites are extruded into liver sinusoids and reach the blood-stream. Merozoites released from merosomes then invade red blood cells and begin the intra-erythrocytic lifecycle of the parasite (Ejigiri & Sinnis, 2009). From the inoculation of sporozoites into the skin to release of the first merozoites into the bloodstream takes 6-16 days depending on the *Plasmodium* species (Antinori et al, 2012). Up to this point, the infection is clinically silent.

1.2.1.3 **Blood asexual stages**

In the red blood cell, the newly invaded merozoite within its parasitophorous vacuole takes on a distinctive “ring” appearance. After approximately 18h (for *P. falciparum*), the parasite begins to digest haemoglobin and produce haemozoin or malaria pigment (Elliott et al, 2008), and from this stage it is known as a trophozoite. As the parasite nucleus begins to divide, the parasite becomes a schizont. Each schizont contains 16-30 new merozoites, depending on the parasite species (Antinori et al, 2012). The infected red cell lyses to release merozoites into the blood where they rapidly invade new erythrocytes.

One cycle in the red blood cell takes between 1 and 3 days depending on the *Plasmodium* species: three of the five species causing human malaria are tertian (~48 hour cycle), *P. malariae* is quartan (72 hour cycle) and *P. knowlesi* has a 24

hour cycle. The synchronous rupture of large numbers of red blood cells, releasing merozoites with whole cell content into the blood stream, is responsible for the periodic fevers characteristic of malaria (Antinori et al, 2012).

The tendency of infected red blood cells to adhere to other cells and the walls of capillaries can result in the formation of clots or occlusions, and this is partly responsible for the symptoms linked with severe malaria, such as cerebral malaria (Hora et al, 2016).

1.2.1.4 **Blood sexual stages**

To be able to complete its lifecycle in the mosquito, *Plasmodium* needs to be ingested within a mosquito blood meal in the form of stage five (mature) gametocytes. Signals contributing to triggering merozoites in the blood stream to develop into sexual gametocytes include high levels of asexual parasitaemia, antiparasitic immune responses, chemotherapeutic agents and other stresses (Carter & Miller, 1979; Dyer & Day, 2000). Each schizont committed to sexual development produces only gametocytes, not a mixture of gametocytes and asexuals (Bruce et al, 1990), and “sexual schizonts” produce only one type of progeny: microgametocytes (male) or macrogametocytes (female) (Smith et al, 2000). *P. falciparum* gametocytogenesis takes ~8 days, which is long compared to other *Plasmodium* species, where gametocytogenesis is usually proportional to the asexual cycle at ~1.5 x the intraerythrocytic asexual cycle (Sinden, 2009).

Gametocytogenesis consists of five morphologically different sub-stages (Carter & Miller, 1979). Only the fifth developmental stage circulates in peripheral blood, waiting to be ingested by a mosquito within a blood meal. Ultimately, gametes derived from these gametocytes in mosquitoes are capable of both self-fertilization and cross-fertilization, making *Plasmodium* a simultaneous hermaphrodite (Baton & Ranford-Cartwright, 2005b).

1.2.2 **Mosquito host stage**

1.2.2.1 **From gametes to zygote**

Gametocytes are ingested during a mosquito blood-meal and taken into the mosquito midgut, where they transform into gametes. This process is triggered by a drop in temperature, and by xanthurenic acid from cells in the mosquito midgut, reviewed in (Kuehn & Pradel, 2010). First the gametocytes swell, increasing their volume, and this leads to erythrocyte rupture. While a single macrogametocyte produces one macrogamete, microgametocytes first have to go through cell division and a process known as exflagellation, which results in up to eight haploid flagellated microgametes. A male and a female gamete then fertilize to produce a diploid zygote stage within the blood meal. Finally, the sessile zygote transforms through a retort-form into a motile banana-shaped ookinete (Baton & Ranford-Cartwright, 2005b).

1.2.2.2 **Ookinete formation and migration**

The ookinetes migrate from the midgut lumen, across the midgut epithelial cells, and eventually develop into sessile oocyst stages on the basal (outer) surface of the midgut epithelium, reviewed in (Baton & Ranford-Cartwright, 2005b).

Ookinetes have to avoid digestion by mosquito proteases required for digestion of the blood meal. They then cross the peritrophic matrix, followed by the microvilli-associated network and the single layer of midgut epithelial cells (Baton & Ranford-Cartwright, 2005a). More detail on the mosquito responses to ookinete invasion is provided in chapter 4.

1.2.2.3 **Oocyst formation**

Oocysts are the stages on the basal (outer) surface of the midgut epithelium in which sporogony (the massive asexual amplification of parasite numbers) occurs, reviewed in (Baton & Ranford-Cartwright, 2005a). Each oocyst can produce thousands of sporozoites. During multiple endomitoses, the nucleus becomes extremely convoluted and lobulized, finally attenuating into numerous small nuclei,

and this is followed by sporozoite formation. Sporozoite building occurs synchronously in the whole oocyst.

1.2.2.4 **Sporozoite migration**

Sporozoites emerge into the haemolymph through small ruptures in the oocyst wall. Sporozoites migrate in the haemolymph to the mosquito head, directed by chemotactic signals triggering active migration, reviewed in (Akaki & Dvorak, 2005; Baton & Ranford-Cartwright, 2005a; Baton & Ranford-Cartwright, 2005b). Most of the sporozoites seem to accumulate in the thoracic salivary gland region, where they pass into the salivary gland lumen by direct invasion and passage through the salivary gland epithelial cells, apparently without the formation of a parasitophorous vacuole. They accumulate in the distal median and lateral lobes of the glands. When an infected mosquito then takes a blood meal, the sporozoites are injected from secretory ducts through the skin of the human host, reviewed in (Baton & Ranford-Cartwright, 2005b).

1.2.2.5 **Dynamics of Plasmodium development in the mosquito**

In the mosquito, the extrinsic incubation period from *P. falciparum* gametocyte ingestion to sporozoites reaching the salivary glands is approximately 14 days at the optimal temperature of 26-30°C. Sporogony is not possible below 16°C and is considerably slowed down above 35°C (Noden et al. 1995).

On average, 5% of the 10 to 10,000 macrogametocytes ingested within one blood-meal successfully develop to the oocyst stage (Alavi et al, 2003). There are significant decreases in parasite numbers in the midgut lumen, with estimates of conversion of only 0.2% of ingested *P. berghei* gametocytes into ookinetes (Alavi et al, 2003), and a 40-fold decrease in the same parasite stage transition reported with *P. falciparum* in *An. gambiae* (Vaughan et al, 1992). Major losses are also incurred in the transition from the midgut lumen ookinete to the oocyst stage - a 69-fold decreases was reported in *P. falciparum* / *An. gambiae* (Vaughan et al, 1992). Within the oocyst there is considerable expansion of parasite numbers. Each oocyst can contain up to 10,000 sporozoites (Beier, 1998), although only a proportion of them successfully reach the salivary glands (approximately 100-100,000) (Beier, 1998). With each mosquito bite, fewer than 100 sporozoites are

injected into the skin (Beier, 1998). Some sporozoites will be killed by an immune system mechanism, such as an innate immunity response (phagocytic granulocytes, macrophages, NK cells or γδT cells) or, in the case of a non-primary infection, by an adaptive immune system response (malaria specific antibody, primed effector T cells). Nevertheless, a single sporozoite reaching the liver is sufficient to establish an infection, reviewed in (Baton & Ranford-Cartwright, 2005b).

1.3 *Anopheles* mosquitoes: Geographical Distribution; Life-cycle

The female mosquito of the genus *Anopheles* is responsible for transmitting human malaria. There are over 450 different species of *Anopheles* mosquito, but only approximately 60 can act as vectors and transmit malaria (Cohuet et al, 2010). Malaria vectors can be found in endemic countries, but also in areas where malaria has been eliminated. Consequently, these areas are at risk of malaria reintroduction (www.cdc.gov). Vector capacity (defined as the efficiency of vector-borne disease transmission) consists of susceptibility to *Plasmodium*, vector host choice, and vector longevity (Cohuet et al, 2010). Dominant malaria vectors with the largest distribution are *An. quadrimaculatus* in North America, *An. darlingi* and *An. marajoara* in South America, *An. gambiae, An. arabiensis* and *An. funestus* in Africa, *An. stephensi* in India and *An. dirus* and *An. punctulatus* in South-East Asia (Kiszewski et al, 2004; Sinka et al, 2010a; Sinka et al, 2011; Sinka et al, 2010b). Due to its large distribution and extremely high vectorial capacity, *An. gambiae* is the principal malarial vector in sub-Saharan Africa.

Anopheles gambiae sensu lato consists of eight morphologically indistinguishable species with variable vectorial capacity. The species are: *An. gambiae s.s., An. coluzzii, An. arabiensis, An. quadriannulatus A, An. quadriannulatus B, An. melas, An. merus*, and *An. bwambae*. *Anopheles gambiae sensu stricto* has the biggest vectorial capacity and was previously divided into two molecular forms M (Mopti) and S (Savannah) (della Torre et al, 2001). Recently the M form has been classified as a separate species, *An. coluzzii*, with the S form retaining the species name *An. gambiae s.s.* (Coetzee et al, 2013). The molecular forms are differentiated by differences in a 4 Mb region on chromosome X (della

Torre et al, 2001; Favia et al, 2001). The distribution of these two forms is uneven across the Africa region (Lehmann & Diabate, 2008).

Malaria is only transmitted by the female mosquito, which needs blood as a source of nutrients for egg production. Like all mosquito species, *Anopheles* has a four stage life-cycle: egg, larva, pupa and adult (Figure 1.2)**.** The duration of each of the stages depends on temperature and humidity. The egg to pupae phase is aquatic, and in ideal conditions takes about 2 weeks. The adult mosquito can live for up to 2 months in captivity, but in nature the lifespan of most will only be 2-4 weeks (Clements, 1992).

On average, a female mosquito lays 50-200 eggs, 3 days after taking a blood-meal. Individual eggs are laid on the surface of the water, where they float due to floats on their sides. Anopheline eggs are not resistant to desiccation. The larvae hatch from the eggs within 2-3 days in ideal conditions 22-27°C, but it can take up to three weeks in colder environments (Clements, 1992; Impoinvil et al, 2007). The larvae feed on microorganisms, such as algae and bacteria on the surface micro layer of the water, using their mouth brushes. They breathe through spiracles on eight segments of their body, which need to be positioned horizontally on the water surface. Larval development consists of four instars, followed by metamorphosis into a pupa. Between each instar the exoskeleton is shed to allow further growth (Clements, 1992). The pupa is round with its head and thorax merged to form a cephalothorax. It has a strong exoskeleton and breathes through trumpets on its dorsal side. Within a day of formation, the pupa splits on the dorsal side of the cephalothorax and the adult mosquito emerges from the water.

Usually the adult mosquito matures within days of emerging. The males feed solely on sugary nectars. The females will also feed on nectar, but additionally require blood for egg development. After a blood-meal, the female will rest for 2-3 days, until her eggs are fully developed and can be laid on the surface of the water. After laying the eggs, the female mosquito seeks another blood-meal. This cycle will repeat until the death of the female mosquito (Clements, 1992).

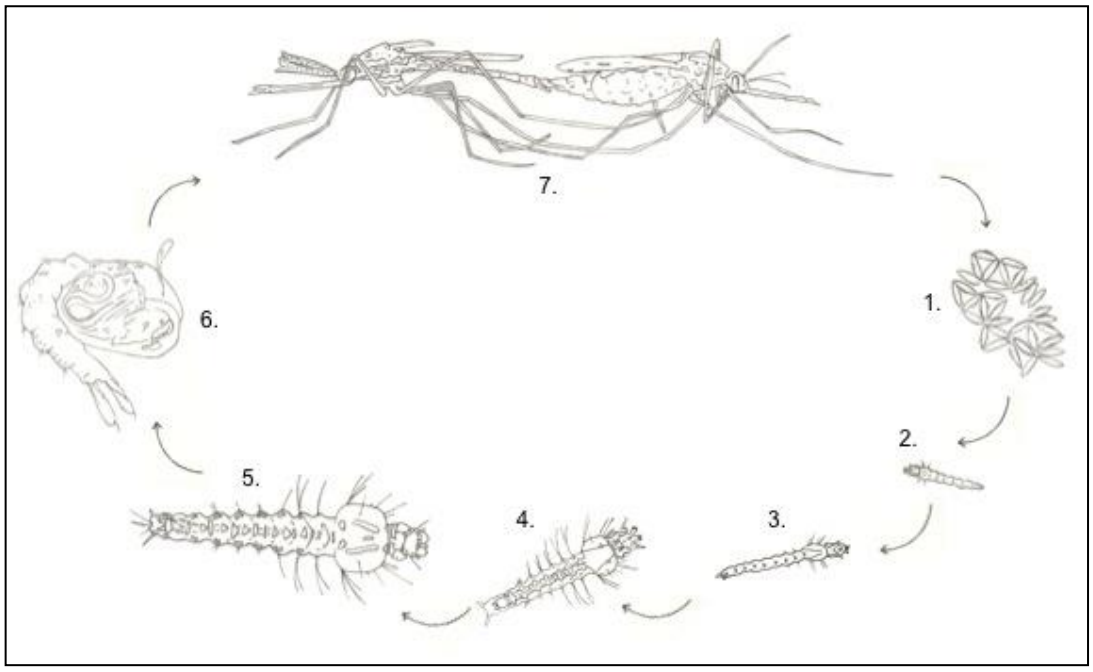


Figure 1.2. *Anopheles gambiae* lifecycle (1.-6.) Mosquito development from egg to pupa is aquatic, in fresh water. In ideal conditions this takes 14 days. (1.) Eggs are laid on the surface of the water. Each egg has a pair of floats on its sides. In ideal conditions, hatching takes 2-3 days. (2.) First instar larva. (3.) Second instar larva. (4.) Third instar larva. (5.) Fourth instar larva. (6.) Pupa. (7.) Mating of adult mosquitoes. The female is ready to mate immediately after hatching, and the male 2-3 days post hatching. Egg development in the mosquito takes 2-3 days following a blood feed. The female mosquito is capable of laying 50-200 eggs every 3 days for the duration of its lifespan (2-8 weeks), if she has access to blood.

1.4 Mosquito factors affecting *Plasmodium* development and infection success

1.4.1 Mosquito species- Vector competence

The intrinsic susceptibility of mosquitoes to malaria parasite infection is termed vector competence. Different mosquito species vary in their susceptibility to infection (Collins et al, 1986; Vaughan et al, 1994; Warren, 1981), and there is also natural variation between individuals within a species. Experiments with 10 species of Anopheline mosquito combined with two *Plasmodium* species, *P. falciparum* and *P. vivax,* demonstrated that there is high variability in the potential to transmit malaria in different combinations of parasite and vector, with a reasonable correlation between the geographical origin of the parasite and that of the vector (Hume et al, 2007; Warren, 1981). The distribution of different species of malaria in different continents and areas is therefore dependent to some extent on the distribution of suitable vectors.

Mosquitoes which are able to reduce or prevent parasite infection are termed refractory. Some refractory mosquito lines can kill most species of malaria parasite, whereas others are species-specific. For example, the *An. gambiae* L3-5 melanising line, selected for refractoriness to the simian parasite *P. cynomolgi*, is also able to melanise human, avian and rodent parasite species (Collins et al, 1986), whereas a line selected for refractoriness to the rodent parasite *P. yoelii nigeriensis* (Hurd et al, 2005) was completely susceptible to *P. falciparum* (Ranford-Cartwright and Hurd, unpublished).

There can also be differences in the ability of a refractory line to recognize and kill different genotypes of the same parasite species (Lambrechts et al, 2005). In the natural situation, because of the variability of host and parasite genotype, different hosts subsets are resistant to different genotypes of parasites. The result of this is that, in nature, there is probably no mosquito that is resistant or susceptible to all genotypes of *Plasmodium.* This variability has to be taken in account in programmes aiming to control malaria using new drugs, vaccines or vector control (Lambrechts et al, 2005). More detail on refractoriness in mosquitoes is provided in Chapter 2.

1.4.2 **Mosquito physical factors influencing infection success**

The body size of mosquitoes correlates with the size of the blood meal they obtain when feeding on an infected host (Ichimori, 1989), and therefore should directly influence the number of gametocytes taken up by a mosquito. The number of oocysts harboured by field-caught *An. gambiae* mosquitoes in Tanzania has been reported to correlate with the size of the mosquito (Lyimo & Koella, 1992), but there was no significant association in the rodent model *P. yoelli nigeriensis* in *An. stephensi* (Ichimori, 1989). This apparent contradiction may reflect differences in immune capacity in larger compared to smaller mosquitoes, with larger mosquitoes better able to mount an immune response.

Mosquito nutritional status may also affect infection, both as a direct effect on parasite growth if there is poor nutrient availability, and an indirect effect on the ability of the mosquito to mount an effective immune response. For example, mosquitoes that had taken a blood meal were more likely to melanise Sephadex beads injected into the haemolymph than unfed mosquitoes (Schwartz & Koella,

2002). However parasite infection rates were boosted in *An. gambiae* mosquitoes which had taken two rather than one uninfected blood meal before a *P. falciparum* infectious meal (Okech et al, 2004), suggesting that increased mosquito nutritional reserves has a positive effect on vector capacity. Vector competence of *An. gambiae* to natural *P. falciparum* parasites does not vary with mosquito age (Okech et al, 2004).

1.4.3 **Mosquito immunity**

Mosquitoes are not passive carriers of malaria parasites, and have a variety of anti-pathogen immune responses which can act to kill developing parasites (Blandin & Levashina, 2004). A major source of immune effector molecules mediating anti-*Plasmodium* responses is believed to be the haemocytes, and the haemolymph is a major site of immune attack (Hillyer & Strand, 2014). Mosquito immunity will be discussed in more detail in Chapter 4.

The mosquito genes that are involved in anti-parasitic responses have been identified from studies using genome-wide transcriptional profiling to compare refractory and susceptible lines of mosquitoes, and by reverse-genetic screening, including gene silencing by RNA interference (RNAi) (Blandin, 2002), reviewed in (Blandin et al, 2008; Yassine & Osta, 2010). From 14,000 genes identified in *An. gambiae*, 282 have been shown to be members of gene families or functional classes related to innate immunity (Christophides et al, 2004). Most of the molecular research has been done in the *P. berghei* / *An. gambiae* combination, which does not represent the same interaction as *P. falciparum* / *An. gambiae*. Genes associated with oxidative stress, apoptosis and cytoskeletal reorganization are differently up/ down-regulated in these two species combinations (Christophides et al, 2004).

1.4.4 **Mechanisms of refractoriness**

Mechanisms of refractoriness have been extensively studied and are described in more detail in Chapter 4. There are three mechanisms of refractoriness towards *Plasmodium* described in *Anopheline* mosquitoes. The three mechanisms are melanotic encapsulation of the ookinete or oocyst, intracellular lysis of the ookinete, and lysis of the ookinete in the midgut lumen.

Melanisation in the L3-5 line occurs 16-24 hours after mosquito midgut invasion (Collins et al, 1986). The ookinetes are recognised by the mosquito and killed prior to melanotic encapsulation (Blandin et al, 2004), but the exact mechanism of recognition and killing is unknown. Genetic mapping studies identified mosquito quantitative trait loci (QTL) controlling the encapsulation response against *P. cynomolgi* in the L3-5 line (Collins, 1997; Zheng, 2003) and also in field-caught *An. gambiae* (Riehle et al, 2006). These *Plasmodium* encapsulation loci were named *Pen1, Pen2 and Pen3*. *Pen1* independently accounts for 60% of the melanisation events (Collins, 1997). However, field-caught mosquitoes rarely melanise *Plasmodium* ookinetes (Niare et al, 2002), suggesting that melanisation is not a common defence against *Plasmodium*.

The lytic refractory mechanism described in the avian parasite *P. gallinaceum* kills ookinetes lying free in the midgut epithelial cell cytosol (Vernick et al, 1995), in the absence of melanotic encapsulation. Genetic crossing of refractory and susceptible lines suggested that this refractory trait was inherited as a single dominant locus (Vernick et al, 1995).

The so called “disappearance” of gametocytes from the midgut lumen was observed in *Anopheles gambiae* when selected for refractoriness to *Plasmodium yoelii nigeriensis* (Hurd et al, 2005).

1.5 *Plasmodium* factors affecting mosquito infection success

1.5.1 Parasite genotype

There is known variation in the ability of different parasite genotypes to infect a given mosquito species, both in laboratory infections (Ranford-Cartwright & Mwangi, 2012) and in natural infections in the field (Morlais et al, 2015). Some of this variability can of course be attributed to the relative ability of different parasite genotypes to produce gametocytes (see below, section 1.5.2).

1.5.2 Gametocyte number

One of the factors determining the intensity of mosquito infection is the number of gametocytes in an infectious blood feed, especially in experimental

infections (Ponnudurai, 1989). The maximum intensity of infection measured by oocyst number is that obtained from gametocyte culture of 0.5% gametocytaemia or above for parasite clone 3D7 (Ranford-Cartwright, unpublished). Ingestion of a higher number of gametocytes than 0.5% by a mosquito does not lead to an appreciable increase oocyst numbers, possibly because of limiting factors such as the limited time frame in which the ookinetes have to leave the midgut before being digested, and the competition for nutrients needed to develop to an oocyst (Lambrechts et al, 2006; Warburg & Miller, 1992). Gametocyte numbers in natural *P. falciparum* infections are usually much lower than those used in experimental infections, but still result in mosquito infection (Schneider et al, 2007). Gametocyte densities adjusted by dilution correlated with oocyst loads in *P. falciparum* / *An. coluzzii* (Da et al, 2015).

The second factor connected with gametocyte numbers is the ratio between male and female gametocytes and gametes. One female gametocyte produces one female gamete, whereas one male gametocyte can produce up to 8 male gametes, which can fertilise up to 8 female gametes to form zygotes. Due to this, a ratio in favour of female gametocyte numbers should in theory lead to higher numbers of zygotes and higher infection intensity. Gametocyte sex ratios in *P. falciparum* are usually strongly female biased, but cannot be experimentally manipulated within a single genotype (Paul et al, 2002; Smith et al, 2000). Gametocyte sex ratios in *P. falciparum* experimental infections using different clones of *P. falciparum* showed a link between higher male sex ratio and infectiousness to mosquitoes (Burkot et al, 1984; Mitri et al, 2009) but this finding was not replicated in other similar studies (Noden et al, 1994). Natural infections in the field also present with variable sex ratios, and the intensity of infection has been reported to increase as the proportion of male gametocytes increased to 50% (Robert et al, 1996). However, apportioning infection success to sex ratio is complicated by the potential variability in infectivity of different genotypes of *P. falciparum* present in these experiments (section1.5.1).

1.6 **Hypothesis and aims of the thesis**

The main aim of this project was to establish a line of *An. gambiae s.s.* refractory to *P. falciparum,* by a mechanism other than melanisation. To date, no

such a line, which could represent natural mechanisms of mosquito refractoriness in this vector-parasite combination, has been generated. As *An. gambiae* is the principal malaria vector in sub-Saharan Africa, and *P. falciparum* is responsible for a vast majority of malaria caused death, such a refractory line would be extremely useful tool for investigating mosquito parasite interaction and developing of transmission blocking approaches.

The fitness associated with refractoriness was proposed to be measured in the selected colony, as it is not possible to measure in the field with mixed populations of mosquitoes. These data are important in understanding whether refractoriness is advantageous or disadvantageous for the mosquito and if refractoriness has a potential to replace susceptibility in nature.

It was not known if the selected line would be refractory only to parasite clone 3D7 used for selection, or if the mechanism would be more general. If refractoriness was parasite clone-specific, a quantitative trait loci (QTL) analysis based on phenotyping of offspring clones from 3D7 x HB3 experimental cross was proposed. This approach would allow us to identify responsible loci for refractoriness in the parasite genome.

2 Chapter: Selection of a new, non-melanising line of *Anopheles gambiae* refractory to *Plasmodium falciparum*

2.1 Aims and objectives

This chapter describes the process of selection of a new non-melanising line of *Anopheles gambiae* refractory to *Plasmodium falciparum* clone 3D7. The aim was to select a non-melanising line of the main mosquito vector of the human malaria parasite, which could subsequently be used in experiments analyzing the underlying mechanism of refractoriness seen in mosquitoes in the field.

Preliminary work had been done to begin the selection of a refractory strain, but the insectaries had suffered from a microsporidia infection. First, selection was needed to re-establish a clean mosquito colony that was free from infection with microsporidia.

The selection for refractoriness of the microsporidia-free colony was then continued. Refractoriness was selected to clone 3D7 of *P. falciparum*. The presence and/or number of oocysts in the midgut 10 days after an infectious blood-meal were used as a marker for infection. Alongside the refractory line, a control line, to control for inbreeding, was developed that had been through the same selection steps but with random choice of adult females to form each generation.

The selected lines GU-REF11 and GU-CON11 were tested for refractoriness against HB3 line of *P. falciparum* as an example of line not used in selection.

The hypothesis tested was that refractoriness of *An. gambiae* to *P. falciparum* is genetically based phenomena, and therefore it is possible to select for it.

2.2 Introduction

2.2.1 Refractory strains of *Anopheles gambiae*

Since 1929 when Huff developed a *Culex pipiens* line, which was refractory to *Plasmodium cathemerium* (Huff, 1929), there have been another 10 successful attempts to produce partially or fully refractory mosquito lines in different combinations of vector and *Plasmodium* species (Table 2.1).

In refractory mosquitoes in the field, melanotic encapsulation is very rare: less than 0.46% of *P. falciparum* oocysts in infected *An. gambiae* mosquitoes from southern Tanzania are melanised (Schwartz, 2002). However the evidence for refractoriness in field mosquitoes is quite strong, manifesting as a low success rate of *An. gambiae* infection by gametocyte carriers of *P. falciparum*. In a comparative study of 930 transmission experiments from Cameroon, The Gambia, Mali and Senegal, the proportion of mosquitoes infected after feeding on gametocyte carriers varied from 4.9-27.3%, leaving 72.7-95.1% of mosquitoes uninfected (Bousema et al, 2012). This suggests a much higher prevalence of refractoriness than that expected from melanisation alone.

Mosquito	Plasmodium	Mechanism (if known)	Reference
Culex pipiens	*P. cathemerium*	-	(Huff, 1929)
Aedes aegypti	*P. gallinaceum*	-	(Ward, 1963)
An. stephensi	*P. gallinaceum*	-	(Frizzi et al, 1975)
An. atroparvus	*P. berghei*	-	(van der Kaay & Boorsma,1977)
An. gambiae	*P. berghei*	-	(Almashhadani et al, 1980)
An. gambiae	*P. cynomolgi*	melanisation of ookinetes	(Collins et al, 1986)
An. stephensi	*P. falciparum*	blocks ookinete invasion	(Feldmann, 1989)
Ae. aegypti	*P. gallinaceum*	not melanisation (not known)	(Thathy et al, 1994).
An. gambiae	*P. gallinaceum*	intracellular lysis	(Vernick et al, 1995)
An. dirus	*P. yoelii nigeriensis*	melanisation of ookinetes and oocysts	(Somboon et al, 1999)
An. gambiae	*P. yoelii nigeriensis*	melanisation of ookinetes, other (unknown) mechanism	(Hurd et al, 2005)

Table 2.1. Mosquito lines refractory to *Plasmodium* infection published in the literature.

Although just 3 out of the 11 selected lines (Table 2.1) have melanisation as the mechanism of refractoriness, these lines, and melanisation, have become the most studied mechanism of parasite killing in the mosquito. Based on the above

field and laboratory data, we suggest that the common mechanism of *Plasmodium* killing by the mosquito is not melanotic encapsulation. Based on this hypothesis, we decided to select a non-melanising *An. gambiae* line refractory to *P. falciparum*.

2.2.2 **Selection of GU-REF/GU-CON lines**

In our laboratory, we had previously selected a new line of *An. gambiae* KEELE, which was refractory to infection through a mechanism of parasite killing that was not melanisation (Ranford-Cartwright, unpublished). Unlike other existing refractory *An. gambiae* laboratory lines, these mosquitoes were selected using *P. falciparum* (clone 3D7). The selection involved feeding the mosquitoes with infectious gametocytes and then selecting the offspring of those mosquitoes with zero or the lowest number of parasites to form the next generation. The offspring of 10-11 females was used to form the next generation in the first 3 generations and this was reduced to the offspring of ~4 females for 4-7 generations. Mosquitoes were selected over 7 further generations and the resultant line, denoted GU-REF(old), was significantly more refractory to infection than the original *An. gambiae* KEELE stock. GU-REF(old) was also significantly more refractory to infection than an inbreeding control line GU-CON(old), which was selected at the same time using randomly selected offspring from the same number of females as those used for the refractory population. Infection prevalence in the KEELE stock and GU-CON(old) was around 80-90%, with up to 500 oocysts per midgut, whereas GU-REF(old) line exhibited very low infection prevalence (~10%) and oocyst intensity (1-4 oocysts/midgut). The mechanism of refractoriness in GU-REF(old) is unknown, although it will involve targeting gametocytes, gametes, ookinetes or very early oocysts, because visible oocysts did not develop in this line. Importantly, no melanisation was observed. Preliminary studies suggested that the GU-REF line did not exhibit the same refractory behaviour to a different *P. falciparum* clone, HB3 (unpublished). Due to microsporidia infection in the mosquito colony after the 7th generation, GU-REF(old) and GU-CON(old) lines were then subjected to selection for absence of microsporidia. This chapter describes the selection of microsporidia-free colonies of GU-REF(old) and GU-CON(old) and their subsequent re-selection for refractoriness.

2.2.3 **Microsporidia infection in mosquitoes**

Microsporidia have been known as pathogens in the mosquito for over 100 years, and, as such, are considered as potential biological control agents (Becnel et al, 2005). Microsporidia are eukaryotic, unicellular organisms belonging to the phylum Microspora, which are now classified as derived fungi (Capella-Gutierrez et al, 2012; Williams et al, 2002). Microsporidia are intracellular in their hosts, which include almost all animal species, including humans where they are opportunistic pathogens in immunocompromised hosts. Microsporidia survive in the extracellular environment as environmentally resistant, long-lived spores. The spore is surrounded by a protein rich exospore, a protein and chitin rich endospore, and a plasma membrane. This structure makes it possible for the spore to survive extreme conditions such as temperatures ranging from -70^0C to 56^0C or pH range 4-9 (Becnel et al, 2005). Spores are also light enough to be carried in the air. In order to be inactivated, the spores have to be autoclaved for 10 min, or exposed to 2% Lysol solution, 10% formalin or 70% ethanol (Becnel et al, 2005).

Microsporidia also affect malaria parasite development and mosquito development. As early as 1959, *Nosema* was identified in a breeding colony of *An. gambiae* in Liberia as blocking experimental infection with malaria parasites (Fox & Weiser, 1959). The infection was found to be transmitted in the colony via cotton feeding wads with sugar water or honey where infected mosquitoes defecated during feeding. One of the few reported, controlled experimental infections was done with *Nosema algerae* (Microsporidia: Nosematidae) in *An. stephensi* and this led to an 85% reduction in *P. yoelii nigeriensis* oocyst numbers, larval and pupal mortality and a reduction in egg batches (Schenker et al, 1992).

Microsporidia are known to be problem pathogens in insectaria. When microsporidia infect a mosquito colony in captivity, it affects the colony fitness, but also makes subsequent Plasmodium infections almost impossible. To recover a mosquito colony in captivity, which has been infected by microsporidia, the whole insectary has to undergo an intensive decontamination process.

2.2.3.1 **Detection and diagnosis methods for microsporidia**

Diagnosis of microsporidial infection in adult and larval stages of mosquitoes is usually by microscopical examination of wet mount preparations from infected tissues. The spores have a characteristic refractile appearance and a posterior vacuole, and can also be stained with Giemsa's stain. Infected larvae may also exhibit visible signs of infection: heavy infections in 4th instar larvae can be seen as white or yellow masses when viewed against a black background (Andreadis, 2007). Fluorescent brighteners such as calcifluor stain chitin in the endospore layer of microsporidia and are also used - spores take up the stain and fluoresce under UV illumination (Vavra et al, 1993; Vavra & Chalupsky, 1982). Molecular detection of microsporidian spores requires the mechanical disruption of the spores, e.g. with glass beads, to release the DNA, which is then amplified, for example using primers to the SSU rRNA locus (Franzen & Muller, 1999; Muller et al, 1999).

2.3 **Materials and methods**

2.3.1 ***An. gambiae* KEELE strain**

The KEELE strain of *An. gambiae sensu stricto* was produced at Keele University, UK in the early 2000s, by balanced interbreeding of four strains: KIL, ZANU, G3 and IFAKARA (Hurd et al, 2005). The KIL strain originated from Marangu, Tanzania 1975. The ZANU strain was isolated from Zanzibar in 1984. The G3 strain was isolated from Gambia in 1975 and IFAKARA was isolated from Tanzania in 1996 (Hurd et al, 2005; Ranford-Cartwright et al, 2016). Balanced interbreeding was performed, by reciprocal crosses of 50 males and females, chosen at random, and sexed as pupae. Initial crosses were done between KIL/IFAKARA and ZANU/G3. Offspring of these crosses were then crossed to produce the KEELE strain (Hurd et al, 2005). A breeding colony of *An. gambiae* KEELE was established in Glasgow University in 2002, with eggs obtained from Keele University.

2.3.2 **Maintenance of the *Anopheles gambiae* colony**

The colony was kept in net cages in an insectarium at 26°C, 80% RH, 12h daylight period. Between blood feeds the mosquitoes had access to glucose feeders containing 5% glucose solution in 0.05% PABA (4-amino benzoic acid). The larval stages were kept in water with sodium bicarbonate (2g w/V) and fed on fish food pellets.

2.3.3 **Culturing of *P. falciparum***

2.3.3.1 **Asexual culture of P. falciparum**

Asexual parasites were cultured in RPMI medium supplemented with 25mM HEPES, 50mg/L hypoxanthine, 0.74µg/mL sodium hydrogen carbonate and 10% (v/v) human serum (heat inactivated), known as complete RPMI. The cultures were maintained at 5% RBC haematocrit, at 37°C, under a gas mixture of 1% O_2, 3% CO_2, balanced N_2. Human blood was obtained from the Glasgow and West of Scotland Blood Transfusion Service as whole blood in citrate-phosphate-dextrose-adenine (CPD-A) anticoagulant, and was washed prior to use to remove white blood cells and anticoagulant. The culture was kept in small (25 cm^3) tissue culture flasks at a total volume of 5 mL, and was maintained at a parasitaemia below 10%. The medium was changed every day and the parasitaemia was monitored using thin smears, staining with Giemsa's solution and examining by microscopy. The parasitaemia was adjusted to maintain the level between 1% and 8% by dilution with fresh uninfected blood 2-3 times a week (Haynes, 1976; Trager & Jensen, 1976).

2.3.3.2 **Gametocyte culture of P. falciparum**

Gametocyte cultures were established from asexual parasite cultures according to standard methodology (Carter et al 1993). Each gametocyte culture was established at 0.5-0.7% parasitaemia, 6% haematocrit in complete RPMI. The culture was set up in a large (75 cm^3) tissue culture flask laid flat and containing 15 mL culture material. The medium was changed every day and once the parasitaemia reached high levels, and the parasites started to look stressed (such as having triangular rings stages and blurred-looking trophozoites and schizonts),

the medium volume was increased to 25 mL with no addition of fresh erythrocytes (Carter et al, 1993). Gametocytes were mature after around 15 days from the start of culturing. For feeding the mosquitoes, 14 and 17 day old cultures were mixed together to increase infection success.

2.3.3.3 **Membrane feeding**

2.3.3.3.1 Mosquito collection

Previously unfed mosquitoes were given their first blood feed as 5 to 7 day old adults. They were kept in waxed paper carton cups covered with netting with a secured side entrance covered in two layers of latex sheet. Mosquitoes were given access to glucose-soaked cotton wool balls lying on top of the netting, which were substituted for water-soaked cotton wool one day before the membrane feed to encourage blood feeding.

2.3.3.3.2 Preparation of infected blood-meal

Glass membrane feeders were covered with stretched baudruche membrane (Goldbeater's skin) and connected to a circulating water bath at 37.1°C. The infectious blood feed was prepared from mixed day 14 and day 17 gametocyte cultures. Each culture was sedimented by centrifugation at 1500 x g (at 37°C). Pelleted cells containing parasites were resuspended in an equal volume of human serum and diluted with fresh blood in serum at 40% haematocrit. The final concentration of gametocytes was adjusted to ~1%. The dilution was based on day 12/15 gametocytaemia calculated from Giemsa-stained thin blood films. During the whole process of the infectious blood feed, the temperature was not allowed to drop below 37°C, as this would have caused premature exflagellation of male gametes and a failure to infect mosquitoes. Approximately 1-1.5 mL of the infectious feed was placed into each membrane feeder, and the mosquitoes were allowed to feed for 20-30 minutes, while a sample of the feed mixture was examined microscopically for exflagellation (phase or Nomarski, 400x magnification), within 10 min from the start of the feed. Mosquitoes which did not feed were removed by aspiration 2-3 hours after the feed and killed (Carter et al, 1993).

2.3.3.4 **Mosquito dissection and identification of oocysts**

Mosquitoes which fed on the infectious blood were dissected 10 days later to look for the presence of oocysts in the midgut. Mosquitoes were killed by chloroform vapour, dipped in 70% ethanol, transferred into 1x PBS and kept on ice. The dissection was done within 30 min of mosquito death. The midgut was dissected from the mosquito and covered by a cover slip under 1 x PBS. Excessive PBS was removed by capillary soaking into dry tissue paper. This process flattened the midgut, allowing for the examination of upper and lower sides. The oocysts were counted under light microscopy at 400x magnification.

2.3.4 **Establishing a Microsporidia-free colony for selection**

2.3.4.1 **Detection of microsporidia**

The published calcofluor staining method was used to detect microsporidial spores (Vavra et al, 1993; Vavra & Chalupsky, 1982). The spores are commonly present in the fat body and intestinal tract, and so midguts were removed from adult mosquitoes, or whole larvae were squashed on to slides, fixed with methanol, and stained with a 0.5% solution of calcofluor white M2R (Sigma Chemical Co., St. Louis, Mo.) for 2-3 minutes, rinsed with water and then counterstained with 0.1% Evan's blue (Sigma) for 1 min at room temperature. After rinsing and drying, slides were viewed under a UV microscope at a wavelength of 395 to 415 nm (observation light of 455 nm) under 1000x magnification. Microsporidia spores appeared as bluish-white ovals.

2.3.4.2 **Decontamination of microsporidia spores in insectaries**

Microsporidial spores are generally resistant to many standard forms of surface decontamination. It was not possible to decontaminate the insectaries using formaldehyde because of the lack of venting in the suites. Therefore after removal and disposal by autoclaving of all mosquitoes and rearing equipment, rooms were exposed to UV decontamination lights for at least 7 days (Marshall et al, 2003). All consumables were replaced with disposable plastics and rigorous quarantine procedures were instigated to prevent recontamination of "clean" rooms.

2.3.4.3 Selection of microsporidia-free mosquitoes to re-establish the GU-REF and GU-CON lines

The GU-REF(old) and GU-CON(old) lines of mosquitoes were diagnosed in 2010 with microsporidia infection, which prevented the malaria parasite from establishing a successful infection. The infection rate in the two lines was established using the calcofluor method above (2.2.3.1). The lines were then re-established from the minority uninfected individuals that were still present in the colonies.

200 GU-REF and 100 GU-CON adult females were separated after a membrane blood feed into individual tubes containing a source of glucose. The mosquitoes were allowed to deposit haematin and were then transferred into a new tube containing water in order to lay eggs 2 days post blood feed. Egg laying mosquitoes were dissected and examined for the presence of microsporidia in midgut tissue and malpighian tubules. Eggs from microsporidia infected mosquitoes were discarded. Eggs from non-infected mothers were reared to establish a second generation. Females arising from each egg lay were kept together as family groups, whereas males were mixed and distributed at random between female families. The second generation of females was given a blood feed and separated into individual tubes. The same process as with the first generation was repeated. This process was continued until all females were negative for microsporidial infection.

2.3.5 Selection for refractoriness to *P. falciparum*

Following reestablishment of microsporidia-free colonies of the previously established GU-CON and GU-REF lines, selection for refractoriness to infection with *P. falciparum* (clone 3D7 was continued for four further generations (generations 8, 9, 10 and 11). The selection involved infecting mosquitoes with infectious parasites and taking the offspring of four to ten mosquitoes with zero parasites at the oocyst stage to form the next generation (Figure 2.1). The control line GU-CON was selected at the same time, as a control for inbreeding, using the same number of females (4-10) for the next generation chosen at random.

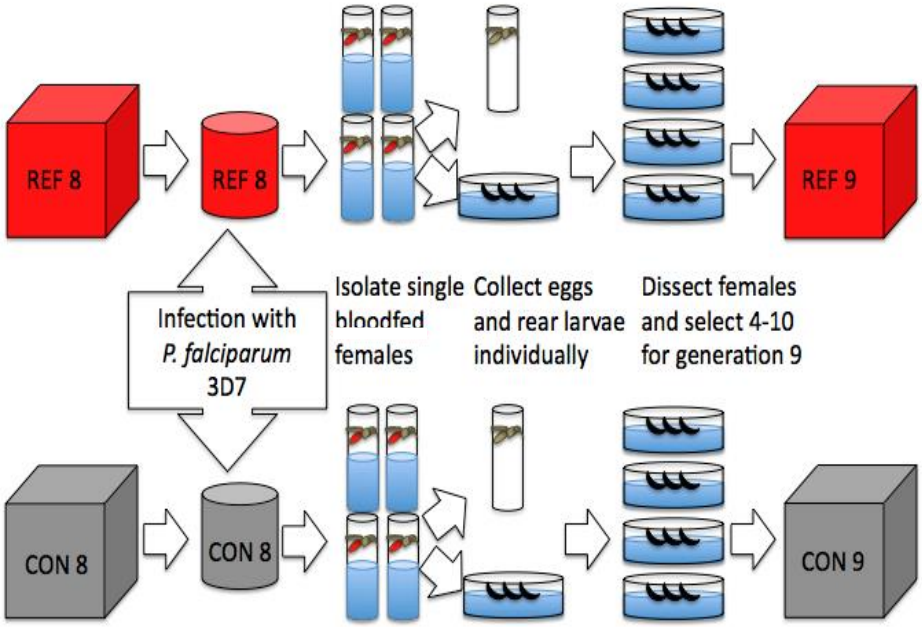


Figure 2.1. **Selection procedure.** 50-200 mosquito females (GU-REF and GU-CON) were infected with 3D7 parasites and separated into individual tubes for oviposition. Ten days after feeding, mosquitoes were dissected and the oocysts on the midgut counted. 4-10 females with zero oocysts were selected to form the next generation of the GU-REF line, and their offspring were grown to adults and used for the next infections. The same number of females was selected at random (regardless of infection level) to provide an inbreeding control (the GU-CON line).

2.3.6 **Statistical analysis**

The difference in oocyst prevalence between the GU-REF and GU-CON lines at each generation was examined using x^2 test or Fisher's exact test according to the sample size. Binomial errors were calculated using R. Oocyst intensities were expressed as medians because of the non-normal distribution. Oocyst distributions between the GU-REF and GU-CON mosquitoes were compared using Kolmogorov-Smirnov tests. All statistics were performed using R (Core Team R, 2013).

2.4 Results

2.4.1 **Selection for microsporidia free colony**

The GU-REF and GU-CON lines were successfully re-established from a minority of microsporidia-negative individual mosquitoes present in the original lines. 200 GU-REF and 100 GU-CON blood fed females were allowed to lay eggs

in separate tubes, of which 70 GU-REF and 37 GU-CON females laid eggs. 11 GU-REF (15.7%) and 13 GU-CON (35.1%) mosquitoes were found to be negative for microsporidia **(**Figure 2.2**)**.

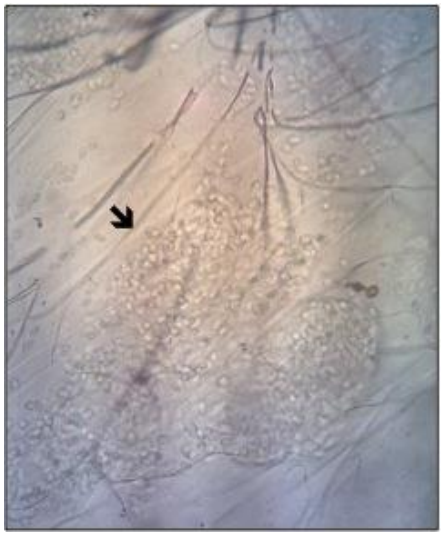


Figure 2.2 Microsporidia spores in mosquito midgut tissue. The arrow shows a tissue cyst full of spores (light microscope, 400x magnification).

Eggs from non-microsporidia-infected mosquitoes were reared on to adults, resulting in 89 GU-REF females (offspring of 11 females) and 64 GU-CON females (offspring of 13 females). These females were blood fed, and 36 females of this GU-REF generation (offspring of 8 G_0) and 24 of GU-CON (offspring of 8 G_0) laid eggs following a blood feed. Females were examined post egg laying for microsporidia and 100% of both GU-REF and GU-CON were found to be negative. Both the GU-REF and GU-CON lines were thus re-established from 8 uninfected mosquitoes (Table 2.2).

Steps	GU-REF line	GU-CON line
Feed #1. No. adult females blood fed	200	100
No. females laying eggs	70	37
No. females negative for microsporidia (%)	11 (15.7%)	13 (35.1%)
Feed # 2. No. adult females blood fed (offspring of feed 1)	89 (11)	64 (13)
No. females laying eggs (offspring of no. female feed#1)	36 (8)	24 (8)
No. females negative for microsporidia (%)	36 (100%)	24 (100%)
Colony recovered from no. females	8	8

Table 2.2. Numbers of female mosquitoes in each step of the re- establishment of GU-REF and GU-CON lines.

2.4.2 **Prevalence and intensities of parasite infection during the selection process for refractoriness**

The prevalence of infection with *P. falciparum* clone 3D7 in generations 8 to 11 was measured following an infectious blood-meal (proportion of mosquitoes with oocysts ten days post infection) (Figure 2.3). There was a significant difference in prevalence between the GU-REF and GU-CON lines by generation 10 (X^2; p=0.013) (Table 2.4Table 2.3). The median number of oocysts per mosquito varied between infectious feeds (Figure 2.5), but no significant trend towards a difference between GU-REF and GU-CON was observed (Table 2.4). The number of oocysts per mosquito in the selections 8-11 are shown in Figure 2.4, and summarised as median oocysts per mosquito in Figure 2.5. No significant difference between intensity of oocyst infection in GU-REF and GU-CON was observed in any generation except generation 9 (Table 2.3).

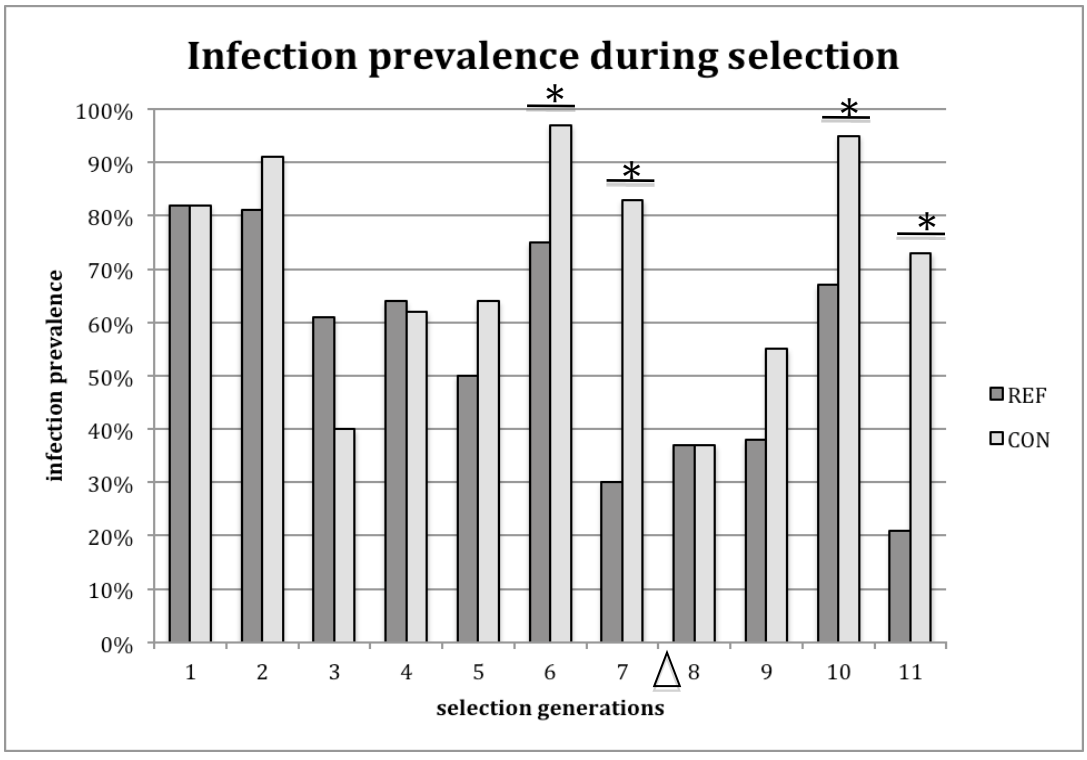


Figure 2.3. *P. falciparum* oocyst infection prevalence in generations 1-11. The asterisks indicate a significant difference in prevalence between GU-REF and GU-CON (X2, p value <0.05). The arrow indicates the occurrence of the microsporidia infection in the colony. Generations 1-7 were selected previously (L. Ranford-Cartwright& L. Peat; unpublished data).The number of mosquitoes examined in the generations 8-11 is shown in Table 2.4.

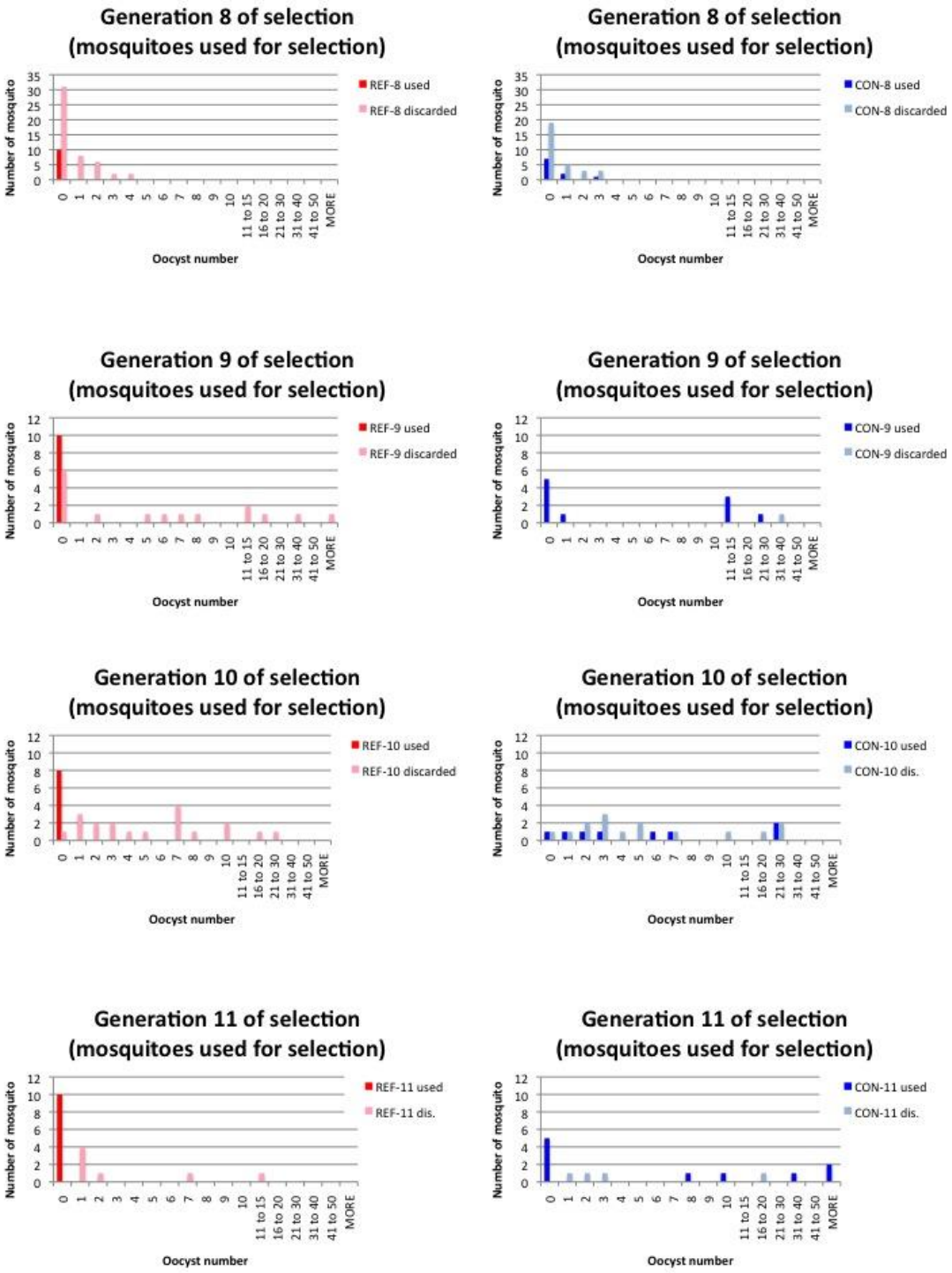


Figure 2.4. Oocyst intensities in dissected mosquitoes during the selection process of generations 8-11. The graphs show the number of mosquitoes with the corresponding numbers of oocysts from dissection on day 10 post-IBF. The mosquitoes used to establish the next generation during the selection process are shown in dark red (GU-REF) and dark blue (GU-CON), whereas those discarded/not used are shown in the lighter coloured bars.

Generation	Median oocyst intensity (range) of infection in GU-REF	n	Median oocyst intensity (range) of infection in GU-CON	n	p value (MWU test)
8	2 (1-4)	49	2 (1-3)	30	0.988032
9	10 (2-58)	26	14 (1-21)	10	0.00214
10	6 (1-25)	27	5 (1-30)	22	0.062886
11	2 (1-5)	17	1 (1-5)	14	0.05943

Table 2.3 Median oocyst intensity in generations 8-11. Median oocyst intensity and range for mosquitoes infected with *P. falciparum* clone 3D7. Oocyst distributions were compared between GU-REF and GU-CON lines for each generation using Mann-Whitney U tests. Significant p values are shown in bold.

Generation of selection	Prevalence of infection in GU-REF	Prevalence of infection in GU-CON	p value
8	36.7% (18/49)	36.6% (11/30)	0.995
9	38.46% (10/26)	54.54% (6/11)	0.366
10	66.6% (18/27)	95.45% (21/22)	0.012
11	21.42% (6/28)	73% (19/26)	0.0004

Table 2.4 *P. falciparum* oocyst infection prevalence in generations 8-11. The proportion of infected mosquitoes is shown in brackets after the percentage of prevalence. Significance was tested by chi-squared or Fisher's exact as appropriate.

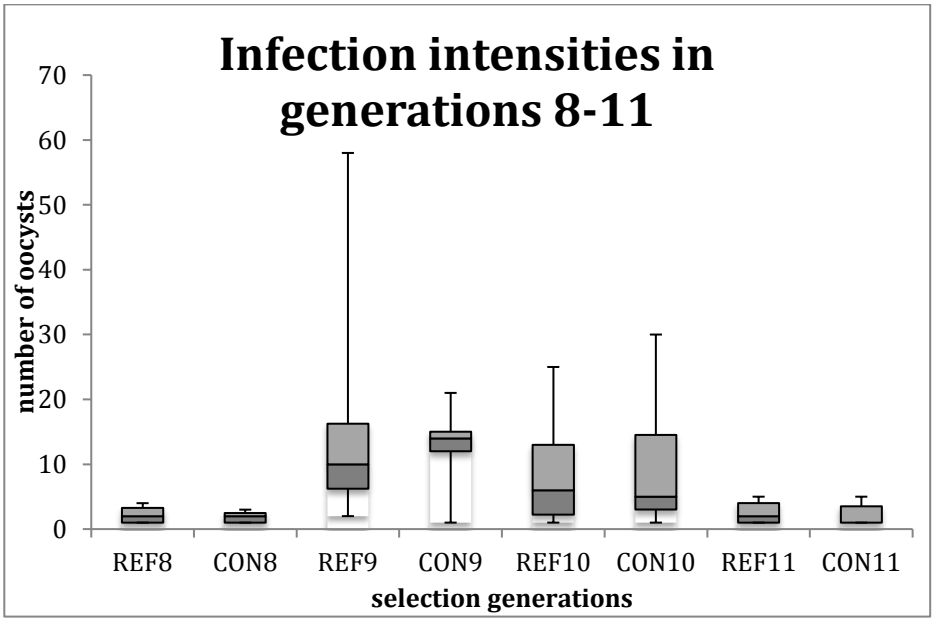


Figure 2.5 Box-plot of oocyst infection intensities in generations 8-11. The box represents the first and third quartiles, with the line within each box representing the median (2nd quartile). The whiskers represent the minimum and maximum values.

2.4.3 **Strain-specificity of refractoriness**

The GU-REF line was selected using *P. falciparum* clone 3D7, and significant resistance to infection with this clone was selected as described above. To establish the specificity of this refractoriness to the parasite genotype, infection levels achieved with the *P. falciparum* parasite clone HB3 were tested. Female mosquitoes from GU-REF11 and GU-CON11 were infected with gametocytes of clone HB3 at the same time as an infection with clone 3D7, and oocyst prevalence and intensity established (Figure 2.5, Table 2.5). Infection prevalence of clone HB3 in GU-REF11 was 3.1% compared to 38.7% for GU-CON11 (X^2 test, p=0.000484), with a median number of oocysts of 1 for GU-REF11 and 3 for GU-CON11 (K-S test, p=0.0128). The experiment was done in without replicates due to problems with GU-REF11 and GU-CON11 colonies at the time.

Generation	Median oocyst intensity (range) of infection in GU-REF	Median oocyst intensity (range) of infection in GU-CON	p value (K-S test)
11	1 (1)	3 (1-14)	0.0128

Table 2.5 Oocyst intensity in generation 11 for HB3 infection. Maximum and minimum oocyst number, in brackets, follows the median number of oocysts per infected midgut. Mosquito numbers dissected were 32 for GU-REF11 and 31 for GU-CON11.

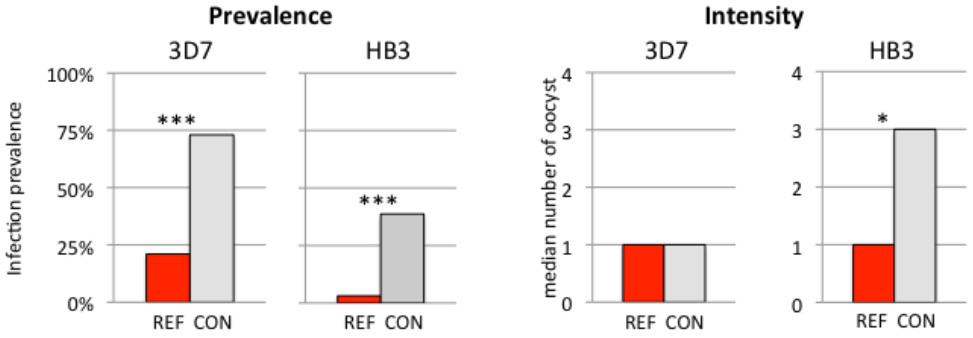


Figure 2.6 Comparison of prevalence and intensity of 3D7 and HB3 in GU-REF11 and GU-CON11. Asterisks (* p< 0.05, **p< 0.005, ***p<0.0005) indicate level of significance. Mosquito numbers n= 32 (GU-REF11), n=31 (GU-CON11).

2.5 **Discussion**

The aim of the work described in this chapter was to select a non-melanising line of *An. gambiae* refractory to *Plasmodium falciparum* clone 3D7.

Mosquitoes were successfully selected to be clear of microsporidia, allowing clean lines of the previously selected GU-REF and GU-CON to be established. However, as shown in Figure 2.3, there was no difference in susceptibility to parasite infection between GU-REF and GU-CON in generation 8, implying that the refractoriness was lost during the microsporidial infection or the microsporidia-free selection steps. The disruption of the refractory phenotype following the microsporidia infection meant that new rounds of selection were required to recover parasite refractoriness. Following the previous selection for seven generations, this project selected a further four generations.

Using prevalence of oocysts 10 days after a blood-meal as a marker of refractoriness, the GU-REF line became significantly more refractory to *P. falciparum* clone 3D7 from the 10^{th} generation, compared to the GU-CON line ($p=0.012$). The difference in infection prevalence further increased in the 11^{th} generation ($p=0.0004$).

Perhaps surprisingly, infection intensity did not change during the selection process, with the exception of the 9^{th} generation. This could be because the mosquitoes selected for the GU-REF generations did not have any oocysts (a sufficiently low prevalence allowed only mosquitoes with zero oocysts to be used for generations 8-11). It is therefore possible that the refractory loci enriched in the GU-REF population influence prevalence (absolute ability to sustain infection) rather than intensity (reduction in the number of parasites establishing).

The fact that it was possible to select for refractoriness in a relatively small number of generations means that refractoriness is probably controlled by a single locus, or a small number of loci. The small number of infected mosquitoes within a refractory population could be the outcome of parental heterozygotes crossing, leading to the representation of a susceptible genetic background.

Moreover, the selected line of GU-REF11 was also refractory to *P. falciparum* clone HB3 which was not used in selection. This suggests that the mechanism of refractoriness is directed towards conserved targets that do not vary between the two parasite genotypes. This precluded the identification of potential targets of the refractory phenotype in the parasites by a QTL analysis approach based on

prevalence differences using progeny of a cross between parasites 3D7 and HB3 (Walliker et al, 1987).

2.6 **Conclusions**

A new line of *An. gambiae* denoted GU-REF has been generated following the recovery of the GU-REF7 and GU-CON7 lines from colonies infected with the fungal pathogen microsporidia. The reselection of the line was necessary because the refractory behaviour (a significant difference in infection before microsporidial infection) had been lost, probably due to bottlenecking during the selection of “clean” mosquitoes.

Following a further four rounds of selection, the 10th generation of GU-REF exhibited significantly greater refractoriness to *P. falciparum* clone 3D7 compared to the control line GU-CON with similar inbreeding. The final 11th generation had a further increase in the difference in the susceptibility to infection.

3 Fitness parameters of GU-REF and GU-CON lines and blood meal digestion pattern as a potential mechanism of refractoriness

3.1 Aims and objectives

The aim of the research presented in this chapter was to establish the fitness of the newly selected GU-REF mosquitoes relative to the GU-CON line. We measured five fitness markers: body size, adult survival, proportion of mosquitoes laying eggs after first blood meal, length of first gonotrophic cycle and number of eggs per egg lay. To further investigate differences between GU-REF and GU-CON that could contribute to refractoriness of GU-REF, the speed of blood meal digestion was also analyzed.

Five research questions were investigated as follows.

1) Did the selection for refractoriness affect adult mosquito body size?
2) Did the selection for refractoriness affect mosquito survival?
3) Did the selection for refractoriness affect the proportion of mosquitoes that lay eggs after the first blood meal?
4) Did the selection for refractoriness affect the number of eggs per oviposition after a first blood meal?
5) Did the selection for refractoriness affect the length of the first gonotrophic cycle (time from blood-meal to oviposition)?

3.2 Introduction

3.2.1 Measures of mosquito fitness

Fitness is usually defined as the relative ability of an individual to leave descendents, and for mosquitoes can be indirectly measured by two traits: reproductive success, and adult survival. Reproductive success is determined by

fecundity and fertility. Fecundity measures the number of gametes produced, which in a mosquito can be translated as the number of mature oocytes (eggs). Fertility measures the number of viable offspring, translated as the number of hatched eggs (Hurd, 1995). To be able to fully establish the effect of selection for refractoriness on the fitness of the population, life-time fecundity, fertility and longevity would have to be combined.

Refractoriness may reduce the overall fitness of mosquitoes, for example if resources are reallocated from reproduction to an upregulation of mosquito immunity. Such trade-offs between insect life-history costs and anti-pathogen responses such as melanisation have been proposed in other insects (Armitage et al, 2003), and bead melanization by mosquitoes has been shown to reduce fecundity (Ahmed & Hurd, 2006; Schwartz & Koella, 2004). Costly refractoriness was not however observed in *An. gambiae* selected for refractoriness to infection by *P. yoelii nigeriensis* (Hurd et al, 2005), where mosquitoes from the refractory line had no survival or fecundity differences compared to the susceptible lines (Hurd et al, 2005). Refractory mosquitoes were however observed to have a lower hatch rate of their eggs (lower fertility), which could not be attributed to reduced insemination. Costs observed in a population of *Aedes aegypti* refractory to *P. gallinaceum* were attributed to the smaller body size of the refractory population rather than to the refractory mechanisms (Yan et al, 1997).

3.2.1.1 **Body size**

Body size of the adult mosquito depends on larval conditions such as crowding (population density), starvation, and food type. Adult mosquito body size is known to be an important factor affecting survival under stress conditions and for reproductive success (Briegel, 1990a; Briegel, 1990b; Takken et al, 1998). Larger mosquitoes also take a larger blood volume during feeding: the blood meal size of large females has been reported to be up to double that of smaller females, with corresponding fecundity increased up to 4-fold (Ichimori, 1989a). The gonotrophic cycle was completed in a shorter time in larger females than in small females which ingested blood of equal volumes (Briegel, 1990b). There is contradictory evidence as to whether smaller blood meal sizes results in lower parasite burden at the oocyst stage. No significant effect of body size/ blood meal

volume with oocyst burden was observed for the rodent parasite *P. yoelli nigeriensis* in *An. stephensi* (Ichimori, 1989b). However the number of oocysts harboured by field-caught *An. gambiae* mosquitoes in Tanzania increased with the size of the mosquito (Lyimo & Koella, 1992).

If selection for refractoriness to produce GU-REF resulted in mosquitoes of a different size (to GU-CON or to the KEELE parent line), the refractoriness could be interpreted as a side effect of size selection. Wing length was used as an estimate of body size as its correlation with whole body size and body mass is clearly established (Briegel, 1990a).

3.2.1.2 **Survival**

Survival is one of the main determinants of mosquito life-time reproductive success: the longer the female mosquito lives, the more reproductive cycles can potentially be completed. The longevity of a female mosquito also determines her ability to transmit malaria, since mosquitoes that die before the extrinsic cycle is completed cannot pass on any infection.

There are several factors affecting the survival of uninfected mosquitoes. The first is nutritional status of the mosquito after hatching, which represents the reserves built up in the larval state (Reiskind & Lounibos, 2009). Smaller mosquitoes with smaller energy reserves do not survive when exposed to sugar starvation (Andersson, 1992); sugar is important for survival and overall fitness, and also contributes to vector capacity (Manda et al, 2007). Blood is also an important source of nutrition, and can replenish a lack of food sources in the larval stage. Under laboratory conditions, mosquitoes live longer when provided with both blood and sugar meals rather than sugar alone (Okech et al, 2003). As stress, temperature and humidity levels are important factors affecting mosquito survival (Christiansen-Jucht et al, 2014), mosquitoes were maintained here under stable environmental conditions.

3.2.1.3 **Proportion of mosquitoes that lay eggs after a bloodmeal**

The first gonotrophic cycle is an important marker of mosquito nutritional status, because mosquitoes with low energy resources can use the first blood

meal to replenish the lack of nutrients from larval stage and produce either no eggs, or a reduced number (Takken et al, 2013). Mosquitoes that do not lay any eggs may also indicate a problem with mating in a colony.

3.2.1.4 **Number of eggs per oviposition**

The number of eggs laid per oviposition is another factor linked with the nutritional status of the mosquito, often manifest as a difference in body size (Lyimo & Takken, 1993). Moreover, although not measured in our experiments, infection of mosquitoes has been shown to reduce egg production by 20-30% in *Ae. aegypti/ P. gallinaceum,* and *An. stephensi/ P. yoelii nigeriensis* (Hurd, 1995). In *An. gambiae/ P. yoelii*, oocyte resorption is increased in infected mosquitoes (Ahmed & Hurd, 2006; Hurd, 1995).

3.2.1.5 **Time from bloodmeal to oviposition**

The length of the gonotrophic cycle influences, along with longevity, the number of cycles a mosquito can achieve during her lifespan. For a fixed longevity, the shorter the gonotrophic cycle, the more offspring can be produced over the time, assuming that offspring per gonotrophic cycle do not vary. Moreover, the length of time taken to mobilise and utilise nutrients for egg production may represent the speed of the processes involved, in individual mosquitoes, strains or even species. The length of the gonotrophic cycle can therefore be an important factor for vectorial capacity.

Gonotrophic cycle length does vary between species of mosquito, and within a species between different environmental conditions such as temperature (Lardeux et al, 2008). The duration of gonotrophic cycle in a population of *An. gambiae* was reported to be shorter during the dry than the wet season (Mala et al, 2014). Mosquito body size has also been reported to be inversely correlated with the length of the gonotrophic cycle (Briegel, 1990b).

3.2.1.6 **Timing of Plasmodium development**

Plasmodium development within mosquito is largely dependent on temperature. Under insectary conditions (26^{o}C, 80% RH) the process from

ingestion of infectious blood to the presence of sporozoites in the salivary glands takes about 14 days (Baton & Ranford-Cartwright, 2005). Within the mosquito midgut the parasite develops through stages known as gametocytes, gametes, zygotes, retort forms and finally ookinetes, which pass through the epithelial cells to encyst on the outer midgut wall. It takes 18-24 hours for *P. falciparum* development from ingestion of the infectious blood meal to the ookinete leaving the mosquito midgut (discussed in detail in Chapter 1).

3.2.1.7 **Blood meal digestion in the mosquito midgut**

During the time of development in the midgut, the parasite is exposed to mosquito midgut proteases, secreted into the posterior gut lumen to digest the blood meal. The main proteases of the mosquito midgut secreted in response to blood feeding are trypsin (peak 28-32h post blood-feed), chymotrypsin (peak 36h post blood-feed) and aminopeptidase (peak 30h post blood-feed) (Billingsley & Hecker, 1991; Rosenfeld & Vanderberg, 1998).

When exposed to proteases of mosquito midgut, Plasmodium zygotes, retort forms and ookinetes show signs of destruction. The ookinetes of *P. gallinaceum* were shown to be sensitive to damage by *Ae. aegypti* gut proteolytic enzymes such as trypsin, especially in earlier stages of development (Gass & Yeates, 1979). Blood meal digestion begins at the periphery of the blood bolus, and these authors suggested that only ookinetes developing within the centre of the blood meal survived. Thus, the speed of blood meal digestion is a critical factor for ookinete survival. If protease levels in mosquito midgut reach high levels before ookinetes leave the midgut lumen, they will be damaged by digestive enzymes. However, the proteases also inactivate complement and macrophages in the midgut, both of which kill parasites (Grotendorst et al, 1986).

The speed of blood meal digestion in mosquitoes is influenced by environmental temperature (West & Eligh, 1957), light and humidity (O'Gower, 1956), and the source of the blood meal (Downe et al, 1963). The speed of digestion may be influenced by differences in the levels of proteases, or the timing of their secretion, and may be responsible for some differences of susceptibility of vector-parasite combinations. For example, *An. albimanus* has faster digestion than *An. stephensi*, leading to the former being more refractory to *P. falciparum*

(Baton & Ranford-Cartwright, 2012). The presence of parasites in the blood meal has a negligible effect on blood-meal digestion (total protein ingested, rate of protein digestion) (Jahan et al, 1999).

3.3 Materials and methods

3.3.1 Analysis of mosquito fitness parameters

Some analyses of fitness were carried out during the selection experiments, and others at the end with the GU-REF11 and GU-CON11 generations of selection. The generation of selection used for each experiment is stated in the relevant section below.

3.3.1.1 Adult body size

Pupae of each line (GU-REF10, GU-CON10 and *An. gambiae s.s.* KEELE) were allowed to emerge and 100 adults of each line were collected for measurement of wing length. The wings were removed from each mosquito under a dissecting microscope (magnification 20x), placed in drop of PBS and allowed to dry on the slide. The length was measured from the axillary incision to the apical margin (Nasci, 1986), using a digital camera imaging system (Moticam 2300) connected to the microscope eyepiece and precalibrated software (Motic images plus v. 2.0).

The wing lengths of the GU-REF10, GU-CON10, KEELE *An. gambiae s.s.* mosquitoes were compared using Kruskal-Wallis pairwise tests and Kruskal-Wallis rank sum tests.

3.3.1.2 Adult mosquito survival

Survival of adult female mosquitoes was investigated under three different conditions, and compared between mosquitoes of the GU-REF11 selected line, the GU-CON11 line (control for inbreeding effects) and the parent *An. gambiae* KEELE line: (1) survival without blood feeding; (2) survival after a single blood-meal; and (3) survival after one infectious blood-meal.

The first condition investigated the nutritional status from the larval stage growth of the selected lines without nutritional supplementation by a blood meal, and could provide information on the nutritional requirements or cost of refractoriness. The second condition represents basal survival in mosquitoes in the absence of stress caused by infection, but with natural nutritional supplementation through a single blood meal. This experiment will determine if maintenance of the refractory state in the absence of parasite challenge is costly. The third condition introduces stress caused by infection, and should indicate if refractoriness confers a survival advantage under parasite challenge.

For each experimental condition, pupae from each line were sexed at the pupal stage based on their terminal genitalia (Moorefield, 1951), and females only were transferred to pupal hatching cages. 100 adult female mosquitoes of each line were then gently transferred to waxed paper cups closed with netting. Glucose was provided on cotton wool pads placed on top of the netting and changed daily. Cups were examined every day and dead mosquitoes were collected from the bottom of the cup daily and numbers recorded. The recording continued until all the mosquitoes had died.

To analyse longevity in mosquitoes allowed to take a single blood meal (condition 2), the mosquitoes were offered a non-infectious blood meal eight days post-emergence. To analyse longevity in mosquitoes allowed to take a single infectious blood meal (condition 3), the mosquitoes were offered a blood meal containing infectious gametocytes (clone 3D7, 1% gametocytaemia) eight days post-emergence. Unfed mosquitoes were removed from the pots after the membrane feeding. The experiments with all conditions were replicated 3 times. The Cox proportional hazard model procedure was used to examine the impact of different feeding conditions on survival data, using the coxph command in the Survival package (Therneau & Grambsch, 2000) in R (Core Team R, 2013).

3.3.1.3 **Proportion of mosquitoes that lay eggs after the first blood meal**

The proportion of mosquitoes that laid eggs was measured during the selection process (generations 8 to 10), and therefore there are no within-generation replicates, and all mosquitoes were given infectious blood meals. Following the infectious blood-meal, unfed mosquitoes were removed and

discarded. The remaining mosquitoes were allowed to deposit haematin, and then 50 female mosquitoes were separated into individual tubes, with water for egg deposition and an individual source of glucose (described in chapter 2). The tubes were checked daily for eggs. Mosquitoes that had not laid eggs after 9 days were discarded and counted as non egg-laying.

The proportion of mosquitoes that laid at least one egg was compared between the GU-REF and GU-CON lines at each of the three generations of selection (generations 8, 9 and 10). Because there were no replicates, the numbers were compared using chi-squared tests assuming a null hypothesis of no difference in the proportion of mosquitoes that laid eggs.

3.3.1.4 **Eggs per oviposition after a first blood meal**

Egg number is a basic factor contributing to mosquito lifetime fecundity. If refractoriness in mosquitoes was linked to decreased egg numbers, mosquitoes with such a trait would be at a disadvantage compared to susceptible ones.

The number of eggs laid was measured in generation 11 for GU-REF11, GU-CON11 and KEELE *An. gambiae s.s*. stock mosquito following an infectious blood meal. The number of eggs was measured in three separate replicates with independent infected blood meals (IBF). The number of eggs laid by each mosquito was counted as described above. The mean number of eggs per mosquito that laid eggs was calculated for each line in each replicate, and the mean numbers compared using a Welch Two Sample t-test.

3.3.1.5 **Length of the first gonotrophic cycle (time from blood-meal to oviposition)**

The time taken post-blood meal for mosquitoes to lay eggs was measured during the selection process (generations 8 to 10), and therefore there are no within-generation replicates, and all mosquitoes were given infectious blood meals. The day of oviposition was recorded for the two mosquito lines (GU-REF and GU-CON) in generations 8, 9 and 10. The original approach was to monitor oviposition over 2 days (as in generation 8). As GU-CON started to take longer to lay eggs after the blood meal during the selection process, the monitoring of

oviposition was extended to 4 days in generation 9 and 7 days in generation 10. The proportion of mosquitoes laying eggs on each day post blood meal was compared for each generation between GU-REF and GU-CON using a chi-squared test, assuming no difference between the two lines. The median day of oviposition was compared using Mann-Whitney U tests.

3.3.2 **Analysis of rate of blood meal digestion**

Based on the early oviposition in GU-REF, we hypothesized that the selection experiment had favoured mosquitoes with faster blood-meal digestion in this line. Faster digestion of the blood-meal may confer refractoriness, because ookinetes may not escape the midgut before protease levels become lethal. Earlier stages of parasite development are known to be more susceptible to protease damage (Gass, 1979).

3.3.2.1 **Dissection of midgut after blood meal and sample preparation**

Female mosquitoes seven days post emergence (GU-REF7 and GU-REF9, GU-CON7 and GU-CON9), were allowed to feed on uninfected blood at 40% hematocrit through a membrane feeder. Every 6h (0h – 54h), five mosquitoes from each group were dissected and the midgut containing the blood meal was assayed for total protein content using the Bicinchonic acid protein assay (Sigma).

3.3.2.2 **Bicinchoninic acid protein assay (BCA) protein assay**

To measure the speed of blood meal digestion as one of the potential mosquito refractory mechanisms, we used the Bicinchonic acid protein assay, which measures the total concentration of protein in the mosquito midgut content post blood feeding, thus indirectly following the activity of midgut proteases. The assay has a wide working range (200–1,000 mg/mL of protein), and the outcome is determined by a colour change (green to purple) in proportion to protein concentration.

Each mosquito midgut and its blood contents were homogenised in 40 μL 1x PBS and stored at -20°C. After thawing, 280 μL of 1 x PBS was added and 50 μL of this solution was used for the assay. 200 μL of the BCA working solution was

mixed with 50 µL final homogenate solution, incubated in 96 well (flat bottom) plate for 30 min in 37°C, and then measured in an ELISA plate reader at 560 nm wavelength. Bovine serum albumin (Sigma) solutions (range of concentration from 100 to 700 µg/mL) were used to create a standard curve. Each standard curve concentration was prepared and measured in triplicate. The concentration of protein present in each sample was calculated from the regression equation of the standard curve. For each time point the mean and standard error were calculated from the five individual mosquito samples for each line.

At each time point, protein levels were compared between GU-REF and GU-CON using X^2 tests.

3.4 **Results**

3.4.1 **Body size**

The median sizes of the GU-REF10, GU-CON10, KEELE *An. gambiae s.s.* original colony are shown in Figure 3.1. Pairwise Kruskal Wallis tests revealed no significant difference between the winglengths in the three groups (GU-REF/GU-CON p=0.3697, GU-REF/KEELE p=0.3357, GU-CON/KEELE p=0.41).

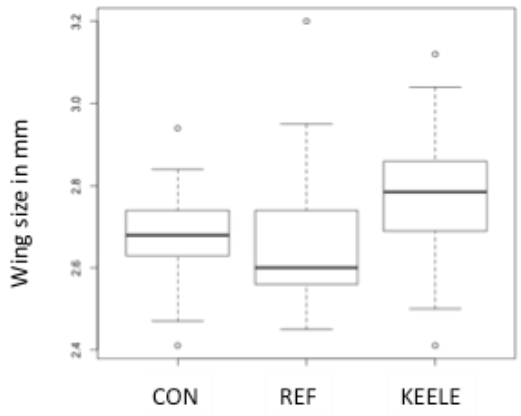


Figure 3.1: Wing size in mm of mosquitoes from the GU-REF10, GU-CON10 and KEELE lines. Each box indicates the first and third quartiles, with the line representing the median (2nd quartile). The whiskers are the 95% confidence intervals, and circles represent outliers. n=50 for each mosquito line.

3.4.2 **Survival**

Under the conditions of glucose-feeding only, GU-REF11 mosquitoes had significantly lower longevity when compared to GU-CON11 mosquitoes, living on average (median) 22 days compared to 29 days for GU-CON11 (Figure 3.2A, p=2e-16). When mosquitoes were allowed a single non-infectious blood meal on day 8, there was no significant difference in longevity (median lifespan: 29 days (GU-REF), 30 days (GU-CON, p=0.4586) (Figure 3.2B), and the longevity of the GU-REF line was restored to that of the GU-CON line. However, if the blood meal contained infectious gametocytes, the longevity of GU-REF was reduced to below that of the GU-CON line (Median lifespan: GU-REF: 27 days; GU-CON: 30 days; p=16.698e-06) (Figure 3.2C).

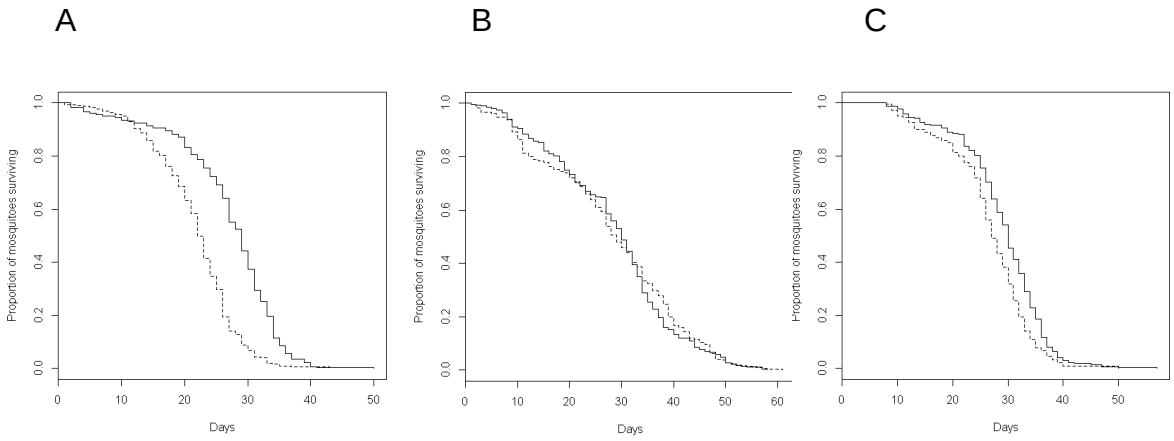


Figure 3.2. Survival of *An. gambiae* GU-REF10 and GU-CON10 mosquitoes. GU-REF10 are shown as a dotted line and GU-CON10 as a solid line. A) Mosquitoes maintained on glucose alone; B) Mosquitoes maintained on glucose but with one non-infectious blood feed 8 days post-emergence; C) Mosquitoes maintained on glucose but with one infectious blood feed on 8 days post-emergence. Each point represented on the graph was calculated as the predicted survival of all replicates with weighting of points depending on variation in the sample size within a replicate. The output was from the model of Survival, Cox Proportional Hazards model (N=600 for each condition).

3.4.3 **Proportion of mosquito that lay eggs after a bloodmeal**

The proportion of mosquitoes that laid eggs during generations 8-10 of the selection are shown in Figure 3.3. Comparisons of the numbers of egg-laying and non-egg laying mosquitoes between the GU-REF and GU-CON samples at each time point revealed that a significantly larger proportion of GU-REF mosquitoes laid eggs compared to the GU-CON control line in each of the three generations studied (x^2 generation 8: p=0.016; generation 9: p=6.9E-13; generation 10: p=0.013).

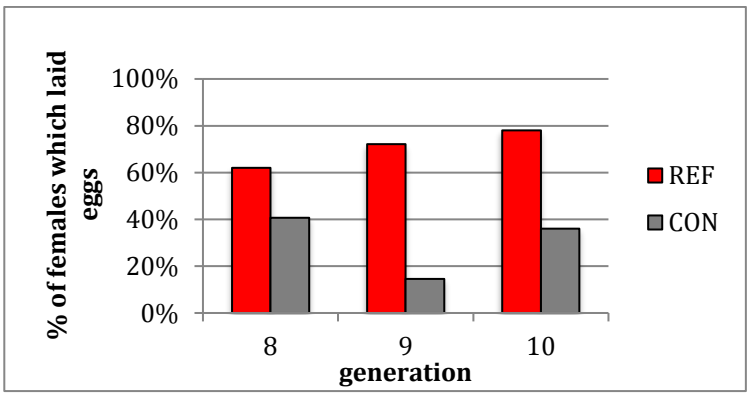


Figure 3.3. The percentage of mosquitoes that laid eggs after an infectious feed. The total number of mosquitoes examined for oviposition was: generation 8: REF n=53, CON n=57; generation 9: REF n=79, CON n=75; generation 10: REF n=50, CON n= 50.

3.4.4 Number of eggs per oviposition in egg-laying females

The number of eggs laid from three (infectious) blood meals is shown in Figure 3.4. The mean number of eggs was the lowest in the GU-REF11 line (mean=40.09), with a small increase (mean=44.74) in the GU-CON11 line, and was highest in the unselected KEELE colony (mean=49.41). Pairwise t-tests revealed no significant difference between GU-REF11 and GU-CON11 (p=0.277), or between GU-CON11 and KEELE (p=0.2695). The mean number of eggs laid by egg-laying females of GU-REF was significantly lower than for the KEELE parent line (p=0.006893).

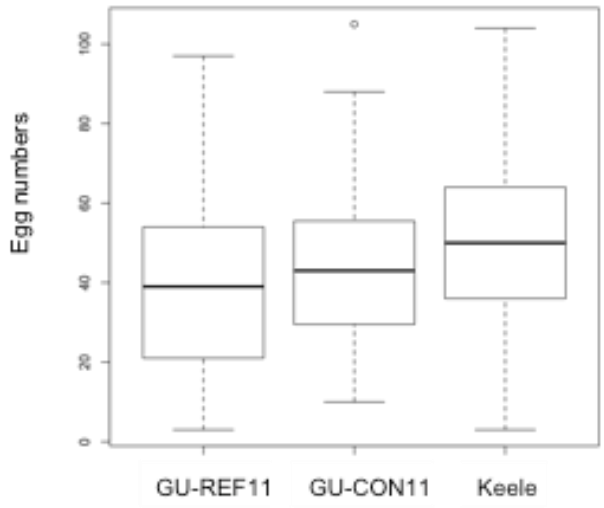


Figure 3.4. Number of eggs per oviposition in mosquitoes laying at least one egg. Each box indicates the first and third quartiles, with the line representing the median (2nd quartile). The whiskers are the 95% confidence intervals, and circles represent outliers.Total sample size was 77 (GU-REF11), 40 (GU-CON11), 81 (KEELE).

3.4.5 Time from bloodmeal to oviposition

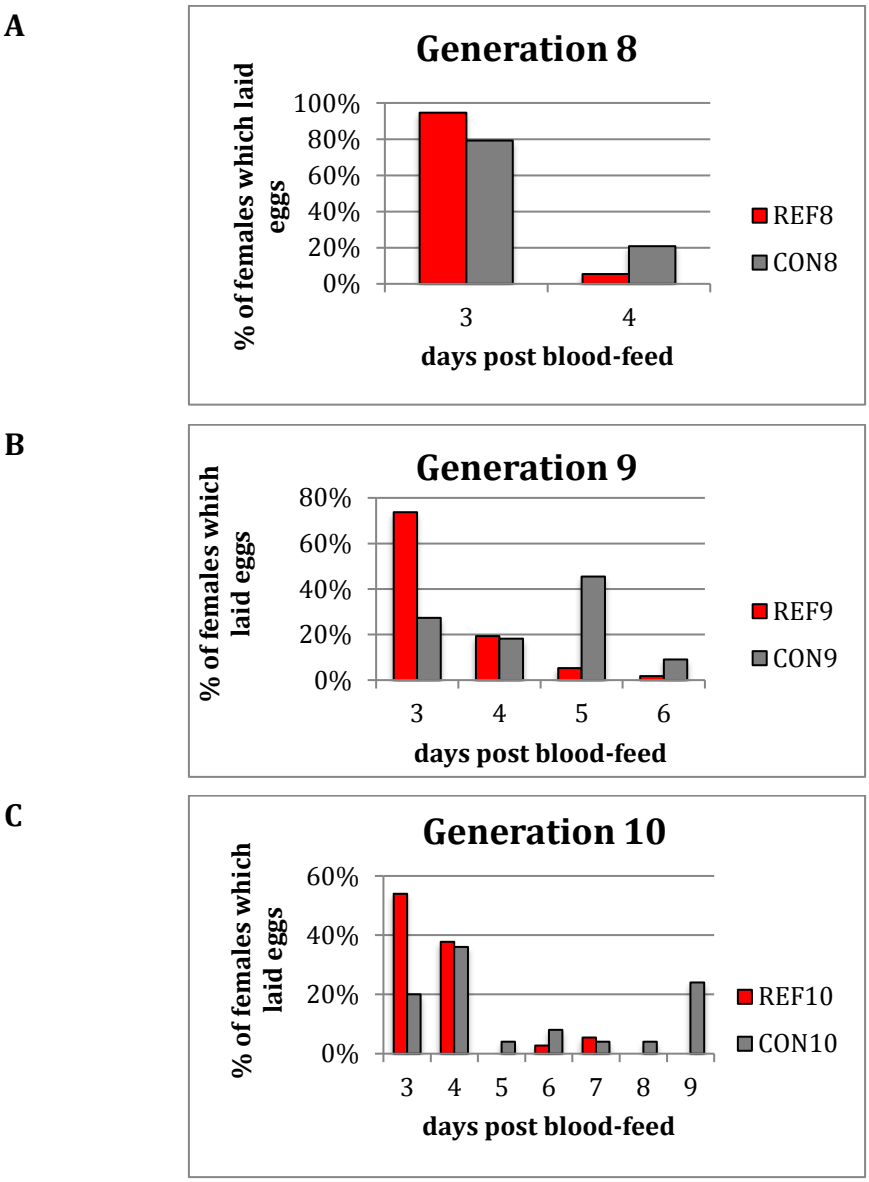


Figure 3.5 Proportion of mosquitoes which laid eggs at different time point post-blood meal. A) Generation 8: n=37 (GU-REF), n= 24 (GU-**CON)**. **B**) Generation 9: n=57 (GU-REF), n= 11 (GU-CON). C) Generation 10: n=37 (GU-REF), n= 25 (GU-CON).

In generation 8, the majority of mosquitoes in both GU-REF and GU-CON lines laid their eggs 3 days after the blood feed (Figure 3.5A) and there was no difference in the egg laying pattern between the GU-REF and GU-CON lines (median day of oviposition REF8 vs. CON8, p=0.3172). From generation 9 (Figure 3.5B), a significant difference was observed in the adult female egg-laying pattern, such that a significantly higher proportion of GU-REF mosquitoes laid eggs on day 3 compared to the GU-CON line (median day of oviposition REF9 vs. CON9, p= 0.00398). A similar pattern was observed in generation 10 (Figure 3.5C), with GU-

REF mosquitoes laying earlier than GU-CON (median day of oviposition) REF10 vs. CON10 p= 0.00108). Rather than GU-REF mosquitoes laying earlier, the data suggest a delay in egg lay for GU-CON, relative to the parent Keele line, which usually lay eggs on day 3 post blood feed (data not shown).

3.4.6 **BCA protein assay time course experiments**

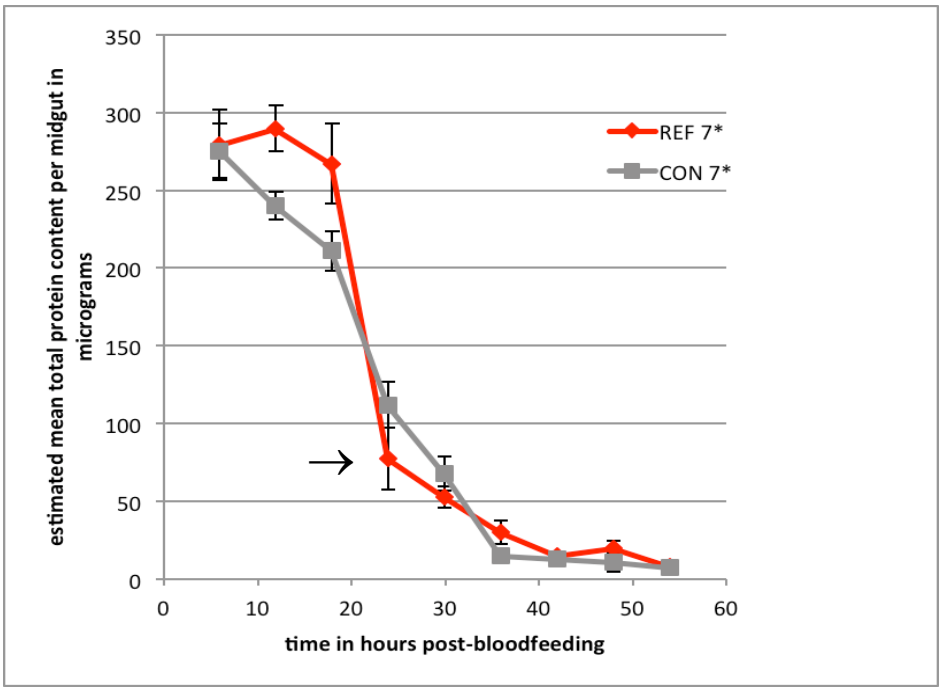
The results from the Bicinchonic acid protein assay for generation 7 (Figure 3.6A) show an identical amount of protein in the midgut at the start of the experiment, followed by an apparently slower rate of protein breakdown, in the REF line compared to the CON line up to 18h post feed (p=0.027 at 12h, p=0.10 at t=18h). However, between 18 and 24 h post feed, the rate of protein digestion increased markedly in the REF line, such that at 24h the amount of protein in REF mosquitoes was lower than in the CON line, although this did not reach statistical significance (p=0.20). By the 9th generation of selection, at the time point 24h after the blood-meal, the GU-REF9 mosquitoes had (almost) significantly less protein in their midguts than the GU-CON9 (p=0.06), suggesting a more rapid increase in the rate of digestion around this time in the refractory line (Figure 3.6B).

3.5 **Discussion**

Previous research has suggested that refractory mosquitoes may have significantly reduced fitness, for example manifesting as reduced fecundity or survival (Voordouw et al, 2009). Of the five measures of fitness examined and reported in this chapter, two showed no difference in the REF line and the inbreeding control: neither body size, nor the number of eggs laid following a blood meal was significantly different between the GU-REF and GU-CON lines. The lack of difference in body size confirms that the selection for refractoriness did not result in smaller mosquitoes.

The GU-REF line did not show fitness costs, compared to the inbreeding control GU-CON, as measured by the proportion of female mosquitoes which laid eggs after a blood-meal. Indeed, the GU-REF line has apparently significantly increased fitness as measured by this parameter of fecundity. Oviposition time was also significantly earlier in the GU-REF line compared to the GU-CON line (p=0.0073). However rather than being a lowering of this parameter in the REF

A



B

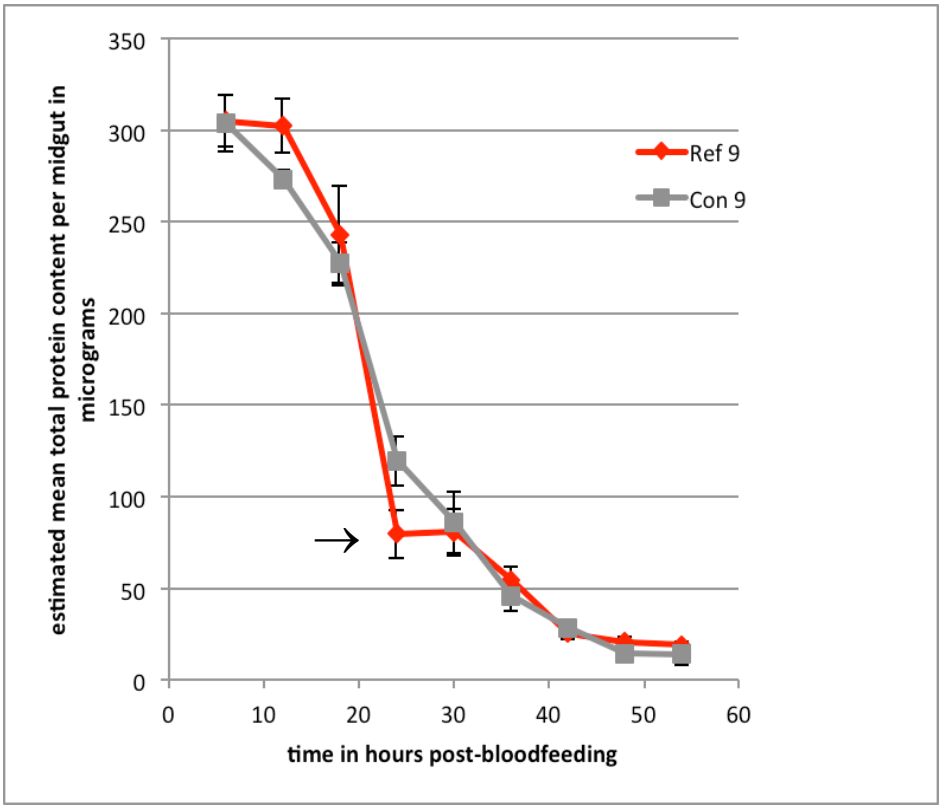


Figure 3.6. Blood meal digestion in the GU-REF and GU-CON lines at generations 7 and 9 of selection. Protein levels per midgut were measured using the Bicinchonic acid protein assay. Each point on the graph represents the mean of five mosquitoes. The error bars show the standard error of mean. The arrow indicates the 24h time point. A) Mosquitoes from generation 7 (* indicates mosquito colony after microsporidia free selection) B) Mosquitoes from generation 9.

line, it appears than the GU-CON line has delayed oviposition, because the REF line is more similar to the parent KEELE line. This is most likely to be an effect of inbreeding in the GU-CON line.

One potential explanation for an increased speed of oviposition could be that mosquitoes have an increased rate of digestion, releasing resources for egg development earlier. This hypothesis was tested: speed of digestion (to 24h) was marginally faster ($p=0.06$) in GU-REF compare to GU-CON lines in generation 9 of selection correlating with earlier oviposition, although this did not quite reach statistical significance.

The survival of mosquitoes presents a more complex picture. Under conditions where mosquitoes are maintained on sugar alone, the GU-REF line has a significantly lower survival than the GU-CON mosquitoes, suggesting a fitness cost to refractoriness in the absence of challenge. However, this fitness cost was removed if mosquitoes were allowed a single blood meal, unless that blood meal contained infectious gametocytes. It appears that the mosquito refractory mechanism has some cost to mosquitoes under infection challenge, reducing their lifespan, albeit by only 3 days. However, this may be compensated by a higher proportion of refractory mosquitoes that lay eggs after the first blood meal.

3.6 **Conclusions**

The overall fitness of GU-REF line compared to the GU-CON line does not appear to have been significantly negatively affected by the selection process for refractoriness.

4 Characterisation of refractoriness in GU-REF *An. gambiae s.s.* mosquitoes

4.1 Aims and objectives

This chapter describes in more detail the effector mechanisms of parasite killing in the mosquito, described briefly in section 1.4.4, and the non-self-recognition and signalling pathways that activate these effectors. The enrichment of candidate genes or alleles associated with refractoriness was investigated in GU-REF *An. gambiae s.s.* mosquitoes.

4.2 Introduction

4.2.1 Recognition of non-self

To raise an immune response requires the recognition of an invading pathogen as foreign. Pattern recognition receptors (PRRs) interact with pathogen-associated molecular patterns (PAMPs, also known as microbe-associated molecular patterns or MAMPs) that are present in microbes but are not found in insects, for example, bacterial peptidoglycans, fungal beta-1, and -3 glucans. Additionally, some PRR-like proteins, such as the Leucine-rich repeat (LRR) protein family regulate immune responses by a direct interaction with host proteins other than classical PAMPs. There is a great diversity of PRRs in different insect taxa, and their activity also varies, and includes direct stimulation of immune effectors like melanisation, activation of intracellular signalling pathways to modulate antimicrobial gene expression, and direct interaction with other host proteins to regulate immune responses.

4.2.1.1 Peptidoglycan recognition proteins (PGRPs)

PGRPs are the best-known insect PRRs; they recognise peptidoglycan, which is mainly found in the Gram-positive bacteria cell wall, and are characterised by a peptidoglycan-recognition domain of ~165 amino acids (Kim et al, 2003). PGRPs can be classified into two groups based on the length of their gene products. Short PGRPs are usually extracellular proteins, and are present in the haemolymph,

cuticle, fat body, epidermal cells, gut, and, to a lesser extent, haemocytes, whereas long PGRP are either intracellular or membrane-spanning proteins and are mainly expressed in haemocytes (Christophides et al, 2002). Activated PGRPs have multiple downstream effects including activation of the prophenoloxidase (PPO) cascade, the Toll pathway (especially for Gram-positive bacteria), the Imd pathway (Gram-negative bacteria) and stimulation of phagocytosis (Gram-negative bacteria).

Seven PGRPs have been identified in the *Anopheles* genome: *S1-S3* encode short AgPGRP*,* and the remaining four genes are differentially spliced to generate six long *AgPGRP*s: *LA1*, *LA2*, *LB*, *LC1*, *LC2* and *LC3* (Waterhouse et al, 2007). Expression of *PGRP*s has been shown to be upregulated in *An. gambiae* on exposure to various bacteria, to bacterial peptidoglycan, and to *P. berghei* challenge (Dimopoulos et al, 2002; Kang et al, 1998). *PGRPLB* is transcriptionally upregulated in *Plasmodium*-infected mosquitoes and remains high throughout infection (Christophides et al, 2004).

4.2.1.2 **Gram-negative binding proteins (GNBPs)**

GNBPs share a conserved β-1,3 -glucan-binding domain, and are usually upregulated following bacteria challenge. The *An. gambiae* genome encodes six GNBPs, *AgGNBPA1, A2GNBP,* and *AgGNBPB1-B4* (Waterhouse et al, 2007). All have an N-terminal signal sequence, three (AgGNBPB1, B2, and B4) have glycosylphosphatidylinositol (GPI)-anchor sequences, and three (AgGNBPA1, B1, and B3) have potential N-linked glycosylation sites. The genes are differentially expressed in the mosquito body and are differentially upregulated in response to bacterial or parasite infection (Warr et al, 2008). AgGNBPB1 was found to be induced in the midgut and salivary glands upon *P. falciparum* infection, but gene silencing of *GNBPA2* had the strongest impact on *P. falciparum* infection. GNBPB3 and GNBPB4 were only upregulated after challenge with *P. berghei,* and gene silencing affected *P. berghei* infection (Warr et al, 2008). AgGNBPs regulate the expression of a number of immune genes including AMPs, *PGRPLC, LRIM1, CLIPB3 and CLIPB3*, suggesting signalling through the Imd and Toll pathways

4.2.1.3 *C-type lectins (CTLs)*

The CTL family is made up of membrane-bound and secreted proteins, which recognise sugar residues via interaction with a characteristic carbohydrate recognition domain (CRD). CTLs are involved in cell adhesion, cell-cell interaction, glycoprotein turnover, and pathogen recognition leading to innate immune responses (Schnitger et al, 2009). 23 CTL genes have been found in the *An. gambiae* genome, separated into four groups: mannose binding (CTLMA), galactose binding (CTLGA), selectins (CTLSE), and other CTLs (Schnitger et al, 2009). Some CTLs share homology regions with scavenger receptors (section 4.2.1.10). Compared to *Drosophila, Anopheles* have an expanded CTLMA group (Christophides et al, 2004).

CTL4 and CTLMA2 are found in haemolymph of *An. gambiae* as a disulfide-linked heterodimeric complex (Schnitger et al, 2009). The two genes were upregulated 24 h after *P. berghei* infection, and gene silencing experiments suggested that they act to protect ookinetes from destruction (Osta et al, 2004). They have also been shown to protect the mosquito from infection with Gram-negative bacteria (Schnitger et al, 2009). CTL4, and an additional CTLGA3, were shown to be upregulated on infection with *P. falciparum* (Dong et al, 2006a).

4.2.1.4 **Thioester containing proteins (TEPs)**

The Thioester- containing proteins (TEPs) are a wide family of proteins in vertebrates and invertebrates, characterised by a specific intra-chain thioester bond. TEPs are classified into two subfamilies: the alpha-2-macroglobulin (A2M) subfamily and the C3 subfamily. In vertebrates, TEPs form part of complement pathway – factors C3, C4 and C5 are all C3-type TEPs. During complement activation, the thioester bond is exposed and forms a covalent (ester or amide) bond with a target surface such as a pathogen, marking it for phagocytosis or lysis by the membrane attack complex.

In insects, TEP members belong to the A2M subfamily, but are functionally similar to the C3 family (Nonaka, 2011). In *Anopheles* TEPs promote phagocytosis of Gram-negative bacteria (Levashina et al, 2001), and have also been shown to

bind to the surface of *P. berghei* ookinetes, promoting their melanisation and death (Blandin et al, 2004).

The *Anopheles* genome encodes 15 TEPs, several of which play a role in vectorial capacity. TEP1, 3 and 4 have been shown to be upregulated after bacterial and parasite infection (both *P. berghei* and *P. falciparum)* (Christophides et al, 2004). TEP1 is produced by haemocytes, and circulates in the mosquito haemolymph as a full-length protein and a processed form, $TEP1_{cut}$, which is stabilised by binding with two LRR proteins LRIM1 and APL1C. TEP1 binding to the ookinete stage marks it for destruction by the LRIM1/APL1C complex with additional components such as TEP1-activated proteases (Clayton et al, 2014).The mechanisms up- and down-stream of TEP1 are still unknown. It has been suggested that TEP1 is responsible for most of the parasite killing in mosquitoes, but other mechanisms also play important role.

Comparison of RNAi between *P. falciparum* and *P. berghei* revealed important differences in mosquito immunity against these two species of malaria parasite (Dong et al, 2006a): TEP1, apolipophorin II/I, WASP, lipoprotein homolog APOD or IRID seem to affect the development of both malaria parasite species, whereas LRIM1, CTL4, CTLMA2 and SRPN2 affect only *P. berghei* (Blandin et al, 2008).

4.2.1.5 **Leucine-rich repeat containing proteins (LRR)**

Leucine-rich repeat proteins (LRRs) are secreted, membrane-bound or cytoplasmic proteins with diverse functions, and are often involved in immune responses in plants and animals. In insects, the LRR domain-containing superfamily, known as leucine-rich repeat immune protein (LRIMs), are known to be involved in anti-pathogen defence. The *An. gambiae* genome was found to have 24 LRIM-like genes (Waterhouse et al, 2010) with similar characteristics of a signal peptide, one or more LRR, specific patterns of cysteine residues, and coiled-coil domains. Mosquito LRIMs were grouped into two subfamilies: Long LRIMs with 10 or more LRRs (e.g. *Ag*LRIM1, *Ag*APL1C), and Short LRIMs with 6-7 LRRs (Waterhouse et al, 2010). LRIM1 was found to be highly upregulated in *P. berghei* infections, and silencing of the gene increased oocyst numbers (Dimopoulos et al, 2002; Osta et al, 2004).

A second member of the LRR family involved in parasite susceptibility was identified from a population genetic analysis of wild *An. gambiae* mosquitoes. Variation in susceptibility to *P. falciparum* infection was linked to a single ~ 10 Mb region in the mosquito genome on chromosome 2L. This was named the Plasmodium-Resistance Island (*PRI*) (Niare et al, 2002; Riehle et al, 2007; Riehle et al, 2006b). Within this QTL, an 18kb locus containing resistance candidate genes was identified and named *APL1* (*Anopheles Plasmodium*-responsive Leucine-rich repeat protein 1) (Figure 4.1). The region contains three separately transcribed *APL1* genes, denoted *APL1A*, *APL1B* and *APL1C*, as well as genes encoding two LRR proteins (LRIM11 and LRIM3) which are not members of the APL1 family (Riehle et al, 2008b). The sequence similarity of the *APL1* genes suggests that they evolved by gene duplication and functional diversification, making them paralogues (Riehle et al, 2008a).

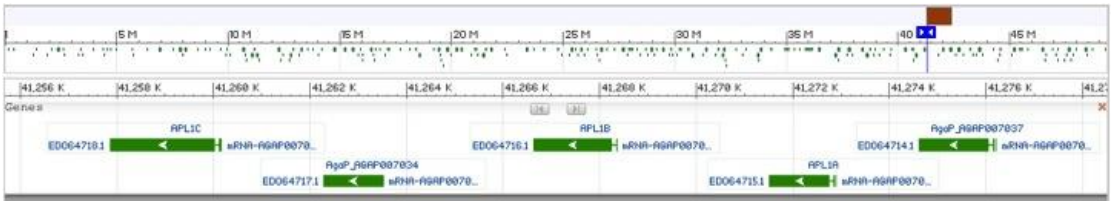


Figure 4.1 The *APL1* locus from *Anopheles gambiae* str. PEST chromosome 2L (41,256K-41,278K). The blue box with white arrows indicates the region on chromosome 2L. Green boxes indicate APL1A, B, C and two more unnamed genes in the region, with the direction of transcription indicated by the while arrows (Image taken from Vectorbase http://www.vectorbase.org/ accessed 1.2017).

4.2.1.5.1 Polymorphism of APL1 gene in *Anopheles gambiae* s.s.

APL1 proteins have the same general structure of a signal peptide which is usually followed by a low complexity PANGGL (Pro-Ala-Asn-Gly-Gly-Leu) region, a Leucine-rich repeat region of around 300 amino acids, and then a CC (coiled-coil) domain containing HLH (helix-loop-helix) motif (Riehle et al, 2008a). The three APL1 paralogues in the *An. gambiae* genome share 50% identity at the amino acid level, with variation occurring in the N and C terminal sequence through the presence or absence of the PANGGL domain, and deletions upstream of the LRR region (Fig. 4.2). The APL1 paralogues also exhibit diversity in field populations and laboratory colonies of *An. gambiae*, especially for APL1A and APL1C (Riehle et al, 2008a).

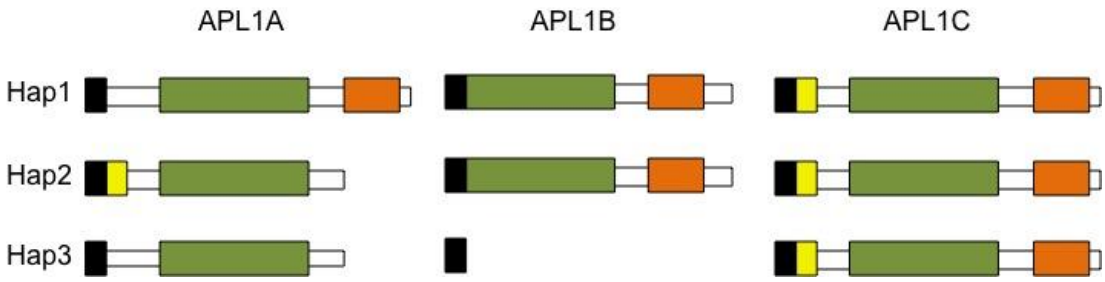


Figure 4.2 Structure of *APL1* genes in *An. gambiae* (NGOUSSO) strain. The predicted peptide domains are shown coded by colour: black – signal sequence, yellow – PANGGL, green – LRR, orange – coiled-coil domain. Adapted from (Holm et al, 2012). Three haplotypes were found in the Ngousso strain.

4.2.1.5.2 Functional significance of *APL1* paralogues and alleles

RNAi knockdown of *APL1C* has been shown to increase mosquito susceptibility to *P. berghei* and *P. yoelii* (Mitri et al, 2009a; Riehle et al, 2006a; Riehle et al, 2008a). *APL1C* transcript and protein abundance are known to be regulated through the Toll /Rel /Cactus pathway (section 4.2.2.1), and silencing of *APL1C* abolishes the Rel-mediated protection for *P. berghei*. APL1C appears to mediate killing of *P. berghei* through the formation of a complex with the LRIM1 (section 4.2.1.5), stabilising TEP1 (section 4.2.1.4) (Fraiture et al, 2009; Osta et al, 2004; Povelones et al, 2009).

Similar experiments with APL1A revealed its relevance for susceptibility to *P. falciparum* (Mitri et al, 2009b). *APL1A* transcription is regulated by the Imd /Rel2 pathway (section 4.2.2.2) (Mitri et al, 2009a). *APL1* allelic diversity was found to correlate with susceptibility of *An. gambiae* to *P. falciparum* infection, with *APL1A*^{2} having the only significant effect (Holm et al, 2012; Mitri et al, 2009a). The presence of the *APL1A*^{2} allele was sufficient to explain the protective effect against *P. falciparum* infection, but it was not possible to determine whether homozygotes of this allele had any further effect over heterozygotes, because of the low frequency of *APL1A*^{2} homozygotes (Holm et al, 2012). The mechanism by which APL1A^2 contributes to mosquito refractoriness to *P. falciparum* is not yet understood: APL1A^2 and APL1A^3 lack the coiled-coil domain, and were also retained in the cytoplasm of haemocyte-like cells, whereas APL1A^1 was secreted from the cell, but it is not clear if secretion of APL1A is linked to its effect on *P. falciparum* (Holm et al, 2012).

However similar *APL* gene silencing studies using a different mosquito line (*An. gambiae* KEELE) and *P*. *falciparum* strain NF54 had different outcomes depending on infection intensity: silencing of *APL1B* and *APL1C* genes increased oocyst numbers at medium or low infection intensities only, and silencing of *APL1A* had no effect on infection (Garver et al, 2012). The authors suggested that the differences could be due to different *APL* sequences in the KEELE and NGOUSSO *An. gambiae* mosquito strains, or to the lower infection intensity of the NGOUSSO strain reported by Mitri (Mitri et al, 2009b).

4.2.1.6 **Galactoside binding lectins (GALEs)**

GALEs are thiol-dependent, β-galactoside-binding lectins. In *Drosophila* GALEs have been shown to play a role in development and immunity (Tanji et al, 2006). The *An. gambiae* genome encodes eight *GALE*s, some of which possess a carbohydrate recognition domain (CRD) (Dimopoulos et al, 1998). *GALE8* was shown to be expressed in the larval stage, and in the midgut of adult mosquitoes, after bacterial or malaria challenge (*P. berghei*), although the up-regulation was "transient and marginal" (Dimopoulos et al, 1997; Christophides et al, 2002).

4.2.1.7 **Fibrinogen-like domain immunolectins (FBNs)**

FBNs, also known as fibrinogen-related proteins (FREPs), are PRRs linked to recognition of microorganisms and to agglutination. The *An. gambiae* genome encodes up to 58 FBNs, possibly reflecting a need to control the midgut microbial flora following haematophagy. Three members of the FBN family, *FBN9*, *FBN23* and *FBN3*, have been shown to be upregulated by *Plasmodium* infection, and gene silencing experiments suggested their involvement in the anti- *Plasmodium* defence (Dong et al, 2006a; Christophides et al, 2004). FBN39 was found to be important only in regulating the mosquito's susceptibility to *P. falciparum*, whereas FBN9 and FBN8 were relevant both to *P. berghei* and *P. falciparum* infection outcomes (Dimopoulos et al, 2000; Dimopoulos et al, 2002; Dong et al, 2006a; Dong & Dimopoulos, 2009).

4.2.1.8 **Immunoglobulin superfamily genes**

Members of the immunoglobulin superfamily mediate pathogen recognition in many organisms. The *An. gambiae* genome has 138 proteins with at least one immunoglobulin domain, and 85 of these were shown to be upregulated in response to bacteria or to *Plasmodium* infection (Garver et al, 2008). Six of these genes, denoted Infection Responsive with Immunoglobulin Domain (IRID) genes, were selected on the basis of upregulation to multiple pathogens and the presence of multiple Ig domains.

IRID 1 (the orthologue of *Drosophila kekkon1*) and *IRID2* (ortholog of the *D-Titin* allele *sallimus*) were significantly upregulated during midgut invasion by *P. falciparum*, whereas *IRID6* (no identified Drosophila orthologue) was strongly downregulated in the gut during *P. berghei* ookinete invasion. Gene-silencing assays resulted in more than two-fold higher oocyst numbers for *IRID4* with *P. falciparum* and *IRID6* for both *P. falciparum* and *P. berghei*. Gene silencing experiments also highlighted a role for *IRID3*, *IRID5* and *IRID6* in limiting bacterial infection (Garver et al, 2008).

The *An. gambiae* Down syndrome cell adhesion molecule (AgDscam) is a hypervariable PRR; massive alternative splicing has the potential to generate 31,000 alternative splice forms that may mediate different pathogen interactions and specificities. AgDscam has been shown to protect mosquitoes from both *P. berghei* and *P. falciparum* infection (Dong et al, 2012; Dong et al, 2006b). The Imd pathway (section 4.2.2.2) regulates alternative splicing (and thus species-specificity) (Dong et al, 2012).

4.2.1.9 **Nimrod proteins**

In Drosophila, Nimrod proteins are known to bind bacteria and stimulate phagocytosis by haemocytes. Homologues exist in *An. gambiae*, and are similarly preferentially expressed in haemocytes, with transcriptional upregulation in response to bacterial infection (Estevez-Lao & Hillyer, 2014). Knockdown of the eater homologue resulted in decreased nitric oxide synthase (NOS) mRNA. Although the influence on *Plasmodium* has not been assessed, an effect on NOS levels (section 4.2.3.4) would be expected to influence parasite infection.

4.2.1.10 **Scavenger receptors (SCRs)**

SCRs are surface glycoproteins which bind to modified LDLs and polyanionic ligands. Members of this family play a role in the recognition of bacteria, and their subsequent phagocytosis (Gough & Gordon, 2000). The *An. gambiae* genome has three structurally different classes of SCR: SCRA (playing a role in mosquito metamorphosis), SCRB (SCRB9 and SCRBQ1-4 orthologues play role in phagocytosis and defence reaction in *Drosophila*) and SCRC (Christophides et al, 2004). There is no evidence to date that SCRs influence *Plasmodium* infection in mosquitoes.

4.2.2 **Signalling pathways**

Pathogen recognition activates signal transduction pathways that regulate the expression of AMPS and other effector mechanisms. Three immune signalling pathways that have been well studied in insects are known as the Toll/ Rel, the Imd/ Rel 2 and the Jak/ Stat pathways (Figure 4.3).

4.2.2.1 **Toll/ Rel 1 pathway**

This pathway involves the activation of the extracellular cytokine-like molecule Spaetzle (six homologues in *Anopheles*) by the pathogen recognition receptor, and its subsequent binding to the cellular receptor Toll (Figure 4.3). Downstream intracellular signalling, involving, for example, recruitment of the death-domain proteins Tube, Pelle or Myd88, results in the nuclear translocation of NF-κB transcription factors such as Rel1. These activate the transcription of antimicrobial peptides and other immune effector genes. The Toll pathway is negatively regulated in the cytoplasm by Cactus, an ankyrin-repeat protein that inhibits the nuclear translocation of NF-κB/Rel proteins. The Toll pathway has been shown to be important against Gram-positive bacteria, viruses, fungi, and *Plasmodium berghei*, reviewed in (Clayton et al, 2014; Hillyer, 2016).

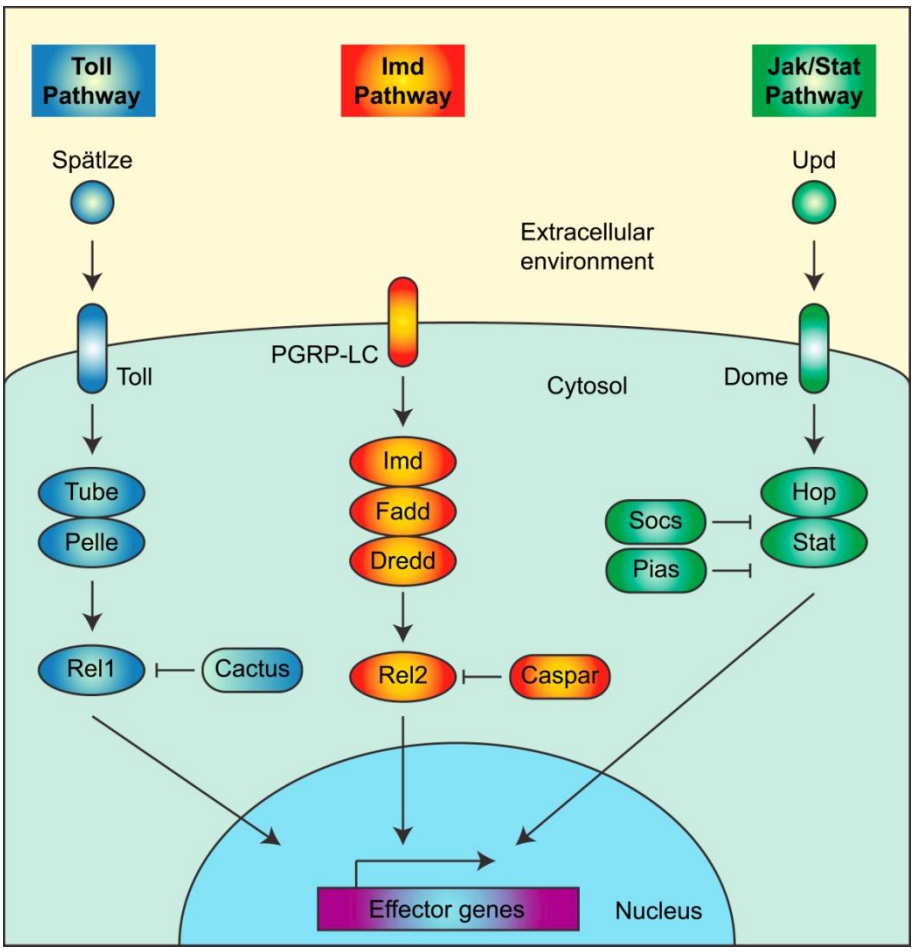


Figure 4.3 Immune signalling pathways in insects. Figure taken with permission from (Hillyer, 2016).

4.2.2.2 **Imd /Rel 2 pathway**

The Imd/Rel2 pathway is activated by binding of the pathogen recognition molecule to the extracellular receptor PGPR-LC (section 4.2.1.1 above), which induces intracellular signalling through the death-domain proteins Imd, Fadd (Fas-associated death domain), Dredd (*A. gambiae* homologue CASPL1) (and others) (Figure 4.3). In this case the NF-κB transcription factors such as Rel 2 are translocated to the nucleus to activate transcription of antimicrobial peptides and other immune effector genes. The Imd pathway is negatively regulated in the cytoplasm by Caspar binding to Rel2. Other antagonists of Rel 2 include the transcription factor Caudal (Cad); silencing of Cad has been shown to decrease *P. falciparum* development in the gut (Clayton et al, 2014). The Imd/Rel2 pathway is important in defence against Gram-negative bacteria, viruses, and *P. falciparum*, reviewed in (Clayton et al, 2014; Hillyer, 2016).

4.2.2.3 **JAK /STAT pathway**

The Jak/Stat pathway is activated, by the binding of the extracellular cytokine Unpaired (Upd) to the cellular receptor Domeless (Dome). The subsequent phosphorylation of Dome by the JAK tyrosine kinase Hopscotch (Hop) recruits Stat, which dimerises and moves to the nucleus (Figure 4.3). Stat activates transcription of antimicrobial genes such as *nitric oxide synthase* and *TEP1*. The Jak/Stat pathway is important in defence against bacteria, viruses and *Plasmodium*, reviewed in (Clayton et al, 2014; Hillyer, 2016).

There are two STAT genes in A*n. gambiae* (*STAT1 /AgSTAT-B* and *STAT2 /AgSTAT-A*), both of which are involved in anti-Plasmodium activity. *Ag*STAT-A activates the transcription of NO synthase (NOS) (section 4.2.3.4), which reduces parasite levels: silencing of *AgSTAT-A* increased mature oocyst development in both *P. berghei* and *P.* falciparum (Gupta et al, 2009), but seems to control earlier stages of infection in *P. vivax* in the south American vector *An. aquasalis* (Bahia et al, 2011).

The JAK/STAT pathway is negatively regulated by proteins such as the Suppressor of Cytokine Signalling (SOCS) and the Protein Inhibitor of Activated STAT (PIAS).

4.2.3 **Effector mechanisms of parasite killing**

4.2.3.1 **Melanisation**

Although melanisation as an anti-malarial response is rarely observed in mosquitoes in the field (less than 1% of cases) (Schwartz, 2002), it is probably the most studied anti-parasitic mechanism.

Melanisation is an acute reaction responsible for wound healing and pathogen killing in insects. In many invertebrates including mosquitoes, melanisation is a humoral but localized acute response. The reaction starts with conversion of tyrosine to quinones and reactive oxygen intermediates leading to the inactivation of a microorganism, crosslinking it with nearby proteins, and the formation of eumelanin leading to melanotic capsule formation, reviewed in (Hillyer, 2016) (Figure 4.4).

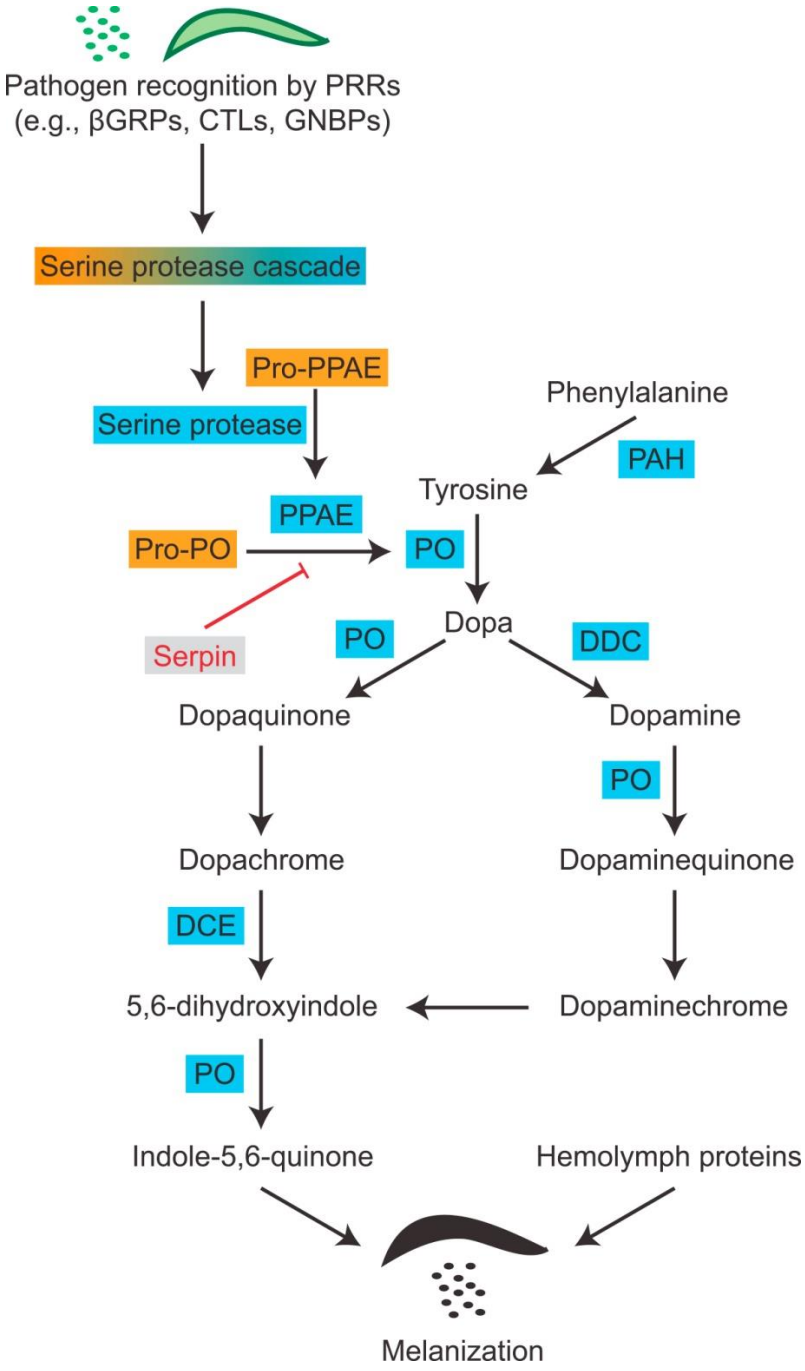


Figure 4.4 Insect melanisation pathway. PRR, pattern recognition receptor; βGRP, β-1,3 glucan recognition protein; CTL, C-type lectin; GNBP, Gram (−) binding protein; PPAE, phenoloxidase activating enzyme; PAH, phenylalanine hydroxylase; PO, phenoloxidase; DDC, dopa decarboxylase; DCE, dopachrome conversion enzyme. Figure taken from Hillyer (2016) with permission.

Tyrosine is oxidated via two pathways, the dopaquinone and dopamine pathways, both using prophenoloxidase (PPO). Phenoloxidases (POs) are secreted into the haemolymph from haemocytes as PPOs (zymogens). PPOs are then activated by phenoloxidase activating enzymes (PPAEs). As the PPOs lack secretory signal peptides, it is suggested that their release from cells is due to

granule exocytosis. In mosquitoes, nine genes encoding PPOs have been found. Moreover *PPO-2*, *-3* and *-9* are induced upon blood feeding (Christophides et al, 2004).

In *Drosophila* the inhibition of the PPO-activating serine proteases (PPAEs) by serpin 27A has a regulatory role in melanisation. This reaction is activated by the Toll pathway, reacting to bacterial or fungal infection (Ligoxygakis et al, 2002). Fourteen *serpin* genes have been identified in *Anopheles*, 10 of which are inhibitory: 3 of the inhibitory genes are orthologous to *Drosophila serpin 27A* (Gulley et al, 2013).*Serpin10* codes for four alternatively spliced inhibitory forms; its expression is localized to midgut epithelial cells, pericardial cells and haemocytes. On top of that, two forms of *serpin10* are induced in female mosquitoes in response to midgut invasion by *Plasmodium* ookinetes (Christophides et al, 2004). Serpins are discussed further in section 4.2.3.6.

Plasmodium melanisation in mosquitoes has been examined in many parasite/ vector combinations. Because melanisation depends on *Plasmodium* species and strain, it is suggested that it is specific recognition based mechanism (Molina-Cruz et al, 2012).

The *An. gambiae* refractory strain L3-5 was generated through selective breeding for melanisation of *P. cynomolgi* (simian malaria) (Collins et al, 1986). Mosquitoes were found to melanise ookinetes after passage through mosquito midgut (16-24h post infective blood meal) (Paskewitz et al, 1988). In addition to melanisation of *P. cynomolgi*, against which it was selected, melanisation also occurs with the avian malaria parasite *P. gallinaceum* (Collins et al, 1986), the human malaria parasites *P. falciparum*, *P. ovale*, and *P. vivax*, simian malarias *P. gonderi*, *P. inui*, and *P. knowlesi*, and the rodent species *P. berghei* (Blandin et al, 2004). Three mosquito quantitative trait loci have been characterized as responsible for 79% of the melanisation based refractory trait: *Plasmodium* encapsulation loci - *Pen1*, *Pen2* and *Pen3*, where the *Pen1* locus has the biggest contribution (Collins, 1997; Zheng et al, 1997).

An *An. dirus* refractory line was selected over 19 generations, to melanise *P. yoelii*, with both ookinetes and oocysts appearing to be melanised (Somboon et al, 1999). However this line did not melanise *P. falciparum* and *P. vivax*, highlighting

the specificity of refractoriness to certain combinations of parasite and vector (Somboon et al, 1999).

Several pathways have been studied in connection with melanisation and its effect on parasite infection in the mosquito. Silencing of thioester-containing protein 1 (TEP1, section 4.2.1.4) and leucine-rich repeat domain-containing protein (LRIM1, section 4.2.1.5) resulted in increased levels of *P. berghei* oocysts, and silencing of C-type lectins (CTLs, section 4.2.1.7) resulted in increased parasite melanisation (Schlegelmilch & Vlachou, 2013; Volz et al, 2006). However with the parasite/ vector combination *P. falciparum/ An. gambiae*, silencing of CTL-4, CTLMA2 and LRIM1 had no effect on oocyst numbers and melanisation, highlighting differences between human *Plasmodium* and its rodent model organism *P. berghei* (Clayton et al, 2014).

Molecular and microarray mRNA expression analysis revealed increased steady-state levels of reactive oxygen species, which favour melanisation of parasites, as well as Sephadex beads (Kumar, 2003; Schwartz, 2002). Moreover, the mechanism of refractoriness to *P. berghei* have been linked with Thioester containing protein (TEP1) (Blandin et al, 2004).

4.2.3.2 **Lytic mechanism**

Lysis of ookinetes of malaria parasites has been described in the combination of *An. gambiae* and the avian malaria *P. gallinaceum. An. gambiae* mosquitoes were selected for refractoriness without melanotic encapsulation. The ookinetes were found to be killed within midgut epithelial cells within 27 hours of midgut invasion. Electron microscopy showed that ookinete death occurred while the parasite lay free in the midgut epithelial cell cytosol, surrounded by an organelle-free zone that consisted of finely fibrillar material (Vernick et al, 1995). The mechanism of ookinete lysis is not known, but TEP1 involvement has been suggested (Fraiture et al, 2009).

4.2.3.3 **Phagocytosis by haemocytes**

Haemocytes represent a cellular component of *Anopheles* innate defence mechanism against *Plasmodium* infection, reviewed in (Hillyer & Strand, 2014).

These cells mostly circulate in haemolymph or are located in the fat body. Several classes of haemocytes have been discovered in *Anopheles* mosquitoes. Oenocytoids are involved in melanisation and phagocytosis. In *Ae. aegypti* sporozoites of *P. gallinaceum* are phagocytosed by circulating oenocytes. In contrast, in *An. albimanus* oenocytoids are embedded in the fat body and are released just after infection. Other circulating haemocytes such as prohemocytes, plasmatocytes and granular cells are believed to play a role in *An. albimanus* in melanisation, cellular encapsulation and phagocytosis (Hernandez et al, 1999).

4.2.3.4 **Other defence mechanisms: nitric oxide**

In *An. stephensi* midguts, *Plasmodium* ookinetes can be destroyed by nitric oxide (NO) and nitrite/ nitrate produced by midgut epithelial cells (Christophides et al, 2004). Elevated expression of NO synthase (NOS) was observed to be present in the midgut and carcass soon after invasion of the midgut by *P. berghei*. This phase of increased NOS activity was however likely primed by rapid bacterial growth after the blood meal. The second phase of NOS expression is correlated to sporozoite release from oocysts. Moreover dietary provision of NOS inhibitors increased parasite numbers in the mosquito, confirming the importance of NOS in anti-*Plasmodium* immunity (Luckhart, 1998).

Production of NO in the midgut columnar epithelia cells, triggering cascades leading to mosquito cell death, is the core of the “time bomb” theory. The initiation of the whole process is ookinete invasion of these cells (Han, 2000).

4.2.3.5 **Humoral effectors and immune killing mechanisms: antimicrobial peptides (AMPs)**

Antimicrobial peptides (AMP) are small (generally 12-50 amino acids), amphipathic molecules that are able to partition into the membrane lipid bilayer, and have a range of antimicrobial activities. Although first discovered in insects (Boman et al, 1974), they exist across a range of vertebrate and invertebrate species.

In insects, AMPs are produced mainly in the fat body, from where are released into haemolymph and distributed throughout the insect body (Clements, 2012).

Other tissues, such as epithelia can also produce AMPs. AMPs are grouped into three classes: linear with amphipathic and hydrophobic α-helices, peptides with cysteine disulfide bonds and proteins enriched in proline and/or glycine residues. Four families of AMPs have been identified in *An. gambiae*: four defensins (DEF1-4), four cecropins (CEC1-4), one attacin and one gambicin (GAM1) (Christophides et al, 2004).

Defensins are significantly upregulated following parasite infection in *An. gambiae* and *Ae. aegypti* (Lowenberger et al, 1999; Richman et al, 1997; Shahabuddin et al, 1998). Whereas over-expression in the fat body of *Ae. aegypti* was effective in decreasing infection of *P. gallinaceum* (Shin et al, 2003), knockdown of Defensin in *An. gambiae* did not affect infection levels with *P. berghei* (Blandin, 2002).

Cecropins were found to be up regulated upon *Plasmodium* infection in mosquitoes from the second day after infection, and inoculation of cecropin into *An. gambiae* after an infectious blood meal produced an increase in oocyst numbers relative to controls (Gwadz et al, 1989), but the high level of AMP used was toxic to the mosquito. Overexpression of *CecA* was found to block development of *P. berghei* by up to 83% in *An. gambiae* (Kim et al, 2004).

Gambicins are class of AMPs identified so far only in *Anopheles* and *Aedes* mosquitoes. *GAM1* is expressed in the mosquito midgut and fat body after challenge with Gram-positive and Gram-negative bacteria, and *P. berghei* ookinetes (Christophides et al, 2004). Knock down of gambicin in *An. gambiae* increased levels of *P. berghei* oocyst infection, but had no effect on *P. falciparum* (Dong et al, 2006a).

A combination of AMPs may be required to completely block parasite transmission: transgenic *Ae. aegypti* co-expressing both *Defensin A* and *Cecropin A* in the fat body were found to block the development of *P. gallinaceum* completely (Kokoza et al, 2010).

4.2.3.6 **CLIP domain serine proteases/ serine protease inhibitors (SERPINS)**

In several invertebrates, CLIP domain serine proteases have been linked to activation of the PPO cascade, reaction to LPS or glucans, and, in *Drosophila*, to

the activation of the Toll pathway upon fungal infection. Serpins are inhibitors of serine proteases, and are known to regulate the PPO and Toll pathways, leading to regulation of melanisation reaction (discussed in melanisation section 4.2.3.1) (Christophides et al, 2004). In *Anopheles,* four groups of CLIPs (CLIPA-D), comprising 41 genes, have been identified in the genome. The CLIPB group is responsible for activation of PPO cascade. After *Plasmodium* infection, *CLIPB14* stays upregulated, whereas *CLIPB15* peaks only during ookinete invasion of midgut (Volz et al, 2005). In contrast another CLIP, *CLIPA6* is induced by bacterial infection, but suppressed by *Plasmodium*.

4.3 Selection of candidate genes investigated in the GU-REF line

The process of selection for refractoriness in the GU-REF line was expected to be the result of enrichment of alleles conferring refractoriness that already existed in the KEELE colony. As an initial screen, candidate genes were selected and their polymorphism, and allele frequencies were investigated in the KEELE and GU-REF lines. The candidate gene selected was *APL1A*, based on the research showing a strong association with infection in the field and laboratory studies described above.

4.4 Materials and methods

4.4.1 DNA extraction

DNA was extracted from individual mosquitoes from the KEELE line (GU-CON and GU-REF) of *An. gambiae s.s.* using the DNeasy spin column protocol (Qiagen). Adult mosquitoes were frozen at -20°C overnight and then processed to extract DNA according to the manufacturer's protocol. Each mosquito generated 200 µL of genomic DNA (Ranford-Cartwright et al, 2016).

4.4.2 PCR

To determine which alleles of the APL1A gene (1,3 or 2) were present in GU-REF and GU-CON, DNA fragments were amplified using published protocols and primer sets (Holm et al, 2012) (Table 4.1).

APL1A1,3 F	gatctaaagcctaagtcgacgta (23)
APL1A1,3 R	tcgatcactggctcacatggat (22)
APL1A2 F	tagctggatcccaagtagtgct (22)
APL1A2 R	cagcgctacgtaattcatgg (20)

Table 4.1 Primers for amplification of diagnostic fragments from APL1A1,3 and APL1A2 alleles. The number in brackets after the primer sequence is the length in base pairs of the primer.Primers are taken from Holm et al, 2012. F= forward primer; R= reverse primer.

The size of expected fragment was 2367 bp for *APL1A*^{2} and 2359 bp for *APL1A*^{1,3}. The PCR program used was as follows: denaturation 95°C for 3 min – (95°C 30 sec – annealing 62°C 45 sec – extension 68°C 3 min) for 40 cycles with final extension 68°C 10 min. PCR reactions were performed in a final volume of 20μl. The reaction mix was as follows: 1μl (1ug) genomic DNA, 1μl (0.1μM) Forward primer, 1μl (0.1μM) Reverse primer, 4μl 10 x polymerase buffer, 1μl (1.1g/mL) DMSO, 1μl (10mM) dNTP's, 0,25μl (2U/μl) Biorad iProof High fidelity polymerase, and 10.75μl H_2O. The PCR reaction products were separated by electrophoresis on 1% agarose gel.

4.5 **Results**

4.5.1 ***APL1A*** **amplification from GU-REF and GU-CON mosquitoes**

The distribution of *APL1A*^{1,3} and *APL1A*^{2} alleles was determined amongst GU-REF and GU-CON mosquitoes in generation 10 of the selection for refractoriness. A total of 90 mosquito were analyzed, 46 GU-REF10 and 44 GU-CON10. The sample consisted of males and females mosquitoes as shown in Table 4.2. An example of the amplification results for three mosquitoes is shown in Figure 4.5.

	APL1A1,3+APL1A2			**APL1A1,3**			**APL1A2**			
	M	F	TOTAL	M	F	TOTAL	M	F	TOTAL	SUM
GU-REF10	15	16	31	2	0	2	5	8	13	46
GU-CON10	20	7	27	2	15	17	0	0	0	44

Table 4.2 Distribution of *APL1A* alleles and genotypes among GU-REF10 and GU-CON10 mosquitoes. (M) male, (F) female. (SUM) is the total number of individuals tested.

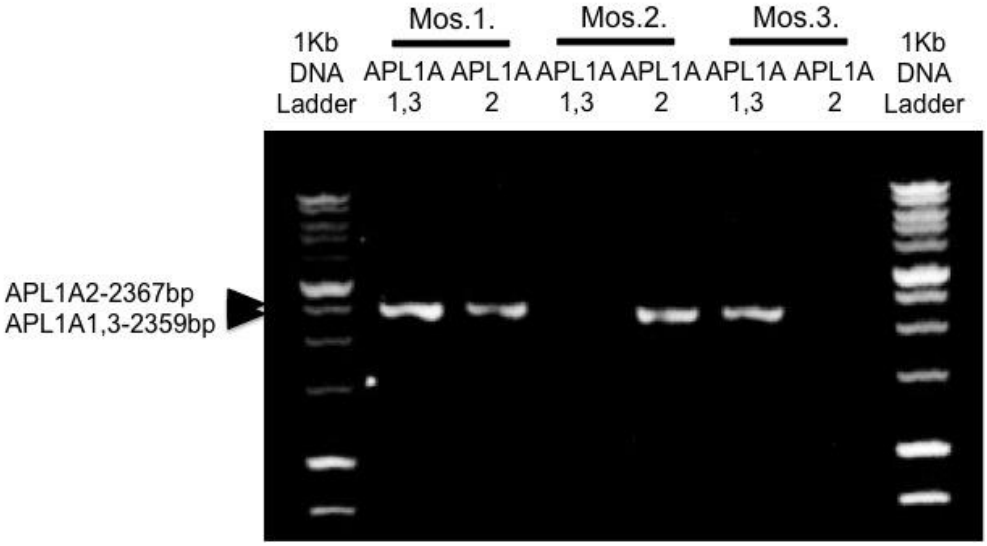


Figure 4.5 Electrophoresis of PCR products for amplification of *APL1A*^{1,3} and *APL1A*^{2} from 3 mosquitoes. Alternative lanes show the amplification products from reactions specific for *APL1A*^{1,3} and APL1A^{2}. Mosquito 1 (Mos. 1.) from GU-REF10 is a heterozygote, mosquito 2 (Mos. 2.) from GU-REF10 is homozygous for *APL1A*^{2}, and mosquito 3 (Mos. 3.) from GU-CON10 is homozygous for *APL1A*^{1,3}. The fragments were separated in 1% agarose gel. Black arrows show sizes of the amplified fragments for APL1A^{1,3} and APL1A^{2}.

4.5.2 Calculation of allele frequencies and testing of Hardy-Weinberg equilibrium

From the data in Table 4.2, the allele frequencies were calculated separately for the GU-REF10 and GU-CON10 lines (all mosquitoes) and for male only and female only mosquitoes, and are shown in Table 4.3.

Allele	Mosquito Sex	Allele frequency	
		GU-REF10	GU-CON10
APL1A-1, 3	Male	0.432	0.545
	Female	0.333	0.841
	Pooled	0.380	0.693
APL1A-2	Male	0.568	0.455
	Female	0.667	0.159
	Pooled	0.620	0.307

Table 4.3. APLA allele frequencies in GU-REF10 and GU-CON10 colonies.

The distributions of alleles in male mosquitoes and in female mosquitoes were compared between the GU-REF10 and GU-CON10 lines using Chi-Squared tests under the null hypothesis of no difference in allele frequency between the GU-REF10 and GU-CON10 lines (Table 4.4). When male and female mosquitoes were pooled together, there was a significant deviation from the frequencies expected under the null hypothesis (p=0.00019). There was no significant

difference in the male allele frequencies (p=0.286), but in the female mosquitoes there were significantly higher frequencies of the *APL1A*^{2} allele in the GU-REF line than expected under the null hypothesis, suggesting enrichment of this allele in the GU-REF10 population in the females (those under selection).

***APL1A* Allele**	**Male and female pooled**			**Males only**			**Females only**		
	GU-REF	**GU-CON**	**total**	**GU-REF**	**GU-CON**	**total**	**GU-REF**	**GU-CON**	**total**
APL1A-1,3	35 (47.1)	61 (48.8)	96	19 (21.5)	24 (21.5)	43	16 (25.6)	37 (27.4)	53
APL1A-2	50 (37.8)	27 (39.1)	77	25 (22.5)	20 (22.5)	45	25 (15.4)	7 (16.6)	32
total	85	88	173	44	44	88	41	44	85
p value	0.00019			0.286			0.000018		

Table 4.4 Comparison of APL1A allele distributions in male and female mosquitoes in the GU-REF10 and GU-CON10 colonies using chi-squared tests. Expected values are shown in brackets.

Considering genotypes, the same comparisons were made and are shown in table 4.5. When male and female mosquitoes were pooled together, there was a significant deviation from the genotype frequencies expected under the null hypothesis (p=0.0000036). There was no significant difference in the male genotype frequencies (p=0.059), but in the female mosquitoes there was a significant difference (p=0.00000018), with higher frequencies of the *APL1A*^{2} homozygotes and lower frequencies of the *APL1A*^{1,3} homozygotes in the GU-REF line than expected under the null hypothesis. This suggests that the selection process in GU-REF has favoured the *APLA*^{2} homozygotes and selected against the *APLA*^{1,3} homozygotes in the female mosquitoes.

***APL1A* genotype**	**Male and female**			**Males**			**Females**		
	GU-REF	**GU-CON**	**total**	**GU-REF**	**GU-CON**	**total**	**GU-REF**	**GU-CON**	**total**
Homozygous *APL1A*^{1,3}	2 (9.7)	17 (9.3)	19	2 (2)	2 (2)	4	0 (7.8)	15(7.2)	15
Heterozygous	31 (29.6)	27 (28.4)	58	15 (17.5)	20 (17.5)	35	16 (12)	7 (11)	23
Homozygous *APL1A*^{2}	13 (6.6)	0 (6.4)	13	5 (2.5)	0 (2.5)	5	8 (4.2)	0 (3.8)	8
Total	46	44	90	22	22	44	24	22	46
p value	0.0000036			0.059			0.00000018		

Table 4.5 Comparison of *APL1A* genotype distributions in male and female mosquitoes in the GU-REF10 and GU-CON10 colonies using chi-squared tests. Expected values are shown in brackets.

4.6 **Discussion**

In this study, the distribution of alleles and genotypes of the candidate gene *APL1A* (alleles *APL1A*^{1,3} and *APL1A*^{2}) were examined in the GU-REF and GU-CON lines of *Anopheles gambiae s.s.*. This gene was selected for analysis over other genes linked with mosquito immunity because of the support for involvement in refractoriness against *P. falciparum*, both in field and from laboratory infections (Holm et al, 2012; Mitri et al, 2009b; Niare et al, 2002; Riehle et al, 2007; Riehle et al, 2006a; Riehle et al, 2008b; Rottschaefer et al, 2011; Waterhouse et al, 2010; Williams et al, 2015). APL1A was never associated with melanisation, which agrees with the lack of melanisation seen in field caught mosquitoes, and in the GU-REF selected mosquito line as a response to *P. falciparum* infection.

The allele associated with refractoriness in the field data was *APL1A*^{2}, and there was no association with *APL1A*^{1} or *APL1A*^{3} (Holm et al, 2012). This hypothesis was tested in the data presented here in *An. gambiae s.s.* mosquitoes selected for refractoriness to *P. falciparum* based purely on the refractoriness phenotype (lack of oocyst development after IBF of *P. falciparum* clone 3D7).

The results showed a significantly higher allele frequency of the *APL1A*^{2} allele, and of *APL1A*^{2} homozygotes in the GU-REF mosquitoes, compared to the GU-CON line. This finding strongly suggests that during selection for refractoriness, mosquitoes with at least one allele of the *APL1A*^{2} gene were selected for, and mosquitoes homozygous for *APL1A*^{1,3} were selected against. There were no *APL1A*^{2} homozygotes found in the 44 mosquitoes tested from the GU-CON10 line, which suggests these mosquitoes may have a lower fitness in the absence of selection than the heterozygous or homozygous *APL1A*^{1,3} individuals.

The differences in allele and genotype frequencies in the GU-REF10 line were however only significant relative to the unselected GU-CON line in female mosquitoes, and not in males. This was surprising, as the selected females produce approximately equal numbers of male and female offspring, which mate to produce the next generation for selection. One explanation could be differential fitness costs in the different sexes of mosquitoes, such that the male *APL1A*^{1,3} homozygotes had higher survival than females. It is noteworthy that the numbers of *APL1A*^{1,3} homozygotes were very similar in the GU-REF10 and GU-CON10

males, whereas *APL1A*^{1,3} homozygotes were not seen in the female GU-REF10 individuals examined.

It was not possible to determine the *APL1A*^{2} distribution in the final generations (GU-REF11, GU-CON11), which had a 51.58% difference in infection prevalence.

The confirmation of *APL1A*^{2} association with refractoriness phenotype here provides further confirmation of the importance of the *APL1A* locus in refractoriness of *An. gambiae* for *P. falciparum*.

4.7 **Conclusions**

To conclude, in this final part of the project, the candidate gene with highest data support to be responsible for refractoriness from the field and laboratory research was selected for further analysis in the *Anopheles gambiae* s.s. GU-REF mosquitoes. The GU-REF mosquitoes in generation 10 demonstrated a very significantly lower infection level (28.85% difference in infection prevalence X^2 p=0.000006) compared to the GU-CON lines. The candidate gene, and specifically allele *APL1A*^{2}, was found to be significantly associated with refractoriness compared to allele *APL1A*^{1,3}. This knowledge is essential for the ability to select an *APL1A*^{2} pure homozygous *Anopheles gambiae* s.s. GU-REF line. With such a pure line, the mechanism of refractoriness can be further studied, and strategies, which enhance the natural immunity of *An. gambiae* to *P. falciparum*, thereby reducing transmission, can be developed.

5 General Discussion

In this chapter, a summary of the major conclusions from my research will be presented: the selection and characterisation of a new refractory *An. gambiae s.s.* mosquito to *P. falciparum*, with its characterization and preliminary analysis of potential refractory immune mechanisms. The implications of these findings for vector control strategies and understanding of vector pathogen interactions will be discussed.

5.1 Principal findings

The key goal of the research project was to select a line of *An. gambiae s.s.* refractory to *P. falciparum,* and to investigate the genetic basis of the trait. The underlying principle was to select a refractory line that represented mechanism(s) of refractoriness common in the field, potentially to multiple *P. falciparum* strains, or that would allow the basis of strain-specificity to be examined. For that reason, and because melanising refractory mosquito strains already exist, melanisation was excluded as a mechanism under selection. The *An. gambiae s.s.* KEELE line had been preselected for partial refractoriness prior to this project, indicating that such a selection was possible.

Unfortunately, microsporidia infection of the colonies at the start of the project required rescue of the colonies by selective breeding from uninfected individuals. This was successful, but the phenotype of partial refractoriness was lost. Reselection for refractoriness was then performed. After a subsequent four generations of selection GU-REF11 and GU-CON11 were established as a final selection generation exhibiting considerable and significant differences in infection prevalence.

Five fitness parameters were followed in selected lines together with speed of blood-meal digestion. The overall fitness of GU-REF line compared to the GU-CON line did not appear to be significantly negatively affected by the selection process for refractoriness.

Candidate gene analysis of *APL1A* showed a significantly higher allele frequency of the $APL1A^2$ allele, and $APL1A^2$ homozygotes in the GU-REF

mosquitoes, providing further confirmation of the importance of the *APL1A* locus in refractoriness of *An. gambiae* for *P. falciparum*.

5.1.1 Selection

5.1.1.1 Selection for Microsporidia free mosquitoes

Initial selection of *An. gambiae* KEELE in the laboratory before the start of my project had resulted in a GU-REF7 with significantly reduced infection prevalence compared to the GU-CON7 inbreeding control line (chapter 2). GU-REF7 was not completely refractory to infection.

Generation 7 experienced a microsporidia infection, compromising the ability of *Plasmodium* to establish infection. The outbreak also affected the parent *An. gambiae* KEELE line; the source was not identified but coincided with the introduction of a new *An. arabiensis* colony from the field. It was therefore necessary to establish microsporidia-free colonies, and there were two options after decontamination: (i) establish a *An. gambiae* KEELE colony from clean eggs, and after its establishment, start the selection process from scratch, or (ii) selectively breed from microsporidia-free individuals from GU-REF and GU-CON, and re-establish the colonies. For reasons of time, the second option was selected, recognising the risk of further inbreeding. The seventh generation went first through two rounds of selection for microsporidia free individuals and was recovered from 8 individuals each as GU-REF7 and GU-CON7 (Chapter 2).

5.1.1.2 Selection for refractoriness

The original hypothesis was that when selected for refractoriness to *P. falciparum* clone 3D7, a difference of susceptibility between the parasite line used for selection and an unrelated clone (HB3) would provide a platform to perform QTL analysis based on offspring of genetic crosses between 3D7 and HB3. It was hypothesised that this would identify parasite targets of refractoriness that differed in the two parasite clones (Ranford-Cartwright & Mwangi, 2012).

A further four rounds of selection were carried out during my thesis project, resulting in GU-REF11 and GU-CON11. At this point infection prevalence in the

colonies differed by ~52% (p=0.0004), although a significant difference in prevalence was apparent in generation 10 (~29% difference in prevalence, p=0.012).

The susceptibility to parasite clone HB3 was tested at this point and a significantly different prevalence was found, with a reduction of ~ 36 % in GU-REF11 compared to GU-CON11 (p=0.000484). This finding supported a hypothesis that the selection was not strain specific, and pointed to a more general mechanism of refractoriness against *P. falciparum*. The QTL analysis planned was therefore no longer an option to identify a parasite locus involved in refractoriness. An alternative candidate gene approach was adopted (Chapter 4).

It did not prove possible to identify the parasite stages killed in the refractory mosquito line using immunofluorescent or Giemsa staining, as described in chapter 2. To examine the stage specificity in the future, alternative techniques, such quantitative detection of stage specific proteins or RNA could be attempted. If the parasite stages killed in GU-REF were identified, this would allow close examination of the mechanisms of killing, narrowing down potential candidate mechanisms.

5.1.2 **Impact of refractoriness on mosquito fitness**

During selection of the refractory line, a negative impact on mosquito fitness could arise because of (i) general inbreeding effects and (ii) refractoriness itself being costly to the mosquito. The GU-CON line went through the same bottlenecks of the population but in the absence of selection for refractoriness, and it is hypothesised that differences between GU-REF and GU-CON therefore identify fitness costs that occur because of refractoriness rather than inbreeding. Five components of mosquito physiology were examined for fitness costs in female mosquitoes of the GU-REF line.

Previous studies, had shown selection for a smaller body size linked with refractoriness (Yan et al, 1997). No significant difference in body size was observed between the selected lines GU-REF10, GU-CON10 and the parent *An. gambiae* KEELE, suggesting that refractoriness in GU-REF10 is not caused by change in body size.

Survival is a key parameter for the mosquito's ability to transmit malaria, and also plays an important role in mosquito lifetime fecundity. Generation 10 of the GU-REF and GU-CON lines were used in survival determination experiments. When kept only on glucose as a nutrient source, the median day of death was significantly lower in GU-REF (22 days) compared to GU-CON (29 days), implying some cost to refractoriness, even in the absence of challenge. However, one non-infectious blood meal on day 8 restored the fitness as measured by survival to that of GU-CON, (median lifespan GU-REF 29 days, GU-CON 30 days (no significant difference)). However, when challenged with gametocytes in the blood meal, survival of GU-REF decreased compared to GU-CON (27 days vs. 30 days), implying that mounting a refractory (immune) response is costly. Such a decrease in survival of infected mosquitoes has been noted in a rodent malaria model (Dawes et al, 2009), but is less clear in human infections (Ferguson & Read, 2002).

Even after the apparently costly infectious blood feed, more mosquitoes from the GU-REF line laid eggs compared to GU-CON. This finding is in agreement with previous research suggesting that infection in non-refractory mosquitoes decreases fecundity (Hurd, 1995), and implies that the cost of refractoriness is less than the cost of infection. The median number of eggs laid was lower, but not significantly so, in the GU-REF11 line after an infectious blood meal compared to the GU-CON11 line. GU-REF mosquitoes did produce a significantly lower number of eggs compared to the parent *An. gambiae* KEELE line, which might indicate a dual effect of refractoriness and inbreeding; inbreeding itself is unlikely to explain the result as there was no significant difference in egg number between GU-CON and the parent outbred line *An. gambiae* KEELE. Overall the lack of a significant decrease in egg production related to infection or refractoriness is in agreement with data suggesting that both infection tolerance and refractoriness have similar fitness costs (Hurd et al, 2005).

During the selection process, susceptibility to infection (GU-CON line) seemed to be connected with a lengthening of the gonotrophic cycle (measured on 1st cycle). In generation 8, where there was no difference in infection intensity in GU-REF8 and GU-CON8, mosquitoes in both lines laid eggs 3 days after the blood feed. In generation 9, GU-REF laid eggs on day 3 whereas GU-CON laid on day 4.

The parental *An. gambiae* KEELE line laid eggs mostly on day 3. I am not aware of any studies of the length of the gonotrophic cycle having direct impact on infection or differences between refractory and susceptible mosquitoes. However, a longer or shorter gonotrophic cycle could reflect a change in blood meal digestion kinetics, which could be relevant to ookinete survival (Gass, 1979). Therefore, blood meal digestion patterns were investigated.

The rate of protein digestion was measured in two generations during the selection process: generation 7 and 9. No significant difference was observed in the speed of protein digestion, although in generation 9, protein digestion was marginally faster in GU-REF compared to GU-CON 24 hours after the blood meal, although this did not reach the significance level (p=0.06). However, given that the GU-REF line had the same length of gonotrophic cycle as the unselected parent line *An. gambiae* KEELE, selection for refractoriness did not select for increased rate of blood meal digestion. Instead, the lengthened gonotrophic cycle in GU-CON is likely to be a result of inbreeding and random selection of mosquitoes with slower digestion.

In summary the GU-REF line did not shown major fitness costs compared to GU-CON line. However all the fitness parameters were measured only in females, and it is possible that male fitness costs exist. The *APL1A*^{2} allele appears to confer differential survival to the two sexes of the mosquito (as discussed below, in section 5.1.3.). Additional fitness parameters such as egg hatching rates, larval survival and pupation rates were not studied.

5.1.3 **Candidate gene approach: APL1A**

From a pool of genes associated with mosquito immunity, we selected *APL1A* as a strong candidate, and examined the allele distribution (*APL1A*^{1,3} and *APL1A*^{2}) in the GU-REF10 and GU-CON10 lines of *An. gambiae s.s.* A significantly higher allele frequency of the APL1A^{2} allele, and of *APL1A*^{2} homozygotes, was found in the GU-REF mosquitoes, suggesting that during selection for refractoriness, mosquitoes with at least one allele of the *APL1A*^{2} gene were selected for, and homozygous *APL1A*^{1,3} mosquitoes were selected against. This finding agrees with data from field, and from laboratory research, linking *APL1A*^{2} to mosquito refractoriness (Holm et al, 2012; Niare et al, 2002). However the investigation did

not extend to other genes with the *PRI* (Parasite Resistance Island) locus, a 15 Mb region on chromosome 2L, which was found to explain the majority of naturally segregating variation in *P. falciparum* in a field study (Riehle et al, 2006). The *PRI* region contains over 70 plausible candidate genes, on the basis of predicted membership of mosquito immune gene families, or up-regulation on infection. One possibility is that the *PRI* region has been selected as a linked set of genes within the GU-REF line. The frequency of recombination in *An. gambiae* genome has been estimated to be 1.2Mb /cM. The PRI locus is region of about 22Mb (Zheng et al, 1997), and therefore can be expected to undergo approximately 1 crossover event in 5 meioses, if the standard recombination rate applies. The observation of enrichment of specific APL1A alleles in the REF line is likely to reflect enrichment of a larger linkage group or haplotype, which will contain additional genes up and down stream, because of the limited number of generations during selection.

Further work is required to investigate the inheritance of other PRI genes in the GU-REF line.

5.2 **Implications of the results**

The ability to select for refractoriness in *An. gambiae s.s.* against *P. falciparum* confirms that refractoriness has a genetic basis and is heritable. The apparent linkage of $APL1A^2$ with refractoriness after selection for refractoriness based on phenotype is an independent confirmation of the importance of this allele with refractoriness observed in the field (Niare et al, 2002). .

It is noteworthy that *APL1A*, and the PRI region in which it is located, lie `within the major chromosomal inversion on the left arm of chromosome 2 (2L+a/2La) (Riehle et al, 2007). Both forms of the 2L+a/2La inversion are found within the parental KEELE colony at approximately equal frequencies (Ranford-Cartwright et al, 2016). It is not known whether the $APL1A^2$ allele is present on both the 2L+a and the 2La chromosomal forms in the KEELE colony. There are three possible scenarios of representation of $APL1A^2$ and $APL1A^{1,3}$ on 2L+a and 2La inversion in *Anopheles gambiae* KEELE (Figure 5.1). The high frequency of the $APL1A^{1,3}$ in the GU-CON10 (0.693) suggests that this allele is likely to be present in 2L+a and 2La forms. The lower frequency of $APL1A^2$ leaves the

possibility of association of this allele with only one of the inversion forms. If *APL1A²* was associated only with one of the inversion forms, selection in the GU-REF line would have resulted in selection of that inversion form. While not reported in this thesis, subsequent work done in the lab did not show enrichment for either the 2L+a or the 2La inversion forms, implying that model A (Figure 5.1) is correct.

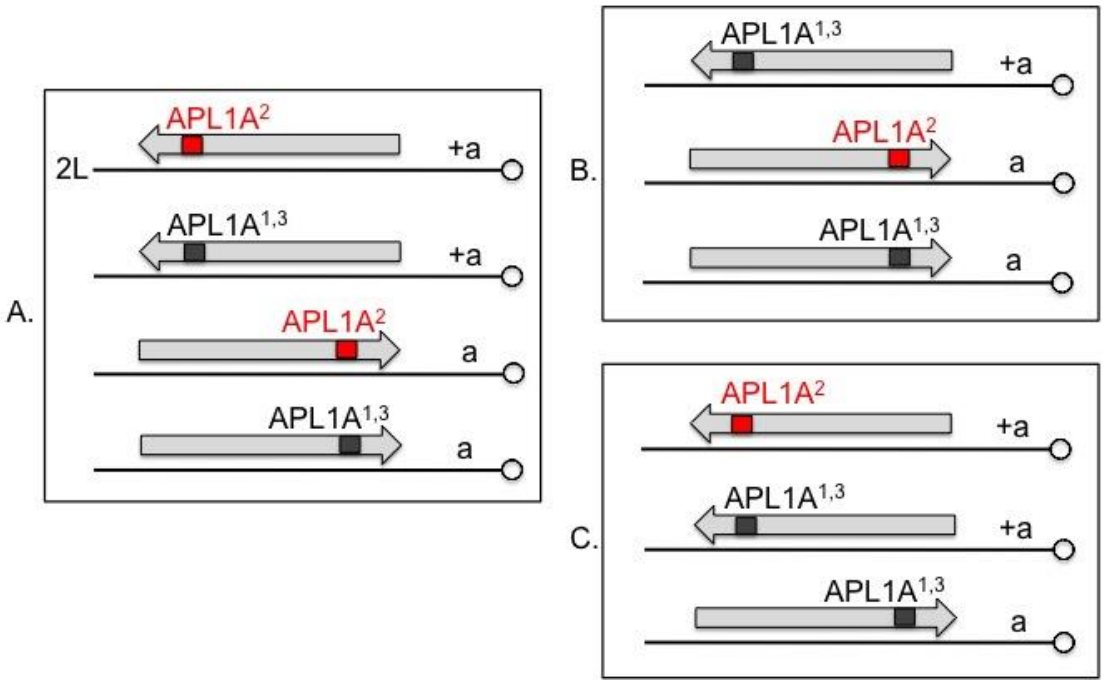


Figure 5.1 Possible scenarios of representation of alleles APL1A2 and APL1A1,3 on 2L+a and 2La inversions in the genome of *Anopheles gambiae* KEELE. The black line represents 2L chromosome, with a circle representing the centromere. The gray arrow indicates the inversion region, with "+a" being wild-type and "a" the inverted version. The dark gray and red boxes indicate the position of the APL1A gene within the inversion region. (A) represents the scenario where all possible combinations of APL1A2 and APL1A1,3 on 2L+a and 2La inversion are present. (B) represents the scenari, where APL1A2 is present only on the inversion "a". (C) represents the scenario where APL1A2 is present only on the wild-type "+a".

The inversion 2La has been associated with resistance to higher temperature and indoor resting behaviour (Ayala et al, 2014). Its prevalence in natural populations iincreased in the dry season and in dry environments. Interestingly a Kenyan population of mosquitoes homozygous for 2L+a were twice as likely to be infected with *P. falciparum* (positive for CSP) than those homozygous for the 2La inverted form, when the 2R inversion was also present (Petrarca & Beier, 1992). In that scenario, the *APL1A²* allele would be likely associated with 2La inversion (scenario A and C), if responsible for refractoriness in the population studied. The

high prevalence of *APL1A*^{1,3} in wild populations suggests that these alleles are represented on both forms of the inversion.

The higher frequency of *APL1A*^{2} homozygotes in females could be explained by a loss of homozygous males in the population before the pupal stage was sampled. As *APL1A* is not linked to the sex chromosome, equal numbers of male and female eggs homozygous for *APL1A*^{2} would be expected. The lower number of males with this karyotype suggests a loss of males, either during egg hatching or the following larval stages. As all the fitness characterisation was made on female mosquitoes, a study of male fitness would be one of the suggested experiments for better understanding of the impact of the *APL1A*^{2} homozygous karyotype in mosquitoes, and its rare occurrence in laboratory colonies such as the *An. gambiae* NGOUSSO and KEELE strains.

5.3 **What are the next steps?**

Additional work to examine enrichment of other alleles within the *PRI* locus is necessary to accept APL1A as the only candidate that has been selected for during selection for refractoriness. Selection of a pure *APL1A*^{2} line (all individuals homozygous *APL1A*^{2} / *APL1A*^{2}) would be beneficial for studies of mosquito immune mechanisms against malaria, as well as in maintaining a stable refractory line, if there is a fitness cost of the APLA^{2} allele, especially in the homozygous state. Attempts to select a pure homozygous line proved difficult, due to a lack of *APL1A*^{2} homozygotes males in the GU-REF11 line. The approach of selecting a pure line for further work, which would not need constant selective pressure to maintain a stable *APL1A*^{2} allele frequency, would probably be very difficult. The alternative would be to genotype mosquitoes at the *APL1A* locus after the experiments, and comparing the allele distribution retrospectively. To examine the phenotype of refractoriness linked to *APL1A*^{2} in the field, the infection of wild-caught mosquitoes, and association of infection rates to *APL1A*^{2} would have to be performed.

To further investigate the parasite stages affected in the refractory line, time course experiments looking at parasite numbers in the blood bolus, and the

mosquito midgut wall in later stages of the infection, could narrow the time frame when the refractory mechanisms acts, and could narrow down potential effectors.

Overall the selected line of non-melanising *An. gambiae s.s.* GU-REF can be used for experiments dissecting mosquito immune mechanisms against *P. falciparum* and in the research field of malaria transmission blocking interventions.

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RESEARCH ARTICLE

Characterisation of Species and Diversity of *Anopheles gambiae* Keele Colony

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Abstract

Anopheles gambiae sensu stricto was recently reclassified as two species, *An. coluzzii* and *An. gambiae s.s.*, in wild-caught mosquitoes, on the basis of the molecular form, denoted M or S, of a marker on the X chromosome. The *An. gambiae* Keele line is an outbred laboratory colony strain that was developed around 12 years ago by crosses between mosquitoes from 4 existing *An. gambiae* colonies. Laboratory colonies of mosquitoes often have limited genetic diversity because of small starting populations (founder effect) and subsequent fluctuations in colony size. Here we describe the characterisation of the chromosomal form(s) present in the Keele line, and investigate the diversity present in the colony using microsatellite markers on chromosome 3. We also characterise the large 2La inversion on chromosome 2. The results indicate that only the M-form of the chromosome X marker is present in the Keele colony, which was unexpected given that 3 of the 4 parent colonies were probably S-form. Levels of diversity were relatively high, as indicated by a mean number of microsatellite alleles of 6.25 across 4 microsatellites, in at least 25 mosquitoes. Both karyotypes of the inversion on chromosome 2 (2La/2L+[a]) were found to be present at approximately equal proportions. The Keele colony has a mixed M- and S-form origin, and in common with the PEST strain, we propose continuing to denote it as an *An. gambiae s.s.* line.

Introduction

Anopheles gambiae sensu lato is the major vector of malaria in sub-saharan Africa, consisting of eight morphologically indistinguishable species. *An. gambiae sensu stricto* exists in two molecular forms, denoted M and S, which can be distinguished by differences in a 4Mb region located centromerically on the X chromosome, including fixed SNPs within 2.3kb intergenic spacer region in the multicopy rDNA located on the X chromosome [1,2], or an M-specific insertion of a short interspersed transposable element (SINE200) [3]. Recently it was proposed

that the M- and S-forms are named as separate species [4], with the M-form taking the name *Anopheles coluzzii* and the S-form retaining the name *An. gambiae s.s.*

The two molecular forms differ in their geographical distribution and ecological niches, as well as in important phenotypic traits such as resistance to insecticides and to desiccation (reviewed in Lehmann and Diabate (2008) [5]). S-form *An. gambiae* are distributed across most of sub-saharan Africa, usually breeding in temporary aquatic habitats, and are associated with rainy seasons. M-form *An. gambiae* have a similar distribution to S-form in West and Central Africa, but are apparently absent east of the Great Rift Valley; they are able to exploit permanent breeding sites such as those associated with human activity, and breed year round [5–8]. The mechanisms driving divergence and speciation of the two molecular forms do not appear to be based on post-zygotic isolation, since laboratory crosses of M- and S-forms produce fully fertile male and female offspring [4,9]. Instead, spatial segregation of the two forms in mating swarms [10–12], or assortative mating behaviour [13] probably contributes to the usually very low rates of hybridisation seen in natural *An. gambiae* populations where the two forms are sympatric [12,14], although hybridisation can reach up to 20% in some sympatric populations [15–17], particularly at the extremes of the geographical distribution of sympatry [18]. Genetic divergence between the two forms in nature has been extensively studied, and was found to be widely distributed across the M- and S-form genomes, supporting the separation of the two forms into species [19–23].

The Keele mosquito strain was developed approximately 12 years ago as an outbred *An. gambiae s.s.* strain for use in experimental selection of malaria-resistant and -susceptible lines [24], and was established in Glasgow in 2002 directly from Keele University, where the line was generated. The chromosomal form has not previously been investigated, and it is usually referred to as an *An. gambiae s.s.* line. Its status now that *An. gambiae s.s.* has been divided into two species is uncertain.

The line was developed by balanced interbreeding of 4 existing laboratory colonies: ZAN U, Ifakara, KIL and G3 [24]. The first three of these colonies originated in East Africa (Zanzibar in 1984; Ifakara (Tanzania) in 1996; Marangu (Tanzania) in 1975 respectively); only the G3 line is from West Africa (MacCarthy Island, The Gambia, in 1975). Therefore, 3 of the 4 strains are expected to have been S-form at their original isolation, because of their East African origin. The G3 line could originally have been M, S or even a mixture, since hybrid M/S forms have been observed in The Gambia [16,18]. The generation of the Keele line involved initial crosses of 50 individuals of each sex between the strains KIL and Ifakara, and ZANU and G3, and the offspring of these two crosses were then mated to produce the Keele strain[24]. Keele mosquitoes are therefore likely to have a mixed origin from M- and S- form parents.

Laboratory colonies of mosquitoes usually exhibit considerable loss of diversity because of small starting populations (founder effect) and subsequent fluctuations in colony size [25,26]. Although the Keele strain was originally developed as an outbred line, the level of diversity of the strain has not previously been characterised. Microsatellites, especially those on chromosome 3 where there seems to be little restriction on gene flow [27], have been used previously to examine diversity in laboratory colonies, including the G3 line [26]. These analyses revealed reduced microsatellite diversity in two laboratory colonies relative to wild-caught mosquitoes from Mali, with an eightfold reduction in mean number of alleles found in eight microsatellite loci on chromosome 3 [26]. Wild-caught mosquitoes also had an abundance of rare alleles (frequency ≤ 0.05) which are less likely to be sampled in the relatively small starting populations for laboratory colonies.

Chromosomal inversions contribute to the substructuring of *An. gambiae* subpopulations and their adaptation to different environments [28–31]. A much-studied large inversion polymorphism on chromosome 2L (2La or 2L+^{a}) has been associated with adaptation to aridity:

An. coluzzii larvae homozygous for 2La have been shown to have enhanced thermal tolerance [32], and *An. gambiae s.s.* adults have enhanced resistance to desiccation [33,34]. Allele frequencies of the 2La/2L+^{a} vary spatially and temporally with respect to the degree of humidity in East and West Africa [35]. Both the 2La and the 2L+^{a} karyotypes are found in *An. gambiae s.s.* and *An. coluzzii*, but with spatial variations in the frequency; the chromosomal arrangements assort independently of molecular form in the field, and probably predate the speciation process [8]. The 2La/2L+^{a} karyotypes present in the Keele line have not previously been established.

Materials and Methods

DNA extraction from mosquitoes

The Keele colony held at Glasgow University is usually maintained at many thousands of individuals, with an average daily pupal collection of between 200 and 500 individuals, and 2000–3000 adults per large mating cage. Mosquitoes are allowed to mate naturally within each cage. Pupae for the study were selected randomly from different pupal trays over several days. 60 pupae were collected initially over 2–3 days for analysis of colony diversity, and an additional 90 pupae were collected for the evaluation of M and S forms at a later time point.

DNA was extracted from individual pupae from the Keele line of *An. gambiae s.s.* using the DNeasy spin column protocol (Qiagen). The sex of each pupa was first determined by examination of the terminalia [36]. Pupae were frozen at -20˚C overnight and then processed to extract DNA according to the manufacturer's protocol. Each pupa generated 200μl of genomic DNA.

Determination of M- and S-forms

Fixed single nucleotide differences in the rDNA intergenic spacer region on the X chromosome are used to define the M- and S- chromosomal forms [1,2,37]. We used a published PCR-RFLP method which amplifies a 390bp product including the polymorphic site at position 581 of the IGS rDNA region [38]; M-forms have a T in this position whereas S-forms have a C. The PCR product was then digested with HhaI (recognition site GCG^C), resulting in fragments of 257bp, 110bp and 23bp from S form, and 367bp and 23bp from M form.

3μl of DNA from each pupa was amplified in a final volume of 28μl containing 1x PCR Buffer, 1mM $MgCl_2$, 0.2mM dNTPs, 12.5ng primer UN (5'-GTGTGCCCCTTCCTCGATGT-3'), 6.25ng primer GA (5'-CTGGTTTGGTCGGCACGTTT-3'), and 1 unit Taq DNA polymerase, using the reaction conditions of an initial denaturation step 94˚C for 3 minutes, and then 30 cycles of 94˚C for 30s, 50˚C for 45s, 72˚C for 60s, with a final extension step of 7 minutes at 72˚C. 12μl of the PCR product was digested at 37˚C overnight with 1U of HhaI enzyme in 1 x NE Buffer 4 and 1 x BSA in a 15μl reaction volume. 7μl of the digested PCR product was run on a 2% agarose gel containing ethidium bromide and visualised by UV transillumination. Undigested PCR product (5μl) was included for comparison to check for digestion; the digested product is clearly smaller than the undigested product for the M-form, and two bands are visible for the S-form (in both cases the 23bp band is not visible on a gel).

Microsatellite analysis of chromosome 3

Four published microsatellite loci (all dinucleotide repeats) on chromosome 3 [39,40] were chosen for analysis of diversity in the Keele colony. The markers were chosen to be spread along the chromosome; their published locations are shown in Table 1. 2μl of mosquito DNA from each pupa was amplified in a final volume of 20μl containing 1x PCR Buffer, 1mM

Table 1. Microsatellite markers on chromosome 3. Distance represents the cumulative genetic distances from the most distal markers, and is taken from [39].

Marker name	Distance (cM)	Forward Primer	Reverse Primer	Amplification conditions
Ag3H93[a]	0	TCCCCAGCTCACCCTTCAAG	GGTTGCATGTTTGGATAGCG	• 95˚C for 5min • 30 cycles of [95˚C for 20s/55˚C for 30s/72˚C for 30s] • 72˚C for 10min
Ag3H119[b]	29.1	GGTTGATGCTGAAGAGTGGG	ATGCCAGCGGATACGATTCG	• 95˚C for 5min • 30 cycles of [95˚C for 30s/53˚C for 30s/72˚C f˚C 30s] • 72˚C for 10min
Ag3H88[b]	61.8	TGCGGCGGTAAAGCATCAAC	CCGGTAACACTGCGCCGAC	as for *Ag3H93*
Ag3H817[b]	93.7	ACTGGTCCGTTGCTGCGCG	ATGAGTGAATGGTGCGCTGG	• 95˚C for 5min • 30 cycles of [95˚C for 30s/53˚C for 30s/65˚C for 30s] • 65˚C for 10min

[a]Primer sequences taken from Lanzaro *et al.*, 1995.

[b]Primer sequences taken from Zheng *et al.*, 1996

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$MgCl_2$, 0.2mM dNTPs, 10nM of each primer and 1 unit Taq DNA polymerase, using the reaction conditions in Table 1. 7μl of PCR product was run for 4–6 hours on high resolution gels consisting of a 4% MetaPhor agarose gel (Lonza UK), containing ethidium bromide, and visualised by UV transillumination. PCR product sizes were estimated by comparison with a 25bp ladder using band size estimation software (Labworks, UVP, UK). Sizes were pooled into bins spanning 4 bp for each locus, taking into account the repeat size of 2 bp in these microsatellites and the estimated resolution for Metaphor Agarose of 3 bp (Lonza, UK).

Analysis of inversions on chromosome 2

The inversion on the left arm of chromosome 2 known as 2La / 2L+[a] was analysed using a published PCR strategy [41]. 2μl of mosquito DNA from each pupa was amplified in a final volume of 20μl containing 1x PCR Buffer (1.5mM $MgCl_2$), 0.2mM dNTPs, 10nM of each primer (Table 2) and 1 unit Taq DNA polymerase, using the reaction conditions of an initial denaturation step 94˚C for 2 minutes, and then 30 cycles of 94˚C for 30s, 55˚C for 30s, 70˚C for 45s, with a final extension step of 10 minutes at 70˚C. 10μl of each PCR product was run on a 1.5% agarose gel containing ethidium bromide and visualised by UV transillumination.

Results

Determination of M- and S-forms

150 *An. gambiae* Keele mosquito pupae were analysed of which 63% were female. A PCR product of 390bp for the IGS rDNA region was amplified and digested from all 150 DNA samples. All digested PCR products were 367bp, indicating that only the M-form of this locus (T at position 581) was present in the colony (M frequency: 100% (95% confidence interval 97.5–100%)). No hybrid individuals were seen.

Table 2. PCR primers used to type the chromosome 2 inversion 2La/2L+[a] [41].

Chromosome 2 Arrangement	Forward Primer	Reverse Primer	Expected PCR product size (bp)
2La	23A2: CTCGAAGGGACAGCGAATTA	27A2: ACACATGCTCCTTGTGAACG	492
2L+a	23A2: CTCGAAGGGACAGCGAATTA	DPCross5: GGTATTTCTGGTCACTCTGTTGG	207

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Table 3. Allele frequencies (freq.) for least and most common alleles at each locus for the Keele colony.

Locus	n[a]	no. alleles	Freq most common allele	Freq least common allele
Ag3H88	25	9	0.16	0.04
Ag3H93	31	7	0.258	0.032
Ag3H817	41	4	0.463	0.073
Ag3H119	39	5	0.487	0.077
mean	**34**	**6.25**	**0.342**	**0.056**
se	3.697	1.109	0.080	0.011

[a]n = number of mosquitoes analysed

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Table 4. Frequency of 2La/2l+[a] genotypes in the Keele colony (n = 161).

		Number	Proportion
Genotype	Heterozygous	84	0.522
	Homozygous 2La/2La	46	0.286
	Homozygous 2L+[a]/2L+[a]	31	0.193
Allele frequency	2La	176	0.547
	2L+[a]	146	0.453

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Microsatellite analysis of chromosome 3

The number of alleles seen at each of the 4 microsatellite markers in shown in Table 3. Some alleles were seen in only one mosquito, giving a low allele frequency of <0.05, but no allele predominated in the Keele colony for any of the 4 microsatellite loci.

Analysis of inversions on chromosome 2

Both the 2La and 2L+[a] chromosomal arrangements were found to be present in the Keele colony, with similar allele frequencies (Table 4). The numbers of homozygotes and heterozygotes was not significantly different to those expected under Hardy-Weinberg equilibrium (χ^2 = 0.446, P = 0.800).

Discussion

Analysis of the Keele colony unexpectedly revealed only the M-form of the rDNA marker on the X chromosome used to distinguish the two forms. Three of the four laboratory strains from which the Keele strain was established originated in East Africa, and therefore were expected to be S-form, although contamination of any of the lines with the opposite rDNA form prior to the development of the Keele strain cannot be discounted. It is unclear when or why the S-form marker on the X chromosome was lost in the Keele line; the initial crosses to generate the line [24] involved progeny of two probable S-form lines mating with the offspring of a cross between a probable M and a probable S-form line: early generations of the Keele line must have had S-form individuals or hybrids. Hybrids of M- and S-forms occur readily in the laboratory, as do as back-crosses to either M- or S- form parents, and the hybrids and their backcrosses were found to be fully fertile, with similar egg batch size, hatching rate, and larval development success under laboratory conditions [9]. Observed fitness differences of M- and S-forms under laboratory conditions include minor differences in the time to hatching of eggs, with S eggs hatching slightly earlier than M [42], higher longevity of virgin female M-forms

[5], and a larger body size of M-form females, which correlated in that study with larger egg batches in M-form than S-form [43]. The latter two factors could, over time, lead to increases in the frequency of M-form mosquitoes in a mixed colony. However in the face of repeated inter-form mating, it is difficult to imagine how the rDNA marker used to discriminate the M- and S-forms would remain linked to the fitness differences unless the genes responsible for these traits were strongly linked to the X-chromosome locus.

Under natural conditions in most of sub-saharan Africa, males form swarms of only one chromosomal form, and mating is generally assortative [10–13]. The mechanism for premating isolation is not fully understood, but differences in wing widths in populations where M- and S-forms mate assortatively lend support to the hypothesis that mosquitoes choose a mate based on wing-beat frequencies [44,45]. However direct measurements from M- and S-form mosquitoes failed to show significant differences in their fundamental harmonic (wing beat) frequencies [46]. Mating of *Anopheles* in the laboratory does not appear to involve typical swarm formation, and adaptation/colonisation involves selecting for mating in the restricted space of a cage (stenogamy) [47,48]. The Keele line within the Glasgow insectaries does form small swarms, and females enter the swarms to mate (unpublished observations), but the majority of mating in a colony probably occurs outside of swarms.

The Keele colony undoubtedly had a mixed M- and S-form origin, and the colony existing today is expected to have a hybrid genome with contributions from the four parent lines. It is similar in this respect to the *An. gambiae* PEST strain, chosen as the first *Anopheles* genome project [49]; the strain was generated in a series of crossing steps between different colonies from Kenya (S-form) and Nigeria (M-form) [https://www.vectorbase.org/organisms/anopheles-gambiae/pest]. Since this strain is commonly referred to as *An. gambiae s.s.*, despite having M- and S-form heritage, we propose that the Keele strain should also continue to be referred to as *An. gambiae s.s.*; the hybrid name *An. coluzzii* × *An. gambiae s.s.* may be more correct, if the rules applied to the nomenclature for inter-species hybrid plants (including the F_1, subsequent generations, back-crosses and combinations of these) were to be followed [50], but is excessively long.

Microsatellite locus analysis of the Keele colony revealed an unexpectedly high level of allelic diversity at 4 microsatellite loci, with an average of 6.25 alleles (range 4–9) seen in the 4 microsatellite loci we examined on chromosome 3. Previous analyses of laboratory colonies e.g. Norris *et al*., 2001 [26], had shown reduced diversity compared to wild-caught mosquitoes, with an average number of alleles of 2.33 (G3 colony, range 1–6) and 3.67 (Mopti colony, range 2–6), using 9 microsatellites on chromosome 3. Two of the microsatellites used in their study, *Ag3H88* and *Ag3H119*, were also used in our analysis of the Keele strain. For *Ag3H88* both the Mopti and G3 colonies (n = 32 for each) had only 2 alleles present in the published study [26], whereas in our study, 9 alleles were observed in 25 mosquitoes of the Keele line. Analysis of *Ag3H88* diversity in wild-caught mosquitoes revealed an average of 8 alleles in populations from 12 African countries (n = 967 mosquitoes), including a population (n = 23) from McCarthy Island (the origin of the G3 line) with 9 alleles at this locus [51]. For *Ag3H119*, previous characterisation of the Mopti and G3 colonies revealed 1 and 6 alleles respectively [26], compared to 5 alleles seen in the Keele colony. A previous study of wild-caught *An. gambiae* diversity in 9 Tanzanian locations found 10 alleles in total for this microsatellite in 638 individuals, with an average of 6.22 alleles per sample site (mosquito numbers sampled per location ranged from 30–106) [52]. Microsatellite diversity in the Keele line therefore appears to be higher than in previously-analysed laboratory colonies, although it does not reach the diversity observed in wild-caught mosquitoes, where large numbers of alleles, many at low frequencies (<0.05) are frequently observed [26,40]. This increased diversity may reflect the generation of the Keele colony by balanced interbreeding of 4 laboratory colonies, with offspring from 50

matings for each of the pairs (KIL x Ifakara and ZAN U x G3) then mated to produce the Keele line [24].

Finally the two karyotypes of the large inversion on chromosome 2 (2La/2L+^{a}) are both present in the Keele colony at approximately equal frequencies, and mosquitoes appear to mate randomly with respect to this marker.

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